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Semiannual Report to
THE ATOMIC ENERGY COMMISSION

MARCH 1960

LEON O. JACOBSON, M.D.
Editor

MARGOT DOYLE, Ph.D.
Associate Editor

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STUDIES ON ERYTHROPOIESIS. XVI. THE RESPONSE TO A SINGLE DOSE OF ERYTHROPOIETIN IN THE POLYCYTHEMIC MOUSE*

By

B. S. Filmanowicz and C. W. Gurney

INTRODUCTION

In the last decade, there has been extensive investigation of the nature of the stimuli which induce erythropoiesis and the role played by the hormone erythropoietin. This work has been summarized in recent review articles by Gordon[1] and Jacobson and associates.[2] Matoth and Ben-Poratt[3] have concluded . . . "erythropoietin is present in erythroid cells. It acts by stimulating the cells to mitotic division." Alpen and Cranmore,[4] and Erslev,[5] have presented evidence that the site of action of this hormone is confined to the stem cell. In their investigations, animals in a state of active erythropoiesis were employed although Erslev used colchicine to inhibit cell division. We believe that much additional information pertinent to the site of action of erythropoietin and the kinetics of red cell production can be obtained by the use of animals in which erythropoiesis has been suppressed. Such a state has been described by Jacobson and associates[6] in the transfusion-induced polycythemic mouse, an animal excellent for this purpose because endogenous erythropoiesis has been suppressed by a mechanism not known to produce any damage to the blood-forming tissues. It is the purpose of this study to investigate the sequence of events initiated by the injection of a highly purified extract of anemic plasma rich in erythropoietin, into such an animal.

MATERIALS AND METHODS

CF No. 1 virgin female mice, weighing 20 to 25 g, were rendered polycythemic by intraperitoneal injections of washed homologous red cells suspended in saline. These red cells were obtained from the jugular vein of ex-breeder females sacrificed by cervical fracture. Heparin was used as an anticoagulant. The red cell suspension was given in 0.5 ml doses (hematocrit approximately 90 per cent) twice daily for 2 days. Studies were begun 6 days after the last injection of red cells, at a time when the hematocrit of the test animals averaged about 75 per cent and erythropoiesis was suppressed as evidenced by the absence of reticulocytes in the peripheral blood smear. Throughout the period of induction of the polycythemic state and the subsequent investigation, the animals were furnished with Rockland mouse pellet diet and water ad libitum.

Hematocrit and reticulocyte counts were performed on blood drawn from one of the tail veins. Blood for hematocrit determinations was collected in heparinized capillary tubes and spun in a micro-hematocrit centrifuge.† Reticulocytes were counted by the direct smear method, using brilliant cresyl blue without counterstain. Determinations were made on the basis of

*This report is based on a paper delivered at the Laurentian Hormone Conference, September 1959. To be published in Recent Progress in Hormone Research XVI, 1959 (in press).

†International Hematocrit Centrifuge, H. S. Aloe Company, St. Louis, Missouri.

number of reticulocytes per 1000 red cells counted, except where the values were below 0.1 per cent, when 5000 red cells were counted.

For the purpose of histologic studies, mice were killed by cervical spinal fracture and sections of spleen prepared. The tissue was fixed in Zenker-Formol, embedded in nitrocellulose, cut at 5 micra and stained with hematoxylin-eosin-azure.

The erythropoietin* used was a highly purified preparation obtained from plasma of sheep rendered anemic by administration of phenylhydrazine and purified by a series of passages through DEAE† and XE97 resins. It appears to be an α-2 glycoprotein, the preparation and characterization of which have recently been described.[7,8]

The material was dissolved in .9 per cent NaCl and its potency, as used in these experiments, was determined in units of activity as defined by Goldwasser and White.[7] In the standard starved-rat assay for erythropoietin, one unit is that amount which elicits a response, as measured by Fe^{59} incorporation by red cells, equal to that produced by injection of 5 micromoles of cobaltous ion in the assay animal.

RESULTS

I. Establishment of a single maximum stimulating dose. Polycythemic mice were given single subcutaneous injections (.5 ml) to determine the lowest dose, in units of activity, which would produce a maximum response, as determined by reticulocyte count. Serial dilutions from 24 units to 3 units were employed and each dosage group in Table 1 represents the average of 4 animals. Several of the values appearing in Table 1 are plotted on Figure 1 which also includes the effect of a single dose of .75 units of erythropoietin injected into another group of animals prepared in the same way at a later time.

Table 1

Dose	Reticulocyte response on days after single erythropoietin injection					
	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5
24 units	0.0	0.0	0.3	1.4	1.3	0.3
18 units	0.0	0.0	0.4	1.3	1.3	0.3
12 units	0.0	0.0	0.4	1.4	0.9	0.1
9 units	0.0	0.0	0.2	1.1	0.6	0.2
6 units	0.0	0.0	0.3	1.3	0.6	0.2
3 units	0.0	0.0	0.3	0.9	0.4	0.0
Saline control	0.0	0.0	0.0	0.0	0.0	0.0

The peak reticulocyte response was obtained on the third day, regardless of the dose, and the lowest maximum stimulating dose was found to be 6 units of activity. This maximum retic-

*Prepared by Armour and Company Research Division under AEC Subcontract #21 under Contract #AT-(11-1)-69.

†Diethylaminoethyl cellulose.

ulocyte response persisted into the 4th day in the animals receiving the higher doses, but significant variation in reticulocyte response was not observed with dosages of 6 to 24 units in the first 3 days.

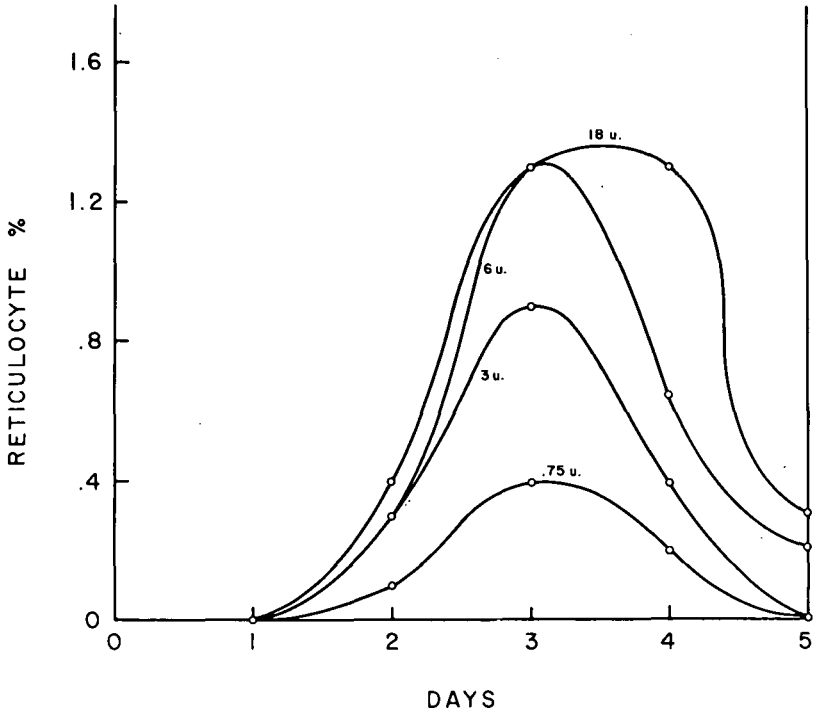

Figure 1. Reticulocyte response to a single injection of erythropoietin in the polycythemic mouse.

II. The morphological sequence of erythropoiesis and subsequent reticulocyte response to a single large injection of erythropoietin. Each of a group of 20 polycythemic mice was given 12 units of erythropoietin on day zero. Four animals were subsequently sacrificed at daily intervals and their spleens removed for sectioning. Saline-injected control animals were sacrificed on days 0 and 4 and spleens sectioned. In all cases, reticulocyte smears of the peripheral blood were made before sacrificing the animals.

Spleen sections were examined and one spleen section from each day was selected for detailed study. Counts were made of the number of nucleated erythroid cells per 5000 nucleated cells in the red pulp. Separate counts were made on the red pulp in the center and in a zone within 500 μ of the capsule since in all sections a greater abundance of nucleated erythroid elements was observed near the capsule.

Erythroid precursors were classified as early or late forms. Counts were made distinguishing between early and later forms of immature nucleated erythroid cells. An early form was considered to be a proerythroblast—i.e., an oval to round cell of about 20 to 30 micra diameter with a large pale nucleus containing only a sparse amount of fine chromatin. The most important differential characteristic of this cell is a strongly basophilic cytoplasm which stains a strikingly intense blue color. The later form was considered to be a normoblast—i.e., a smaller round cell of about 10 to 20 micra diameter with a more darkly staining chromatin-clumped

nucleus, and cytoplasm varying in color from blue to the gray characteristic of a mature erythrocyte.

It was observed (see Table 2) that while erythropoiesis was suppressed in the saline-injected control animals, a wave of erythropoietic activity passed through the spleen of the erythropoietin-injected mice. On the first day after injection, an increase in early erythroid forms was

Table 2

ERYTHROID CELL COUNTS (PER 5000 NUCLEATED CELLS) IN POLYCYTHEMIC MOUSE SPLEEN AND PERIPHERAL RETICULOCYTES (PER CENT) FOLLOWING A SINGLE INJECTION OF ERYTHROPOIETIN

Day	0	1	2	3	4	5	6	Day 4 Saline control
Early erythroid cells								
Central	7	37	58	1	11	2	5	6
Subcapsule	12	50	41	1	8	0	6	15
Late erythroid cells								
Central	0	0	215	390	24	0	0	0
Subcapsule	0	0	325	570	35	0	0	0
Reticulocytes	0	0	.3	1.9	1.3	4	0	0

noted, although no normoblasts were observed at this time. There was a striking increase in the number of later forms; i.e., normoblasts on the second day after injection. By the third day, a great abundance of late erythroid cells was present and the reticulocyte count in the peripheral blood had risen to its peak value. By the fourth day, splenic erythropoiesis had fallen precipitously while the peripheral reticulocyte count was only beginning to fall, and on the fifth day, splenic erythropoiesis had returned to its original inactive state, while the reticulocyte count was very low but had not yet returned to zero. This sequence, as followed in the subcapsular areas of the spleen and in the peripheral blood, is illustrated in Figure 2.

DISCUSSION

It is our belief that the polycythemic mouse is an ideal preparation for the study of many aspects of red cell formation. Here erythropoiesis is depressed by a "physiologic" mechanism, which acts neither as a toxic or nutritional insult to the marrow, nor as an impairment of the metabolic processes of the animal, but rather because the stimulus to erythropoietic activity, erythropoietin, presumably has been removed.[6,9] If this assumption is correct, then one would expect an orderly and predictable sequence of erythropoiesis to be initiated when this stimulus was again administered. The responses obtained can reasonably be attributed directly to the influences of erythropoietin: it is not necessary to make any assumptions or corrections accounting for endogenous erythropoiesis. Indeed, the simplicity and reproducibility of the results obtained suggest that these experiments may have widespread applications in areas not intimately related to erythropoiesis. Using the model employed here, many of the fundamental aspects of cell growth, differentiation and maturation can readily be investigated.

It is well known that the spleen is an erythropoietic organ in the mouse. The present ex-

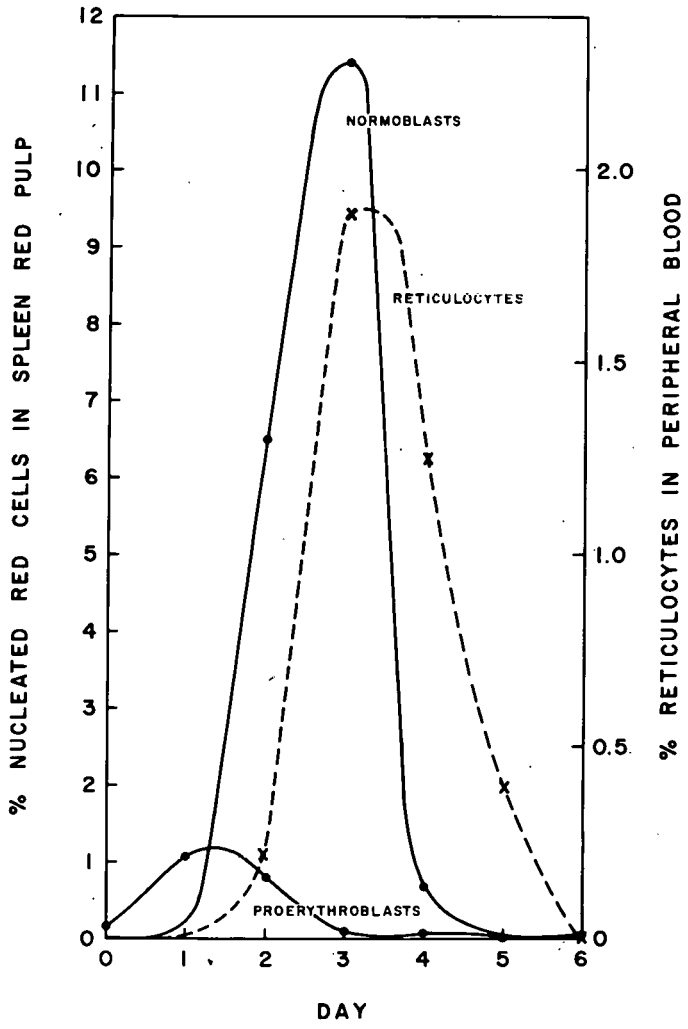

Figure 2. Erythropoietic response in spleen and peripheral blood following a single injection of erythropoietin in polycythemic mice.

periments, insofar as the histologic studies are concerned, have been designed and interpreted on the assumption that changes in erythropoiesis observed in the spleen are representative also of the state of erythropoietic activity in the marrow. Marrow and spleen specimens were examined and compared, though not quantitated, during preliminary studies, and no striking qualitative differences were observed. Spleen is preferred to marrow in these studies only because of the ease with which the spleen can be obtained and the larger amount of readily available hematopoietic tissue it yields.

From Figure 1 it can be seen that as small a dose as .75 units of erythropoietin elicits a reticulocyte response in polycythemic mice of average weight 23 g (32 units/Kg), while a single dose of 6 units (260 units/Kg) produces a maximum response. Although it is realized that marked species differences in responsiveness to erythropoietin may exist, these data are of interest because they indicate the limits within which adequate dosages for clinical testing may lie. It is thus not surprising that we have not yet observed significant response in patients given 600 units

daily (10 units/Kg) of the fractions used in this experiment,[8] since these data suggest this may well be below the minimum dose required to induce a response in a sensitive animal.

In this study, erythropoietic activity has been expressed in the unit as defined by Goldwasser and White.[7] Biological activity might also be defined as the basis of the smallest amount of material necessary to produce a maximum reticulocyte response in the polycythemic mouse 72 hours after injection. Future investigation will be necessary before the validity of the various bioassay procedures used in defining a unit of erythropoietin-stimulating activity can be estimated.

The histologic sequence of response to a single injection of erythropoietin is much as one would expect. An orderly wave of erythropoiesis is seen to pass through the spleen. Using the techniques employed in this study, it is possible to estimate the time intervals involved in the process. In polycythemic mice, even prior to stimulation, one can very occasionally find a cell which so closely resembles a proerythroblast (Table 2) that it cannot definitely be identified otherwise, and it has therefore been classified as an early cell in the erythroid series. In the subcapsular area where these cells occur in greater abundance than elsewhere in the spleen, they still account for less than 0.3 per cent of the nucleated cells of the red pulp. After a single injection of erythropoietin, proerythroblasts increase in numbers within 24 hours, presumably by multiplication of the few proerythroblasts present at the time of injection, by differentiation from primitive stem cells, and by subsequent division. By 72 hours, proerythroblasts are absent. This suggests that the duration of this phase is less than 72 hours. Normoblasts were not found in the 24-hour specimen, but were noted at the 48-hour interval. One would expect that some might be found in specimens examined between the 24- and 48-hour intervals. The peak normoblast concentration is noted at 72 hours and is followed by a precipitous decline, very few being present at 96 hours. This rapid decline is presumed to be the result of their maturation and release from the bone marrow as reticulocytes. The normoblast phase is quickly followed by the reticulocyte phase—a total of less than 4 days duration. Since it is believed reticulocytes do not divide, this is consistent with an average reticulocyte survival time of 2 days. These data clearly demonstrate that the total time from onset of differentiation to release of the young red cells is, except for a very few cells, less than 96 hours. After maturation of the reticulocytes, no evidences of the wave of erythropoiesis can be distinguished.

It was noticeable that there was generally a greater abundance of nucleated erythroid cells near the capsule than in the more central areas. Also at 24 hours, the proerythroblasts appeared to be arranged in clumps near the capsule, whereas this clumping tendency was not so evident in the central area. We have no information that might suggest any explanation of these conditions.

A question of fundamental interest to us has been the mechanism by which erythropoietin stimulates erythropoiesis. The early work of Jacobson and his associates[6] clearly indicates that erythropoietin is instrumental in inducing differentiation of primitive stem cells. Alpen and Cranmore[4] and Erslev[5] have more recently presented evidence suggesting that this is the only action of the hormone, although Stohlman[10] suggests that more than one mechanism may be involved. It is our opinion that the techniques employed in the present experiments may serve to elucidate this problem, and such investigations are contemplated in the near future.

It is of interest to consider this investigation as it relates to the observations of Linman, Bethell, and Long.[11] These investigators believe there are at least 2 erythropoietins, one of

which is water-soluble, probably protein in nature, and active in augmenting hemoglobin production, while the other is said to be lipid in nature and is thought to be the factor regulating cell division. They also believe that the latter factor, when acting alone, stimulates the production of cells deficient in hemoglobin. In the experiments reported herein, a single preparation, highly purified and glycoprotein in nature, and resembling the first substance described by Linman, Bethell, and Long, is capable of activating differentiation and subsequent maturation of red cell precursors from primitive mesenchyme. The standard assay of this material depends on Fe^{59} incorporation of developing erythrocytes of the starved rat.[8] Clearly, this one erythropoietic factor alone is sufficient to bring about both differentiation and maturation and no lipid fraction is required. If another factor is already present in abundance in the animal under study, it does not appear to induce differentiation or stimulate proliferation. Undoubtedly many nutritional factors are required for orderly and optimal erythropoiesis. However, our experiments suggest that one factor, glycoprotein in nature, is sufficient to induce differentiation from the reticulum. Once differentiation is initiated, orderly multiplication and maturation proceeds. The number of new cells produced, and the iron utilized, as measured by Fe^{59} incorporation,[8] are proportional to the amount of this factor administered. All the data we have accumulated to the present time are adequately explained by the existence of the single regulating factor, which we know as erythropoietin.

SUMMARY

The response to a purified preparation of erythropoietin as measured by counts of erythrocyte cells in the spleen, and of peripheral reticulocytes, was studied in the polycythemic mouse. Following a single injection of erythropoietin, an orderly wave of erythropoiesis was observed to sweep through the spleen. Graded responses were obtained for doses ranging between .75 and 6 units. Although the peak reticulocyte count persisted longer following large doses, no higher reticulocyte count was obtained in doses above 6 units. Implications of these observations are discussed.

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TRANSFUSION-INDUCED POLYCYTHEMIA AS A MODEL FOR STUDYING FACTORS INFLUENCING ERYTHROPOIESIS*

By

L. O. Jacobson, E. Goldwasser, and C. W. Gurney

INTRODUCTION

Our entry into the study of the control of erythropoiesis was originally related to our continuing interest in the problem of recovery from radiation injury, and we naturally investigated any possible means of stimulating the hematopoietic system. Our first problem was to design an assay system for the plasma erythropoietic factor(s) (hereafter referred to as erythropoietin) that required neither a vast amount of time or effort, nor a large volume of plasma or plasma extracts for a single assay.

Before 1955, assay for erythropoietin was confined largely to the study of the erythropoietic response of normal rodents and rabbits. A positive erythropoietic response to repeated plasma injections was considered to be demonstrated by an increase in reticulocyte, hemoglobin, and erythrocyte values of the peripheral blood, an increase of the red cell mass, or histologic evidence of bone marrow hyperplasia. Simultaneous use of all of these parameters was usually not employed by all investigators.

This general approach to assay was originally introduced by Carnot and Deflandre,[1] and with certain modifications continues to be useful. If these parameters are employed, erythropoietin titer of the sample being tested must be high, in order to obtain unequivocal differences from control preparations. In 1955, we demonstrated that injection of active plasma into normal rats would elevate the rate of iron^{59} incorporation into the circulating red cells from about 35 per cent in the controls to from 45 to 50 per cent in experimental animals. As a regular assay procedure, we administered three 2-ml plasma injections on 3 successive days; on the third day, a few hours after the last plasma injection, 1 μc of Fe^{59} citrate was injected intravenously, and 18 to 20 hours later, a 1-ml sample of blood was withdrawn and counted in a well-type crystal scintillation counter. Results were expressed as per cent of injected counts per minute incorporated into the total red cell mass.[2]

The influence of hypophysectomy on erythropoiesis. It had been shown that the total red cell mass of the rat slowly fell to approximately one-half its original value after hypophysectomy.[3,4] In a study designed to determine the mechanism of this reduction in erythropoiesis, we found that within 2 weeks after hypophysectomy of the adult rat, the reticulocyte value and the Fe^{59} red cell incorporation of the peripheral blood were reduced by a factor of nearly ten, and remained thus depressed for several weeks. These hypophysectomized rats with a markedly reduced rate of erythropoiesis showed increased responsiveness to the injection of exogenous erythropoietin. Their reticulocyte response and Fe^{59} red cell incorporation when injected with

*This report forms part of a paper presented at the Proceedings of the Ciba Foundation, London, England, February 1960, to be published in book form by Messrs. J. & A. Churchill, Ltd., England and distributed by Messrs. Little Brown & Co., U.S.A.

anemic plasma was several-fold greater than that observed after normal plasma or saline injection (Figure 1).

This led us to inquire whether the hypophysis produced a substance that acted directly on erythropoiesis. We were able to demonstrate that the plasma of hypophysectomized rats made anemic by bleeding after hypophysectomy, contained an increased titer of erythropoietic activity comparable to that observed in normal rats subjected to phlebotomy.[5] Gordon had previously reported that a reticulocytosis would appear in hypophysectomized rats in response to

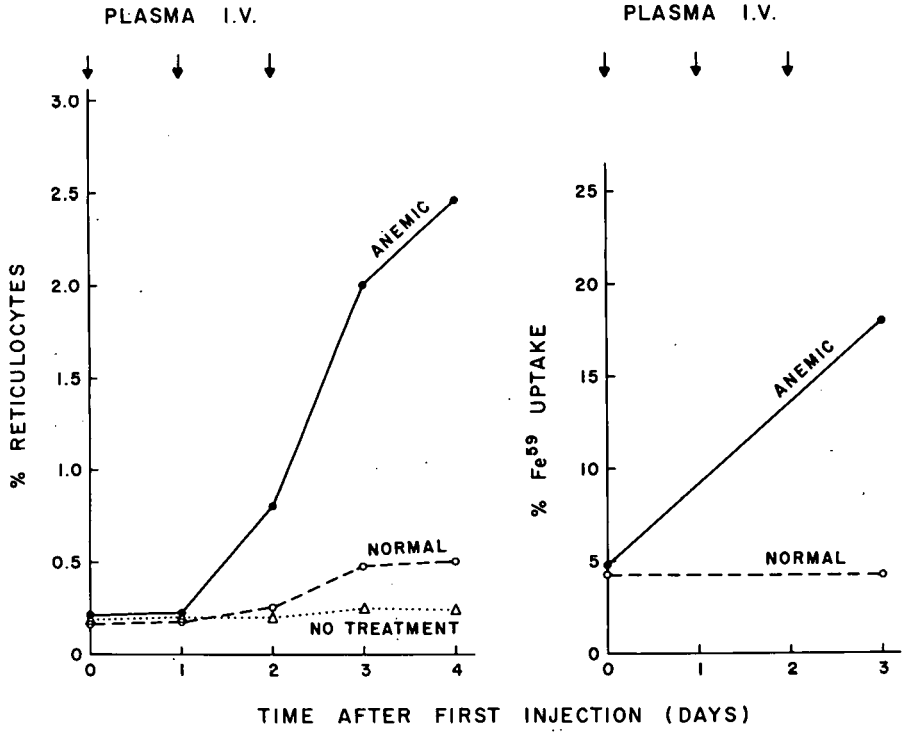

Figure 1. Assay of anemic plasma in hypophysectomized rats.

hemorrhage.[6] These results in our own laboratory and those of Dr. Gordon, largely eliminated the hypophysis as a source of an erythropoietic substance with a direct action on erythropoiesis; a theory which had previously been advanced and subsequently withdrawn by others.[4]

In an attempt to explain the rapid reduction in erythropoiesis and the marked responsiveness of the hypophysectomized rat to exogenous erythropoietin, we postulated that upon loss of the pituitary hormones after hypophysectomy, the metabolism of the animal fell rather abruptly to a lower level and thus the over-all metabolic oxygen requirement was reduced. The red cell mass, on the other hand, fell slowly after hypophysectomy; and thus before the red cell mass reached its new, and lower, equilibrium, the oxygen-carrying capacity or supply was greater than required. Consequently, production of erythropoietin fell to a much lower level than normal and the rate of erythropoiesis was reduced accordingly. This postulate was strengthened by the observed responsiveness of the hypophysectomized animal to exogenous erythropoietin and suggested that erythropoietin production was controlled by the relationship between the supply of oxygen available to the tissues of the body and the tissue demand for oxygen. We were thus led to consider other experimental conditions in animals in which either the oxygen de-

mand was increased without a change in the oxygen supply, or in which the oxygen demand was unchanged but the oxygen supply was increased. Accordingly, we studied hyperoxia,[7] acute starvation,[8] and hypertransfusion[9] and found that in each of these conditions erythropoiesis was rapidly and drastically reduced, while responsiveness to exogenous erythropoietin, as measured by reticulocyte increase in peripheral blood or increased red cell incorporation of Fe^{59}, was markedly increased. These observations provided illuminating information on the mechanism of the over-all control of erythropoiesis, as well as several relatively simple assay methods for erythropoietin. The most reliable of these assay methods have proved to be those using the acutely-starved rat and the transfusion-induced polycythemic mouse. Our original hypothesis on the mechanism of suppression of erythropoiesis in this condition[5] is supported by the work of Crafts and Meinecke[10] who showed that the oxygen requirement of the hypophysectomized rat is actually reduced.

Preparation and methods of use of the polycythemic mouse for erythropoietin assay. As stated above, the most sensitive test for erythropoietic activity of which we are aware is the transfusion-induced polycythemic mouse. The strain of mice used, their weight or age are not important factors, but we regularly use the female CF No. 1 mouse, age about 10 weeks, weighing 20 to 25 g. The mouse is routinely prepared for assay by the intraperitoneal injection of 0.5 ml of washed, packed, homologous red cells on 3 consecutive days, and repeated on day 5. At the end of this time, the hematocrits are in the neighborhood of 75 per cent, the reticulocytes are at 0.00 per cent, and all evidence of active erythropoiesis in the bone marrow and spleen has ceased. This state can be maintained indefinitely by giving additional red cell transfusions. Beginning on the 6th day, the plasma or other preparation for erythropoietin assay is given in 0.5 ml amounts subcutaneously or intravenously for 4 consecutive days. The reticulocytes of the peripheral blood are sampled daily. Figure 2 shows the response to an assay of a purified preparation of anemic sheep plasma. By 2 days after the initial injection of exogenous erythropoietin, erythropoiesis is already active in the marrow and spleen, even though the reticulocytes of the peripheral blood have only reached about 0.2 per cent: the daily injection of the same dose of erythropoietin maintains a relatively constant reticulocyte level in the peripheral blood. Upon cessation of erythropoietin injections, the reticulocyte value returns to zero in 3 to 4 days.

Normal plasma and saline are regularly used as controls. Occasionally after four 0.5-ml injections of normal plasma, the reticulocytes reach 0.1 per cent. Saline produces no response. When normal plasma is continued daily for 6 to 7 or more days, a slight increase in reticulocyte response of 0.1 to 0.3 per cent is usually observed. This strongly suggests that sufficient erythropoietin is present in normal plasma to produce a reticulocyte response in this sensitive assay system.

Another method of assay using the transfusion-induced polycythemic mouse is to begin red cell transfusion and the injection of the material for erythropoietin assay on the same day.[9] Here again the response to plasma with an active erythropoietin titer levels off at about 2.5 per cent by 6 to 8 days, when normal plasma is at about the 0.2 per cent level and the saline controls have fallen to zero. Figure 3 shows the erythropoietic response of the spleen at 8 days.

Using either of the techniques described above, one can substitute Fe^{59} red cell incorporation as the indicator of increased erythropoietic activity. The method is essentially that previously described in the starved rat assays.[2]

It is of interest to note that Dahl, Blaisdell and Beutler[11] working in our laboratories, found

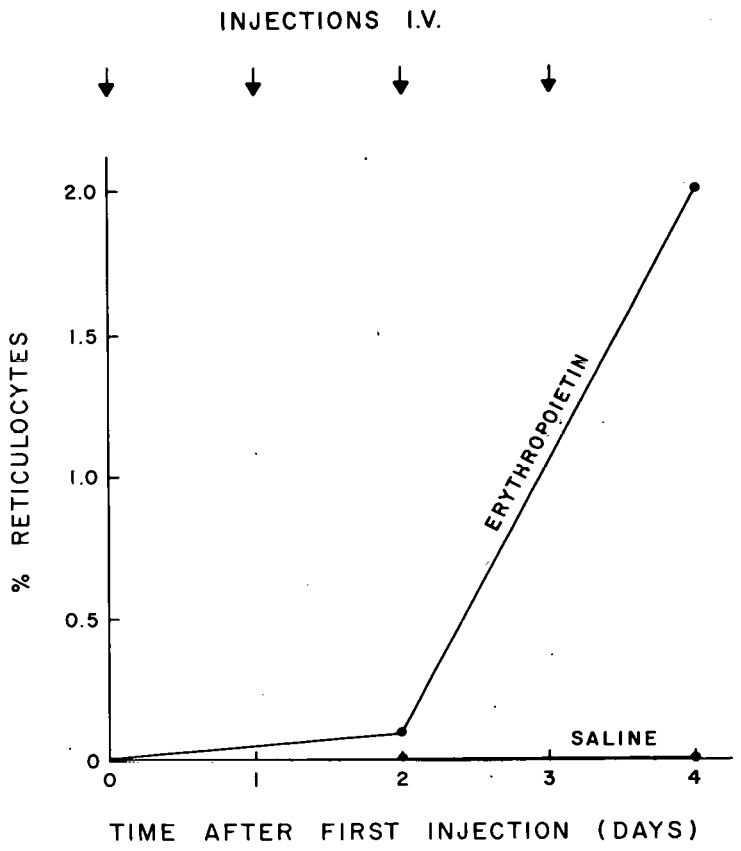

Figure 2. Reticulocyte response of polycythemic mice to purified sheep erythropoietin.

that hypertransfusion of the rat almost invariably produced gastric ulceration and hemorrhage. In our original studies on hypertransfusion as a means of suppressing erythropoiesis, we found it difficult to maintain the hematocrit at circa 70 to 75 per cent in the rat, whereas the mouse presented no special problem, and in no mice have we observed gastric or other gastrointestinal ulceration or hemorrhage. Dahl's observations thus eliminate the hypertransfused rat for erythropoietin assay. We have no explanation of this species difference in response to hypertransfusion.

Some studies using the transfusion-induced polycythemic mouse.

A. The effect of continued polycythemia on the capacity to reinitiate erythropoiesis.

One can easily prolong polycythemia in the mouse by giving repeated red cell transfusions as needed to maintain the hematocrit between 70 and 75 per cent. In this way erythropoiesis can be suppressed completely for an indefinite time, normally appearing again when the hematocrit is allowed to fall to or below the normal range.

B. Transplantation of polycythemic marrow

Transplantations of marrow and spleen cells from polycythemic mouse donors to lethally-irradiated recipients of the same strain showed that erythropoiesis began in the recipient at the same time after transplantation as in irradiated recipients that had received normal bone marrow or spleen. In point of fact, there was no evidence to sug-

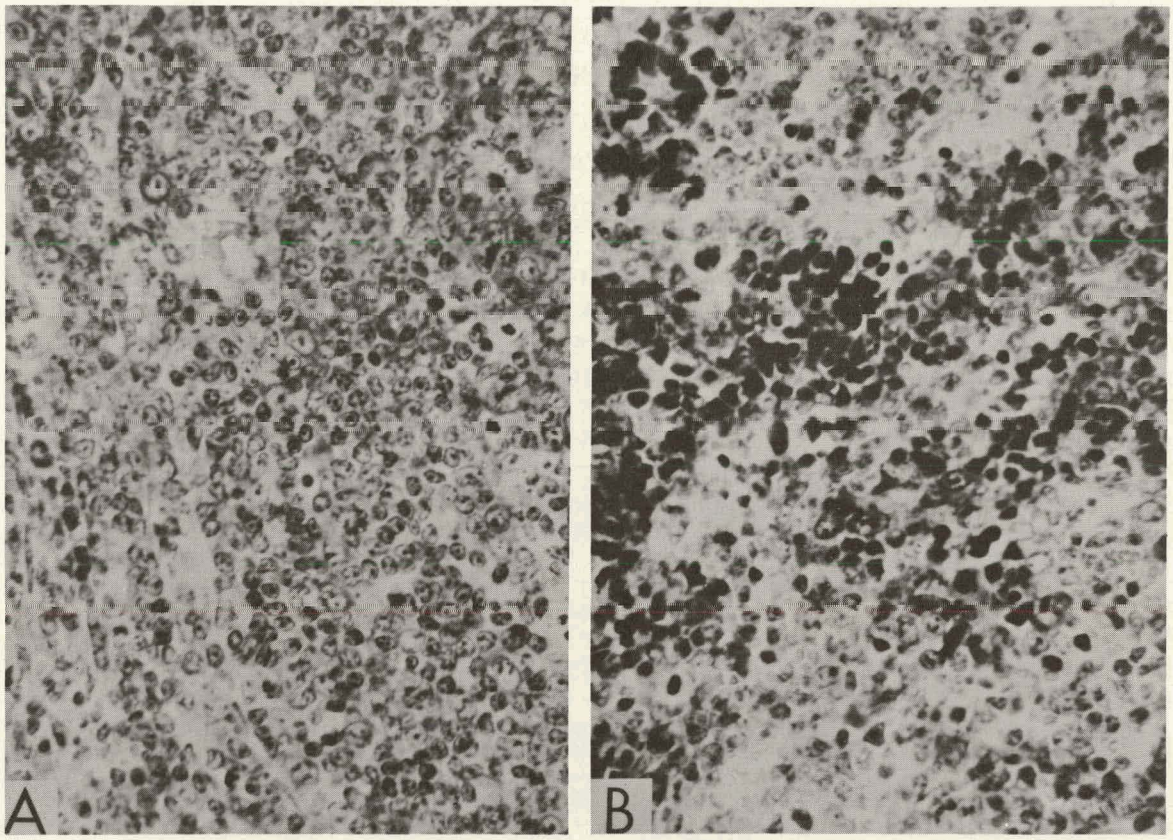

Figure 3. Histologic effect of four 0.5-ml injections of rat plasma on splenic erythropoiesis in mice with a transfusion-induced polycythemia. Tissues taken 8 days after first injection of plasma. A. Spleen of normal plasma-injected mouse. Red pulp is devoid of erythropoietic foci. B. Spleen of mouse injected with plasma from anemic rat. Marked erythropoietic activity in the red pulp.

gest that such marrow transplants had any appreciable, differential effect, beneficial or the reverse, on growth of any of the cell types of the transplanted tissue (Table 1).

Although these findings are not unexpected, they serve to illustrate that the red cell precursor can apparently remain dormant for long periods of time or that erythropoiesis can originate at any time from the mother reticular tissue or multipotential stem cells, in response to a suitable stimulus.

C. The effect of an atmosphere containing 10 per cent oxygen on the erythropoietic response of polycythemic mice.

The exposure of normal mice to an atmosphere having a lower than normal concentration of O_2 produces an increase in erythropoietin titer and an erythropoietic response comparable to that observed in rats similarly exposed. Stohlman and Brecher[12] have shown that exposure to a simulated high altitude (hypoxic anoxia) for 8 to 24 hours increases the plasma erythropoietin titer of rats.

In Table 2, the effects of exposures of groups of polycythemic mice to an atmosphere of 10 per cent oxygen for 0, 8, and 12 hours are compared. It is evident that no significant reticulocyte response was observed. The most obvious explanation is that

Table 1

EFFECT OF BONE MARROW FROM NORMAL AND TRANSFUSION-INDUCED POLYCYTHEMIC MICE ON THE LEUCOCYTE, HEMATOCRIT, AND RETICULOCYTE VALUES OF CF NO. 1 FEMALE MICE EXPOSED TO 750 r TOTAL-BODY X-RADIATION

Time in days	0	3	7	10	14	18	21	24	28
Leucocytes (cu. mm.)									
Controls (untreated)	11600	10200	12100	10800	8750	12000	18450	9750	12800
Normal bone marrow	15500	480	1650	3450	6750	8150	7900	8100	8200
Polycythemic bone marrow	15500	435	2310	3200	3350	3900	7400	6800	6150
X-ray only	15300	375	430	650	dead	-	-	-	-
Hematocrit (per cent)									
Controls (untreated)	52	50	52	52	50	53	44	50	52
Normal bone marrow	51	51	45	38	39	41	37	34	36
Polycythemic bone marrow	51	51	44	35	36	40	42	39	38
X-ray only	51	50	41	21	dead	-	-	-	-
Reticulocytes (per cent)									
Controls (untreated)	3.0	3.9	5.1	5.9	4.9	4.2	6.7	5.8	4.0
Normal bone marrow	3.1	0.0	3.8	9.8	13.7	10.5	10.1	6.3	8.7
Polycythemic bone marrow	3.7	0.0	2.3	9.0	16.3	12.6	8.3	7.0	6.0
X-ray only	3.8	0.0	0.01	0.05	dead	-	-	-	-

the increased red cell mass effectively compensates for this degree of anoxia and therefore no stimulus for erythropoietin production occurs. Another possibility is that the polycythemic state renders the site of formation insensitive to the anoxia. If this is so, then the condition must be reversible since upon rapid reduction of the hematocrit to below the normal value, erythropoiesis invariably recurs in the expected time of 3 to 4 days.

D. The effect of cobaltous chloride on erythropoiesis in the polycythemic mouse.

Goldwasser, Jacobson, Fried and Plzak[13] had established the fact that the injection of 5.0 micromoles cobaltous chloride would produce a minimal erythropoietic response in the starved rat. Assuming the starved rat and the polycythemic mouse to be equally responsive to cobalt ion, one might expect the polycythemic mouse to show an erythropoietic response to a daily injection of 0.5 micromoles or less. We felt that a determination of this might reveal a minimal reproducible value that would serve as a

Table 2

EFFECT OF EXPOSURE TO 10 PER CENT 0_2 FOR 8 AND 12 HOURS ON THE RETICULOCYTE VALUES OF MICE WITH TRANSFUSION-INDUCED POLYCYTHEMIA

No exposure to 10 per cent 0_2						8-hr exposure to 10 per cent 0_2						12-hr exposure to 10 per cent 0_2					
Mouse No.	0 hr	2 d.	3 d.	4 d.	5 d.	Mouse No.	0 hr	2 d.	3 d.	4 d.	5 d.	Mouse No.	0 hr	2 d.	3 d.	4 d.	5 d.
Reticulocyte values																	
1	0	0	0	0	0.01	11	0	0	0	0	0.01	21	0	0.01	0.02	0.1	0.01
2	0	0	0	0	0	12	0	0	0	0.01	0	22	0	0	0	0	0
3	0	0	0	0	0	13	0	0	0.01	0	0	23	0	0	0	0	0
4	0.01	0	0	0	0	14	0	0	0	0	0	24	0	0.01	0.01	0	0
5	0	0	0	0	0	15	0	0	0	0.15	0.01	25	0.01	0	0	0	0
6	0	0	0	0	0	16	0	0	0.03	0	0	26	0	0	0.01	0	0
7	0	0	0	0	0	17	0	0	0	0	0	27	0.01	0	0	0	0
8	0.01	0	0	0	0	18	0	0	0	0	0	28	0	0	0	0	0
9	0	0	0	0	0	19	0.01	0	0	0	0	29	0	0	0.01	0	0
10	0	0	0	0	0	20	0.01	0	0.02	0.1	0.01	30	0	0	0	0	0
Hematocrit values																	
1	78	70	69	63	64	11	77	69	65	64	62	21	73	69	62	57	57
2	77	72	70	59	61	12	75	63	63	66	62	22	74	66	63	55	50
3	79	70	68	61	63	13	72	68	61	61	46	23	70	68	64	64	61
4	72	68	65	57	61	14	76	73	72	70	66	24	74	70	65	65	64
5	74	72	65	63	64	15	73	63	60	55	55	25	74	58	64	65	61
6	76	72	67	64	55	16	74	68	70	54	59	26	68	71	68	55	61
7	75	76	71	65	61	17	77	73	69	69	68	27	75	68	70	61	62
8	78	72	63	62	62	18	78	72	67	65	71	28	78	73	70	62	66
9	76	74	72	66	68	19	81	75	75	71	70	29	80	72	67	67	65
10	75	73	69	69	64	20	80	63	58	61	55	30	78	71	72	67	72

basis in establishing a unit of erythropoietic activity.

As shown in Table 3, polycythemic mice were divided into groups and given daily intraperitoneal injections of cobaltous chloride ranging from 0.2 to 4.5 micromoles. Reticulocyte counts were made daily, but no response was observed for these quantities. In another experiment reported by Krantz, Goldwasser and Jacobson,[14] a dose of 5.3 micromoles for 3 days increased the reticulocyte count from 0.0 to 0.1 to 0.4 per cent. Administration of this dose for longer periods resulted in death of all the animals. In yet another experiment,[15] we administered a dose of 5 micromoles of cobaltous chloride subcutaneously to a group of normal mice 8 or 9 hours before we collected the blood.

Table 3

EFFECT OF INTRAPERITONEAL INJECTIONS OF COBALTOUS CHLORIDE ON THE RETICULOCYTE RESPONSE OF CF NO. 1 FEMALE MICE WITH A TRANSFUSION-INDUCED POLYCYTHEMIA

Number of mice used	Amount of $CoCl_2$ (μM)	Days of administration	Reticulocyte response
8	0.2	39	0
10	1.0	24	0
10	1.5	21	0
10	4.5	8	0

The plasma from these animals, which was essentially devoid of free Co^{++} ion, produced an erythropoietic response (reticulocytes) in polycythemic mice comparable to that observed from anemic plasma of known potency (Table 4). Furthermore, it was demonstrated that plasma harvested from rats injected with 250 micromoles of cobaltous chloride produces an erythropoietic response in polycythemic mice. This fivefold difference in the erythropoietic response of starved rats and polycythemic mice to cobalt ion is striking and deserves further investigation. It is possible that the polycythemic state renders the site of erythropoietin production relatively insensitive to cobalt

Table 4

EFFECT OF 4 SUBCUTANEOUS INJECTIONS OF PLASMA FROM COBALT-INJECTED MICE AS COMPARED WITH THAT OF NORMAL MOUSE PLASMA WITH COBALT ADDED, ON THE RETICULOCYTES OF MICE WITH A TRANSFUSION-INDUCED POLYCYTHEMIA

	Hematocrit (per cent)		Reticulocytes (per cent)	
Time in days after first plasma injection	0	4	0	4
Normal plasma with cobalt	76	68	0	0
Plasma from cobalt-injected mice	76	64	0	2.5

ion. On the other hand, we have learned that it is difficult if not impossible to achieve a significant polycythemia in mice by cobalt ion administration, in doses up to 4.5 micromoles for 69 days. This may be due in part to the toxicity of cobalt in mice.

The response of polycythemic irradiated mice to rat bone marrow.* When polycythemic mice are subjected to a lethal dose of X radiation, some will survive if injected with heterologous bone marrow. This procedure provides a model for the study of the effect of suppression of erythropoiesis on the viability of a heterologous transplant, and of the effect that differentially delaying the growth of a part of a transplant has on the eventual recovery of the hematopoietic system of the irradiated recipient.

Mice (CF No. 1 females) were transfused with washed red blood cells of the same strain. On the 7th day after beginning the transfusions, when the reticulocyte number had fallen to zero, the mice were exposed to 800 r whole-body X radiation and 100 million rat bone marrow cells were given intravenously. The red cell transfusions were continued to maintain the hematocrit at circa 70 per cent to prevent red cell formation, either by spontaneous regeneration in the tissue of the recipient mouse or from the transplant of rat marrow. The hematocrit could be reduced at will by bleeding the animal or by allowing natural red cell decay to occur. This allowed us to observe the course of red cell formation as the natural stimulus for erythropoiesis was restored. The results are based on studies in which more than 100 mice were used.

The phenomena relative to erythropoiesis which one might consider operative as the hematocrit falls are: first, that no erythropoiesis occurs; second, that only rat erythrocytes are produced; third, that both rat and mouse erythrocytes are produced; and fourth, that only mouse erythrocytes are produced.

The responses observed are briefly as follows:

Figure 4 represents a polycythemic mouse that received rat bone marrow after exposure to 800 r total-body X radiation. The hematocrit was maintained above 65 per cent for 18 to 20 days. With the fall in hematocrit, as illustrated, the number of reticulocytes rose. As far as could be determined by alkaline phosphatase staining, granulopoiesis of rat origin had already begun by 8 days after the transplantation. This condition still obtained at 42 days. It is obvious that suppression of erythropoiesis for 18 days after the rat-cell transplant did not interfere with the formation of rat red cells when the stimulus for erythropoiesis was applied. This suggests that specific rat red cell precursors remained dormant for 18 days, or that the production of red cells originated under these circumstances from multipotential rat cells present in the marrow.

Figure 5 reveals another type of response. In this instance, no additional red cell transfusions were given after irradiation and rat bone marrow transplantation. The hematocrit began to fall as expected and hematopoiesis was observed by the 8th day postirradiation. Evidence for production of red cells and white cells of both rat and mouse origin was present. It is of interest but not unexpected that the formation of all rat cells had ceased some time after the 33rd day. Before death this animal presented the usual clinical appearance so characteristic of "late immune" disease, and death may be attributed to the crisis of this syndrome.

Figure 6 illustrates another variation in response. As the hematocrit fell only mouse red

*This section forms part of a paper entitled "Some observations on the mechanism of recovery from radiation injury" by L. O. Jacobson, E. K. Marks, E. O. Gaston, and E. L. Simmons, presented at the 9th International Congress of Radiology, held at Munich, West Germany, July 1959 and published in the Proceedings of the Congress.

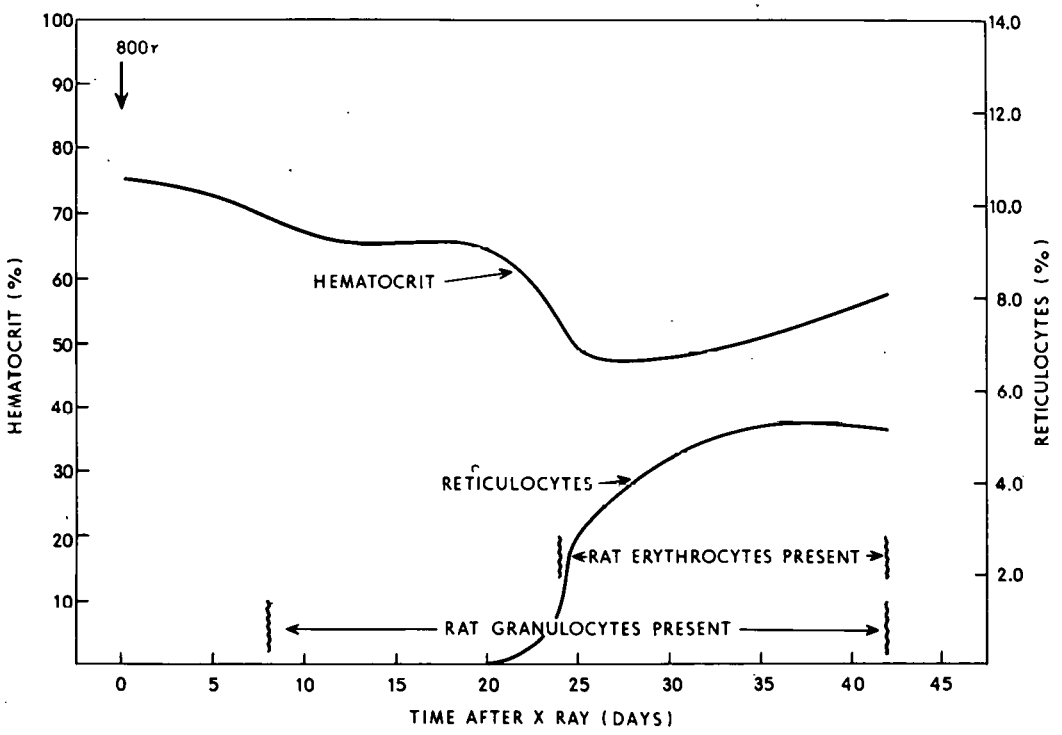

Figure 4. Response of polycythemic X-irradiated mouse to rat bone marrow (living 50 days). This mouse produced both rat granulocytes and rat erythrocytes.

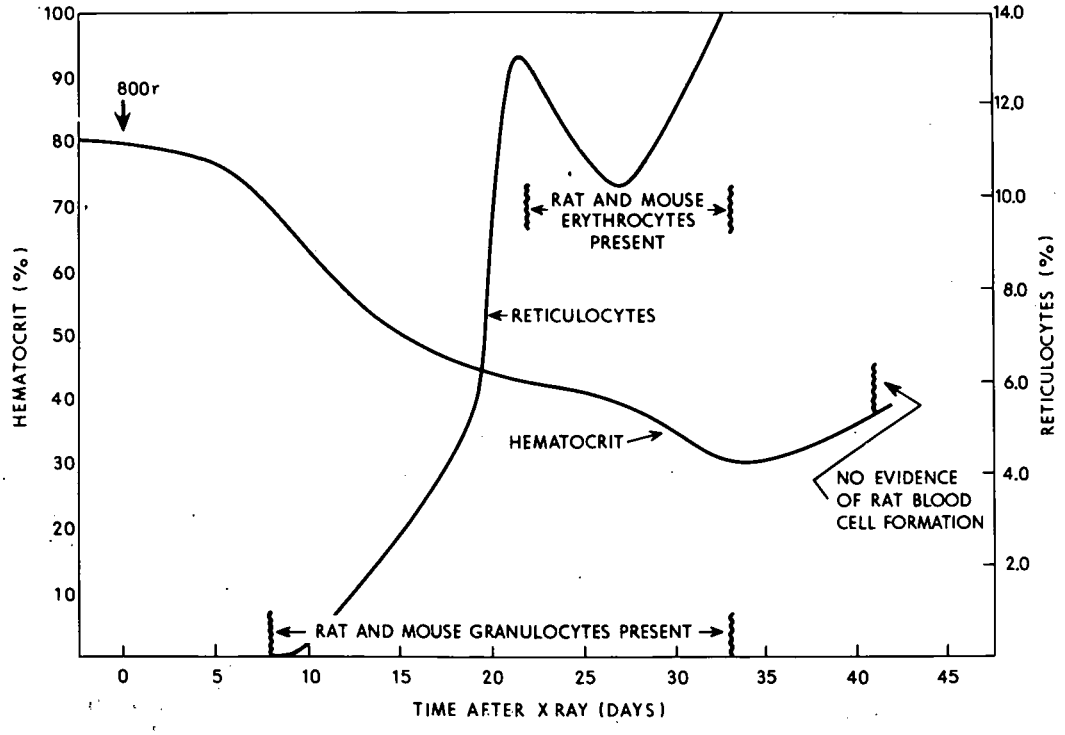

Figure 5. Response of polycythemic X-irradiated mouse to rat bone marrow (dead on day 44). In this mouse, both rat and mouse granulocytes and erythrocytes were produced.

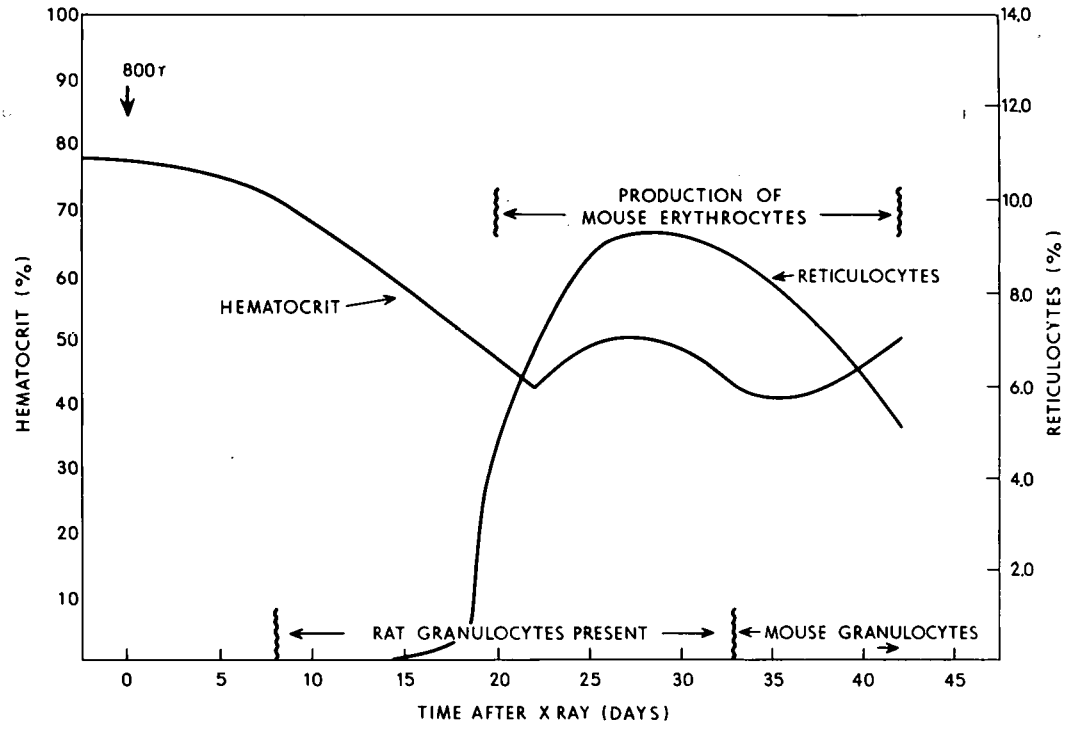

Figure 6. Response of polycythemic X-irradiated mouse to rat bone marrow (living at 42 days). This mouse produced mouse erythrocytes and rat granulocytes. It was sacrificed on day 163 postirradiation.

cells were formed, although rat granulocytes began to be produced at the expected time after the transplant. Rat granulocytes apparently disappeared between the 33rd and the 42nd day, and only mouse granulocytes were present. In this instance, it is possible that during the period of suppression of erythropoiesis the mouse bone marrow began to resume its normal production of mouse red cells. It is interesting that mouse granulocytes were not detected until more than 10 days after production of the mouse red cells had been restored. One might speculate that in this instance the recipient destroyed the graft. This animal was sacrificed 163 days after irradiation.

Figure 7 illustrates a response that one might expect to encounter occasionally at this irradiation dose level. Mouse erythrocytes and granulocytes were produced in the recipient. Cells of rat origin were never detected. For some reason, possibly non-specific irritation, regeneration of the mouse hematopoietic tissue occurred and the mouse survived on this basis. Possibly the effective regeneration of mouse hematopoiesis provided an immunological barrier to the growth of the rat graft.

One may tentatively conclude that 1) "physiologic" suppression of erythropoiesis in polycythemic mice given lethal X radiation and a rat bone marrow transplant does not interfere with the "take" of the transplant since rat granulocytes generally appear at the expected time, and 2) although erythropoiesis may be suppressed for at least 20 days, upon release of this suppression the production of rat red cells only, of mouse red cells only, or of both, may begin. The data suggest that the heterologous transplant contains dormant erythroblasts capable of being activated, or that erythropoiesis may arise under these circumstances from a multipotential cell in the rat marrow transplant.

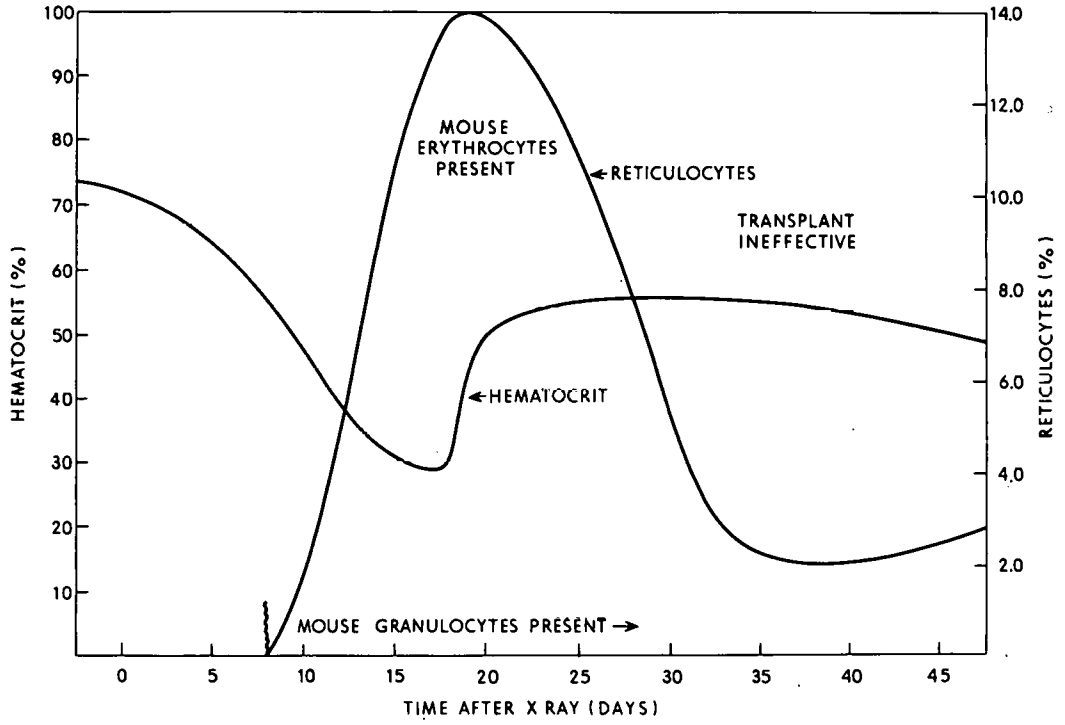

Figure 7. Response of polycythemic X-irradiated mouse to rat bone marrow (living at 55 days). No evidence of a transplant was found in this animal.

Establishment of a unit of erythropoietin. The variability in the erythropoietic potency of purified fractions prepared from the same batch of anemic plasma but using different methods of extraction and purification is widely recognized. In addition, a marked variability in the loss of erythropoietic activity may and usually does occur even when the same batch of anemic plasma is processed by the same chemical procedure at different times.

These facts necessitate the arbitrary establishment of a unit of erythropoietin in order that various end products of plasma processing may be compared. The work of Goldwasser, Jacobson, Fried, and Plzak,[13] established the fact that cobalt ion produced an erythropoietic response in animals by virtue of the production of erythropoietin. On the basis of this work, Goldwasser and White[16] studied the effect of single 5 and 10-micromole dosages of cobaltous chloride on the Fe^{59} red cell incorporation of the starved rat and observed responses substantially greater than the saline control. They established, in addition, that purified fractions from anemic sheep plasma gave slopes comparable to that of cobalt in the same response range. Cobalt therefore was accepted as the primary standard for low-level responses with the 5 micromole response designated as one unit.[17] At the same time, it is acknowledged that the relationship between the responses to cobalt and erythropoietin may not hold true at higher levels or with different injection schedules.

Site of action of erythropoietin. The difficulty universally encountered in classifying the precursor cells in the blood-forming tissue remains with us. Nevertheless, progress has been made in delineating the site of action of the erythropoietic hormone. Erslev[18] and Alpen and Cranmore[19] have, on the basis of distinctly different approaches, concluded that erythropoietin acts on the

"stem cells" of the marrow, producing differentiation which culminates in the well-known red cell maturation series. In studying the problem in our laboratory, Filmanowicz and Gurney[20] have taken advantage of the physiologic suppression of erythropoiesis which occurs in the transfusion-induced polycythemic mouse. In this preparation, which gives no evidence of active erythropoiesis as judged by the absence of reticulocytes in the peripheral blood and no activity in the erythroblast series of the blood-forming tissue, the response to purified erythropoietin previously standardized in cobalt units can be studied after single and repeated injections. The erythroblastic response of the hematopoietic tissue and the reticulocyte response observed in the peripheral blood can be studied simultaneously. A single injection of purified erythropoietin produces an orderly differentiation and maturation of erythrocyte precursors terminating in a peak reticulocytosis at 3 days. The reticulocytes have fallen again to zero by the 5th day after the single injection.

SUMMARY AND CONCLUSIONS

The origin of some current procedures for assay of erythropoietin as they developed in our laboratories is described. Those assay systems that utilize Fe^{59} red cell incorporation or reticulocytes as a measure of the erythropoietic response include the normal rat, the hypophysectomized rat, the starved rat and the transfusion-induced polycythemic mouse. The responsiveness of the last 3 subjects to erythropoietin injection is reflected in the relative or absolute polycythemia so induced. We have postulated that these artificial states reduce erythropoietin production by virtue of a change in the relationship of tissue oxygen demand to the oxygen supply available. The assay procedure using the starved rat is technically the simplest and least time-consuming; that utilizing the transfusion-induced polycythemic mouse most nearly represents a physiologic suppression of erythropoiesis, and is the most sensitive and reproducible. In addition to its usefulness for purposes of erythropoietin assay, the polycythemic mouse lends itself admirably to many physiologic studies of erythropoiesis.

In the polycythemic mouse, the entire blood-forming tissue remains apparently devoid of erythropoietic activity, or this function is dormant until exogenous erythropoietin is administered, or endogenous erythropoietin production is restored either by reducing the hematocrit to its normal range, or by the administration of a stimulus such as cobalt ion. The stimulus may act upon any one of a number of cells, including the dormant primitive erythroblasts, the red cell precursors, and the multipotential cells in the bone marrow.

The bone marrow of these polycythemic mice, which is apparently dormant or inactive insofar as erythropoiesis is concerned, retains the capacity to initiate erythropoiesis equally as effectively as normal marrow, when transplanted into isologous or homologous supralethally-irradiated mice. Heterologous (rat) marrow may be transplanted to the polycythemic supralethally-irradiated mouse and suppression of erythropoiesis maintained by postirradiation transfusion. Such suppression does not interfere with the growth of other transplanted cell types, and the mouse survives. On reduction of the hematocrit or red cell mass to, or below, the normal range, rat red cell precursors multiply and repopulate the mouse hematopoietic tissue. These studies suggest that the isologous, homologous or heterologous transplant contains dormant erythroblasts capable of being activated or that erythropoiesis may arise under these circumstances from multipotential cells in the transplant.

An arbitrary definition of one unit of erythropoietin has been formulated. As a primary stand-

ard in the low threshold response range, one unit is defined as the erythropoietic response of the starved rat tissue to an injection of 5 micromoles of cobaltous chloride.

Using the polycythemic mouse as a subject, the site of action of erythropoietin has been explored and appears to involve principally those undifferentiated precursors which may be proerythroblasts or even still more primitive cells.

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PURIFICATION OF SHEEP ERYTHROPOIETIN*

By

W. F. White† and E. Goldwasser

In a previous report[1] we outlined a three step method for the large scale production of a concentrated erythropoietin preparation derived from anemic sheep plasma. While the material (called by us Step 3 erythropoietin), satisfied several criteria for homogeneity, it soon became evident that the active hormone was in fact only a minor constituent, and that the properties of Step 3 erythropoietin that we described were those of the major contaminant. This report is a description of our efforts in further purification of the hormone.

Before the results of a purification procedure can be expressed in quantitative terms, it is necessary to define a unit of erythropoietic activity. We have formulated such a definition based upon the fasted rat assay method for erythropoietin.[2] This method is as follows:

Male Sprague Dawley rats, 125-170 g in weight, are deprived of food 20 to 30 hours before the start of the assay. Two ml of test substance are injected intravenously on two consecutive days; on the third day 1 μc of Fe^{59} as ferric citrate is injected intravenously, and 16 to 18 hours later, a 1-ml sample of blood is withdrawn by cardiac puncture and counted in a well-type scintillation counter. Samples are assayed in 5 rats. Results are expressed as per cent of total injected counts per minute incorporated into the total circulating volume of blood which had been previously determined in similar rats by the Cr^{51} method of blood volume measurement, and found to be approximately 5 per cent of the body weight. Control rats, injected with saline, show an incorporation of 3.5 to 5.5 per cent. One unit of activity is equal to that increase in response (over control level) seen in rats tested by the above method when 5 micromoles (2.5 micromoles each day) are administered subcutaneously.

Further purification of Step 3 material: Step 4. Step 3 erythropoietin was chromatographed on the carboxylic resin XE-97 by the method of Schmid et al.[3] which easily separates α-1 glycoproteins from α-2 glycoproteins. In this method the α-1 glycoproteins are not retained by the column at pH 5.2 in 0.05 sodium citrate buffer while the α-2 glycoproteins are retained and can be eluted by the application of 0.10 M sodium citrate buffer at pH 5.72. Judged by Schmid's results, our previous experiments with XE-97 resin suggested that erythropoietin would behave as an α_2-glycoprotein. Accordingly, Step 3 material was applied to an XE-97 column under the conditions Schmid described.

As shown in Figure 1, a large peak of optical density (A) passed directly through the column in the pH 5.2 buffer, while a much smaller peak (B) was eluted by the pH 5.72 buffer. On analysis, peak (A) accounted for more than 90 per cent of the optical density of the initial sample, and little or no erythropoietic activity, while peak (B) showed about 3 per cent of the original optical density and most of the activity. This separation has been repeated many times and is referred

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*This report with some modifications forms part of a larger paper to be published in Recent Progress in Hormone Research XVI, 1959 (in press).

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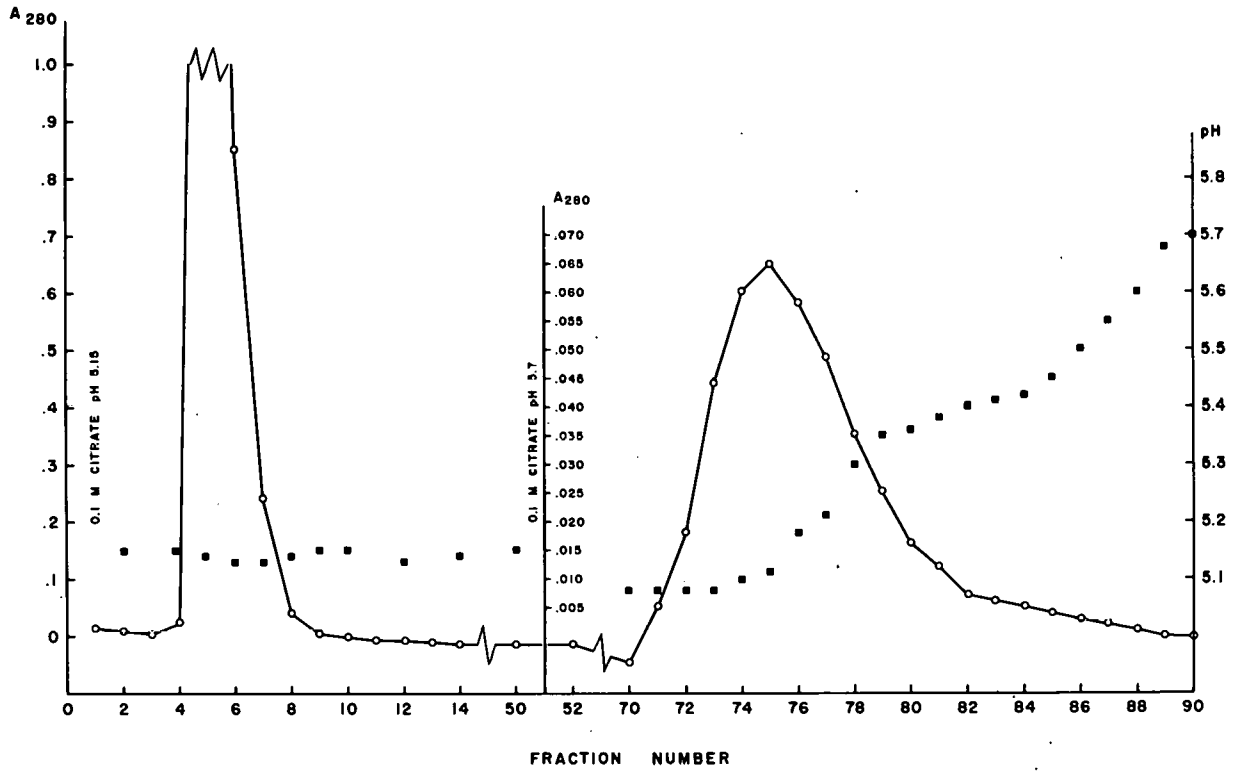


Figure 1. Chromatography of Step 3 erythropoietin on XE-97 resin (Step 4). Size of column: 30 cm high x 0.9 cm diam. Twenty mg sample in 5 ml buffer. Vol. fractions = 3 ml. Rate = 0.5 ml/min. Recoveries of optical density: fraction A, 87%, fraction B, 5%. Recoveries of biological activity: fraction A, none; fraction B, 75%.

to as Step 4, with the respective fractions retaining the designations A and B as shown in the figure.

The early Step 4 fractions were assayed immediately after dialysis without reduction to the dry state. Essentially all of the original activity was recovered in Fraction B and estimates of the potency of this fraction, based on the Lowry protein determinations, were of the order of 40 to 100 units per mg. However, lyophilization of the thoroughly dialyzed Fraction B frequently resulted in substantial losses of activity. Preliminary analyses of the dry product for amino acids, carbohydrates, hexosamine, and sialic acid accounted for only about 65 per cent of the weight, leading to the suspicion that some of the buffer was not being removed. This was confirmed by direct analysis for citrate. Accordingly, all later samples of Fraction B have been electrodialyzed to remove the last traces of citrate. Losses of activity have been rare since the institution of this procedure. Recoveries of total activity to the completely de-salted dry product have been in the range of 70 to 100 per cent.

Electrodialysis is performed in a modified A. H. Thomas apparatus. The cells are separated in this case by cellophane and have a volume which is just under 100 ml. Before electrodialysis is attempted, the step 4B fraction is thoroughly dialyzed against distilled water. The resulting solution is transferred to the electrodialyzer and 300 volts are applied. The progress of desalting is followed in the rise and ultimate fall in the amperage reading. During electrodialysis a precipitate forms and by the end of the process has settled to the bottom of the cell. Tests show

that this precipitate is inactive, and that substantially all the activity is regularly recovered from the clear supernatant. The precipitate appears to be a glycoprotein, differing little from the supernatant material in gross chemical analysis.* The supernatant fractions have conductivities below 2×10^{-5} mhos indicating less than .01 mg citrate per ml. Thus, at least 90 per cent of the citrate is removed in the electrodialysis process.

The potencies of the final dry step 4B fractions have been in the range of 20 to 100 units/mg with the exact value appearing to depend largely on the potency of the original plasma.

PROPERTIES OF THE HIGHLY POTENT ERYTHROPOIETIN FRACTION

In studying the chemistry of the highly potent Step 4B fraction, the inert Step 4A fraction has been carried along as a reference glycoprotein. Since the parent Step 3 material already closely resembled the α_1-glycoprotein of human plasma, it was assumed that Step 4A represented the normal α_1-glycoprotein of sheep plasma. The total weight of the active B fraction separated in Step 4 is very small and, therefore, the chemical properties of Step 4A vary little from those of the parent Step 3 material.

Information has been obtained on the following properties of the Step 4 fractions.

1. Isoionic point. Assuming that all ions are removed in the electrodialysis procedure which is used routinely on the Step 4B fractions, the pH of the solution should indicate the isoionic point. This value is about pH 4.5 for the Step 4B fraction. The corresponding value for the α_1-glycoprotein, both human and sheep, is about 4.0.

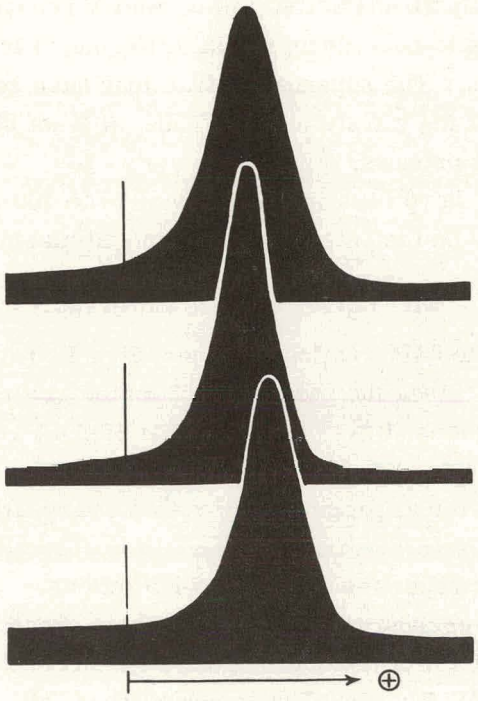
2. Electrophoresis. As shown in Figure 2, both Step 4B and Step 4A fractions gave essentially single peaks in free-electrophoresis at pH 4.5. Within the limits of error of the method, both have the same mobility at this pH, namely -2.5×10^{-5} cm^2/volt/sec. When the two Step 4 fractions are mixed in equimolar proportions and run at pH 4.5, a single peak again results. The same mixture run at pH 8.6 either in veronal or in veronal-borate† buffers gives in each case a single peak with a mobility of -5.8×10^{-5} cm^2/volt/sec.‡ Thus, at least for the two pH's used thus far, the Step 4 fractions are inseparable.

3. Ultracentrifugation. The Step 4 fractions have been studied in the ultracentrifuge. In sedimentation velocity runs the $S_{20,w}$ values at zero concentration were 3.0 for Step 4A and 4.7 for Step 4B. Figures 3 and 4 show the patterns for the individual fractions and Figure 5 shows the picture for a mixture of the two fractions. The single run on the mixture of the two fractions gave a single peak with an S value squarely on the line for Step 4A rather than midway between the two lines. Further mixed runs will be needed to provide a satisfactory explanation for this abnormal result. The highly active Step 4B fraction was examined in the ultracentrifuge by the Archibald technique and gave a molecular weight of 39,900 ± 4,000. The corresponding Archibald

*The following analytical values have been obtained from the precipitate: total nitrogen - 10.6 per cent; hexose - 7.7 per cent; sialic acid - 9.9 per cent; hexosamine - 7.1 per cent. In addition, the precipitate is essentially homogeneous in electrophoresis at pH 4.5 and at pH 8.6, showing mobilities of -2.8 and -5.6×10^{-5} cm^2/volt/sec.

†Borate was added in the hope that separation of the two components could be effected by a differential complexing effect of borate with the carbohydrate components of the two fractions. Goldwasser and Matthews[4] have shown that the introduction of borate into the buffer increases the electrophoretic mobilities of some carbohydrate-containing proteins.

‡In a comprehensive electrophoretic study of normal sheep plasma, Koenig[5] et al. assigned a mobility of -5.3×10^{-5} cm^2/volt/sec to the α_1 peak. Thus the mixed Step 4 erythropoietin fractions have a mobility somewhat higher than that of the α_1 peak of whole plasma.



step 4A

$\mu = -2.45(10)^{-5}$ cm.^{2}/v./sec.

step 4B

$\mu = -2.52(10)^{-5}$ cm.^{2}/v./sec.

step 4A +4B

$\mu = -2.75(10)^{-5}$ cm.^{2}/v./sec.

Figure 2. Electrophoretic analysis of erythropoietic fractions. (Descending patterns; pH = 4.5; $\Gamma/2$ = 0.1 acetate; 200 min; 3.3 v/cm).

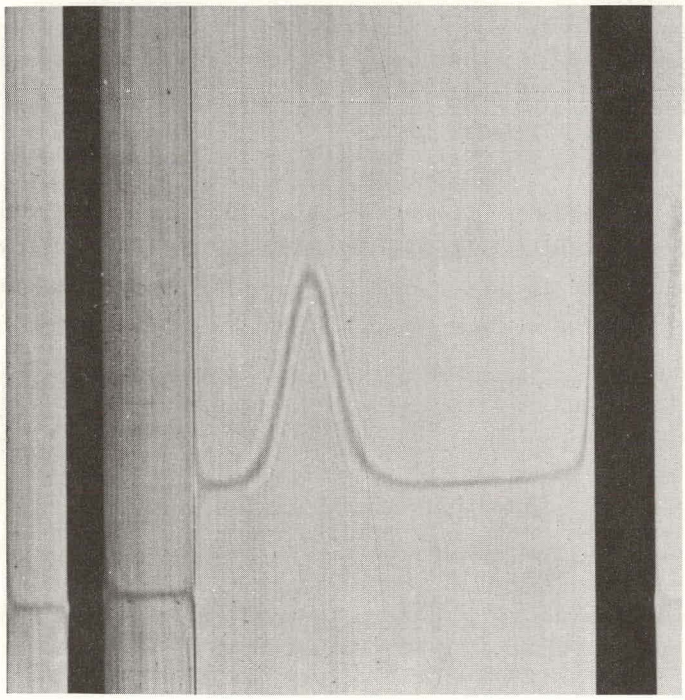


Figure 3. Ultracentrifuge patterns of Step 4 fractions: Step 4A at 9.7 mg/ml, picture at 112 min. Runs in pH 4.5 acetate buffer.

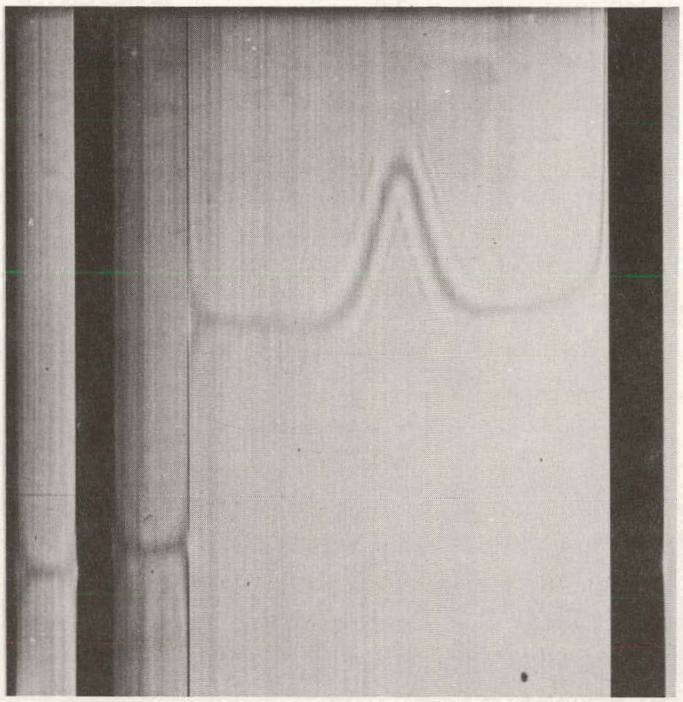


Figure 4. Ultracentrifuge patterns of Step 4 fractions: Step 4B at 9.5 mg/ml, picture at 112 min. Runs in pH 4.5 acetate buffer.

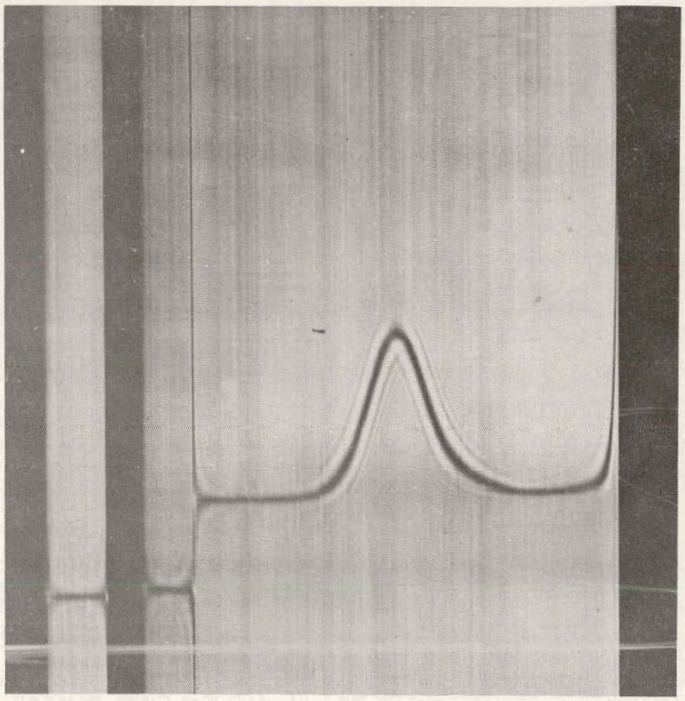


Figure 5. Ultracentrifuge patterns of Step 4 fractions: Mixture of equal volumes "a" and "b." Picture at 96 min. Runs in pH 4.5 acetate buffer.

value for Step 4A fraction was 42,400 ± 5,000.

4. Ultraviolet absorption. Step 4B fraction, directly off the XE-97 column, shows a nondescript spectral-transmittance curve with no peak in the characteristic 275 mμ range. However, after electrolytic desalting, a typical protein curve emerges. Presumably the true curve is masked by the contaminant which precipitates during the desalting process. Spectrophotometric values still vary slightly from batch to batch. However, the average $E^{1\%}_{1\ cm}$ value at pH 6.8 is about 4.5 and the A_{280}/A_{250} value is about 1.35. By contrast, the corresponding values for Step 4A are 9.6 and 2.3. As expected, the values for Step 4A are very close to those reported for human α_1-glycoprotein.[6-9]

5. Chromatography. The purity of the Step 4B fraction has been assessed by re-running it on XE-97 under the same conditions as those used for Step 4. At the stage of purity of the Step 4B fraction, losses in chromatography on XE-97 are frequently high. However, in one recent run 15 per cent of the optical density passed through the column at pH 5.1 (Step 4A position) and 66 per cent was eluted in a single symmetrical peak in the "B" position. In this case the recovery of activity in the "B" fraction was good. Thus within reasonable limits the Step 4B fraction retains its physical and biological integrity in rechromatography on XE-97.

Since our early work indicated that DEAE-cellulose was limited in its ability to fractionate erythropoietic activity, it was of interest to rerun the highly purified Step 4 fractions separately on that adsorbent. The samples were applied to the column at pH 4.5 and low salt concentration. In both cases, all the optical density was held by the column at low salt and in both cases the optical density was quantitatively eluted in a single, symmetrical peak by the application of an NaCl gradient. A careful study of the conductivities of the eluate fractions showed that both samples were eluted at exactly the same salt concentration, namely: 0.07 M sodium chloride. Our previous failure to separate the activity from the α_1-glycoprotein by means of DEAE-cellulose was thus explained on the basis of the identical behavior of the two fractions.

6. Gross chemical analysis. The chemical properties of the Step 4 fractions are summarized in Table 1. For comparison, our data on human α_1-glycoprotein are also shown.* It is evident that both fractions from Step 4 are glycoprotein in nature. The analytical values for the biologically inert Step 4A fraction are almost identical with those for the α_1-glycoprotein of human blood. On the other hand, the highly active Step 4B fraction shows marked differences from the α_1-glycoproteins. Particularly notable are the lower values for total hexose, sialic acid and tyrosine and the higher values for serine and threonine.

7. Proteolytic Digestion. It has been reported[9,10] that the erythropoietic activity of relatively crude concentrates from anemic plasma is destroyed by the action of proteolytic enzymes. Thus it was of interest to study the action of these enzymes on our highly purified Step 4B fraction. Figure 6 shows the plots of alkali uptake during the chymotryptic and tryptic digestions of Step 4B fraction. In both cases the autotitrator† was set at pH 8.0. On the assumption that the amino groups of the released peptides are titrated to the extent of 50 per cent at this pH, the number of bonds that are split is twice the number of moles of alkali taken up. It will be noted that the action of both enzymes levels off when approximately ten bonds have been split. At this end point, both digests show a complete loss of biological activity. On the basis of the amino acid

*The human α_1-glycoprotein sample was kindly supplied by Dr. Karl Schmid of Harvard.

†Type TTT 1a, Radiometer, Copenhagen.

Table 1

ANALYTICAL SUMMARY OF STEP 4 FRACTIONS

	α1-glycoprotein*	Step 4A	Step 4B
Protein % (Lowry)	85	85	88
Total hexose %	12.5	12.2	8.2
Hexosamine %	11.1	9.8	7.1
Sialic acid %	10.9	11.8	6.9
E 1%, 280 mμ, pH 7	8.1	9.6	4.5
A_{280}/A_{250}	2.5	2.3	1.35
Isoionic point	4.0	4.0	4.5
Electrophoretic mobility, $cm^2/v/sec. \times 10^5$ pH 4.5 $\Gamma/2$ 0.1	-	-2.45	-2.52
$S_{o,20,w}$	-	3.0	4.7

*Sample human α_1-glycoprotein courtesy of Dr. Karl Schmid.

composition, Step 4B fraction has a total of about 10 aromatic amino acid residues and about 16 basic amino acid residues (Table 2). Therefore, with both enzymes a high percentage of the available bonds are split. Step 4A fraction by comparison, is not appreciably split by either chymotrypsin or trypsin. This finding agrees with the results obtained by others[9] with human α_1-glycoprotein.

8. Analysis for N-terminal amino acids. For N-terminal analysis, the dinitrofluorobenzene method of Sanger[11] was used. The DNP-derivatives of the Step 4 fractions were prepared by reaction with excess 2,4-dinitrofluorobenzene at pH 8.0, pH being maintained by the addition of standard base from the microburet of the autotitrator. The reactions were allowed to proceed until the curve resulting from a plot of volume base vs time reached a constant slope. The DNP-proteins were recovered as usual and acid hydrolyzed. The hydrolysates were extracted with ether and both the ether-soluble and water-soluble yellow DNP derivatives were chromatographed on paper for identification. In the case of Step 4A fraction, no α-DNP-amino acid was found, whereas Step 4B fraction yielded only DNP-serine.* In accordance with the results of others[12,13] for N-terminal serine polypeptides, the yield of DNP-serine from Step 4B fraction was much less than one mole. However, by limiting the hydrolytic period for the DNP-protein, the yield has been increased to .32 mole per mole of protein. In addition, we have found, as have other investigators[14] that addition of glycoprotein to DNP-amino acids results in severe losses of the latter during hydrolysis. By application of the proper correction factor in the case of the Step 4B fraction, all or most of one residue of serine is accounted for per mole of protein.†

*In the case of both of the Step 4B fractions, the expected E-DNP-lysine was found in the aqueous layer and dinitrophenol and DNP-glucosamine were found in the other layer.

†Working with the carboxypeptidase molecule, E. O. P. Thompson[15] found traces of DNP-serine in addition to the DNP-derivative of the true N-terminal aspartic acid. He attributed the presence of DNP-serine to a partial rupture of peptide linkage during the reaction with DNFB. Thus, until confirmed by an independent method, the possibility exists that serine is not N-terminal in Step 4B fraction. However, the findings of DNP-serine with Step 4B fraction is characteristic and serves to differentiate that fraction from the α_1-glycoproteins.

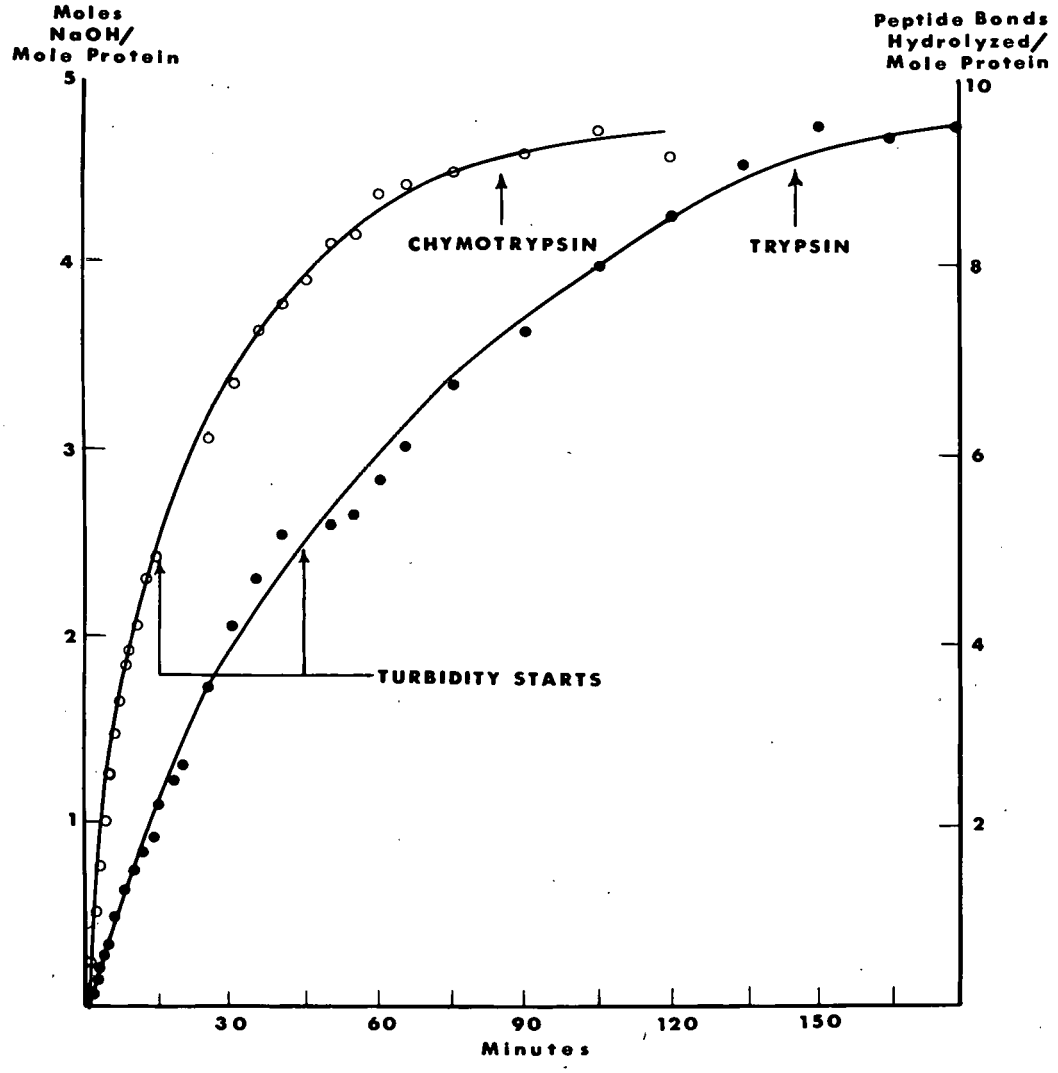


Figure 6. Proteolytic hydrolysis of Step 4 erythropoietin.

9. Summary of properties of Step 4 fractions. Table 1 summarizes the properties of the Step 4 fractions. It is clear that Step 4A is the α_1-glycoprotein of sheep plasma and that Step 4B is markedly different. At this point, it was again tempting to conclude that Step 4B fraction was the pure hormone, particularly since it appeared to be more active than any other preparation previously described in the literature. Some of the Step 4B fractions gave significant responses in the Fe^{59}-uptake assay at a total dose of 10 gamma of solids. Again, however, there was a definite variability in the potency of the final product and the potency of the Step 4B fraction still appeared to depend on the potency of the particular plasma pool from which it was made. The simplest explanation of this phenomenon was that the bulk of the final product was still merely a carrier for the true hormone and that the carrier was perhaps a normal constituent of sheep blood.

10. Step 4 fractions from normal sheep plasma as references. In order to test the possibility that the Step 4B fraction was still not the pure hormone, a large pool of normal sheep plasma was carried through the entire 4-step procedure. The yields at the first three stages were

Table 2

AMINO ACID ANALYSES OF STEP 4 FRACTIONS

	Step 4A	α_1-glycoprotein*	Step 4B
Lysine	5.1	5.1	3.7
Arginine	3.3	3.4	2.6
Tyrosine	4.2	3.7	0.8
Valine	1.9	1.6	4.6
Methionine	0.7	0.7	0.0
Isoleucine	2.9	3.1	5.3
Leucine	3.8	3.9	7.6
Phenylalanine	2.9	3.3	2.6
Aspartic	5.3	4.9	4.4
Glutamic	8.7	8.1	11.7
Serine	1.5	1.9	5.4
Glycine	1.3	1.3	1.6
Threonine	3.2	3.0	5.8
Alanine	2.6	2.5	1.8
Cystine	1.3	1.6	1.5
Histidine	2.2	2.2	1.3
Proline	2.6	2.8	1.5
Total	53.5	53.1	62.2

*Sample human α_1-glycoprotein courtesy of Dr. Karl Schmid.

similar to those with anemic plasma. Thus the solution of the problem depended entirely on the fourth step. Here, surprisingly, the yield of fraction 4B was greater, rather than less, than that from anemic plasma. Moreover, it soon became evident that the properties of the Step 4B fraction from normal plasma were the same, within the limits of experimental error, as those for the corresponding fraction from anemic plasma. Among the properties studied thus far are: $E^{1\%}_{1\ cm}$, A_{280}/A_{250}, % sialic acid, % hexose, % hexosamine and N-terminal residue.

Thus, even at the Step 4B stage, the bulk of the fraction still appears to be a normal constituent of sheep plasma. This conclusion again raises the question of the relationship of the Step 4B fraction to the α_2-glycoproteins which Schmid[16,17] has shown to be normal constituents of human plasma.* It is true that the electrophoretic mobility of the Step 4B fraction is in the α_1 rather than the α_2 range, but the possibility exists that the mobilities of the glycoproteins are modified during purification. The answers to these questions await the accumulation of analytical data on the α_2-glycoproteins, particularly those of sheep plasma.

Additional doubt concerning the purity of Step 4B fraction has arisen recently as a result of preliminary chromatographic experiments. In this work, Step 4B fraction has been resolved into a series of fractions having variable potencies, some as high as several hundred units per mg.

*This is not to imply that one of the known α_2-glycoproteins might be the hormone itself. This possibility has been dispelled by assay of samples of both the zinc and barium α_2-glycoproteins of human plasma (kindly supplied by Dr. K. Schmid).

Chemical characterization of these fractions awaits the accumulation of the several hundreds of liters of anemic plasma which will be necessary to yield reasonable quantities of final product.

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THE CREATION OF DEEP CEREBRAL LESIONS BY SMALL BETA-RAY SOURCES IMPLANTED UNDER GUIDANCE OF FLUOROSCOPIC IMAGE INTENSIFIERS (AS USED IN THE TREATMENT OF PARKINSON'S DISEASE)*

By

S. Mullan,† R. D. Moseley, Jr.,‡ and P. V. Harper, Jr.

The creation of cerebral lesions in the control of abnormal movements and rigidity is one of the major neurosurgical interests of the present decade. The pyramidal tract has previously been sectioned at the cortex, the internal capsule, in the cerebral peduncles and in the spinal cord, but at present the main targets are the juxtapyramidal basal ganglia—the globus pallidus and the thalamus. The close proximity of these structures to one another demands a precision of destruction which will obviate inadvertent damage to structures other than the target (Figure 1). Such precision will increase both the safety of the procedure and the accuracy of physiologic interpretation of the results.

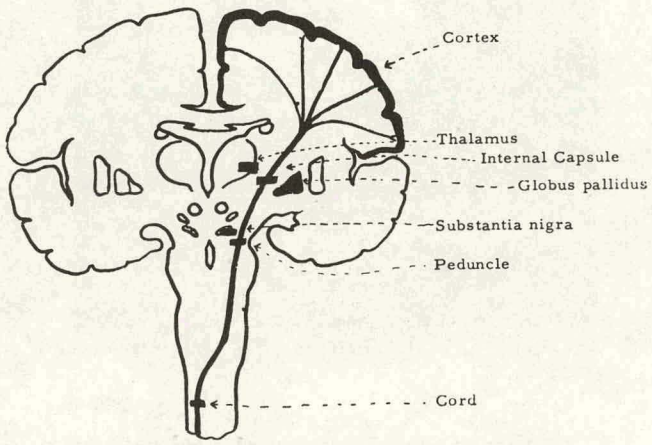

Figure 1. Diagram of various sites of surgical attack in the treatment of Parkinson's disease.

Methods currently in use are chemical cautery,[1] electrical cautery,[2] surgical ablation,[3] and ultrasonic destruction,[4] each of which has its intrinsic defects.

These sources of destruction (except the ultrasound) are applied at the end of a needle or other slender instrument which may be directed to the target by roentgenographic or by stereoscopic guidance. The latter has no inherent advantage because, unfortunately, the heads of our

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*This report is taken from a paper appearing in the Am. J. Roentgenol. Rad. Therapy, Nuclear Med., 82:613, 1959.

†Division of Neurological Surgery, University of Chicago.

‡Department of Radiobiology, University of Chicago. This work has been aided by a grant from the Simms Foundation.

patients are not standard; they are long and short, broad and narrow, high and low. Their brains, particularly in the elderly group, may show amazingly different degrees of atrophy. Stereotaxic instruments, irrespective of the skill of construction and manipulation, because of biologic variations can direct the source only to the presumed target.

The ideal technique must be simple, safe and under perfect control at all times. The lesion should develop slowly so that, should any undesired change commence, its presence can be detected at an early removable stage. Experience with the use of beta emitting isotopes over several years and with the image intensifier roentgen ray machines for a shorter period has suggested to us that a beta emitting source placed on the target under the guidance of biplane image intensifiers would provide an approximation to the ideal technique which we sought.

Palladium 109 was chosen because of its short half life (13.5 hours) and the convenient metallic form in which it is available. In animals wire sources 3 mm long and weighing 2.8 mg with an activity of approximately 20 mc will produce lesions which are first visible after forty-five minutes irradiation and will reach their maximum of 5-6 mm diameter in ninety minutes (Figure 2). A small amount of swelling may be noted during the following week but there is no extension of the histologic lesion over a period of a two year observation.[5]

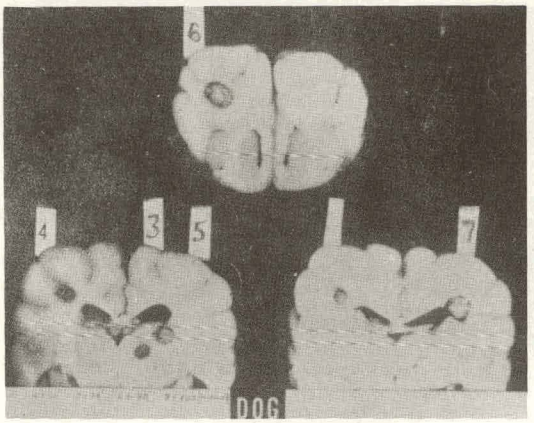

Figure 2. Lesions produced by Pd^{109} (20 mc) in dog. Duration of application: (3) forty-five minutes, (4) sixty minutes, (5) seventy-five minutes, (6) ninety minutes, (7) one hundred and five minutes. Animal sacrificed ten days later.

TECHNIQUE

The first problem is to relate the target to a fixed bony landmark—the tip of the posterior clinoid process. Twenty-five to thirty cubic centimeters of air are introduced into the lumbar or cisternal subarachnoid space without removal of fluid, and the anterior and posterior commissures, the floor of the third ventricle and often the mammillary bodies are identified. Using these reference points, the site of the chosen target is plotted and its distance from the tip of the posterior clinoid is measured in three dimensions. The tube to skull distance is 72 inches and a correction factor is not employed. A burr hole is then made in a convenient location(usually behind the hair line at 5 cm from the midline).

Several days later, using local anesthesia, the isotope is implanted under the guidance of the image amplifiers (Figure 3). These provide an enlarged image sufficiently bright to be viewed without dark adaptation. The principles of these machines are outlined in Figure 4 and

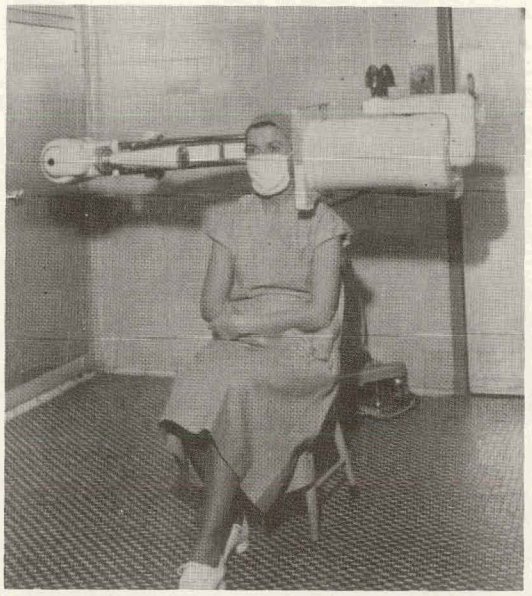

Figure 3. "Surgex" image amplifier (Phillips). Two such instruments are used at right angles to one another to provide biplane visual control of insertion of the needle.

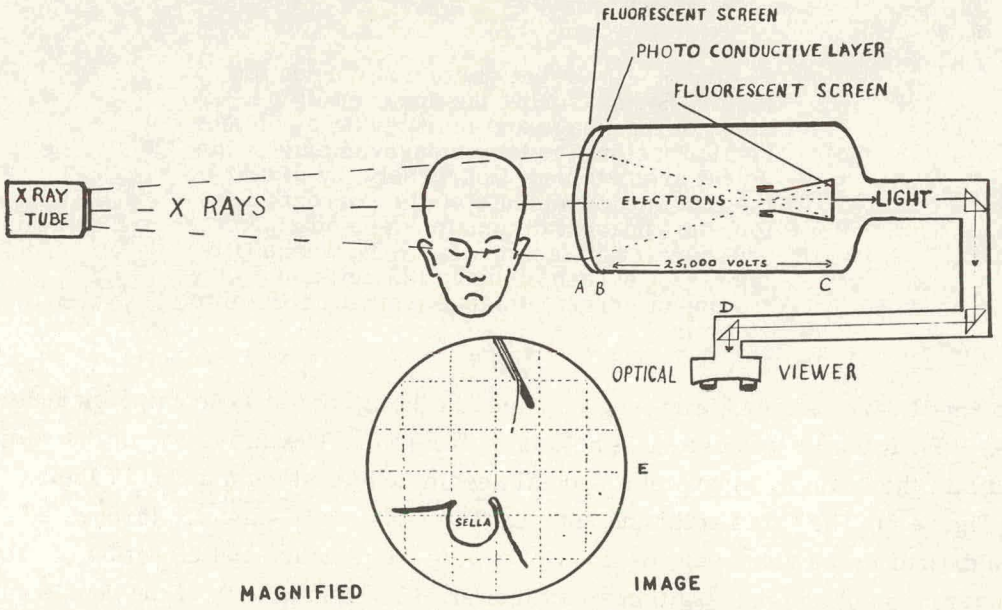

Figure 4. Principles of the image amplifier. Roentgen rays fall upon fluorescent screen A causing light to strike photoconductive layer B. Electrons are emitted, accelerated by a 20,000 volt potential and condensed onto a small fluorescent screen C. This gives a bright image which is magnified and transferred by an optical system to the viewer D.

are well described in the literature.[6,7] The image of the introducing needle and sella turcica has, superimposed upon it, the image of a wire grid placed in front of the screen. By this means point to point distances on the image are measured in grid squares, but to obtain the true dimensions of the object it is necessary to correct for divergent distortion. Since in these machines the target screen distance is fixed, the only variable is the object screen distance and the correction factor is readily obtained from a graph (Figure 5). The chosen target is thus a precise number of grid squares or fractions thereof (corresponding to a precise number of millimeters) from the tip of the posterior clinoid process.

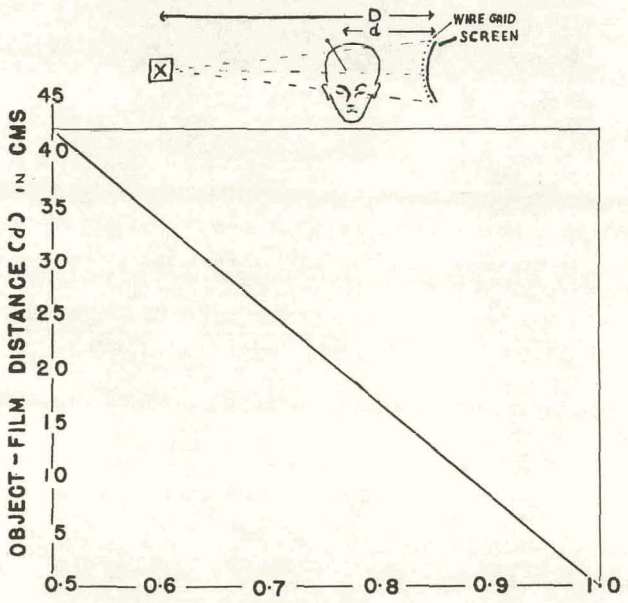

Figure 5. Divergent distortion correction factor. By measuring the apparent dimensions of the image and multiplying by the correction factor, the true measurements of the object are obtained. Conversely, by dividing the true measurements by the correction factor, the apparent dimensions are obtained. The correction factor = D - d/D, where D is the target screen distance (83 cm) and d is the object screen distance (varying from 0 to 40 cm).

The small wire isotope 3 mm long is placed in the tip of a plastic catheter under water (B. D. $N_{444}T$). If the fit is loose, it is held in by bone wax. The active end of the catheter is introduced into the brain by means of a special needle fashioned from a No. 17 lumbar puncture needle (Figure 6). The tip is solid and smooth. The catheter is extended through a hole which has been drilled in the side near the tip. By varying the distance and direction of protrusion of the catheter, a point anywhere within a centimeter of the line of axis of the needle can be reached by the isotope. The initial placement of the needle need not, therefore, be absolutely accurate. Having guided it towards its target by fluoroscopic control, biplane roentgenograms are taken and the precise direction and extent of extrusion of the isotope are calculated and then accomplished.

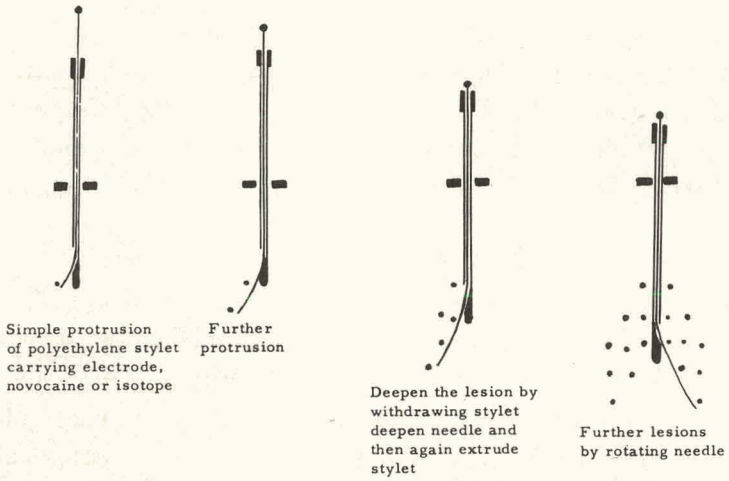

Figure 6. The versatile needle. By selective extrusion, any point within 10 mm of the axis of the needle may be reached.

As a rule, the mere placement of the needle has caused an alteration of function (e.g., improvement of rigidity, tremor or both). More rarely, these changes advance while the isotope catheter and needle remain in place for ninety minutes. They have not been observed to advance beyond this period. If a more extensive lesion is required, the isotope is repositioned but, as a rule, this is not necessary. If symptoms return, as they sometimes do, a few days later a further lesion is made (Figure 7).

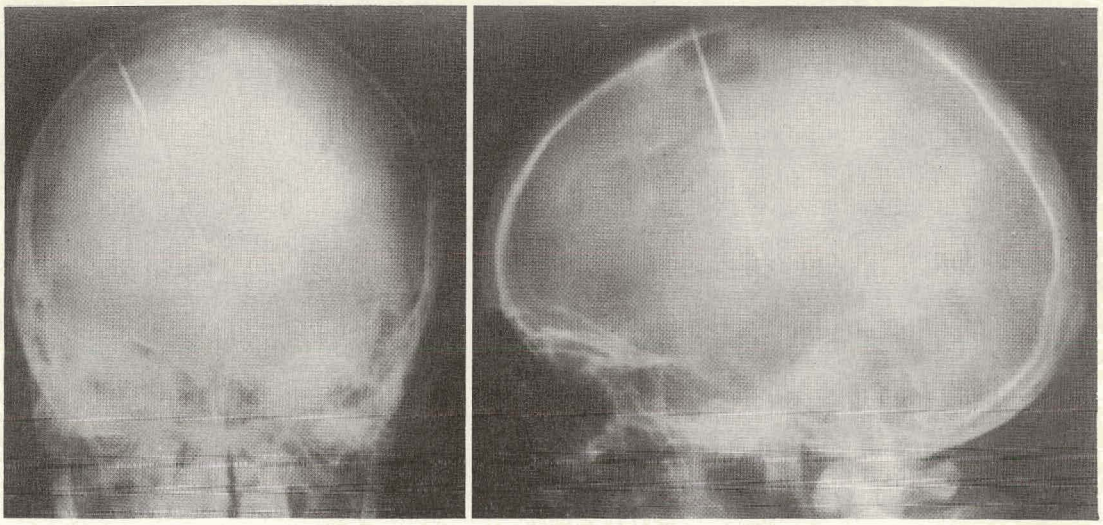

Figure 7. Roentgenogram of needle in position. The small isotope is scarcely visible beyond and a little to one side of the tip of the needle.

In order to estimate the exposure dose received by the neurosurgeon, neuroradiologist and other operating room personnel during the procedure, isodose curves of scattered radiation were plotted and photographic dosimetry of chest, hand and ring badges was carried out. Although two machines are used in a biplane position, they are never "on" simultaneously and the

total use of the roentgen-ray beam during the implantation is less than five minutes. Fluoroscopic factors are 75 kv. peak and 3 ma. The skin dose for the patient (entrance dose) is approximately the same as for a gastroduodenal examination (25 r). The dose outside the lead apron of the surgeon and radiologist averages 20 mr per procedure (recommended limits are 300 mr per week). We have found that it is unnecessary to have the hands in the field at any time.

DISCUSSION

Palladium 109 has now been employed in this manner in the treatment of 8 patients, in 2 of whom the insertion was bilateral. The use of fluoroscopic guidance eliminates the complexities of stereotaxic instruments, most of which require rigid fixation to the bony skull to be of value. Its superiority over serial roentgenograms lies mainly in the elimination of the delay that processing of these requires. The special needle makes multiple empirical punctures unnecessary. The beta source provides a very accurately controlled lesion. Its small size and gradual development over forty-five to ninety minutes are both factors of safety. If a single lesion proves too small, it can be easily enlarged at a later date. The short half life of palladium 109 and virtual freedom from gamma radiation make handling a simple process. This short half life of 13.5 hours is, however, a significant disadvantage in that hospitals some distance away from a nuclear reactor cannot be supplied. Ag^{111} of half life 7.5 days is now under investigation and may prove to be a better beta source. It has been used in the treatment of 2 patients (bilaterally).

SUMMARY

The ideal method of creating deep cerebral lesions should be safe, simple, and under rigid control at all times. The lesion should develop slowly so that any undesired change can be detected and arrested at the earliest possible stage.

Palladium 109 in the form of a metallic wire 3 mm long, weighing 2.8 mg and with an activity of 20 mc produces in brain a necrotic lesion of 5-6 mm diameter. It is first visible after forty-five minutes application and reaches its maximum size after ninety minutes.

With the aid of fluoroscopic image intensifiers and a special needle, this convenient metallic source (Pd^{109}) can be accurately placed in deep cerebral tissues under local anesthesia. The fluoroscopic radiation to which the patient is exposed approximates that of a gastrointestinal examination.

Satisfactory alleviation of the tremor and rigidity of Parkinson's disease has been achieved by this method.

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THE RETICULOENDOTHELIAL SYSTEM IN ANTIBODY FORMATION*

By

R. W. Wissler, F. W. Fitch,† and M. F. LaVia‡

INTRODUCTION

The important role played by mesenchymal cells in combating infection and forming antibody is now generally accepted.[1-6] There can be little debate about the following basic observations:

A. Macrophages (fixed and free) avidly ingest, and frequently kill and digest living microorganisms.

B. "Antibody-forming cells" arise from more or less primitive cells (reticular cells) and under some conditions, develop into plasma cells which contain antibody.

C. Small or medium-sized lymphocytes have an important function in the immune process and in "delayed hypersensitivity."

Beyond these generally accepted concepts many questions remain. For example, if one accepts the coexistence of two kinds of primordial reticuloendothelial cells, one of which is phagocytic (fixed macrophage) and one of which is not (primitive reticular cell), it still remains to define the interrelationships and the roles played by them in resisting infection and forming antibody. It is not yet clear how a mesenchymal cell is stimulated to form antibody, whether mitoses are commonly or only rarely necessary for antibody formation, or what is the usual fate of the antibody-forming cell.

Some idea of the state of flux that has characterized ideas relating cells to antibody formation may be gained by a brief consideration of the rapid shifts of scientific opinion during the past 25 years (Table 1). Evidence has been advanced to support theories of the formation of antibody by macrophages (histiocytes), by lymphocytes and by plasma cells or their precursors, but most of this evidence has been indirect. There has been a lack of agreement on the nomenclature and interrelationships of the various mesenchymal cells. While this difficulty remains, it is probably less of a hindrance to progress than it was two decades ago.

Burnet's recent lectures on "The Clonal Selection Theory of Acquired Immunity"[1] outline the problems faced. He points out that any theory of how cells fabricate antibody must take into account the important chronological and quantitative differences between the "primary" and "secondary" immune responses as well as other phenomena of the anamnestic response, and that it should explain the presence of local tissue immunity, adjuvant action and immunological tolerance.

The main purpose of the present paper is to re-evaluate the problem of how cells form anti-

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Table 1

SUMMARY OF EVIDENCE IMPLICATING VARIOUS CELLS IN ANTIBODY FORMATION

1. Macrophages
   a. Cells which ingest antigen might logically be expected to form antibody.
   b. Blockade of RE system decreases antibody response.
   c. Cytoplasmic shedding of macrophages correlates with antibody formation.
   d. Tissue depots with large proportions of macrophages show high concentration of antibody.
2. Lymphocytes
   a. Lymphocytes increase in tissue during infection.
   b. Antibody titer increases acutely when lymphocytes break up (ACTH, Cortisone).
   c. Antibody can be extracted from lymphosarcoma.
   d. Antibody concentration is higher in lymph node than in serum.
   e. Antibody-forming capacity can be transferred by lymph node cells.
3. Plasma Cells
   a. Plasma cells increase in chronic infection.
   b. Hyperglobulinemia correlates with number of plasma cells in tissue.
   c. Hyperimmunization of rabbits increases number of plasma cells in tissue.
   d. Proliferation of immature plasma cells in spleen and lymph node of rabbit occurs during antibody formation.
   e. No plasma cells are present in agammaglobulinemia.
   f. Antibodies have been demonstrated inside plasma cells using fluorescein-labeled antigen.

body. Recent observations will be correlated with "The Clonal Selection Theory." An effort will be made to correlate observations from different laboratories, to clarify interrelationships among the cells involved, and to project these observations into a meaningful picture which may improve understanding and help guide future investigation.

INTERRELATIONSHIPS OF CELLS

It is impossible to reason in this field without some concept of the interrelationships existing among the mesenchymal cells involved in antibody-formation. The chart presented here (Figure 1) has many points in common with the one recently proposed by Marshall[7] but in many areas it is presumptive rather than definitive. In agreement with Marshall this scheme presents the fibroblast, osteoblast, erythrocyte, granular leukocyte, and plasma cells as "end stage cells" incapable of reversing or of progressing to another cell type. On the other hand, it differs from Marshall's diagram in that the lymphocytes are regarded as multipotential cells capable of developing into macrophages and/or plasma cells and possibly into "hemocytoblasts." This difference is important since observations in our laboratory suggest that the small or medium-sized lymphocyte is the "messenger" that carries information throughout the body regarding previous experience with antigens. Later it will be shown that such a link is necessary and that the plasma cell is unlikely to play this role. In the rat spleen, following a single intravenous injection of particulate antigen, the main steps of the cellular progress accompanying antibody formation ob-

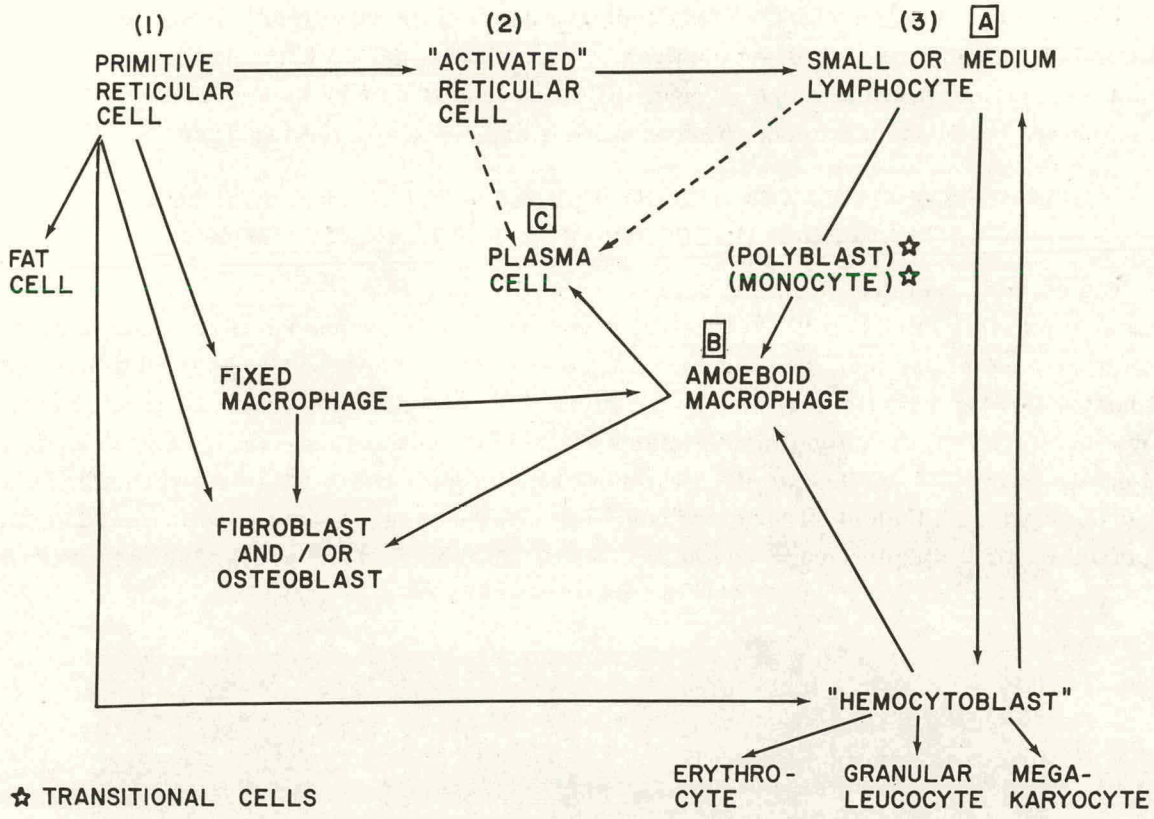


Figure 1. Interrelationships among mesenchymal cells. Solid arrows indicate transformations for which there is general agreement. Broken arrows, suggested transformations. The numbers designate the cell progression from 1 to 3 which has been observed in the rat spleen following a single intravenous injection of a particulate antigen. The letters are the suggested cell transformations from A to C occurring following a second (or repeated) injection of antigen.

served repeatedly, are from reticular cell to "activated" reticular cell to small or medium-sized lymphocyte (1 to 2 to 3) (Figure 1). In contrast, the sequence described by others during the "secondary" reaction or using other types of antigens, is from small or medium lymphocyte to macrophage to plasma cell (A to B to C) (Figure 1).

FACTORS INFLUENCING THE CELLULAR PATTERNS IN ANTIBODY FORMATION

A number of factors influence the type of cellular reaction that follows antigenic stimulation. Among these, total-body irradiation,[8,9] chronic protein deprivation,[10] acute interference with protein synthesis,[11] cortisone administration,[12,13] etc., have been extensively investigated. Further study of other factors may greatly alter the interpretation of this pattern of cellular response. These include: the physical state of the antigen; the chemical nature of the antigen; the tissue participating in antibody formation, such as the spleen (intravenous injection) or lymph node (subcutaneous injection); the time interval that elapses after the first injection or injections of a given antigenic substance (or substances) before the reaction is studied; and the species being used. In general, there have been very few studies in which these parameters have

been adequately evaluated. Here we will compare some of the results obtained using one set of experimental conditions with those obtained by other investigators using quite different experimental designs. At present, some of these different results appear to defy analysis; but in other instances, it is possible to construct a rational explanation of the variations in cell response.

OBSERVATIONS IN THE RAT SPLEEN FOLLOWING A SINGLE INTRAVENOUS INJECTION OF A PARTICULATE ANTIGEN

The cellular reaction of the rat spleen to a single antigenic stimulation has been studied extensively in this laboratory.[8-14] The rat spleen was chosen for several reasons; namely: 1) with the dose of particulate antigen used it appeared to produce most if not all of the humoral antibody following a single intravenous injection;[15,16] 2) the antigen localized very quickly thus providing a brief, accurately-timed stimulus;[8,10] 3) this initial tissue localization was definite and easily traced;[8,10] 4) the cellular reaction could be interpreted with little difficulty by means of closely-spaced histological observations;[14] and 5) the weight and chemical composition of the organ were fairly simply measured because of the encapsulated and accessible nature of the organ.[17]

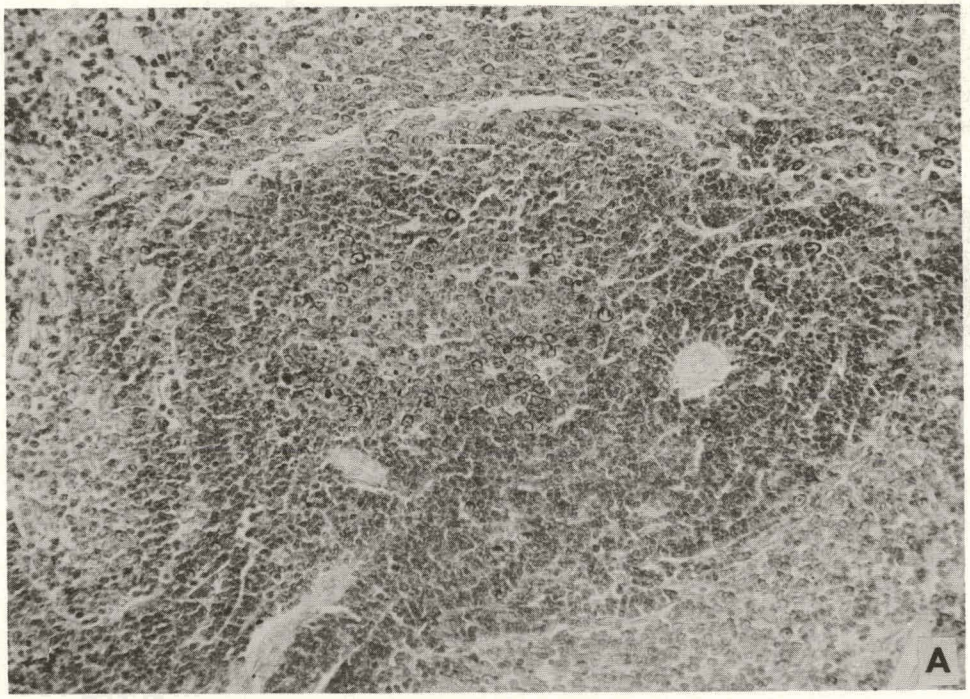
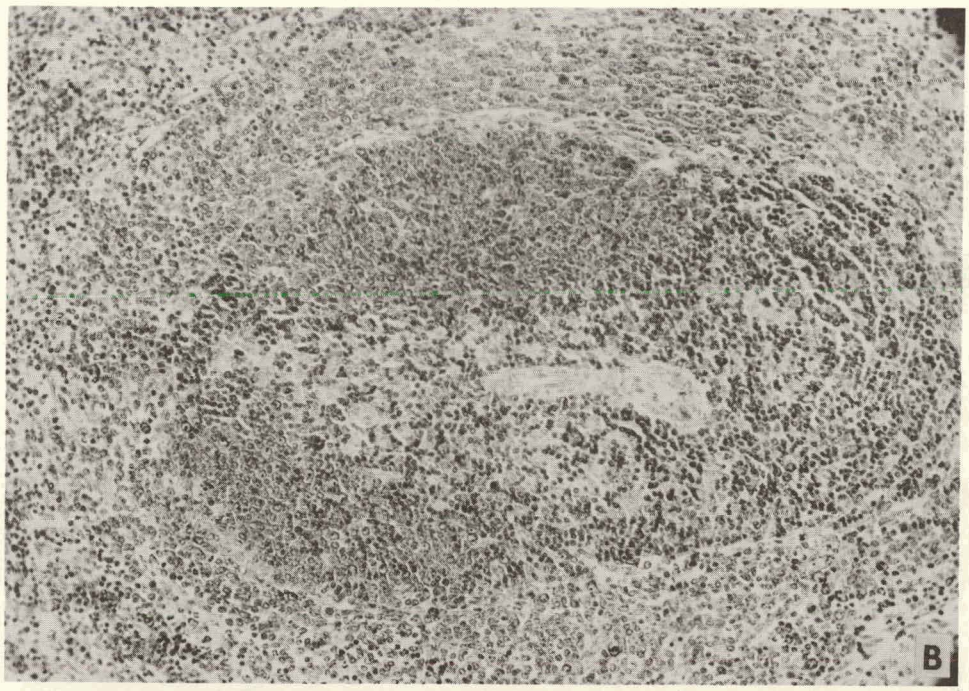
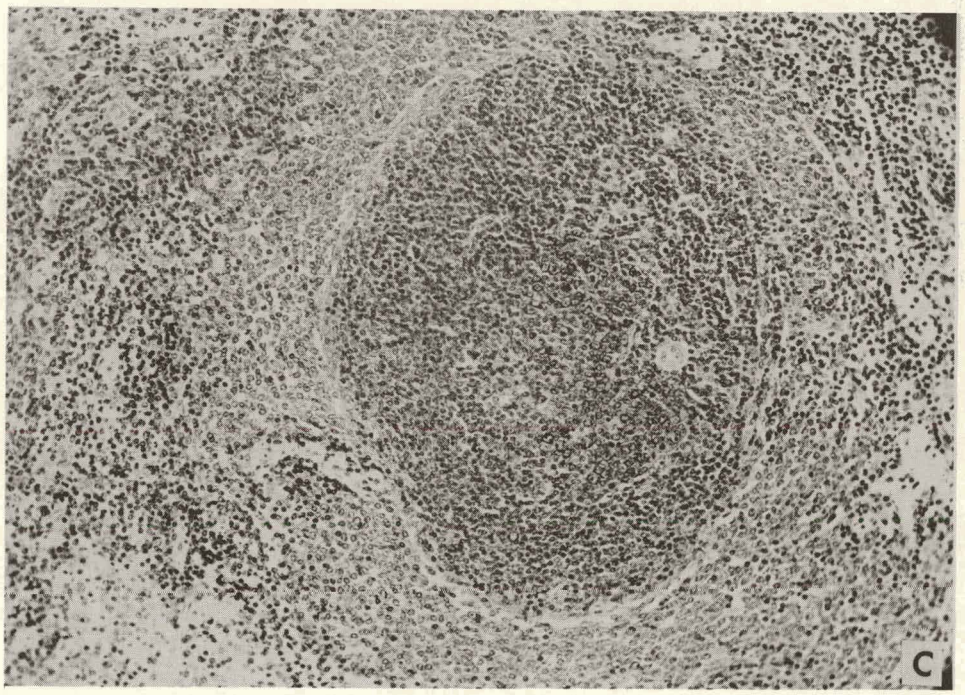


Figure 2. Lymphoid follicle of the rat spleen. Methyl green-pyronine. Magnification X 200. A. From a normal rat showing "resting" follicle. B. From a rat killed 12 hours after typhoid vaccine intravenously showing the great follicular activity including some decrease in small lymphocytes in the central portions of the follicle and some increase in relatively pyroninophilic lymphoblasts which are near the edge of the follicle. C. From a rat killed 12 hours after sheep erythrocytes intravenously showing little or no follicular activity. Spleens from animals from the same experiment killed at 4 days after injection of the same antigen showed many pyroninophilic cells in the red pulp.





During the past 8 years we have noted only one important variation in the cell pattern of response in the spleen following a single injection of one or another of several different particulate antigens. This consists of the presence or absence of very acute reactions in the splenic follicle,[17] and is illustrated in Figure 2 (A, B, C). Here it is evident that when a suspension of an endotoxin-containing-organism, such as killed S. typhosa is injected intravenously, there is considerable reaction of the Malpighian corpuscle within the first 24 hours. This reaction is ab-

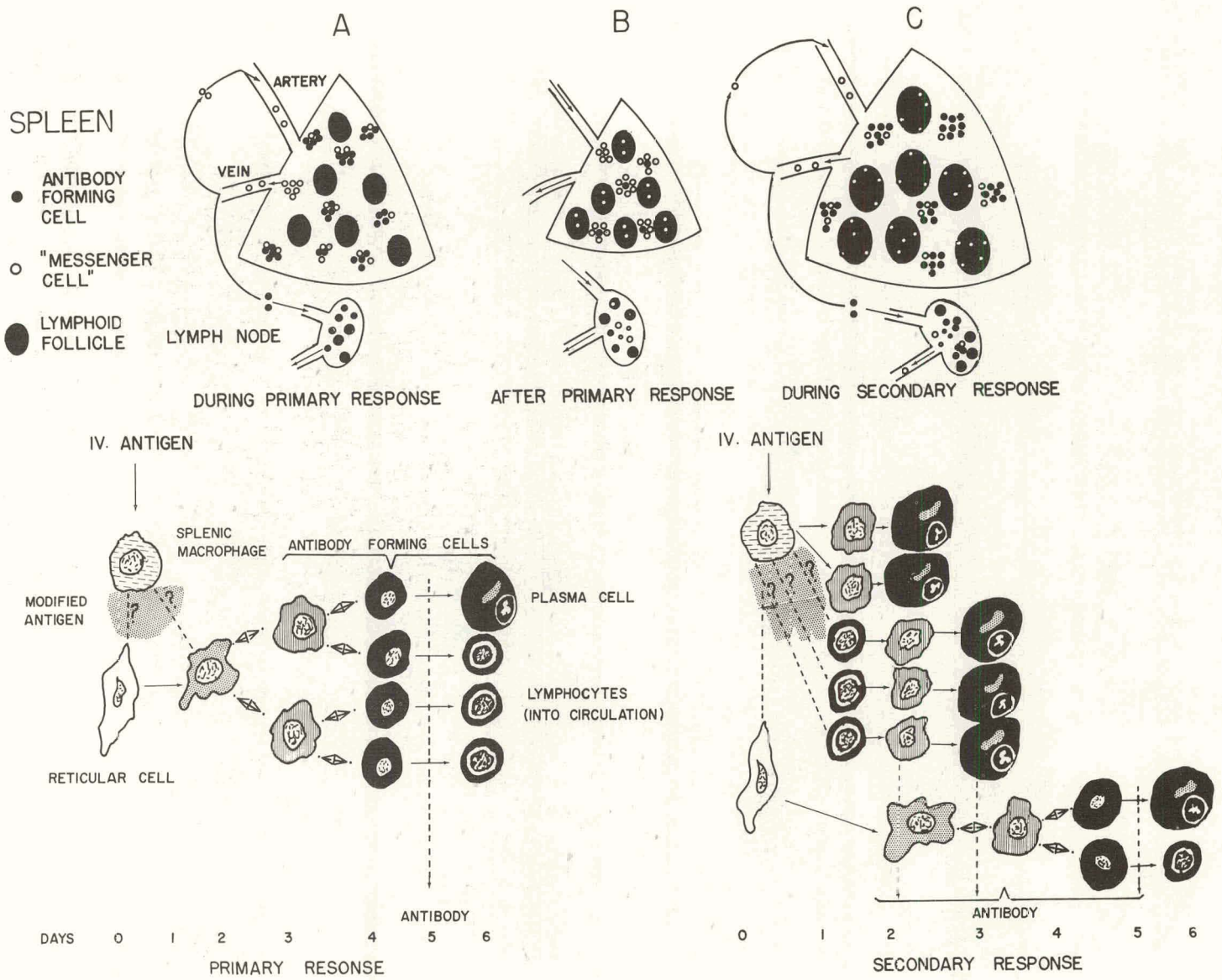


Figure 3. Proposed cellular patterns during "primary" (A) and "secondary" (C) immune response following intravenous particulate antigen injection. The diagrams of the spleen indicate the relative participation of the splenic red and white pulp during (A and C) and between (B) these two types of response. The proposed cell transformations relative to mitotic activity, the types of cells implicated, and the timing of the various reactions relative to antibody release are indicated in the cell sequences shown under the spleens labeled A and C.

sent when a "bland" antigen such as washed sheep erythrocytes is used. In both instances, however, peak humoral antibody titer (agglutinins to H antigen, and hemolysin to sheep cells), the timing and shape of the antibody curve, and the other cellular reactions are quite similar. Therefore, we have assumed that the reaction of the follicle is not necessary for antibody response in the rat. When it occurs it probably reflects an effect of liberated toxic substance (endotoxin?) rather than an integral part of the antibody response. Spleen weights correlate well with these observations[14] and the presence of numerous neutrophilic leukocytes in the sinuses of the splenic red pulp during the first 12 to 24 hours after the injection of toxic antigens,[17] supports this interpretation.

Excluding this very acute follicular reaction following a single injection of antigenic material, the rat spleen response to different particulate antigens is quite consistent and is limited to the red pulp. It is shown diagrammatically in Figure 3A.

The evidence indicates that most if not all of the antibody is formed in the spleen, and that the antigenic particules are engulfed by macrophages (histiocytes) which promptly digest them, at least to the extent that an I^{131} label is released. At this stage, none of the antigen is deposited in the white pulp. Radioautographic evidence indicates that all of it is deposited in the red pulp, about half in the marginal zone at the edge of the follicles and about half in the rest of the red pulp.[8,10]

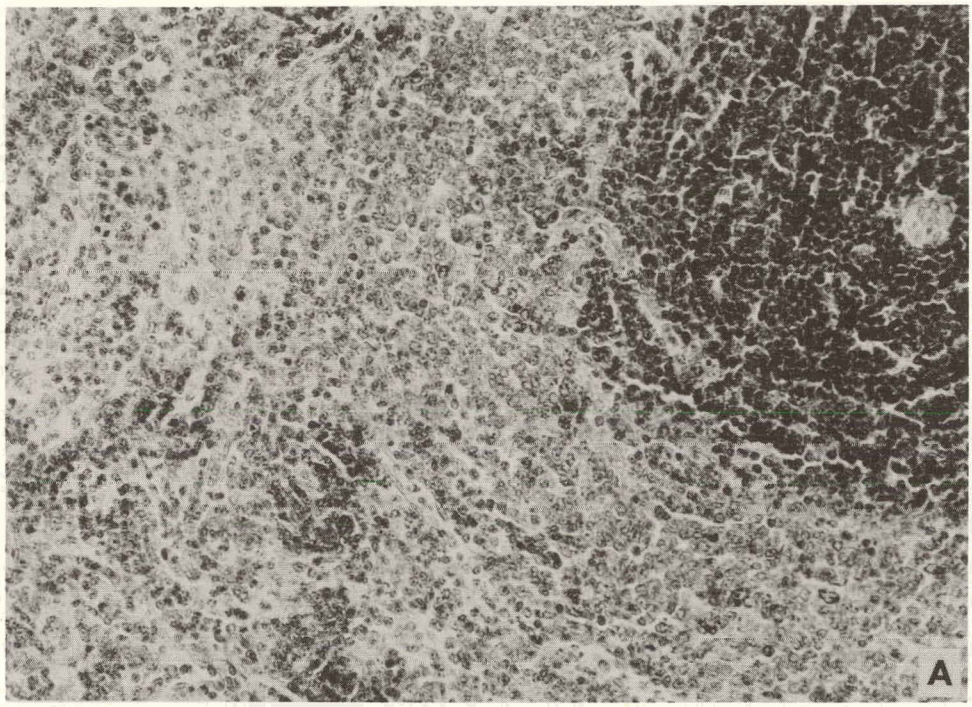
The illustrations that follow (Figure 4) are from experiments recently performed by Kattlove, La Via and Warren[18] using a typhoid flagellar preparation as the intravenous antigenic stimulus. The results are similar to those obtained previously with more complex antigenic mixtures.[14]

Except for the inconstant acute follicular reaction, there is very little evident histological change for the first 36 hours, and the spleen resembles that of a control rat (Figure 4a). Mitotic activity then begins in the red pulp. The cells that divide are not the macrophages that ingested the antigen, but are primitive reticular cells. It is not known where these cells receive their stimulus, but it may come from a soluble digestion product of the antigen derived from the macrophage.[19,20] Mitosis reaches its peak in 3 to 4 days and then gradually subsides. In the meantime, with sufficient antigenic stimulation, the red pulp becomes stuffed with large cells that have abundant pyroninophilic cytoplasm and large vesicular nuclei (Figure 4b). The spleen weight reaches its peak by day 4 and by this time may have doubled. The cellular reaction then subsides, and the weight declines. Beginning about day 5, and continuing to about day 8, there is a transient appearance of many "small dark cells" adjacent to the few remaining pyroninophilic cells in the red pulp (Figure 4c). These cells apparently are derived from the large basophilic cells by cytoplasmic loss and nuclear condensation. They do not seem to break up but leave the spleen rapidly and have been identified in the blood stream.[17] The serum antibody reaches its peak at about day 6. By 8 or 10 days after the antigenic stimulation, the entire spleen resembles that of a control animal, the only noticeable difference being a slight increase in number of mature plasma cells in the red pulp (Figure 4d).

CELLULAR REACTION FOLLOWING TWO (OR MORE) INJECTIONS OF ANTIGEN

One may assume that the cells or their derivatives which proliferated in response to the first injection of antigen, migrate into the blood stream and then colonize the lymph nodes, spleen,

A



B

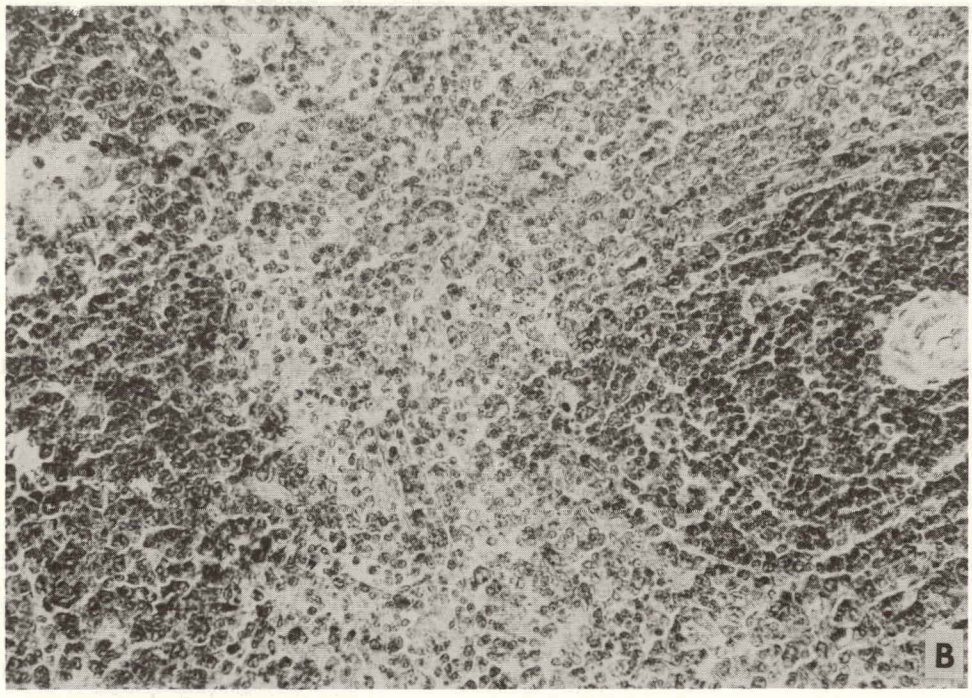
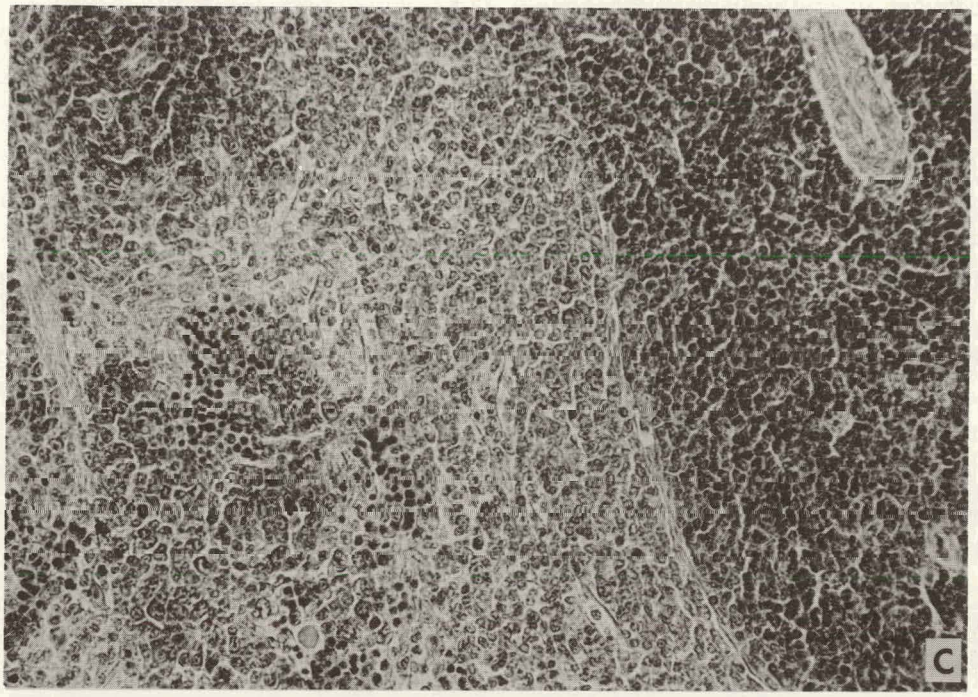
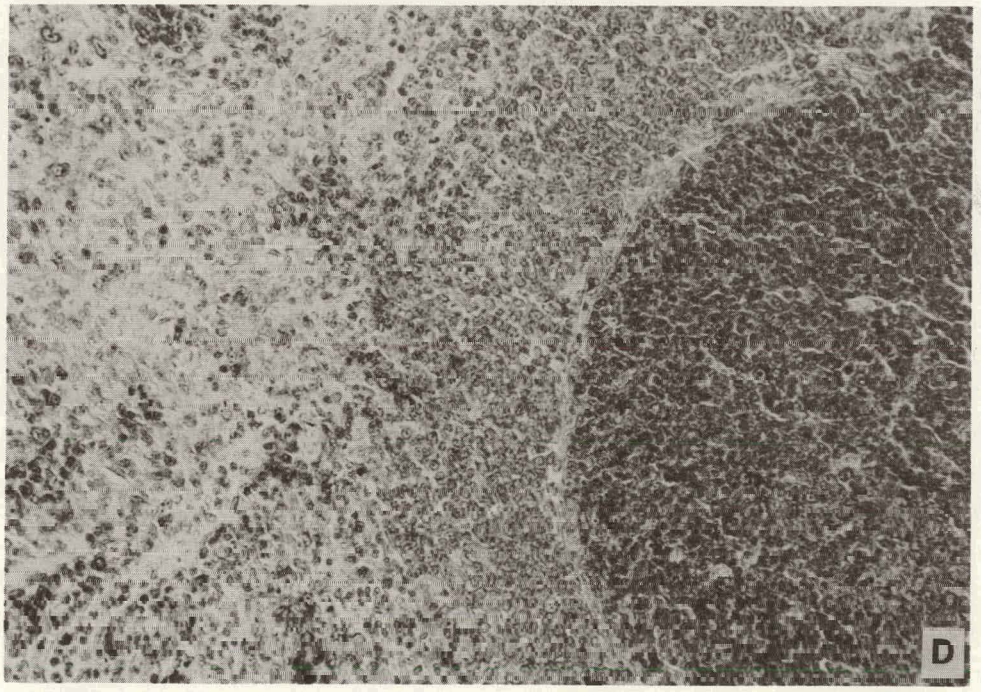


Figure 4. Rat spleen (edge of follicle, marginal zone and red pulp) at different intervals after intravenous injection of a flagellar preparation from S. typhi. Most of the changes are in the red pulp with little significant activation in the white pulp (follicles). Methyl-green pyronine. Magnification X 280. A. Normal rat—note the relative emptiness of the red pulp. B. Four days after injection. The red pulp contains many newly proliferated large cells with pyroninophilic cyto-

C



D



plasm. Mitotic figures are common. Note the similarity of the follicle and the marginal zone to that of the control spleen. C. Six days after injection. The red pulp shows many small dark cells and a great reduction in the large pyroninophilic cells as compared to 2 days earlier. The follicle is not altered. D. Eight days after injection. The red pulp has returned to the preinjection state (4A) except for the presence of an occasional plasma cell.

bone marrow and possibly many other parts of the body (Figure 3b). It is interesting to speculate on their potentialities and their response to a second injection of antigen. There is some evidence that lymphocytes from the blood stream may be potential macrophages.[21,22] Recent cell transfer studies[23,24] suggest that lymphoid cells from a previously immunized rabbit can mature into antibody-forming cells (presumably plasma cells) without apparent mitotic activity. Other studies indicate that the cells from a peritoneal exudate rich in macrophages also have this potentiality.[25,26]

It is likely that some of the cells released during the primary response return from the blood stream to the spleen and settle out in the follicles as well as in the red pulp,[27,28] and some of these cells may then take part in the secondary reaction. The observations of Leduc, Coons, and Connolly[29] indicate that germinal center cells may contain antibody after secondary stimulation. The finding of gamma globulin in germinal centers might be similarly interpreted.[30]

The results of experiments reported by Fagreaus[2] suggest that there may be great mitotic activity during the response to repeated antigenic stimulation. On the other hand, it seems likely from cell transfer studies[24,26] that mitotic activity is not necessary for antibody formation during the secondary response.

RELATION OF OBSERVATIONS TO THE CLONAL SELECTION HYPOTHESIS

In his recent lectures, Burnet[1] has developed the concept, first proposed by him in 1957[31] that the self-replicating unit necessary to explain many of the phenomena of antibody formation is cellular rather than intracellular (enzymatic). Similar suggestions were made independently by Talmage[32] and others[14] in 1957. The crux of the "clonal selection theory" as stated by Burnet,[1] is that "in the animal there exist clones of mesenchymal cells, each carrying immunologically reactive sites corresponding in appropriate complimentary fashion to one (or possibly a small number of) potential antigenic determinants. This provides a population of cells which, when an appropriate stage of development has been reached, are capable of producing the population of globulin molecules which collectively provide the normal antibodies. When an antigen is introduced it will make contact with a cell of the corresponding clone, presumably a lymphocyte, and by so doing stimulate it to produce in one way or another more globulin molecules of the cell's characteristic type. The obvious way of achieving this is to postulate that stimulation initiates proliferation as soon as the cell in question is taken into an appropriate tissue niche—spleen, lymph node, or subacute inflammatory accumulation."

As suggested above, many of the cells that proliferate in response to a primary antigenic stimulus do not mature into plasma cells but instead enter the blood stream[33,17] or lymph stream[34] as small or medium-sized lymphocytes. There is evidence that these cells are able to colonize many tissues including the lymph follicles of the organ in which they were originally formed.[27] This migration provides a large, widely dispersed population of cells with the probable potential capacity to form antibody. The prompt participation of these cells in antibody formation would explain the heightened and more generalized response when antigen is injected for the second time and this helps to support Burnet's clonal selection theory. These observations may also explain why antibody-containing cells are present in the lymph follicles after multiple injections of antigen but not after one injection. If one accepts the numerous observations which indicate that lymphocytes can mature into macrophages (phagocytes),[21,22] then it is apparent

that some of these previously stimulated cells may ingest the antigen after the second injection. Whether lymphocytes as well as macrophages can mature directly into plasma cells is still in doubt (Figure 1), but some recent evidence seems to provide additional support for this concept.[24] Either the prompter processing of the antigen by these "prepared" macrophages or the direct maturation of these cells into plasma cells could explain the greatly shortened lag period in the secondary response. The preservation of clones of lymphocytes as potential antibody-forming cells resulting from previous antigenic stimulation gives a cellular foundation for the anamnestic reaction. Plasma cells are rarely seen in the circulation of mammals and there is general agreement that they do not have the capacity to differentiate into other cell types. Thus it is unlikely that they could carry immunological information from one part of the body to another or that they could function to preserve this information for future accelerated and augmented reactions to a repeated stimulus.

SPECIAL PROBLEMS WITH REFERENCE TO THE CELLULAR REACTION TO ANTIGENIC STIMULATION

Earlier in this discussion, it was stated that a particulate antigen delivered rapidly to the spleen would result in a brief, localized stimulus. The cellular reaction following this type of stimulus would be expected to follow a clear-cut and orderly sequence. The alternative reactions produced by other common methods of immunization may be considered. When a soluble antigen is given intravenously it circulates for a considerable period of time and is only gradually localized in the antibody-forming tissue. This continued circulation is likely to result in a prolonged stimulus to the antibody-forming tissue which may last for several days. Assuming the same sequence of cellular events as that observed in response to a particulate antigen, it is not difficult to visualize the result. There would be a prompt development of antibody-forming cells, followed by their transformation into small lymphocytes, which would be released into the circulation, and hence returned to the red and white pulp presumably within a period of one week. There might also be continuous stimulation during subsequent days by additional antigen from the blood or lymph. Under these conditions one might expect a rather prolonged primary type of cellular response together with a superimposed secondary response. These would be difficult to interpret histologically. The soluble antigen would probably be distributed much more widely among tissues (as well as within a given tissue) than the particulate antigen; thus it would be more difficult to establish a definite histological relationship between antigen localization and cellular response. These and other considerations may help to explain the relatively greater (and later) participation of the follicles of the rabbit spleen following intravenous injection of soluble antigen.[35]

The reaction in regional lymph nodes following subcutaneous antigen injection is probably equally complex. Not only is the antigen delivered to the lymph node over a considerable period of time, but inflammation at the site of injection may contribute "antibody-forming cells" which mature and migrate to the lymph node in time to set up a "secondary" cellular response while the prolonged "primary" response is still in progress. This type of problem may have influenced the results reported by McNeil.[33,36] The reaction of the lymph node or the spleen to a homograft, a tumor, or an infection, may be similar since the cellular response may be prolonged by continuously elaborated antigens. Furthermore, local inflammation may supply previously stimulated cells to the lymph node or spleen, thus superimposing a "secondary" type of reaction upon a "primary" reaction.

An entirely different problem which has received little attention is that of species differences in cellular response. It is not known whether the rat,[14] the fowl,[37] the rabbit,[29,35] the man[6] all have essentially the same cellular reaction or whether they exhibit differing histological responses to the same stimulus. Some observations of the cellular pattern correlated with antibody formation exist in each of these species but they are very difficult to compare because of differences in experimental design, including the antigen used and the tissues studied. Preliminary observations in this laboratory* comparing the histological reaction of the rat spleen and the rabbit spleen to the same particulate bacterial antigenic stimulus suggest some similarities and some differences. The comparison of histological response to the same soluble antigen may prove to be much more informative since the rat fabricates little antibody following injection of any one of many purified proteins commonly used as antigens, even after multiple stimuli.[38]

SUMMARY

The evidence implicating several cell types in the antibody-forming mechanism has been reviewed. Mesenchymal cell interrelationships have been outlined. An effort has been made to indicate how the recorded observations of cellular progressions during "primary" and "secondary" immune reactions fit into this scheme of cell organization. In particular, the histological patterns observed in the rat spleen after a single particulate antigen injection have been compared with those in the spleen and elsewhere after multiple antigenic stimuli. The relation of these observations to Burnet's clonal selection theory is emphasized. The cellular sequence of events is discussed in the light of the possible implications of the physical state of the antigen, the route of injection, the presence of reproducing antigens (bacterial and cellular) and the species studied.

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THE EFFECT OF L-TRIIODOTHYRONINE ON RADIATION SENSITIVITY*

By

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In a previous paper we have briefly discussed some of the agents which have been used to effect a change in the reaction of tissues to radiation.[1] For example, an increase of oxygen tension in a given tissue at the time of irradiation leads to an increase in the radiation effect.[2,3] Similarly it has been shown that the hyperthyroid state hastens mortality in mice subjected to acute lethal doses of total-body irradiation.[4,5] In this connection, it seemed possible that the increased metabolic rate characteristic of the hyperthyroid state might be reflected in an increase in the radiosensitivity of selected local tissues, whether normal or neoplastic.

Experiments were undertaken to assess the effect of x-rays on tumors in experimental animals and clinical patients in which the hyperthyroid condition had been induced by L-triiodothyronine, the potent fraction of the thyroid hormone that acts rapidly at the cellular level.[6] The present report is an extension and confirmation of this work.[1]

EXPERIMENTAL

Three abnormal tissues were studied, two rat tumors and one mouse tumor. The first used was the solid, non-metastasizing, non-systemic, myeloid, chloroleukemia rat tumor, already reported on.[1]

The second tumor was melanoma mouse tumor S-91, derived from the DBA/2 strain mouse from the Jackson Memorial Laboratory, Bar Harbor, Maine, and introduced subcutaneously into the hind leg of an animal of the same strain. These 25-g animals were subjected to daily injections of 30 μg of triiodothyronine for 3 days, and on the fourth day when the metabolism was plus 40 per cent, radiation was administered. Excellent immobilization during irradiation was obtained by the use of a specially-designed lead box and the design was further elaborated so that 15 animals could be treated at the same time on a rotating board. Single doses of 1000 to 4000 r air were used. (Radiation was delivered as follows: 250 KV, H.V.L., 1.5 mm Copper, F. S. D., 58 cm).

The last described method of immobilization and treatment was used on the 250-g rats tested in the third set of experiments. Using a Walker 256, carcinoma-sarcoma, rats were prepared by introducing the tumor into the hind leg. When single doses of 1000 to 4000 r air were used, the higher doses produced complete necrosis of the hind leg in both experimental and control animals. Fractionation was then used as follows: 200 μg of triiodothyronine were given on the first, third, and fifth day, 1000 r air on days two, four, and six. The control group of animals received like doses of radiation on alternate days.

As previously reported, the chloroleukemia disappeared from euthyroid rats when a tumor

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*Summary of a paper delivered at the American Roentgen-Ray Society meeting in Cincinnati, September 1959.

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dose of 1000 rads ± 200 rads was given. In the hyperthyroid rats, this tumor disappeared following a dose of 500 rads ± 200 rads.

In the melanoma-bearing animals, tumor growth could not be halted even at the highest doses used, whether triiodothyronine was used or not. It was noted however, that the animals treated with triiodothyronine lived somewhat longer.

Using fractionated treatment in the Walker 256 tumor-bearing animals, only one of a group of 25 animals survived 4 months without tumor, when treated with x-ray alone. In a like group treated with triiodothyronine and x-ray, 5 of 25 animals survived 4 months without tumor. In an untreated control group of 25 animals, all animals were dead of tumor in 21 days.

CLINICAL

Initially, patients with far advanced cancers able to tolerate an elevated metabolic rate were selected for treatment. Their basal metabolic rates (BMR) were elevated 35 to 40 per cent above normal by oral doses of triiodothyronine beginning with a dose of 200 μg. This dose was increased daily by 50 μg, until the desired BMR was obtained and maintained thus throughout the period of radiation. X ray was given in daily fractionated doses. Toxicity due to triiodothyronine and manifested by a sudden decrease in caloric intake, apathy, lethargy and prostration, was controlled by decreasing the dosage by 100 to 150 μg, when there was an immediate regression of the unfavorable symptoms. In several patients, the drug was stopped abruptly without untoward reaction, and the BMR returned to normal within 24 hours.

Two patients with well-differentiated squamous cell bronchogenic carcinomas having extensive subcutaneous metastases, one of whom had symmetrical supraclavicular metastases, were selected for treatment. After induction of the hypermetabolic state the metastases on one side were treated. At a tumor dose of 1500 rads, there was marked regression and treatment was suspended at 3000 rads, a dose not ordinarily considered carcinocidal. The overlying skin also demonstrated an inordinately early and severe radiation response which regressed rapidly at completion of the treatment. This suggests that the skin reaction was also enhanced to some extent. After the hyperthyroid condition had been allowed to return to normal, the metastases on the opposite side were treated in a similar fashion. At autopsy three months later, the side subjected to the treatment of a combination of drug and x-ray, showed only fibrosis, while the tumor was still present on the side treated by x-rays alone. Similar observations were made in the second patient.

A patient with extensive lung metastases due to melanoma was subjected to the combined drug and x-ray treatment. No response was observed when a calculated tumor dose of 2000 rads was delivered to the lung lesions. Because of the negative results obtained in the experimental animals with melanoma, a higher dose of radiation was not attempted.

Since normal brain tissue is not markedly affected by the hyperthyroid state, two patients with biopsy-proven, well-differentiated, adenocarcinoma brain metastases with unknown primary lesions were treated with the combined therapy. These brain lesions responded dramatically to 3000 to 4000 rads tumor dose, our usual palliative dose. Although this is not ordinarily carcinocidal for this type of tumor, there has been no recurrence of the brain lesion as observed by clinical evaluation and by isotope localization. In both cases, however, the primary lesion very rapidly became manifest, either during the course of the treatment or very shortly thereafter. This observation has been made in a number of other cases, and suggests that only those

patients with inoperable, well-localized tumors considered incurable by conventional radiation therapy should be treated in this way.

In summary, triiodothyronine can be used both experimentally and clinically to produce a qualitative change in the radiosensitivity of certain selected tissues. In the case of melanoma, the administration of L-triiodothyronine did not increase radiation sensitivity to a degree sufficient to control this tumor. The ease with which the metabolic state can be controlled and modified, both experimentally and clinically, by this drug makes it a useful tool. The fact that unradiated lesions have a higher growth rate when a patient is in the hypermetabolic state makes the selection of patients for this method of treatment a matter of great importance. It may also indicate one of the mechanisms of increased radiation sensitivity, since rapidly growing tissue is usually considered to be more radiosensitive.

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THE REGULATION OF IRON ABSORPTION
I. A SEARCH FOR HUMORAL FACTORS*

By

E. Beutler and E. Buttenwieser

Since the demonstration of McCance and Widdowson[1] that the body does not excrete iron selectively, it has been recognized that the regulation of absorption must be the factor that controls the amount of iron in the body. Indeed, it has been demonstrated repeatedly that iron-deficient subjects absorb a larger proportion of a test dose of iron than do normal subjects.

The many studies of iron absorption that have been reported in the past several decades have been reviewed carefully by Josephs.[2] The nature of the mechanism by which the body controls iron absorption has never been elucidated experimentally. Granick,[3] as a result of his classic studies of ferritin, suggested that anemic subjects absorbed more iron than normal, because the anoxia of the bowel resulting from anemia tended to promote dissociation of iron from ferritin, and thus made more apoferritin available to receive iron from the lumen of the bowel. However, the role of ferritin in iron transport has never been demonstrated clearly; its participation in iron transport was suggested on the basis of now repeatedly discredited[4,5] observations concerning a "mucosal block" mechanism. It has been suggested[6] that ferritin in the bowel may represent merely a storage form, such as occurs in other tissues. Furthermore, the clinical studies of Bothwell and his associates[7] suggest that in anemias associated with decreased bone marrow activity, iron absorption is not increased, but may even be decreased.

Among theories proposed to explain the increased iron absorption that occurs in iron deficiency and under certain other circumstances, are two very plausible suggestions by Bothwell and associates,[7] and by Gillman and Hathorn.[8] Gillman has suggested that the absorption of iron might be controlled by the level of certain iron enzymes in cells. We now realize that many iron enzymes are in a dynamic state in tissue cells[9] and that their levels are influenced by a great many pathologic states, including iron deficiency.[10-12] Bothwell and associates[7] have emphasized the remarkable correlation between erythroid marrow activity and iron absorption and the need for an explanation of the mechanism whereby the needs of the marrow are felt by the mucosa.

In the present investigation, a search has been made for a humoral factor that might provide such an explanation.

MATERIALS AND METHODS

A. Recipient animals—measurement of iron absorption:

1. Mice—Fasting, CF No. 1 mice, weighing 20 to 25 g, were used as experimental animals. Iron absorption was measured by total-body counting immediately and 2 to 4 days after the intragastric administration of a 5-ml solution of Fe^{59} as $FeCl_3$, as described by Krantz and associates.[13]

*This report is based on a paper appearing in J. Lab. Clin. Med., 55:274, 1960.

Table 1

THE EFFECT OF PLASMA ON IRON ABSORPTION OF IRON-DEFICIENT AND NORMAL RATS

Experiment no.	Material injected	Route of injection	Time of injection relative to the intragastric administration of iron	Recipient animal	Number of animals	Amount of iron given intragastrically	Average per cent absorbed (±1 standard error of the mean when 5 or more animals per group. Otherwise individual values are given.)
1	4 ml 0.9% NaCl	Subcutaneous	- 30 min	Normal rat	2	1 mg	8.0, 7.6
	No injection			Normal rat	2	1 mg	4.5, 6.0
	4 ml plasma from normal donor rat	Intravenous	- 30 min	Normal rat	1	1 mg	4.1
		Intraperitoneal	- 30 min	Normal rat	1	1 mg	6.0
		Subcutaneous	- 30 min	Normal rat	2	1 mg	6.0, 4.0
	4 ml plasma from iron-deficient donor rat	Intraperitoneal	- 30 min	Normal rat	1	1 mg	5.8
		Subcutaneous	- 30 min	Normal rat	2	1 mg	6.7, 4.5
2	4 ml 0.9% NaCl	Intravenous	- 1 hr	Iron deficient rat	3	1 mg	23.3 (24.9, 20.2, 24.7)
	4 ml normal rat plasma	Intravenous	- 1 hr	Iron deficient rat	3	1 mg	25.3 (34.7, 20.1, 21.1)
3	0.4 ml 0.9% NaCl	Intravenous	- 1 hr	Normal mouse	6	0.1 mg	10.4 ± 2.3
	No injection			Normal mouse	10	0.1 mg	7.9 ± 0.8
	0.4 ml normal rat plasma	Intravenous	- 1 hr	Normal mouse	7	0.1 mg	7.3 ± 1.0
	0.4 ml iron-deficient rat plasma	Intravenous	- 1 hr	Normal mouse	5	0.1 mg	9.2 ± 3.0
4	0.5 ml 0.9% NaCl	Intravenous	- 1 day + 1/2 hr	Normal mouse	5	0.1 mg	6.8 ± 1.8
	No injection			Normal mouse	8	0.1 mg	6.0 ± 1.6
	0.5 ml normal rat plasma	Intravenous	- 1 day + 1/2 hr	Normal mouse	7	0.1 mg	7.8 ± 1.5
	0.5 ml iron-deficient rat plasma	Intravenous	- 1 day + 1/2 hr	Normal mouse	6	0.1 mg	5.6 ± 0.9

Table 1 (continued)

5	0.4 ml 0.9% NaCl x 2	Intravenous	- 1 day - 1 hr	Normal mouse	9	0.01 mg	20.2 ± 2.4
	No injection			Normal mouse	9	0.01 mg	20.4 ± 3.7
	0.4 ml normal rat plasma x 2	Intravenous	- 1 day - 1 hr	Normal mouse	9	0.01 mg	24.8 ± 4.0
	0.4 ml iron-deficient rat plasma x 2	Intravenous	- 1 day - 1 hr	Normal mouse	10	0.01 mg	27.3 ± 3.0
6	0.5 ml 0.9% NaCl x 5	Subcutaneous	- 4 days - 3 days - 2 days - 1 day - 1 hr	Normal mouse	6	0.1 mg	4.0 ± 0.3
	No injection			Normal mouse	6	0.1 mg	4.1 ± 0.5
	0.5 ml normal rat plasma x 5	Subcutaneous	- 4 days - 3 days - 2 days - 1 day - 1 hr	Normal mouse	6	0.1 mg	3.4 ± 0.5
	0.5 ml iron-deficient rat plasma x 5	Subcutaneous	- 4 days - 3 days - 2 days - 1 day - 1 hr	Normal mouse	6	0.1 mg	6.2 ± 1.0
7	0.5 ml 0.9% NaCl x 5	Subcutaneous	- 4 days - 3 days - 2 days - 1 day - 1 hr	Normal mouse	5	0.1 mg	7.2 ± 1.1
	No injection			Normal mouse	5	0.1 mg	9.6 ± 0.9
	0.5 ml normal rat plasma x 5	Subcutaneous	- 4 days - 3 days - 2 days - 1 day - 1 hr	Normal mouse	5	0.1 mg	10.9 ± 1.2
	0.5 ml iron-deficient rat plasma x 5	Subcutaneous	- 4 days - 3 days - 2 days - 1 day - 1 hr	Normal mouse	5	0.1 mg	11.1 ± 0.6

Table 2

THE EFFECT OF ORGAN EXTRACTS ON IRON ABSORPTION

Experiment no.	Material injected	Route of injection	Time of injection relative to the intragastric administration of iron	Recipient animal	Number of animals	Amount of iron given intragastrically	Average per cent absorbed (± 1 standard error of the mean when 5 or more animals per group. Otherwise individual values are given.)
8	0.5 ml 0.9% NaCl x 2	Intravenous	- 1 day - 1 hr	Normal mouse	3	0.1 mg	7.4 (3.1, 11.0, 8.0)
		Intraperitoneal	- 1 day - 1 hr	Normal mouse	2	0.1 mg	1.9 (2.3, 1.5)
	No injection			Normal mouse	4	0.1 mg	3.11 (3.4, 4.5, 2.6, 2.0)
	Bone marrow from iron-deficient rat. 0.4 ml 10% homogenate x 2	Intravenous	- 1 day - 1 hr	Normal mouse	2	0.1 mg	6.8 (9.4, 4.1)
		Intraperitoneal	- 1 day - 1 day	Normal mouse	1	0.1 mg	5.1
	Spleen from iron-deficient rat. 0.5 ml 10% homogenate x 2	Intravenous	- 1 day - 1 hr	Normal mouse	3	0.1 mg	5.6 (8.0, 3.2, 5.6)
		Intraperitoneal	- 1 day - 1 hr	Normal mouse	2	0.1 mg	2.2 (2.4, 2.2)
	Liver from iron-deficient rat. 0.5 ml 10% homogenate x 2	Intravenous	- 1 day - 1 hr	Normal mouse	2	0.1 mg	5.8 (6.8, 4.8)
		Intraperitoneal	- 1 day - 1 hr	Normal mouse	2	0.1 mg	2.3 (1.6, 3.1)
	Kidney from iron-deficient rat. 0.5 ml 10% homogenate x 2	Intraperitoneal	- 1 day - 1 hr	Normal mouse	2	0.1 mg	7.1 (5.5, 8.8)
9	0.5 ml 0.9% NaCl x 3	Intraperitoneal	- 2 days - 1 day - 1 hr	Normal mouse	6	0.1 mg	4.1 ± 0.7
	No injection			Normal mouse	6	0.1 mg	4.9 ± 0.9
	Kidney from normal rat. 0.5 ml 10% suspension x 3	Intraperitoneal	- 2 days - 1 day - 1 hr	Normal mouse	6	0.1 mg	5.5 ± 0.9
	Kidney from iron-deficient rat. 0.5 ml 10% suspension x 3	Intraperitoneal	- 2 days - 1 day - 1 hr	Normal mouse	6	0.1 mg	3.3 ± 0.5

2. Rats—Fasting rats were anesthetized by intraperitoneal injection of pentabarbital. One ml of the same iron solution was introduced into the stomach of each animal, using a small bore, flexible rubber catheter. After one-half hour, the rat was placed in a wide-mouth bottle, 10 cm in diameter, and radioactivity counted over a lead-shielded, thallium-activated sodium iodide crystal, cylindrical in shape, 1-1/2" x 2". Preliminary studies indicated that if the rat remained curled at the bottom of the bottle, its exact position did not appreciably influence the number of counts obtained. Equivalent quantities of Fe^{59} administered intravenously to other rats gave counts essentially identical with equal doses given intragastrically. The second total-body count was carried out on anesthetized animals 2 to 4 days after the intragastric dose had been given.

B. Donor animals:

All tissues and plasma used were obtained from female, Holtzman strain rats. Iron-deficient and control rats were produced by placing weanling rats on iron-poor diet, the control rats being given 25 mg of iron as iron-dextran* intramuscularly.[12] In experiment 6, control plasma was obtained freshly from stock rats by drawing blood by cardiac puncture into a heparinized-syringe and removing red cells and buffy coat by centrifuging. All plasma used was fresh except that in experiments 1 and 2, which had been stored at 4° C overnight. Ten per cent tissue homogenates in 0.9 per cent saline solution were prepared from freshly killed rats and centrifuged at 35 g for 5 minutes. The supernatant was used for injection. In experiment 9, kidney, previously frozen for 1 and 2 days, was used for injections given at time -1 day and -1 hour.

EXPERIMENTS AND RESULTS

In experiments 1, and 3 through 7 summarized in Table 1, attempts were made to stimulate iron absorption in mice and rats by the administration of plasma from iron-deficient animals. Various routes of administration and dosage schedules were employed. The dosage of iron given intragastrically was also varied. No consistent stimulation of iron absorption could be demonstrated.

In experiment 2 (Table 1) the possibility that the normal regulatory mechanism was an inhibitory one was considered and an attempt was made to demonstrate such an inhibitory substance in normal rat plasma. No such inhibitory influence on iron absorption in iron-deficient rats could be detected. This experiment demonstrated also that the quantity of iron absorbed by iron-deficient rats was 3 to 6 times that absorbed by normal animals.

In experiment 8 (Table 2) crude extracts of liver, kidney, spleen, and bone marrow from iron-deficient rats were injected intravenously and intraperitoneally into mice. Kidney homogenates injected intravenously immediately killed all the mice. This was also found to be true of kidney homogenate from normal animals. Saline solution injected intravenously appeared to enhance the absorption of iron slightly. None of the organ homogenates used, with the possible exception of kidney homogenate injected intraperitoneally, increased iron absorption beyond the normal range. Further study of the effect of kidney homogenate injected intraperitoneally in a larger number of animals (experiment 9, Table 2), however, failed to show any significant effect on iron absorption.

*Supplied through the courtesy of Lakeside Laboratories, Milwaukee, Wisconsin.

DISCUSSION

Moore,[14] and Bothwell and associates[7,15] have pointed out the striking parallel between bone marrow activity and absorption of iron. This correlation has been confirmed recently by Krantz and co-workers[13] and by Field and associates.[16]

There are several possible explanations for these findings. First, the conditions that give rise to increased marrow activity, viz., anemia, anoxia, and cobalt administration may also affect the mucosal cell directly and thus give rise to increased iron absorption. Moore,[14] however, has observed that iron-depleted dogs absorb iron as well under atmospheric conditions as in a 70 per cent oxygen atmosphere, and that transfusion of iron-deficient women to normal level failed to affect appreciably the amount of iron absorbed. These observations, with those of Bothwell showing decreased iron absorption in severely anemic subjects with inactive bone marrows, suggest that anemia and anoxia are not primary controlling factors.

Secondly, it is possible that both iron absorption and marrow activity are stimulated by the same humoral factor, namely: erythropoietin. However, Krantz and co-workers[13] have shown that plasma and plasma extracts rich in erythropoietic activity fail to stimulate iron absorption significantly in polycythemic mice. A third possibility is under investigation in the current study, namely: that the bone marrow makes its needs felt in the bowel by producing a humoral substance that stimulates iron absorption. Failure to demonstrate the presence of a hormone does not necessarily prove that one is not present. However, our inability to demonstrate any activity in plasma and hematopoietic organs of animals capable of absorbing greatly increased amounts of iron casts some doubt on the hypothesis that the marrow exerts a humoral control over the mucosal absorption of iron, and should lead to more serious reconsideration of an alternate hypothesis, such as that of Gillman and Hathorn.[8] Further observations and critical evaluation of the data already available are needed if we are to solve this challenging problem in physiology.

SUMMARY

No humoral stimulation or depression of iron absorption could be demonstrated under any of the following conditions: 1) the injection of plasma from iron-deficient rats into normal mice and normal rats; 2) the injection of plasma from normal rats into iron-deficient rats; 3) the injection of crude extracts of rat liver, spleen, kidney, and bone marrow into normal rats.

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STUDIES ON ANTIBODY PRODUCTION BY SPLEEN EXPLANTS MAINTAINED IN VITRO*†

By

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While a considerable body of work has accumulated on various aspects of the use of tissue culture in the study of antibody synthesis,[1-7] the evidence presented to date in support of the in vitro initiation and continuation of antibody production is still inconclusive.

On the other hand, most of the studies involving the culture of the spleen or other organs removed from animals sensitized with one or more antigen injections, report a net synthesis of antibody.[8-16]

In rats, antibody response to a particulate antigen is largely confined to a series of cellular changes in the spleen beginning approximately 48 hours after antigen administration.[17]

The present work was undertaken to study these changes between 48 and 144 hours (2-6 days) after a single intravenous injection of a bacterial antigen. It was hoped that by use of a tissue culture system the histologic changes could be further characterized and correlated with some of the biological reactions, particularly those involving the nucleic acids, in the course of antibody formation.

MATERIALS AND METHODS

Young adult male albino rats of the Sprague-Dawley and Holtzmann strains were used throughout the study. They were immunized by a single dose of formalinized, H strain, Salmonella typhi vaccine†† given in the tail vein 48-72 hours before the culture was begun. On the morning of culture, the rats were bled out from the heart under ether anesthesia and a sample of serum taken for antibody titers. The spleen was removed aseptically, a sample taken for histologic study, and the remainder rapidly weighed and minced into 1-2 mm^3 fragments with sterile cuticle scissors. The chief requirements sought for the tissue culture system were: a) the maintenance of small tissue fragments on an artificial support to avoid laborious plasma clot or similar preparations; b) the direct "harvest" of the tissues without their falling into the medium; c) the ability to stir the medium at will and for prolonged periods.

A 55-mm Petri dish was provided with 3 equidistant indentations about 9 mm from the bottom which formed points of support for a ring-mounted sterile filter.‡‡ A magnetic bar*** was

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‡‡Type SS filters with 3-μ pore size, mounted on nylon rings and sterilized by gamma radiation. Millipore Filter Corporation, Watertown, Massachusetts.

***Teflon covered magnetic bars. Made to order by Arthur S. LaPine and Co., 6001 S. Knox Avenue, Chicago, Illinois.

placed on the bottom of the dish and the dish was partially filled with medium, care being taken to wet the filter uniformly. Tissue fragments were placed on the filter and medium added until it was covered to a depth of 1 mm. Ten dishes so prepared were set in a circular aluminum tray and covered by a mold culture flask the bottom of which had been removed. This was provided with a rubber stopper pierced by glass tubing through which a mixture of 95 per cent O_2 and 5 per cent CO_2 humidified by bubbling through water and filtered through sterile glass wool, was introduced into the culture flask. Aeration was carried out for 2 or 3 hours at a time, two or three times a day, except for experiments in which it was continuous. Sterilization of the assembled tray, cover, and glass filter was by autoclave.

The aluminum tray containing the dishes was placed on a support holding 10 electric motors, one to each dish. The free ends of the motor shafts were provided with small magnets each lying directly beneath a dish. Motors were separately controlled so that individual dishes could be agitated at will. The apparatus is illustrated in Figure 1.

Eagle's medium[18] supplemented with 100 mg per cent of glucose and normal rat serum in varying proportions from 30 to 50 per cent, was used for incubation. Penicillin and streptomycin (50 units or 50 γ/ml respectively) were added to the medium. Phenol red (0.05 g/L) was used as an indicator. Finally, to each dish was added 1 μc of glycine-1-C^{14} dissolved in 0.1 ml of sterile physiologic saline (0.85 per cent NaCl). The spleen samples were cultured in this medium for 48 to 72 hours.

At the end of the incubation period, the tissues were removed from the filters and frozen, a sample having first been taken for histologic examination. Antibody synthesis was measured as follows. Cells were removed from the tissue culture fluid by centrifuging at 2500 rpm. The supernatant was carefully decanted and added to a "thick" suspension of bacteria equivalent to about 5 ml of the suspension used for immunization. This preparation was then incubated for 30 minutes at room temperature, a treatment considered sufficient to remove all the antibody present by specific reaction with the antigen. After incubation the organisms were centrifuged off, washed with glycine until the washings were free from radioactivity (3 times), plated on steel planchets and counted in a windowless, gas-flow Geiger-Mueller-type counter. All counts were corrected for self absorption.

A 1.0-ml sample of the fluid was occasionally taken before processing, and frozen for agglutinin titration, the procedures for which have been described elsewhere.[19]

Tissue samples were fixed in Carnoy's solution, embedded in paraffin, and stained with methyl green-pyronine.[20]

Control experiments were run on tissue from uninjected animals cultured as above.

Further, spleens of immunized rats, homogenized and extracted with saline, were tested for antibody as measured by agglutinin up to the third day after antigen injection.

RESULTS

At the time of sacrifice, antibody was usually absent from the serum. After the tissue culture period, a positive titer was observed in the medium whenever agglutinin titrations were performed. Since this was usually comparable to titers observed during in vivo antibody studies, agglutinin titrations were not carried out in all experiments. The criterion for synthesis of antibody in this study was the extent of specific uptake by the typhoid organisms (antigen) of radioactivity (antibody) from the culture medium. This was always low in the culture medium from

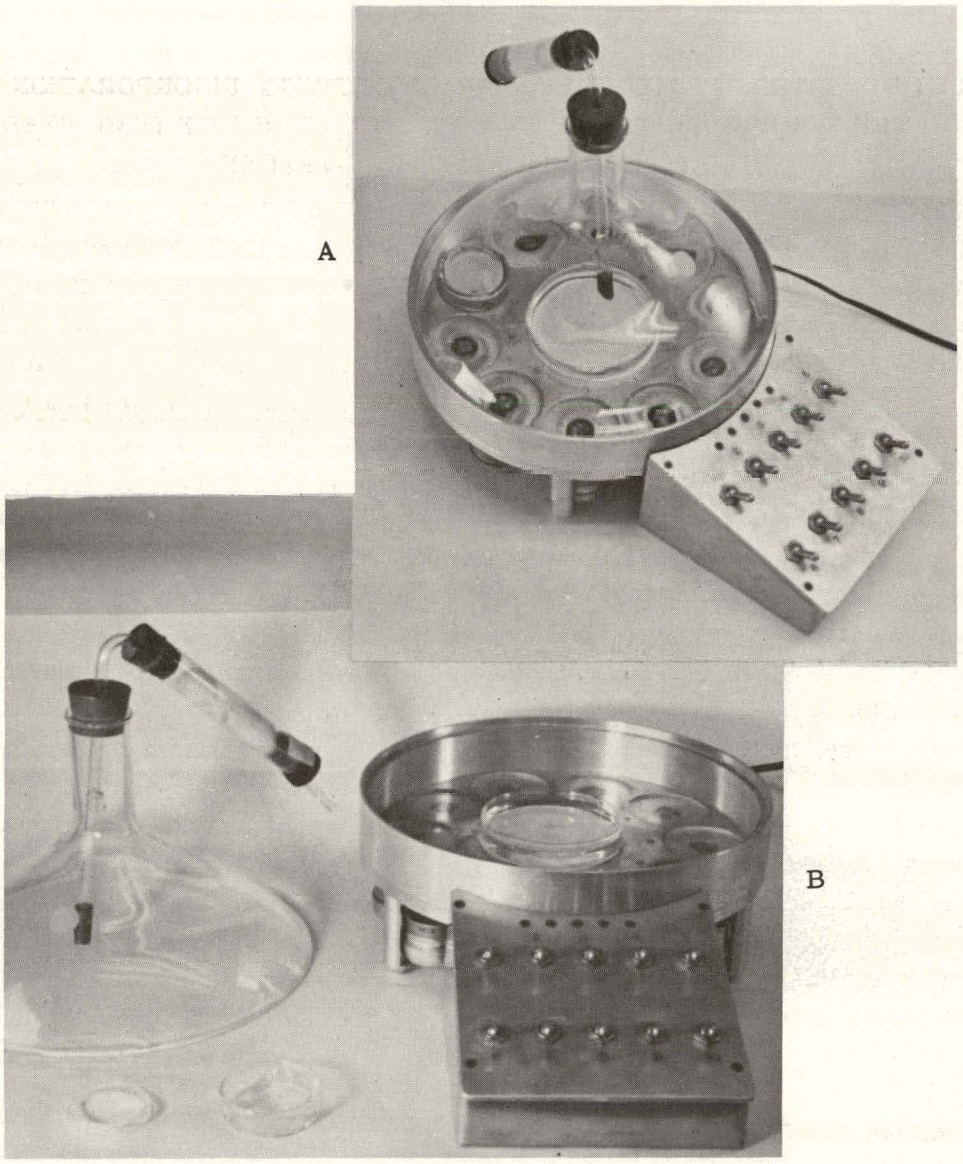


Figure 1. Incubation apparatus. A. Assembled and ready for use. B. Disassembled to show the various parts.

non-injected animals. Since this might represent in part nonspecific, cross-reacting protein material[21] and in part nonspecific adsorbed radioactive glycine, the results are expressed as the ratio of counts per minute in antigen-bound material from the culture medium of injected rat spleen, to the counts obtained from the medium of the non-injected controls. The latter value being equal to 1, the ratio was always greater than 2 and might be as high as 10.6 (Table 1, experiment 7).

Figure 2 summarizes the results from 9 preliminary experiments. Each average figure is calculated from values obtained from the culture medium of at least 4 different animals. Table 1 illustrates the results obtained from radioactivity counts and agglutinin titration. As there always seemed to be satisfactory agreement between these two measures of antibody synthesis

Table 1

AGGLUTININ TITERS IN MEDIUM AND RADIOACTIVITY INCORPORATION INTO ANTIGEN BOUND MATERIAL IN TISSUE CULTURES OF IMMUNIZED AND NON-IMMUNIZED RAT SPLEEN

	Exp. no.	Counts/minute in antigen bound material		Agglutinin titer	
		Injected	Control	Injected	Control
48 + 48	1	117 159	39	70 70	0
	2	324 427	171	x	
48 + 72	1	248 262	74	70 150	0
	2	421 407	107	x	
72 + 48	3	3881 6198	938	x	
	4	2612 3194	929	x	
	5	4231 2113	746	60 30	0
72 + 72	6	158	40		
	7	246 141	23	x	
	8	1229 1434 1266	233	x	
	9	860	368	x	

[x]Titration not done.

when both were performed, agglutinin titration was not invariably carried out.

Comparison was made of histologic preparations of the spleens of injected and non-injected rats, fixed at the time of sacrifice and after different periods of culture. As shown in Figure 3 the cells described by Wissler et al.[17] as antibody forming cells could always be detected in the culture from injected animals. When these were compared to the preparations made from injected spleens before culture, it was clear that definite differentiation had taken place, as witnessed

by the more advanced stage of maturation of the cultured cells judged by appearance of nucleus and cytoplasm. The increase in cytoplasmic pyroninophilia and the smaller more chromatin-rich nucleus are considered to be particularly significant. No such cells were present in preparations from non-injected animals. Mitotic figures were never observed. Technical difficulties prevented nucleic acid extraction and analysis.

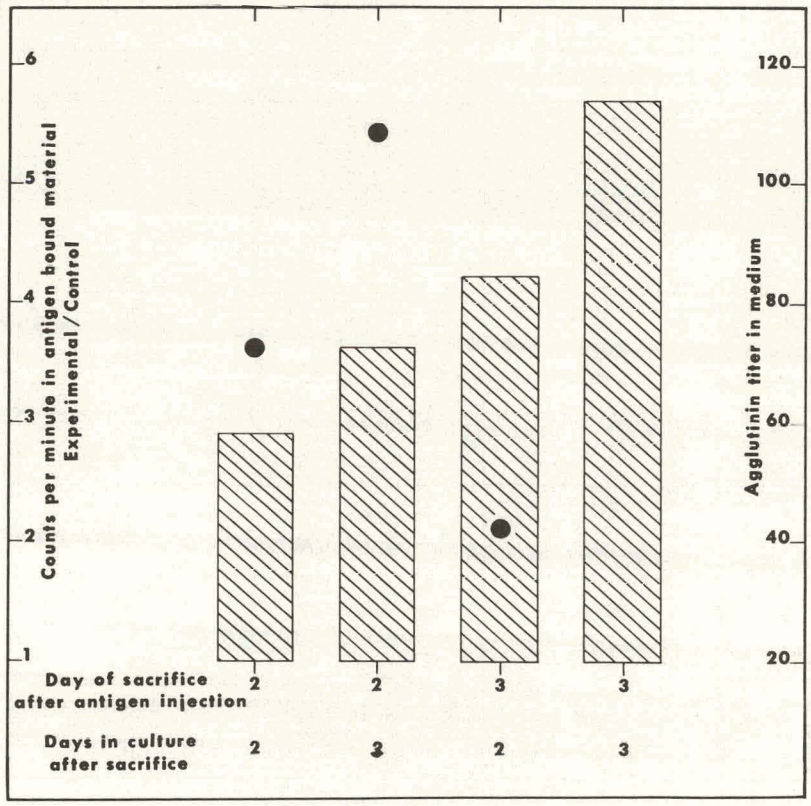


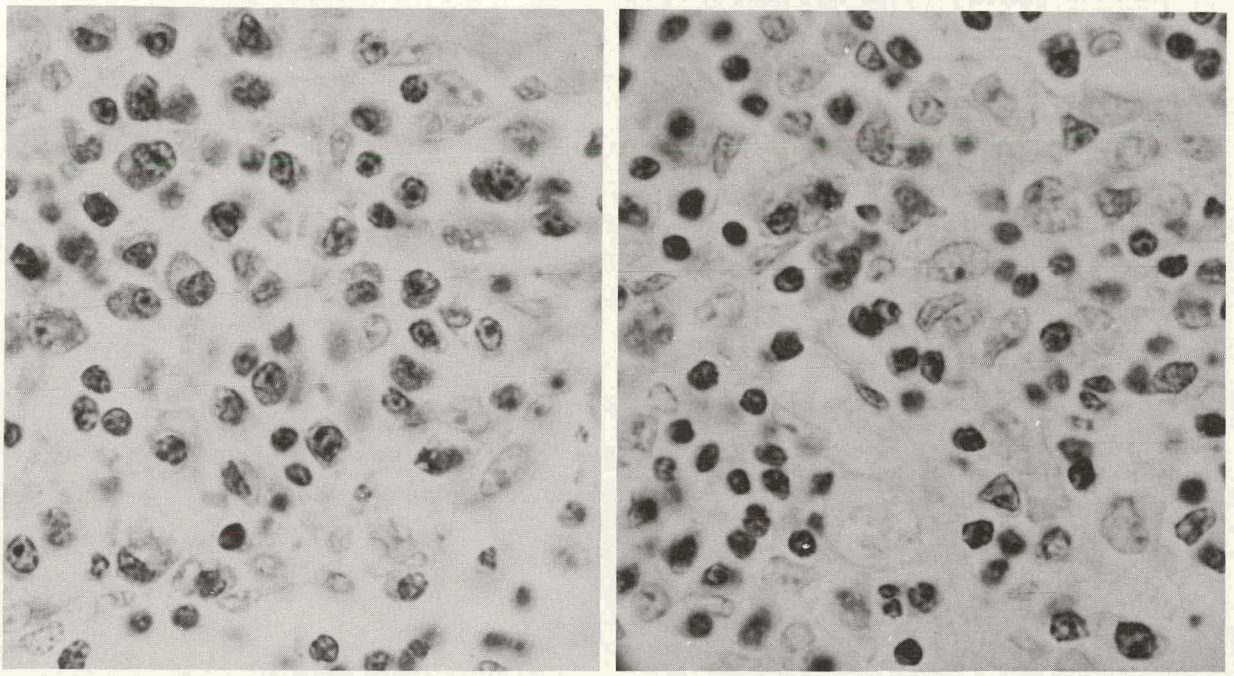
Figure 2. Incorporation of radioactive glycine into antigen bound material (cpm in total tissue culture medium). The black dots represent agglutinin titer levels. No titration was done in the fourth group of experiments.

DISCUSSION

A net synthesis of antibody has been observed when spleen fragments of immunized rats removed 2-3 days after immunization were maintained in a tissue culture system for 2 to 3 days. The synthesis was measured by the net uptake of a radioactive amino acid by material that binds specifically to the antigen, and by the agglutinin titers in the medium which rose to levels comparable to those observed in vivo. These observations suggest that the synthesis of antibody initiated in vivo can proceed in vitro and that antibody can be produced by tissue fragments which contained none at the time of explant.

The discrepancy between the levels of antibody detected by agglutinin titer as compared to the uptake of radioactive material as shown in the third group of experiments (Table 1, 3 to 5) has two possible explanations. One is that the final antibody titer results from a combination of

the metabolic processes of antibody protein, and that labeled nonspecific antigen-binding substances are present which attach to the antigen. On the other hand, labeling in this series may be more efficient so that lower amounts of antibody may have a higher label.



A B

Figure 3. A) Spleen fragment from an injected animal cultured for 2 days. Note the numerous large basophilic cells (antibody forming cells). B) Spleen fragment from a non-injected rat cultured for 2 days. Only lymphocytes and a few fibroblasts are present.

The cell types appearing during the maturation which proceeds during culture resemble those in vivo during the antibody formation. The early stages of antibody production in vivo are characterized by rapid cell multiplication and the cells produced in response to the antigenic stimulus appear to produce antigen while undergoing differentiation. Mitoses were not observed in the tissue culture system and the antibody production is therefore carried out presumably in the course of development of those cells which have divided immediately prior to the initiation of the culture. It is also possible that cultures derived from spleens removed soon after immunization (say 48 hours) will have relatively fewer cells immediately derived from mitoses and so produce less antibody than those removed later. The fact that the addition of antigen to a tissue culture system in which mitosis is not very active fails to initiate antibody production may be explained in this manner.

Studies of antibody synthesis by cells transferred from a stimulated donor to an irradiated recipient[22] appear to confirm this hypothesis. In these cells, mitotic activity is low after transfer and antibody levels are below those expected in normal donors.

It might be postulated that only under conditions favoring cell division could the addition of antigen to a tissue culture system result in antibody production.

Finally, it seems unlikely that the antibody present in the system at the end of the experiment is "released" from the cells rather than "synthesized" by them. The fact that no antibody can be detected in the tissues before culturing, and that the antibody forming cells are observed to mature during the process furnishes indirect evidence that net synthesis accounts for the increase in titer that occurs.

SUMMARY

A maintenance tissue culture system has been designed for the study of the spleen of rats given one intravenous dose of a particulate antigen. Antigen was synthesized as evidenced by a rise in antibody content in the tissue culture medium and incorporation of a radioactive amino acid by material specifically bound to antigen. This synthesis was accompanied by cellular differentiation similar to that described in vivo during antibody synthesis but mitoses were not observed.

ACKNOWLEDGMENTS

The authors wish to thank Drs. R. W. Wissler, F. W. Fitch, and A. G. Johnson for their very valuable criticism during the course of the experiments reported in this paper.

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THE METABOLISM OF PROGESTERONE AND ITS RELATED COMPOUNDS IN HUMAN PREGNANCY*†

By

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Prior to the availability of isotopically labeled steroids and their precursors, little was known about the metabolism of progesterone in the human. No more than 10 to 30 per cent of a sizable amount of progesterone administered to a patient could be accounted for as urinary metabolic end products such as pregnanediol and related compounds. Virtually nothing was known about the fate of the remaining 70 to 90 per cent of the administered dose, its absorption from the injection site, its distribution in blood plasma and tissue, and its possible routes of excretion other than the urine.

It was the pioneer work of Konrad Bloch[1] that opened the door to a new and dynamic approach to the study of steroid hormone metabolism in human reproduction. In 1945, he demonstrated the conversion of cholesterol labeled with deuterium, the stable isotope of hydrogen, to urinary pregnane-3(α), 20(α)-diol in a pregnant woman (Figure 1). The result of this first trac-

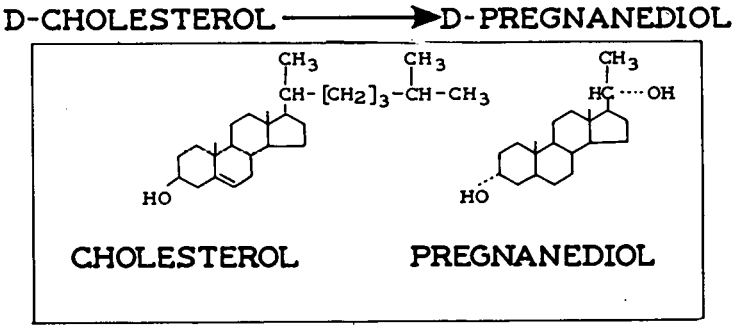

Figure 1. Konrad Bloch's classic experiment (1945) demonstrating the conversion of tagged cholesterol to tagged pregnanediol.

er study in endocrinology in the human provided strong evidence that cholesterol was an important precursor of steroid hormones, a concept that was later substantiated by numerous in vivo and in vitro studies. During recent years, we have used precursors and steroid compounds labeled with unstable (radioactive) isotopes such as tritium and carbon 14 to study the synthesis and metabolism of the key pregnancy hormone, progesterone, in the reproductive cycle and human pregnancy.[2-5]

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The present report is concerned with the investigation of the metabolic fate of tagged progesterone and 17(α)-hydroxyprogesterone caproate administered to pregnant women. The latter compound has recently attracted the interests of clinicians and biochemists because of its interesting biologic properties. Unesterified 17(α)-hydroxyprogesterone has little or no progestational action. However, this minimal activity is magnified to an unexpected degree by the esterification of this steroid with caproic acid to produce 17(α)-hydroxyprogesterone-17-n-caproate, first reported by Karl Junkmann in 1954.[6,7] Moreover, the esterified compound possesses the unique property of markedly prolonged progestational action following its intramuscular administration.[8,9] However, the search for urinary metabolic end products recognized as metabolites of the free (unesterified) 17(α)-hydroxyprogesterone or of progesterone did not disclose any appreciable increase in the excretion of pregnanediol, pregnanetriol, etiocholanolone, and related compounds. It was thought important, therefore, to study the metabolic fate of radioactive 17(α)-hydroxyprogesterone caproate administered to pregnant women, and, more especially, to compare it with that of tagged progesterone.

METHODS

The following carbon 14-labeled compounds were used in these studies (Figure 2): (1) progesterone labeled in the steroid ring at carbon atom 4, (2) progesterone tagged at carbon atom 21 of the side chain, and (3) 17(α)-hydroxyprogesterone caproate labeled at carbon position 4. These compounds were purified by chromatographic technics and repeated crystallization. The steroids were administered to a group of nonpregnant and pregnant patients. Cancer of the cervix, cardiac disease, multiple sclerosis, cancer of the breast, and other serious maternal complications made therapeutic interruptions of pregnancies necessary. The amounts of radioactivity administered were approved by the University of Chicago Clinics' General Authorization for Human Use and the Clinics' Radioisotope Committee.

Samples of tissues, feces, urine, and blood plasma were oxidized to carbon dioxide in a vacuum combustion line and counted in an ionization chamber with a vibrating reed electrometer, according to the method of Brownell and Lockhart.[10] Unconjugated and conjugated steroids were obtained from the plasma by the method of Bradlow and Gallagher.[11] Samples of expired air were collected in polyethylene bags for periods of 5 to 10 minutes. These samples were transferred to a gas collection apparatus and measured. The carbon dioxide was trapped in aqueous sodium hydroxide. The addition of acid liberated the carbon dioxide from an aliquot of the sodium hydroxide which was measured and transferred to an ionization chamber.

The methods used for the isolation, identification, purification, and radioassay of steroid compounds excreted in the urine are described in detail elsewhere.[12,13]

RADIOACTIVITY IN URINE, FECES, EXPIRED AIR, AND TISSUE

Progesterone. The early experiments of Venning and Browne[14] demonstrated that the kidneys are important excretory organs for progesterone metabolites such as pregnanediol and pregnanolone. They reported the following interesting observation. In the presence of active sources of progesterone (corpus luteum, placenta), pregnanediol excretion accounted for 20 to 40 per cent of an administered dose of progesterone, whereas less than 15 per cent of the injected steroids was excreted as urinary pregnanediol in patients with amenorrhea, during the preovulatory phase of the menstrual cycle, or following bilateral removal of the ovaries. More-

over, Guterman[15] postulated that the percentage of progesterone converted to pregnanediol is higher in viable than in nonviable early pregnancies, that were subsequently terminated by spontaneous abortion. Thus, the conversion rate of progesterone to pregnanediol seemed to serve as a useful guide for the prognosis of threatened abortion.

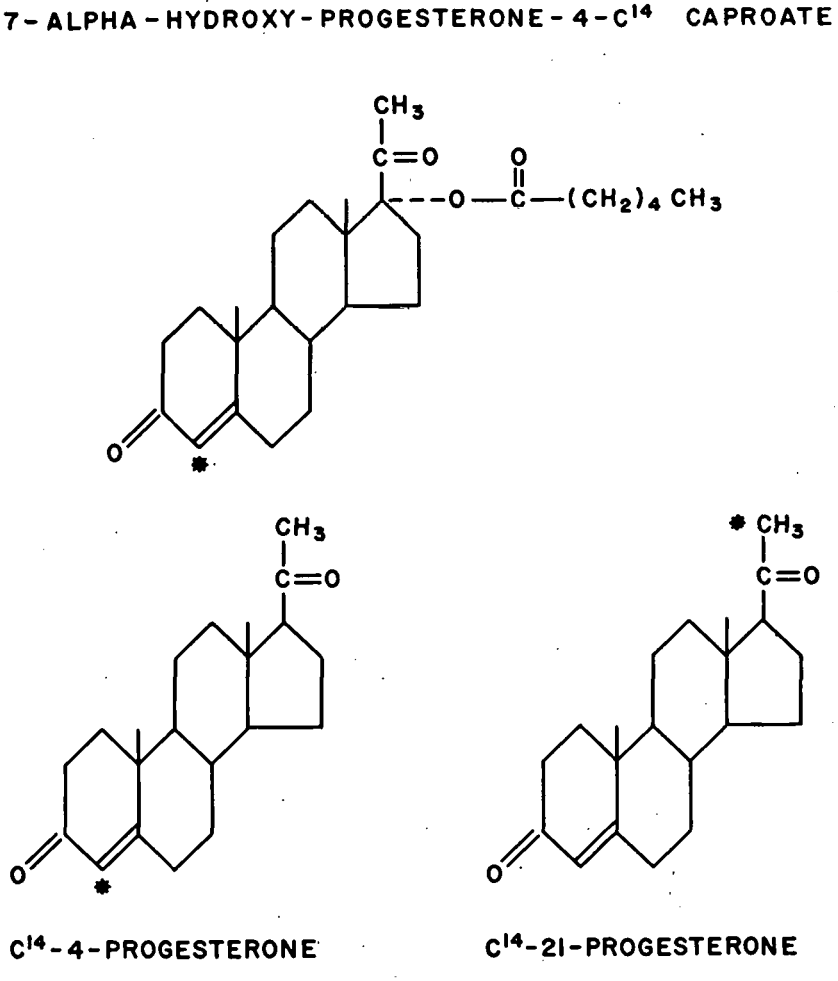

Figure 2. Carbon-14 labeled progestins used in the study.

Following the intramuscular injection of progesterone-4-C^{14}, we studied the recovery of radioactivity in the urine of three groups of patients: (1) 11 pregnant patients scheduled for therapeutic abortion, (2) 5 patients following interruption of pregnancy, and (3) 6 nonpregnant patients. The pregnant patients excreted an average of 39.6 per cent of the administered dose during a period of 6 to 8 days following the injection (Figure 3). There was no relationship between the amounts excreted and the stage of pregnancy or the clinical condition that had made the interruption of pregnancy necessary. Five of these patients received a second single intramuscular injection of progesterone-4-C^{14} 5 to 9 days following surgery. As shown in Figure 3, there was no appreciable difference between the average urinary output of radioactivity before and after termination of early pregnancy (39.6 and 33.5 per cent, respectively). In only one individual was a decrease in the urinary recovery noted following abortion. Six nonpregnant pa-

tients excreted an average of 34.1 per cent of the original radioactivity into the urine, again indicating no significant difference between the result obtained in this group and that obtained in the two other groups of patients. The total urinary output (50 per cent) of radioactivity in 1 patient with missed abortion was within the range observed in normal early pregnancy.

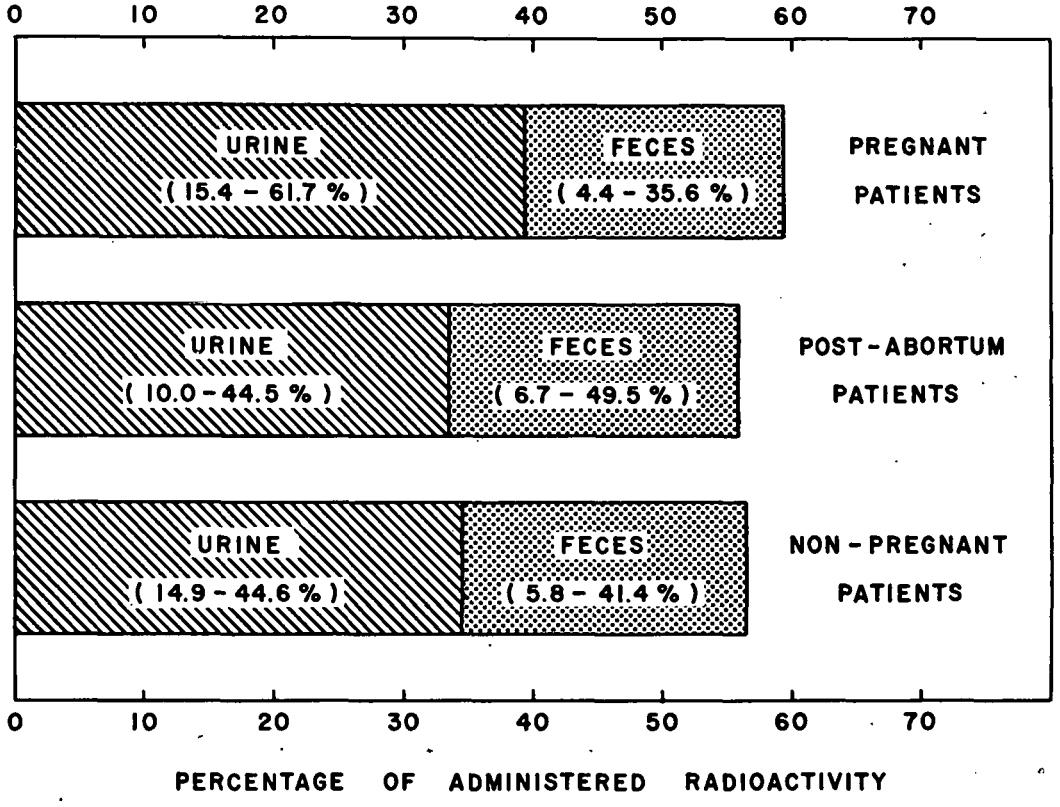

Figure 3. Radioactivity in urine and feces following a single intramuscular administration of progesterone-4-C^{14}.

The wide variation in the total recovery values in our three groups of patients is noteworthy and points to an individual excretion pattern for progesterone that is not influenced by the presence or absence of adequate luteal function. Indeed, Quilligan and Rothschild[16] recently reported that they have seen no relationship between the conversion percentages of progesterone to pregnanediol and the presence or absence of luteal function or the viability of early pregnancies, challenging the concepts of early investigators of this interesting problem.

The use of labeled progesterone in the investigation of excretory pathways of progesterone finally established the gastrointestinal tract as a second important pathway for the excretion of progesterone metabolites in the human.[2-5] There is a considerable variation in the excretion of total radioactivity derived from progesterone-4-C^{14} in pregnant, nonpregnant, and postabortal patients (Figure 3).

The following experiments were designed to determine whether substantial amounts of radioactivity may be excreted by way of the respiratory tract into the expired air, a possibility that would indicate a breakup of the steroid molecule into smaller compounds such as carbon

dioxide. In our first study we were unable to recover any radioactivity in the carbon dioxide of the expired air of a patient, 11 weeks pregnant, who had received progesterone-4-C^{14}, suggesting that ring A of the hormone is not split into such small fragments during its metabolism.

However, when a relatively large dose (28.3 μc) of progesterone tagged at carbon 21 of the side chain was administered intravenously to another patient during the eleventh week of gestation, substantial amounts of radioactivity were recovered in samples of expired air (Figure 4).

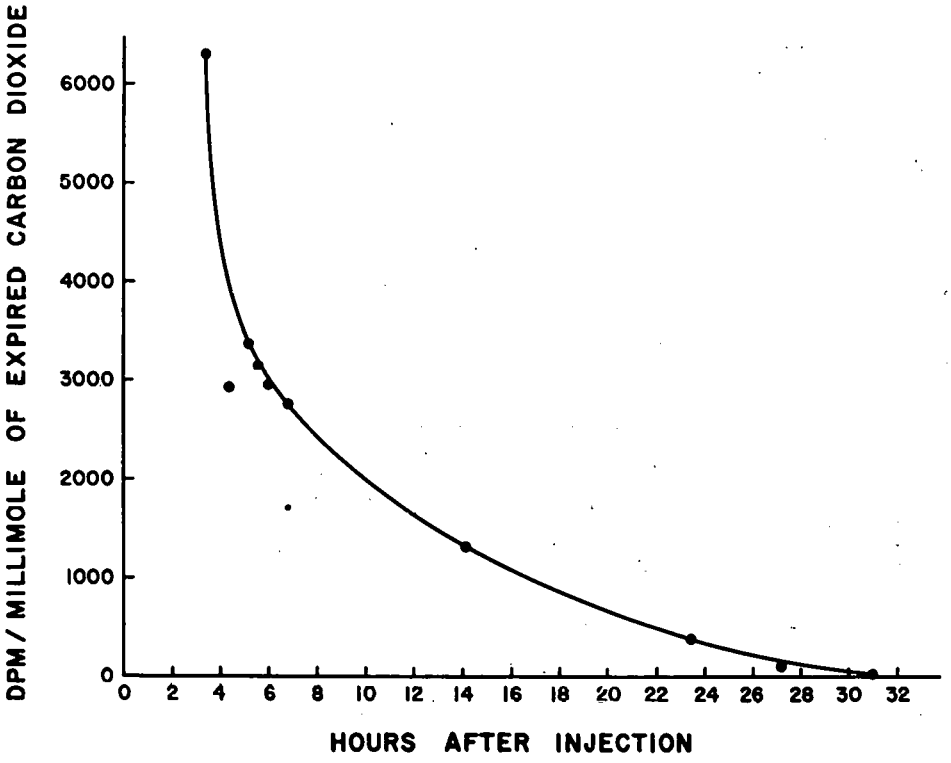

Figure 4. Concentration of radioactivity in samples of expired carbon dioxide following intravenous administration of 28.3 μc of progesterone-21-C^{14}.

The concentration of radioactivity in the expired carbon dioxide declined rapidly during the first 14 hours, and no appreciable amounts could be recovered after 31 hours. A rough calculation of these data[17] revealed that approximately 18 to 19 per cent of the administered steroid was excreted by way of the lungs within 31 hours after the injection. This experiment finally established the lungs as the third important excretory organ for progesterone metabolites in the human.

We determined the total excretion of radioactivity in the urine and feces of this patient and found 22.62 per cent and 29.74 per cent, respectively. Thus, we can account for a total of almost 71 per cent of the administered dose of progesterone in urine, feces, and expired air. This amount was eliminated during a 6-day interval following the injection. At the end of this period a therapeutic interruption of pregnancy was performed. Maternal and fetal tissue obtained at surgery were assayed for the presence of radioactivity derived from the intravenously injected progesterone-21-C^{14}. The highest concentration was found in a fat specimen removed from the abdominal wall. Assuming an even distribution of radioactivity in the fat compartment of the

body, about 9.1 per cent of the administered steroid was still present in the fat-containing cells of the organism 6 days following the single intravenous injection of tagged progesterone (Figure 5). Thus, a total of 80 per cent of the administered dose could be accounted for in the excreted urine, feces, expired air, and the fat compartment of the body. The results of a comparable study

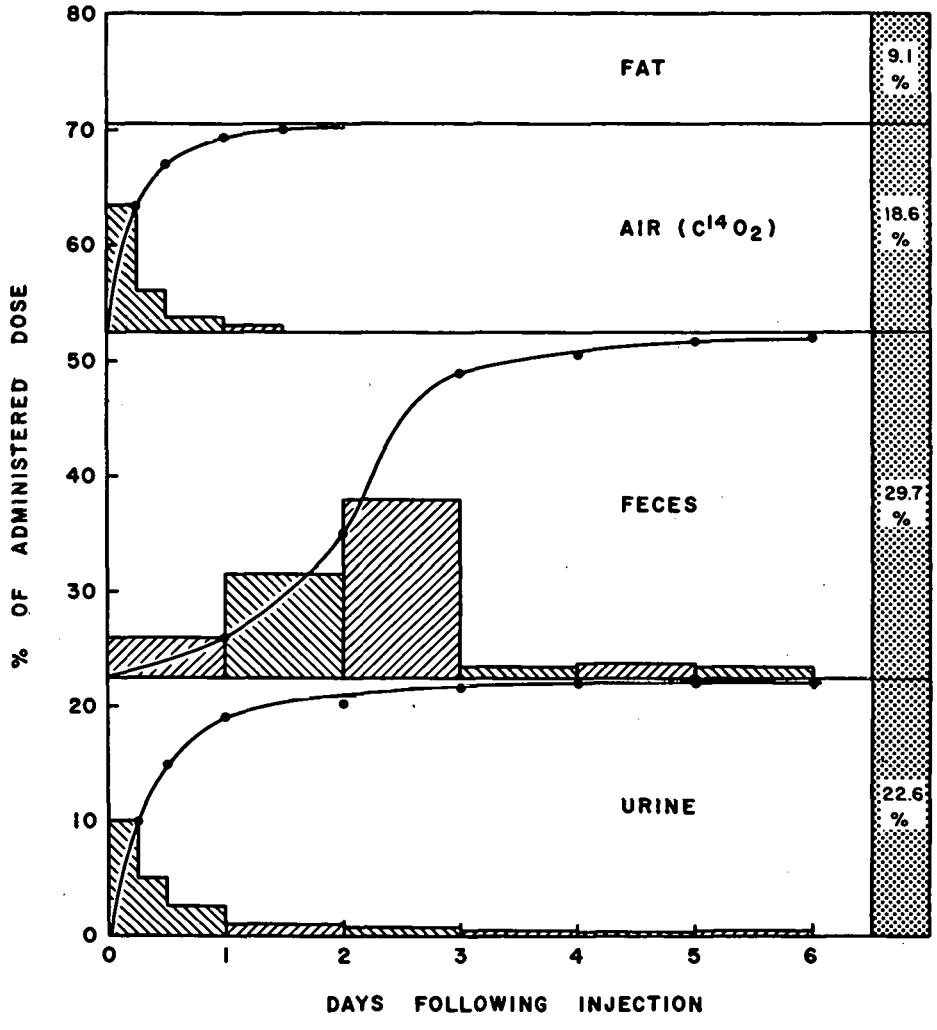

Figure 5. Recovery of radioactivity from urine, feces, expired air, and body fat following intravenous administration of progesterone-21-C^{14}.

in a second patient to whom a single intramuscular injection of progesterone-4-C^{14} had been administered are shown in Figure 6. At the end of a 134-hour period somewhat less than 70 per cent of the injected dose was accounted for in the excreted urine, feces, and fat compartment.

Figure 7 summarizes the results in 9 pregnant patients who had received a single intramuscular injection of progesterone-4-C^{14} and had undergone surgery at varying time intervals following the injections. The findings demonstrate clearly that progesterone and/or its metabolites diffuse promptly into the fat compartment of the body and are apparently retained there for a considerable length of time. This was particularly true in 1 patient, 14 weeks pregnant, who excreted relatively small amounts of radioactive end products in the urine and feces during

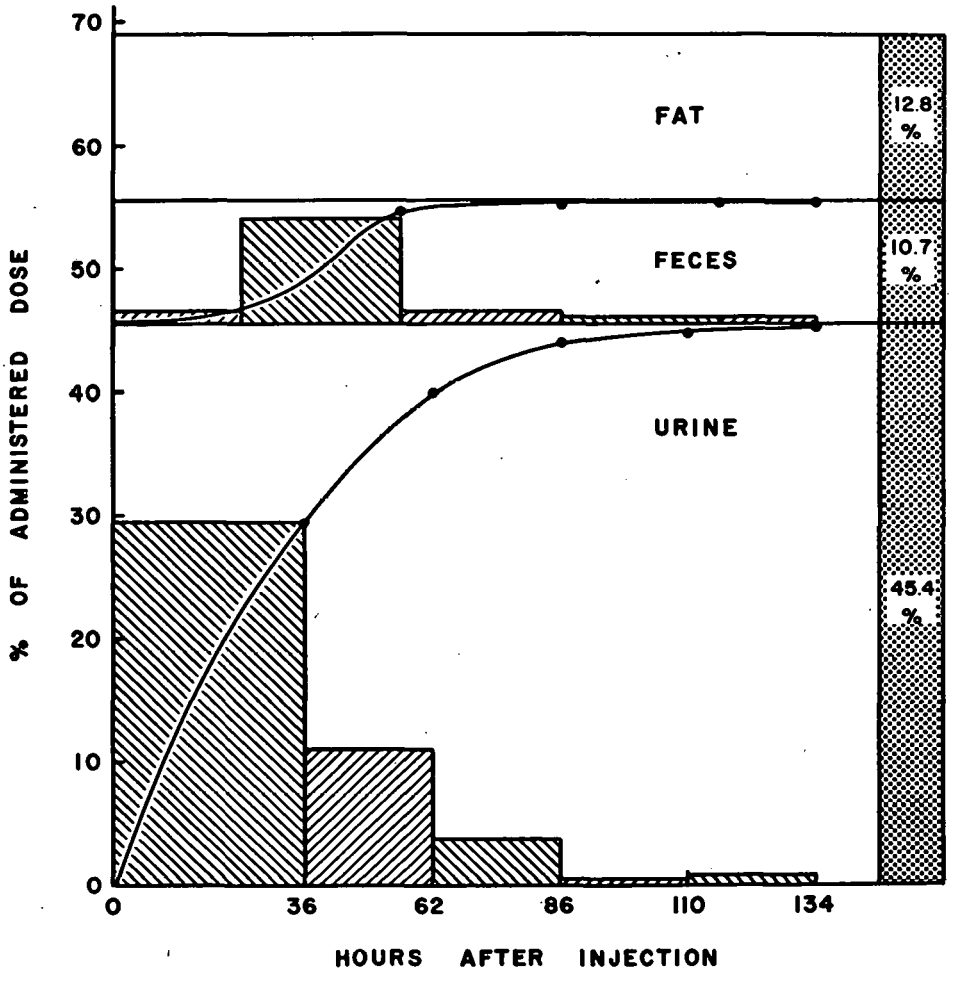

Figure 6. Recovery of radioactivity from urine, feces, and body fat following intramuscular administration of progesterone-4-C^{14}.

a 6-day period following the administration of the tagged hormone (Figure 8). When surgery was performed at the end of this period, considerable quantities of radioactivity were still present in the fat compartment (almost 40 per cent of the administered dose). Nine days following surgery a second intramuscular injection was given to this patient. Again, almost the same small amounts of radioactivity were excreted in the urine and feces, indicating a metabolic pattern of progesterone in this individual that differed considerably from that usually observed and which was not altered by the pregnant state.

The rapid disappearance of free progesterone from the circulation was furthermore demonstrated in the following study. Following the intravenous injection of a single dose of progesterone-21-C^{14} during the tenth week of gestation the concentration of radioactivity in the plasma was determined at various intervals. The steroids present in the plasma were partitioned in chloroform extractable steroids (free or unconjugated steroids) and those that remained in the aqueous phase (conjugated steroids). The concentration curve of radioactivity (Figure 9) declined rapidly during the first 2 hours and only minimal amounts of activity were present in the uncon-

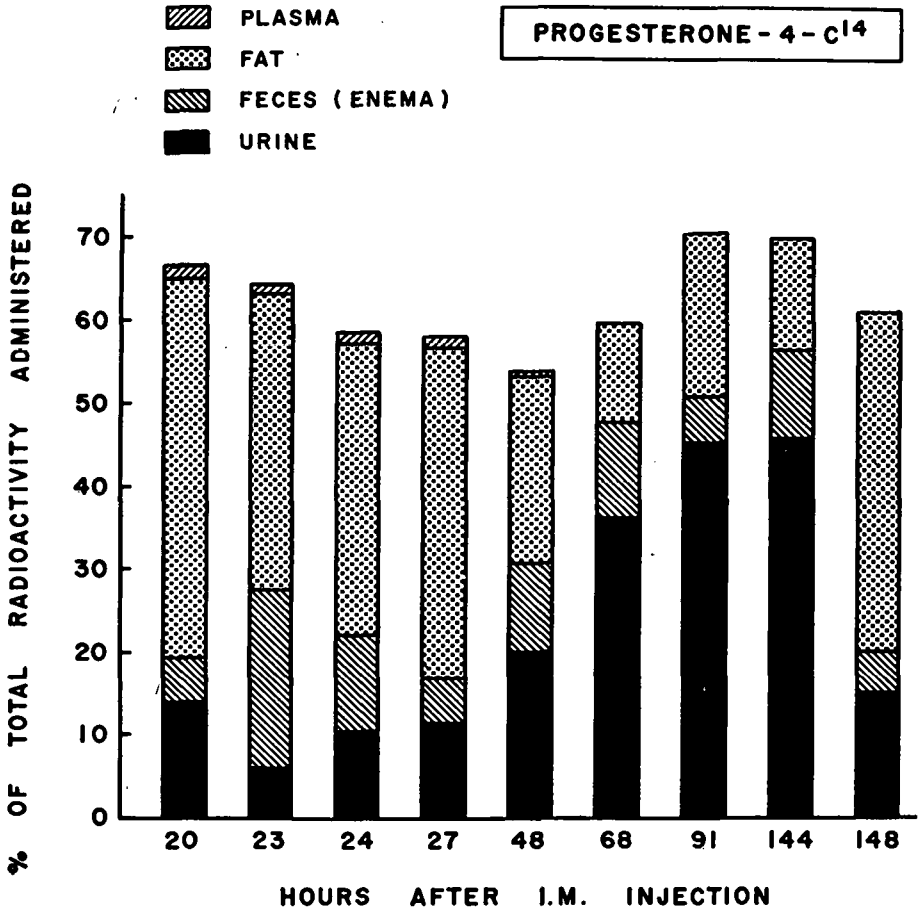

Figure 7. Radioactivity in urine, feces, plasma, and body fat following intramuscular administration of progesterone-4-C^{14} to nine pregnant patients.

jugated steroid fraction 37 hours after the administration of the hormone. At this time the free steroids had practically disappeared from the plasma. Figure 10 shows the radioactivity present in the total plasma volumes at various time intervals (plasma volume = 5 per cent of body weight). Twenty-five minutes after the intravenous injection only 1.83 per cent of the injected dose was present in the free steroid fraction, whereas 5.75 per cent was found in the conjugated steroid fraction. Thus, within 25 minutes the conjugated steroid level was three times that of the free steroids. This factor increased to 18 and 25 at the 129- and 399-minute intervals.

However, when a single injection of tagged progesterone was administered intramuscularly to a patient, the peak concentration of radioactivity in the plasma was reached approximately 25 hours later (Figure 11). The radioactivity present in the total plasma volume at this time was about 1.3 per cent of the injected dose. Thereafter, the plasma activity declined rapidly. Relatively small amounts were found at the end of 4 and 9 days. No free (unconjugated) steroids, using the method described above, could be detected in the plasma following the intramuscular injection of progesterone. The results of these studies demonstrated the rapid disappearance of the free steroid from the circulation into the fat compartment and an extremely fast conjugation

of the free steroid hormone, presumably in the liver and kidneys.

In contrast to the high concentration of radioactivity derived from tagged progesterone in fat, only moderate amounts of radioactivity were demonstrated in the myometrium and the decidua of 2 patients with advanced pregnancies (17 and 18 weeks), whereas a relatively higher concentration was found in these tissues of a patient who was 11 weeks pregnant (Table 1). The

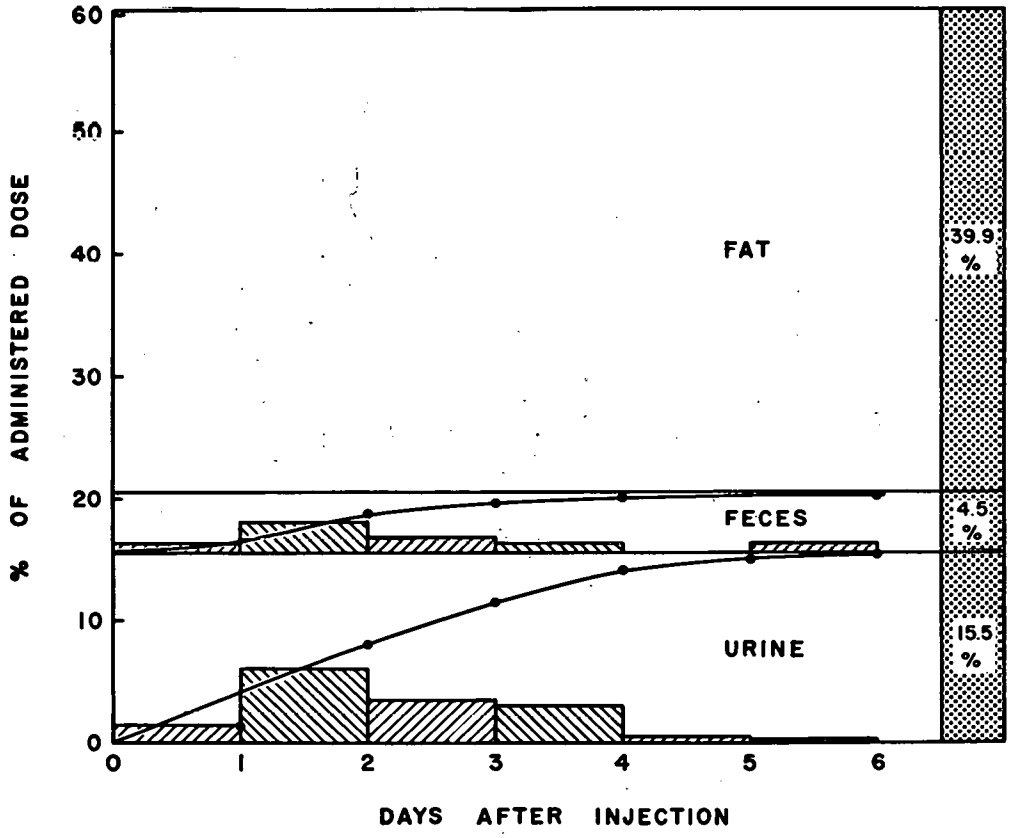

Figure 8. Retention of considerable amounts of radioactivity in body fat following intramuscular administration of progesterone-4-C^{14}.

concentration of radioactivity in the decidua and myometrium of the latter patient, however, was still considerably lower than that in the fat. Similar low concentrations of radioactivity were present in the myometrium of nonpregnant patients. It is of great interest to note that extremely low concentrations of radioactivity could be found in the endometria of 2 women who were operated upon during the pre- and postovulatory phases of their normal menstrual cycles. On the other hand, no radioactivity whatsoever was demonstrable in the atrophic endometria of 2 other patients. It is well known that endometrium that has not been primed by estrogens remains unresponsive to the typical biologic effects of progesterone. Our findings seem to indicate that, in the absence of estrogen activity, progesterone and/or its metabolites do not reach the cells of the mucosal layer of the uterus. Furthermore, they demonstrate that only minimal amounts of progesterone are necessary to induce its biologic effect at the cellular level of the target organ, the endometrium.

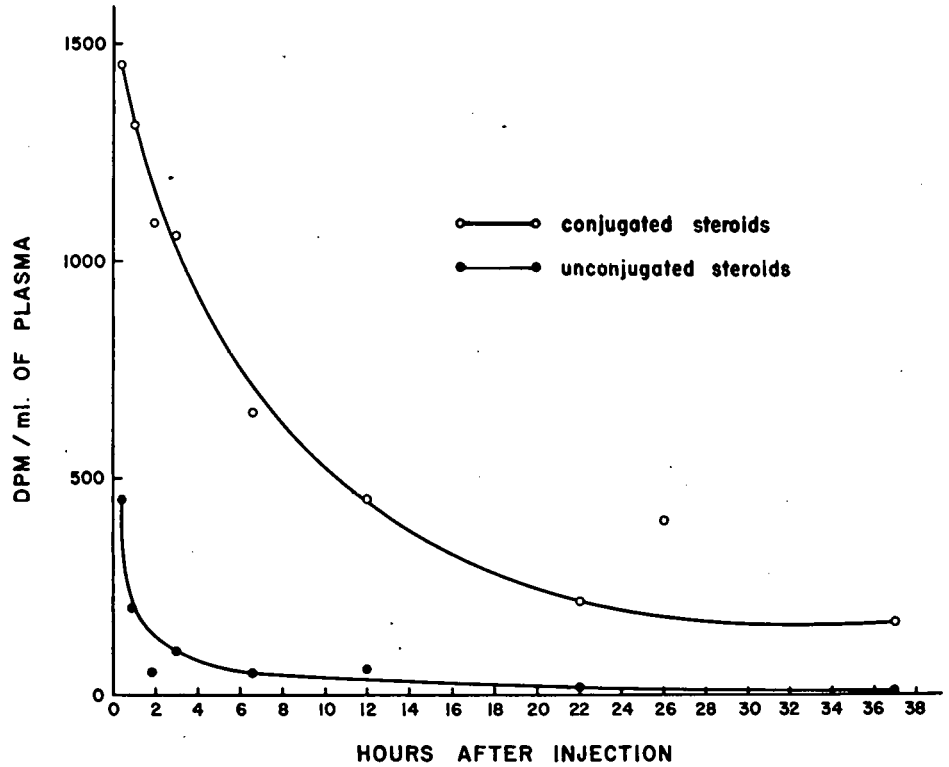

Figure 9. Plasma concentration of radioactivity following intravenous administration of 28.3 μc of progesterone-21-C^{14}.

Table 1

CONCENTRATION OF RADIOACTIVITY IN TISSUE FOLLOWING THE ADMINISTRATION OF C^{14}-4-PROGESTERONE

Patient	Fat	Endometrium	Myometrium	Myoma
	(DPM/g of wet tissue)			
C. C.	1754	594 (Decidual)	809	
J. O.	809	136 (Decidual)	90	
M. P.	502	101 (Decidual)	196	
G. S.	1072	85 (Proliferative)	300	630
N. B.	346	18 (Secretory)	77	218
A. F.	249	0 (Atrophic)	163	
E. W.	733	0 (Atrophic)	13	113

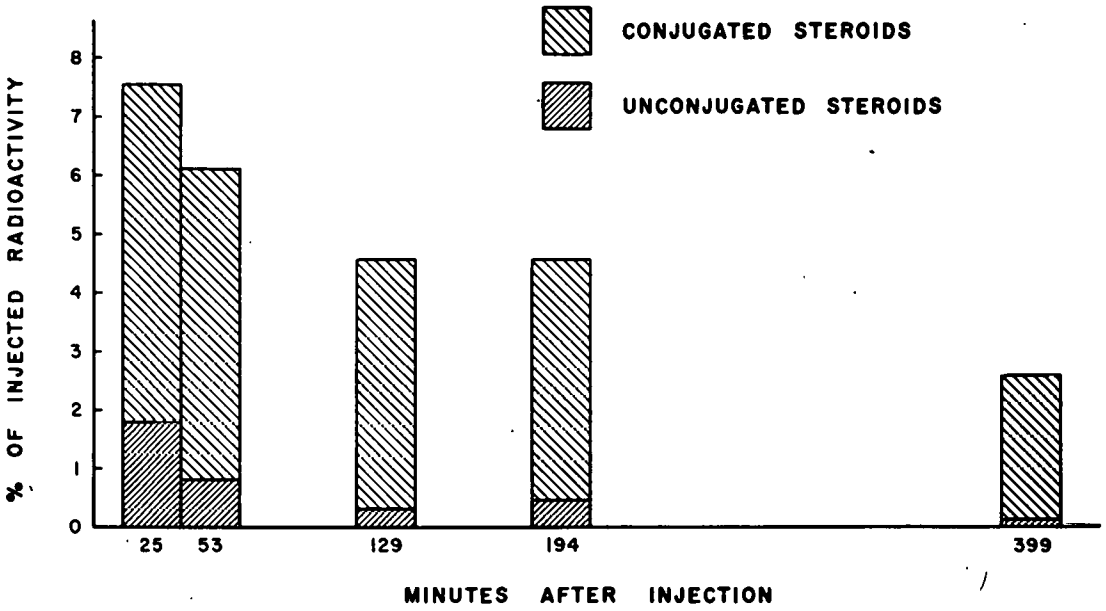

Figure 10. Radioactivity in total plasma volume following intravenous administration of progesterone-21-C^{14}.

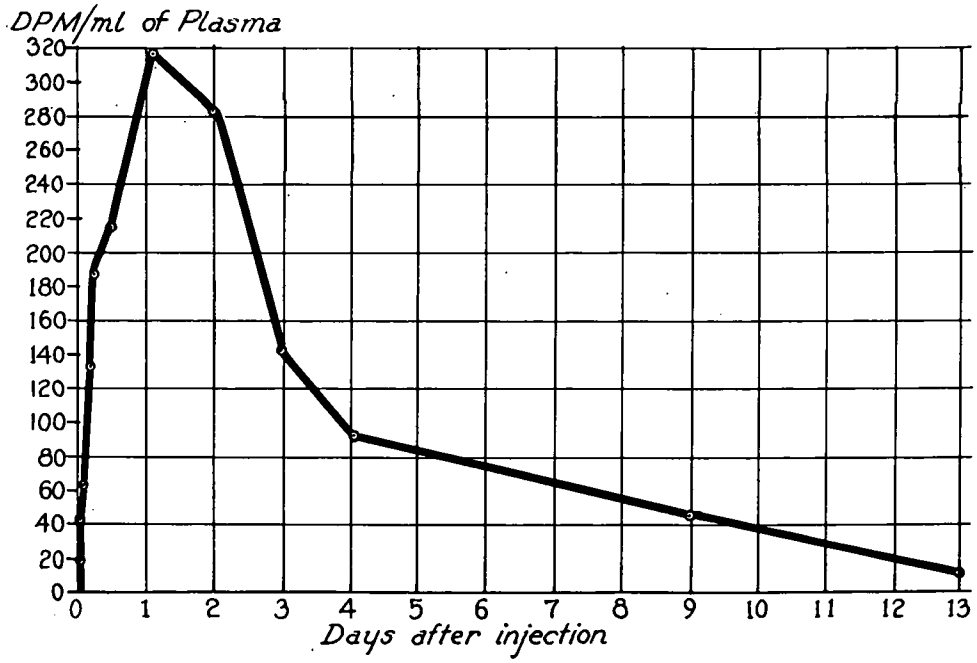

Figure 11. Plasma concentration of radioactivity following intramuscular injection of progesterone-4-C^{14}.

17(α)-Hydroxyprogesterone caproate. Following single intramuscular injections of 17(α)-hydroxyprogesterone- 4-C^{14} caproate to 4 pregnant patients the urines were collected for a period of 7, 12, 14, and 15 days, respectively. The doses of radioactivity administered varied from 25.9 to 28.8 microcuries. An average of 29.7 per cent (ranging from 27.6 to 29.8 per cent) of the

administered dose was recovered from the urine collected for 12 to 15 days (Figure 12). The fourth patient excreted 15.3 per cent of the injected radioactivity during a collection period of 7 days.

The daily urinary excretion pattern of radioactivity derived from tagged 17(α)-hydroxyprogesterone caproate differed considerably from that seen following the intramuscular or intravenous administration of radioactive progesterone (Figure 13). A plateau in the excretion curve was reached within 36 to 48 hours after the injection. In the succeeding 5 to 6 days almost equal

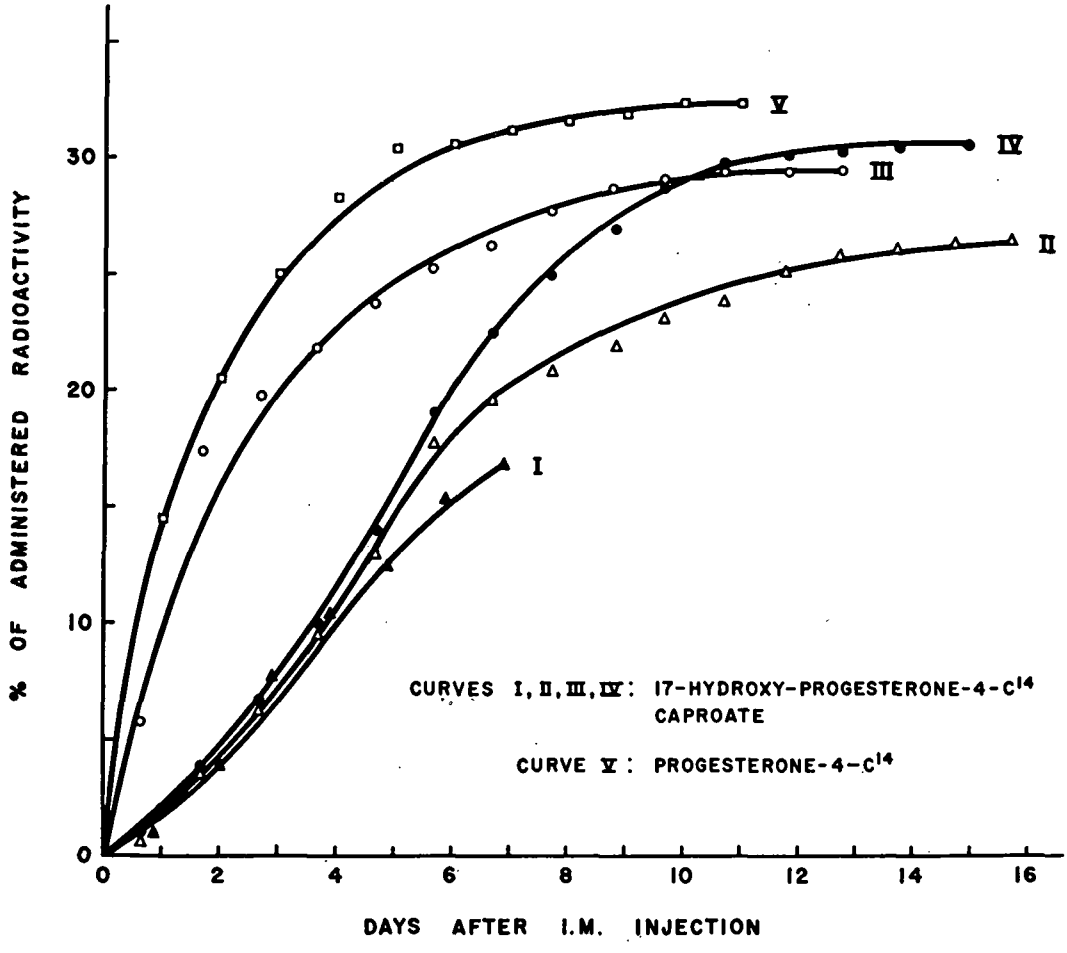

Figure 12. Cumulative excretion curve of radioactivity following intramuscular administration of C^{14}-labeled progestins to pregnant patients.

amounts of radioactivity were present in the urine of 3 patients. Thereafter, the daily urinary excretion of radioactivity derived from the esterified compound declined rather slowly. The fourth patient excreted a relatively large amount of radioactivity from the fourteenth to thirty-eighth hour after the injection (10.9 per cent of the administered dose). Following this single peak (demonstrated by the sharp rise of the cumulative excretion curve IV shown in Figure 12), the daily urinary excretion declined very slowly in the following 8 days.

Relatively large amounts of radioactivity were found in the feces following the intramuscu-

lar administration of 17(α)-hydroxyprogesterone caproate. In comparison with the fecal excretion of radioactivity derived from labeled progesterone, there appeared to be a distinct tendency toward an increased excretion of metabolic end products into feces of patients who received the esterified steroid (Figure 14). The largest amounts of radioactivity were found in the feces expelled between 48 and 142 hours following the injection of the caproate.

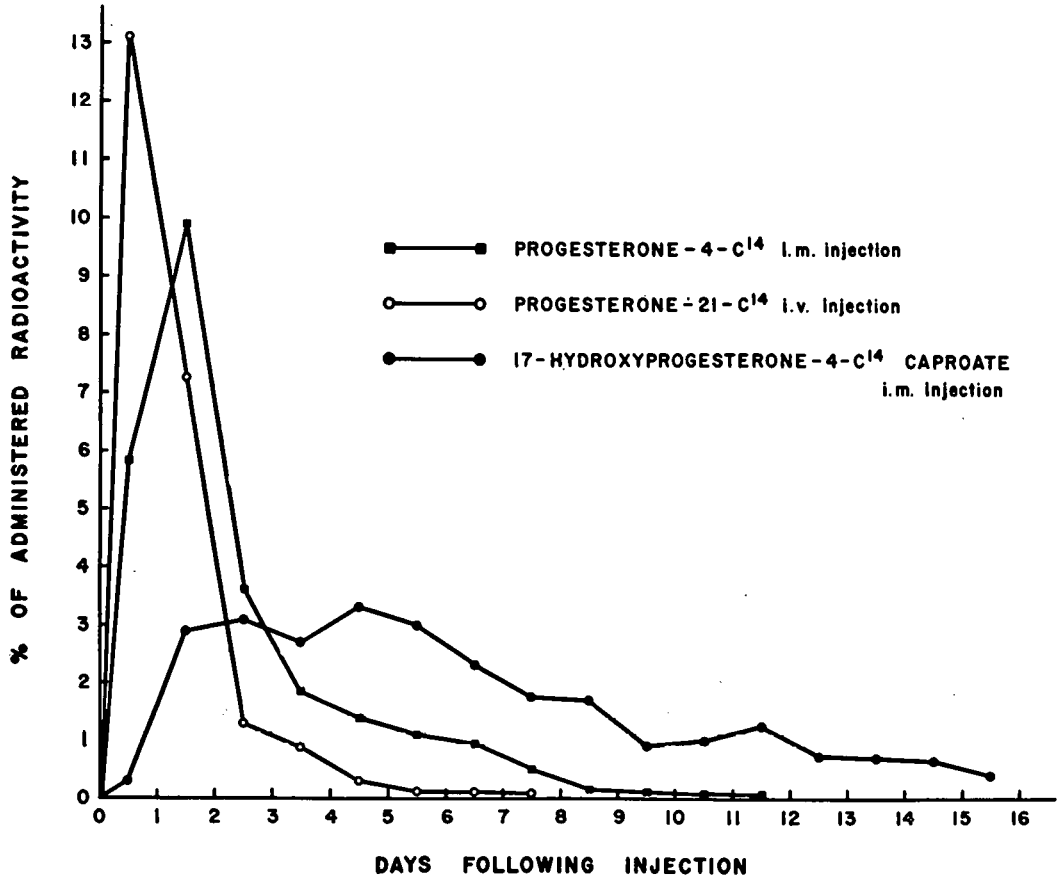

Figure 13. Pattern of urinary excretion of radioactivity following administration of C^{14}-labeled progestins.

The delayed excretion of radioactivity following a single intramuscular injection of 17(α)-hydroxyprogesterone caproate may be the result of retarded absorption of the steroid ester from the muscular injection site or temporary storage of the hormone and/or its metabolites somewhere in the body, presumably in the fat compartment. We know from the results of our studies with progesterone-4-C^{14} that this free steroid hormone is rapidly absorbed from the injection site and stored in the fat compartment. Consequently, various maternal and fetal tissues obtained at the time of surgery were assayed for radioactivity. The highest concentration was found in the fat specimens of the 6 patients who had received the esterified steroid. However, a comparison between the amounts of radioactivity stored in the total fat compartment derived from progesterone-4-C^{14} and that derived from 17(α)-hydroxyprogesterone-4-C^{14} caproate revealed an interesting finding. In Figure 15 the data obtained on total radioactivity in the

fat compartment are plotted against the time intervals between injection and surgery in 6 patients who had received the tagged caproate and in 12 patients who had received a single intramuscular dose of progesterone-4-C^{14}. There is a distinct difference between the two groups of patients. Relatively smaller amounts of radioactivity were retained in the fat compartments of patients to whom the caproate ester had been administered as compared with the progesterone-

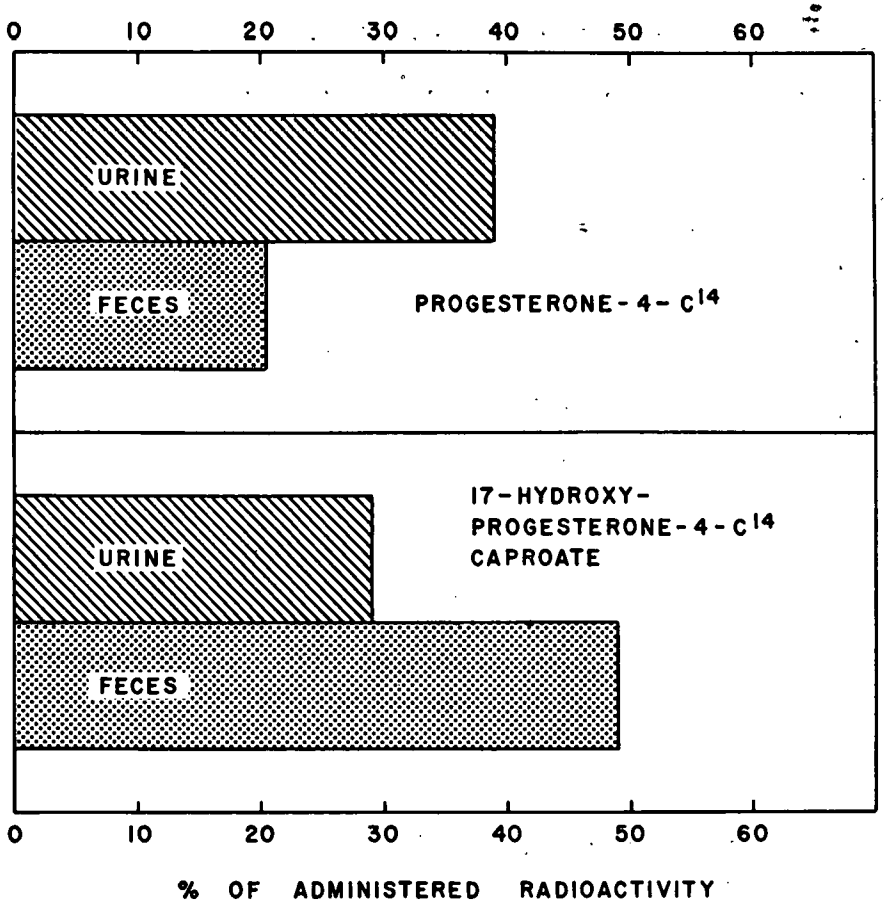

Figure 14. Radioactivity in urine and feces of pregnant patients following intramuscular administration of C^{14}-labeled progestins.

treated group. If unchanged 17(α)-hydroxyprogesterone caproate is absorbed into the blood circulation, one should expect a higher concentration of radioactivity in body fat since this steroid compound has very high solubility in oils and fats.

This finding may indicate that the prolonged biologic action of the caproate is not due to a slow release of the active principle from fat storage but rather to a retardation in the absorption from the injection site. The data shown in Figure 16 help to substantiate this view. About 88 hours following the injection of 17(α)-hydroxyprogesterone caproate we were able to account for only 40 per cent of the administered dose in urine, feces, and fat. Another patient, who was operated upon 24 hours after an injection of this steroid, had by that time excreted only 5.5 per cent of the administered dose in urine and feces (enema) and 17 per cent of the administered dose was present in the fat compartment at the time of surgery. Corresponding values for 2 oth-

er patients were 42.6 and 45.5 per cent, respectively, in urine, feces, and fat. Compared with the data obtained from patients who had received tagged progesterone (Figure 7), these values are much lower. Again, these findings lend support to the idea that the prolonged action of the caproate may be due to its slow absorption from the injection site.

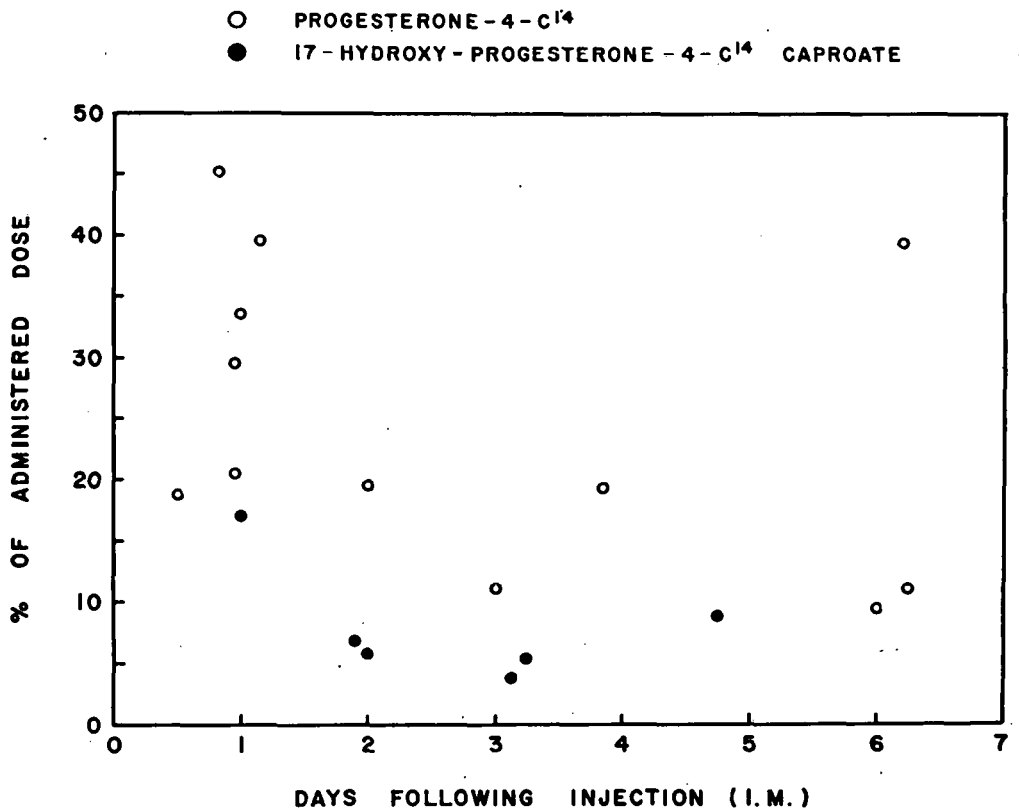

Figure 15. Radioactivity in the fat compartment of the body at various time intervals following intramuscular administration of C^{14}-labeled progestins.

Studies of the radioactivity concentrations in the blood plasma following the intramuscular injection of 17(α)-hydroxyprogesterone caproate demonstrated a slow rise in the amount present in total plasma volume (Figure 17) until the fifth day, followed by a slow decline thereafter. Again, this curve differs distinctly from that exhibited in patients who had received progesterone-4-C^{14} intramuscularly (Figure 11). When the steroids present in the blood plasma were partitioned into organic and aqueous phases, about 1/10 of the radioactivity was found in the chloroform extractable material, most likely representing free unconjugated steroids.

Only relatively small amounts of radioactivity were found in the myometrium and decidua of the patients who had received the esterified compound, when compared with the higher concentration in the fat. It was of interest to compare these data with those obtained following the injection of labeled progesterone. To make the data comparable, we calculated the amounts of steroids in gammas per gram of wet tissue following a hypothetical dose of 100 mg of the progestational substance. This calculation is based on the assumption that the active principle at the periphery was the unchanged hormone administered to patients. The data were plotted against

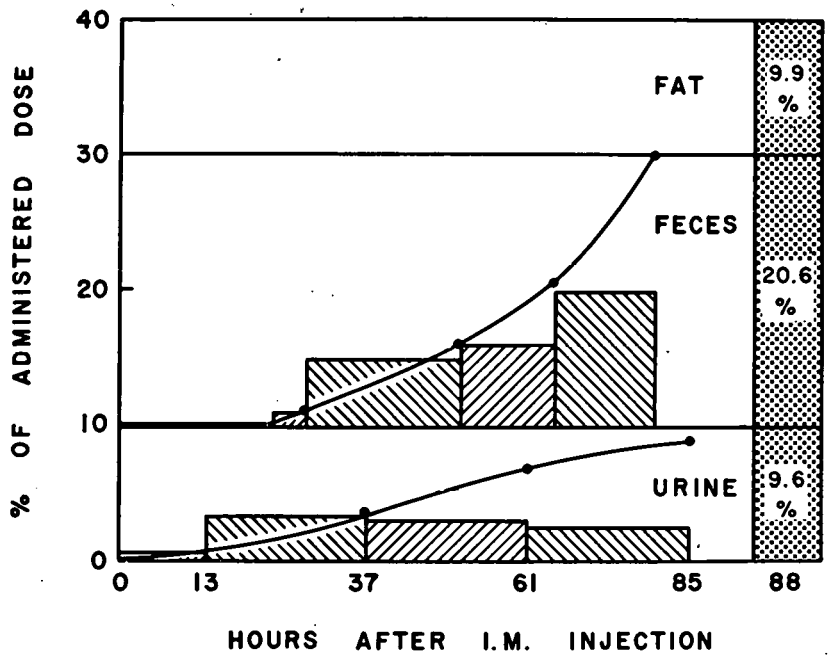

Figure 16. Radioactivity excreted in urine, feces, and body fat 88 hours following intramuscular administration of 17(α)-hydroxyprogesterone-4-C^{14} caproate.

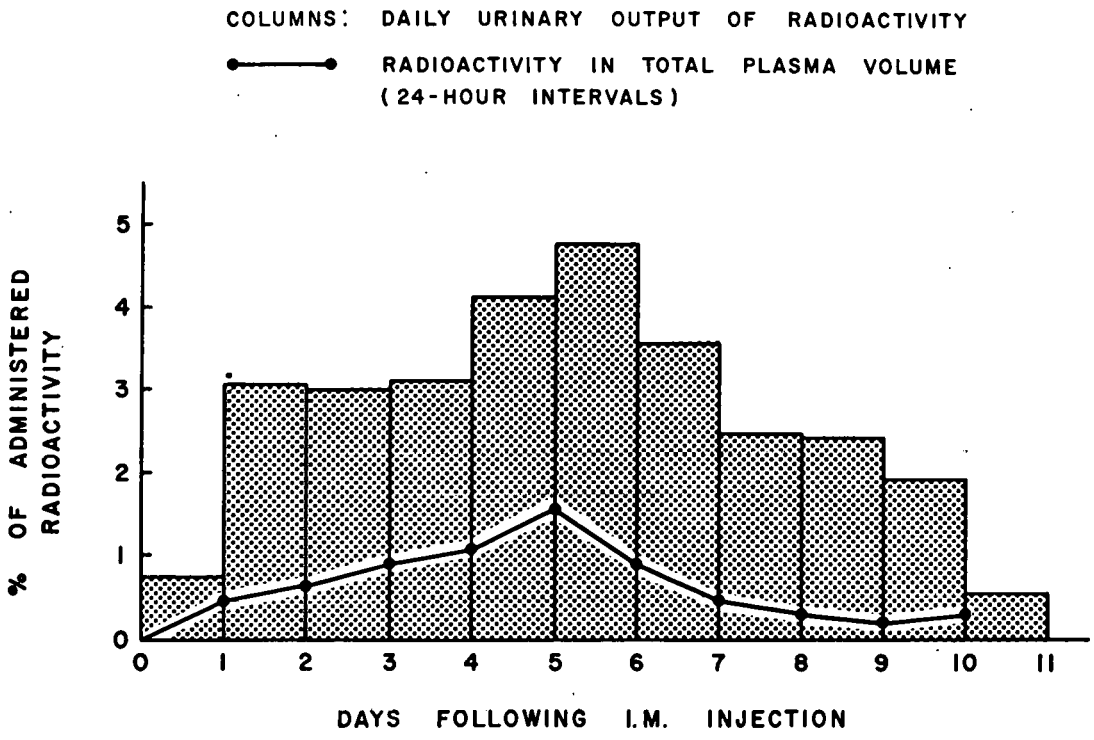

Figure 17. Radioactivity in total plasma volume at various time intervals following intramuscular administration of 17(α)-hydroxy-progesterone-4-C^{14} caproate.

the time intervals between injection and removal of the tissue (Figure 18).

Two significant conclusions can be drawn from this graph. (1) Surprisingly small amounts of steroids were available in endometrium, myometrium, and placenta following a relatively large dose of exogenous hormones. The amounts did not exceed 1/10 of a gamma in one gram of wet tissue for progesterone. This observation substantiates the view that only minimal amounts of hormonal substances are necessary to alter certain metabolic processes and individual enzyme reactions, generally known as "progestational activity." (2) Following the same dose of 17(α)-hydroxyprogesterone caproate we calculated a definitely higher concentration of

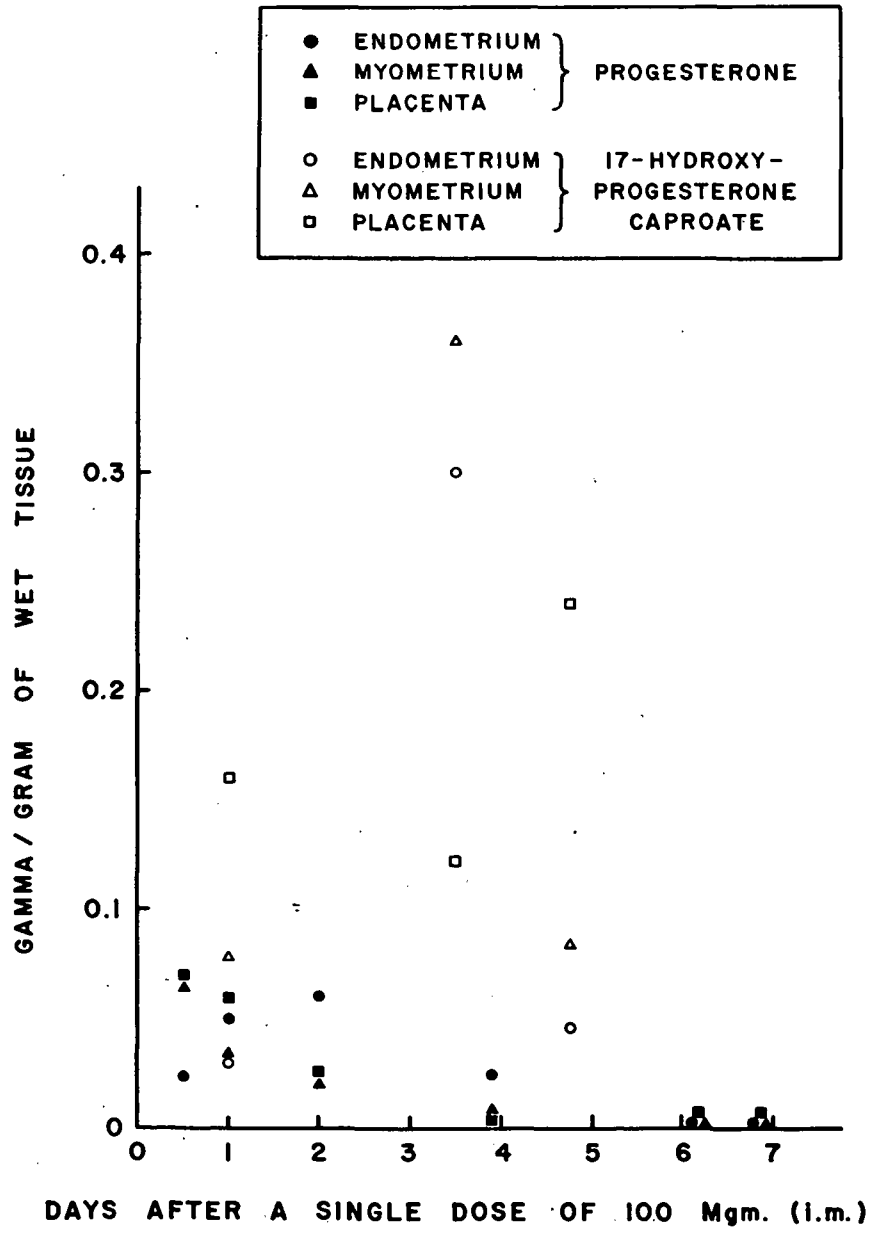

Figure 18. Amounts of steroids present in tissues following a hypothetical dose of 100 milligrams of progesterone and 17(α)-hydroxyprogesterone caproate.

steroid substances. It therefore appears likely that a successful attempt to raise the concentration of the active principle of a given progestational compound in a target tissue for a prolonged period of time will produce a more pronounced biologic effect of this particular progestational agent.

METABOLIC END PRODUCTS

Progesterone. Progesterone as such is not excreted into the urine of pregnant or nonpregnant women. Two pregnane compounds, pregnane-3(α), 20(α)-diol and pregnane-3(α)-ol, 20-one (commonly called pregnanediol and pregnanolone), have been recognized as the principal urinary metabolic end products of progesterone. These steroids have no progestational activity and are excreted in large and increasing amounts as water-soluble glucuronides in human pregnancy. They are accompanied by lesser amounts of various pregnanes and allopregnanes (pregnanedione, allopregnanedione, allopregnanolone, allopregnanediol).

There is good evidence that the side chain of some progesterone molecules is removed during its metabolism, resulting in the formation of steroids containing only 19 carbon atoms, possibly of the type of Δ^4-androstenedione. This possibility was suggested by the fact that radioactive carbon dioxide was found in the expired air following the intravenous administration of progesterone labeled with carbon 14 at position 21 (Figure 19). In 1 of our patients who had received progesterone-4-C^{14} androsterone and etiocholanolone were isolated in pure form from the urine and found to be radioactive. Both compounds are known metabolites of androstenedione, but it must be emphasized that the yield of such a conversion appears to be rather small. The diagram shown in Figure 20 demonstrates the presently known end products of progesterone metabolism.

17(α)-hydroxyprogesterone. The addition of an hydroxyl group to progesterone at position 17 results in 17(α)-hydroxyprogesterone and the loss of most of its progestational activity when administered to human subjects.[18] In contrast to progesterone, this steroid is not metabolized to pregnanediol or pregnanolone in appreciable amounts, but the administration of 17(α)-hydroxyprogesterone to human subjects results in the increased excretion of pregnane-3(α), 17(α), 20(α)-triol and certain 17-ketosteroids such as androsterone and etiocholanolone.[19,20] On the other hand, 17(α)-hydroxyprogesterone does not appear to have a significant androgenic action in the human.[21] The presently known metabolites of this compound are shown in Figure 20.

17(α)-hydroxyprogesterone caproate. Several attempts to recognize the urinary metabolites of 17(α)-hydroxyprogesterone caproate have failed thus far to establish a consistent metabolic pattern.[22,23] There is no definite change in the excretion of pregnanediol or pregnanolone following the administration of large amounts of this highly active steroid. There is no increase in the excretion of pregnanetriol. Furthermore, the excretion pattern of 17-ketosteroids is not altered in most individuals.

As already demonstrated, considerable amounts of radioactivity were recovered in urine and feces following intramuscular injections of the caproate. In order to determine the distribution of free (unconjugated) and conjugated steroids in the urine, an aliquot of 10 to 20 ml of urine was partitioned between chloroform and aqueous 10 per cent sodium carbonate. Figure 21 shows the results obtained from our study of the daily urines of 1 patient and Table 2 summarizes the results in the total urine collection of 3 patients. Consistently, the aqueous phase (Fraction II) contained most of the activity in all urine specimens and about one fifth of the radioactivity present in the original urine specimens was recovered in the organic phase (Fraction I). This finding

may indicate the presence of the unchanged steroid caproate in Fraction I, since it is easily extractable by chloroform when added to a fresh urine specimen. However, we were not able to identify unchanged 17(α)-hydroxyprogesterone caproate in urine or feces.

In another experiment various hydrolytic procedures for the cleavage of the conjugated urinary steroids were successively applied to the urine. As shown in Figure 22, 40 to 54 per cent of the original activity present in the urine became extractable following hydrolysis with

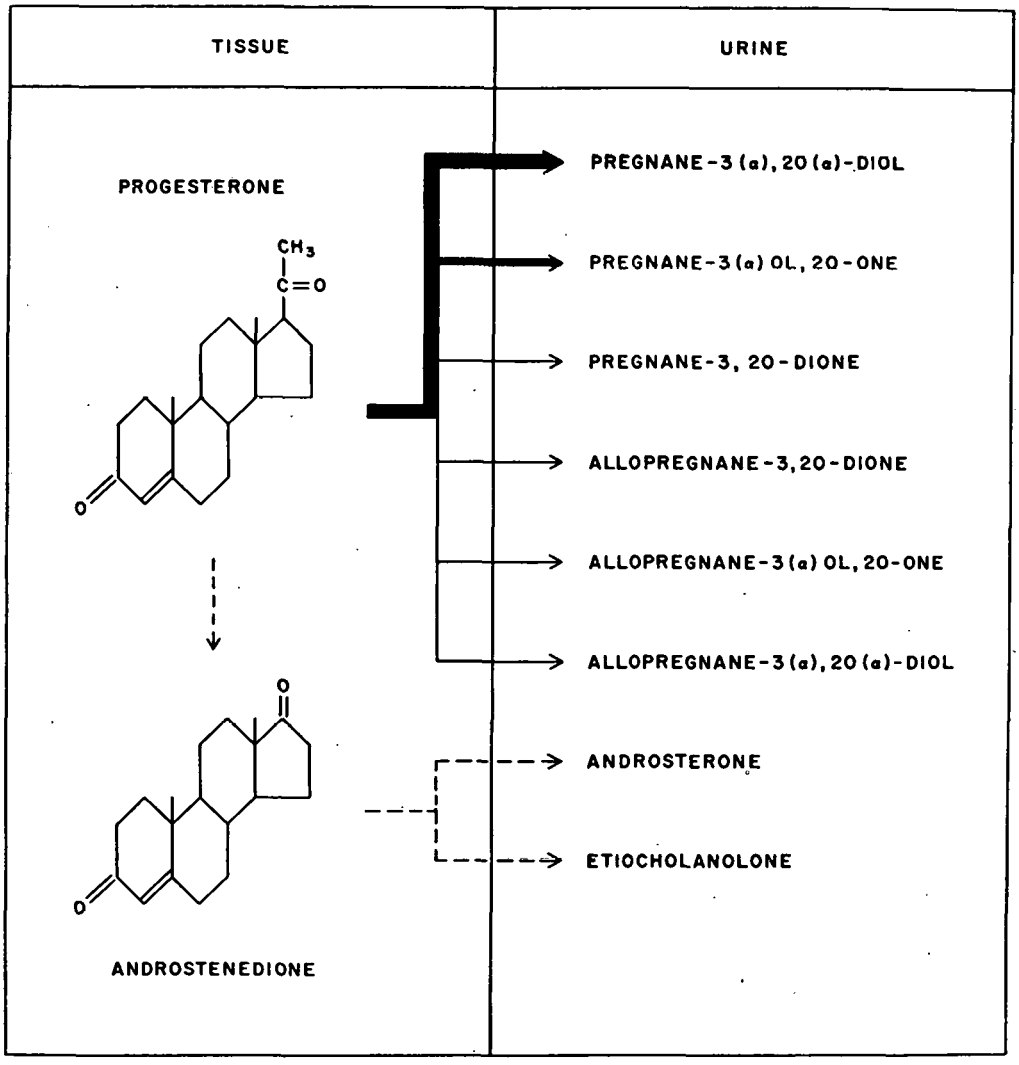

Figure 19. *Urinary metabolites of progesterone.*

β-glucuronidase. Since no attempts were made to extract the free steroids prior to hydrolysis, about half of the radioactivity would be derived from conjugated steroids such as glucuronides and the other half from free steroids (see previous experiment). An additional 15 to 22 per cent of the original activity was found in the ether extract following mild acid hydrolysis at pH 1. From the known behavior of steroid sulfates toward pH 1 hydrolysis, it must be presumed that about one quarter of the conjugated urinary metabolites of 17(α)-hydroxyprogesterone caproate was excreted as esters of sulfuric acid. The amount of conjugates hydrolizable by hot acid

ranged from 1.5 to 11.0 per cent of the original activity. Thus, only 56.6 to 82.6 per cent of the urinary radioactivity was extractable following all hydrolytic procedures in this particular patient. In another patient 66.7 per cent, and in a third patient only 39 per cent, of the original activity was extracted under the same conditions.

These observations indicate the presence of an unknown mode of conjugation of urinary steroid metabolites following the administration of 17(α)-hydroxyprogesterone caproate. Com-

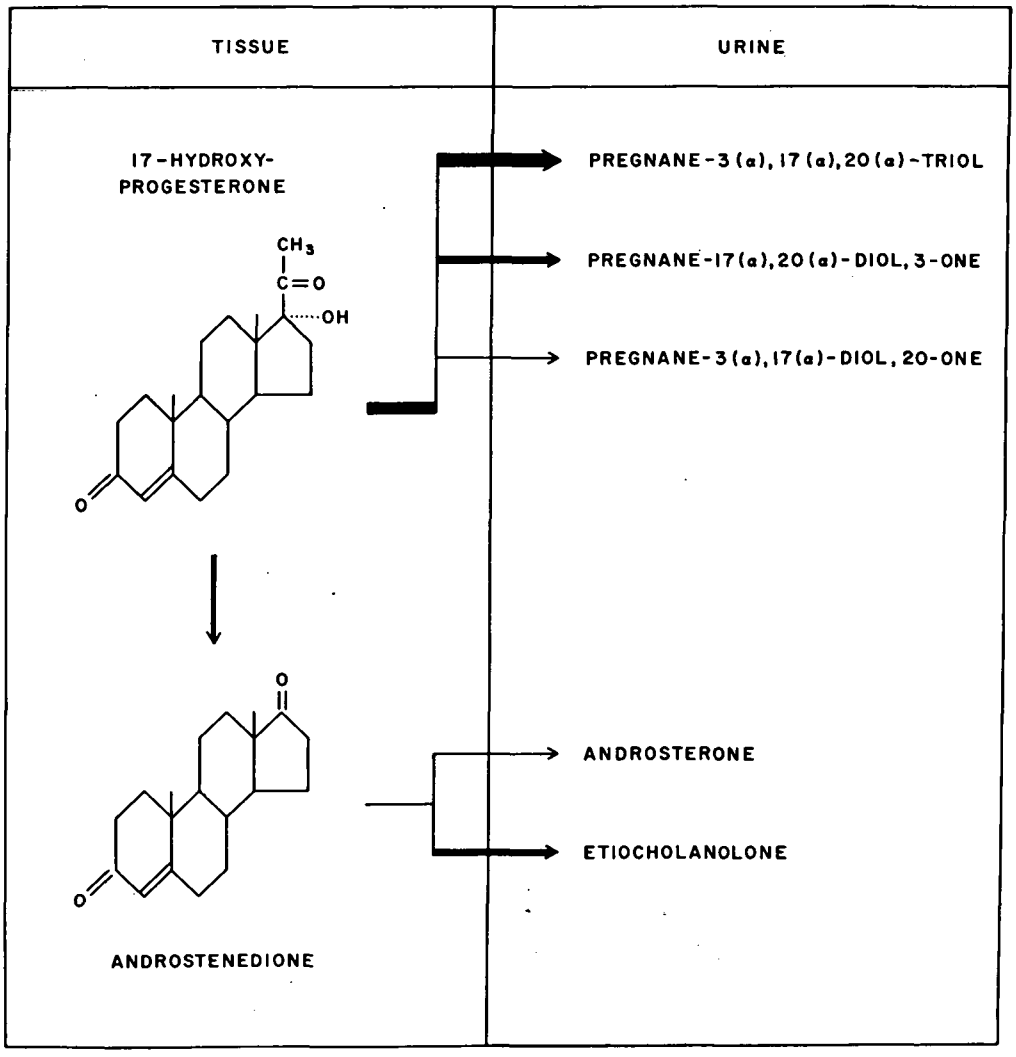

Figure 20. Urinary metabolites of 17(α)-hydroxyprogesterone.

parably larger amounts of glucuronides are present in the urine following the injection of progesterone, averaging about 90 per cent of the original activity, indicating a profound difference in the type of inactivation between the two steroids.

In our third experiment, the crude lipid ether extract obtained from the urine following hydrolysis was partitioned into neutral, phenolic, and acidic fraction. The neutral fraction contains pregnanediol, pregnanetriol, pregnanolone, androsterone, etiocholanolone, dehydroisoandrosterone, 11-ketoetiocholanolone, tetrahydrocortisone, and other steroid metabolites. The phenolic

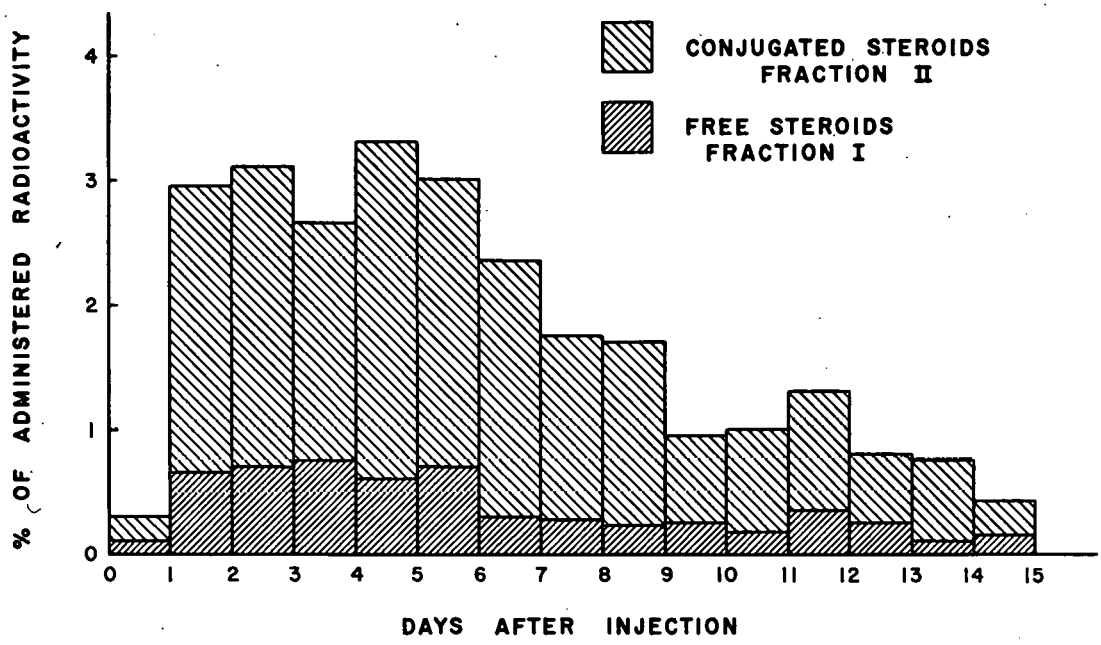

Figure 21. Distribution of radioactivity in free and conjugated steroids in the urine following intramuscular administration of 17(α)-hydroxyprogesterone-4-C^{14} caproate.

fraction contains estrogens such as estrone, estriol, and estradiol, while organic acids are found in the acidic fraction. In contrast to the neutral fraction, the phenolic and acidic fractions are less fully explored. Surprisingly, the major portion of the radioactivity derived from 17(α)-hydroxyprogesterone-4-C^{14} caproate was found in the phenolic and acidic fractions, whereas only 4.1 to 11.0 per cent of the original activity was recovered from the neutral fraction (Table 3). In contrast, about 90 per cent of the urinary radioactivity is found in the neutral fraction following the administration of progesterone-4-C^{14}. The results of these findings indicate that the major portion of the metabolic end products of 17(α)-hydroxyprogesterone caproate is not iden-

Table 2

RECOVERY OF RADIOACTIVITY IN THE URINE OF PREGNANT PATIENTS FOLLOWING A SINGLE INTRAMUSCULAR INJECTION OF 17(α)-HYDROXYPROGESTERONE-4-C^{14} CAPROATE

Length of gestation	Dose given μc	Collection period	Total activity[a]	Fraction I[b]	Fraction II[c]
			(Percentage of administered dose)		
12	28.2	349 hr	27.59	5.43	21.17
12	26.2	302 hr	29.21	4.18	23.65
10	25.9	141 hr	15.26	2.60	11.37

[a]Total radioactivity determined by the combustion method.

[b]Fraction I: Chloroform extractable portion (gas-flow counter).

[c]Fraction II: Steroids remaining in aqueous phase (gas-flow counter).

tical with the known end products of progesterone or 17(α)-hydroxyprogesterone.

It appeared of great interest to investigate the metabolites found in the feces of patients who had received 17(α)-hydroxyprogesterone caproate. The combined stool specimens of 1 patient were homogenized, adjusted to pH 1, and extracted with 100 per cent butanol. About 35 per

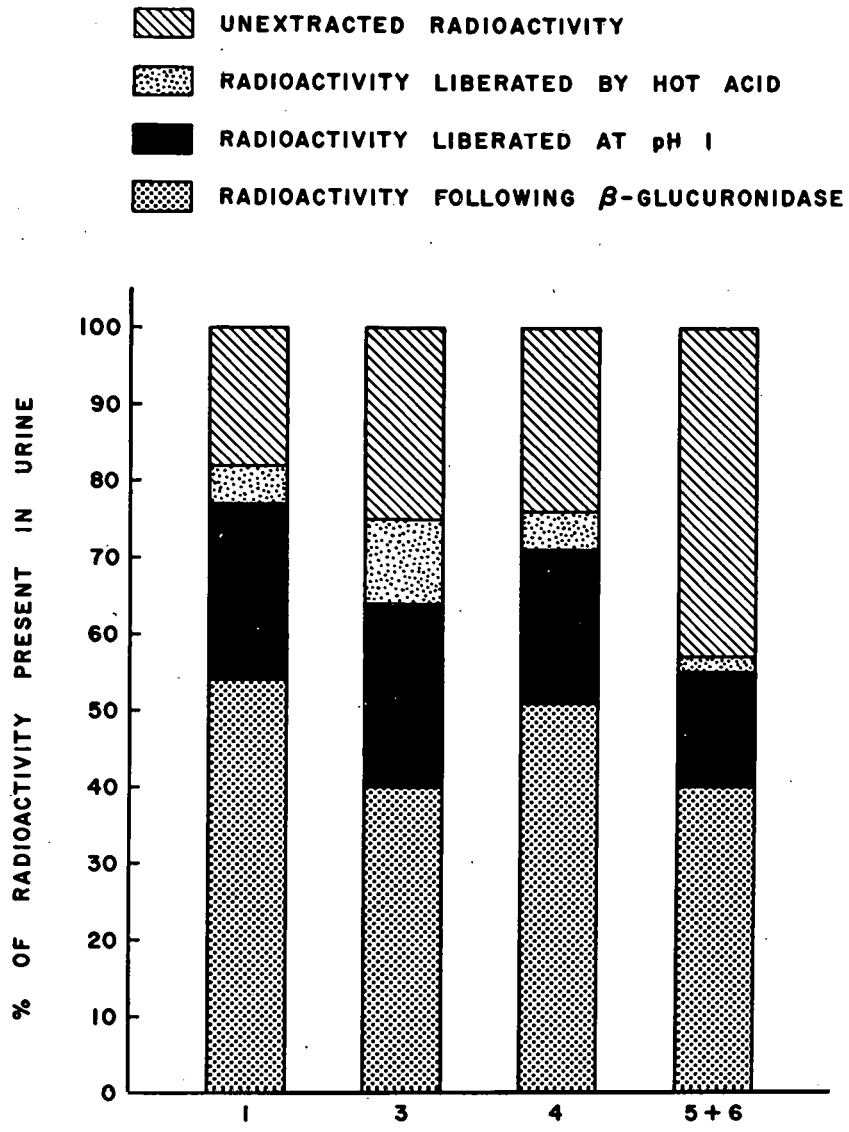

Figure 22. Distribution of radioactivity in urinary ether extracts obtained after various hydrolytic procedures following intramuscular administration of 17(α)-hydroxyprogesterone-4-C^{14} caproate.

cent of the total original radioactivity was extractable. From the known behavior of steroids toward pH 1, hydrolysis, and extractability with butanol, this extract presumably contains all free steroids as well as those present as glucuronides and sulfates. Thus, we cannot account for 65 per cent of the original activity present in the feces. It seems proper to assume that most fecal metabolites of the caproate are present as water-soluble conjugates that cannot be hydrolyzed

Table 3

RADIOACTIVITY RECOVERED IN THE URINARY NEUTRAL STEROID FRACTION FOLLOWING SINGLE INTRAMUSCULAR INJECTION OF LABELED PROGESTINS

Length of gestation	Steroid (dose)	Hydrolysis used		Radioactivity (% of administered dose)
12	17 (α)-hydroxyprogesterone-4-C^{14} caproate, 28.2 μc	β-glucuronidase and pH 1		4.1
12	17 (α)-hydroxyprogesterone-4-C^{14} caproate, 26.2 μc	β-glucuronidase and pH 1		3.5
10	17 (α)-hydroxyprogesterone-4-C^{14} caproate, 25.9 μc	β-glucuronidase and pH 1		11.0 0.025
11	Progesterone-4-C^{14}, 28.1 μc	β-glucuronidase and pH 1	#1	90.2
			#2	95.0
			#3	83.0
			#4	88.0

by the customary procedures.

Subsequently, the crude butanol extract obtained from the feces was partitioned in the neutral, phenolic, and acidic fractions. Similar to the findings in the urine, a relatively small amount of activity was found in the neutral fraction (6.9 per cent of the dose present in the butanol extract), whereas a surprisingly large amount was found in the phenolic fraction (81.9 per cent) and smaller amounts in the acidic fraction (11.1 per cent). These observations substantiate the view that the major portion of the metabolic end products of 17(α)-hydroxyprogesterone caproate are not identical with the known end products of free steroid hormones.

Continued investigation of the nature of the radioactive material excreted in urine and feces of patients who have received the labeled caproate are underway in our laboratory. We wish to report one preliminary finding. Struver and Lupu[24] isolated radioactive estriol from the phenolic fraction of the crude butanol extract of the feces (Table 4). It is well known that progesterone exerts its typical biologic effect on the endometrium when it is properly prepared by estrogens. Thus, the marked progestational effect of 17(α)-hydroxyprogesterone caproate could be partly explained by its conversion to estrogens. This speculation would be more convincing if we could prove that this conversion would provide sufficient amounts of estrogens available to the target cells. Our preliminary data, however, appear to indicate that the conversion rate of progestin to estrogen is rather low.

SUMMARY AND CONCLUSIONS

We have described a number of studies in which radioactive progesterone and 17(α)-hydroxyprogesterone caproate were administered to women during their reproductive life span. An average of almost 60 per cent of the administered radioactivity was excreted in the urine and feces during a 6- to 9-day period following the administration of progesterone labeled with carbon 14

Table 4

SPECIFIC ACTIVITY OF C^{14}-ESTRIOL (ISOLATED BY CARRIER METHOD) FOLLOWING THE ADMINISTRATION OF 17(α)-HYDROXYPROGESTERONE-4-C^{14} CAPROATE

Compound	Mg counted	DPM[a]	Free estriol DPM/mg
Estriol acetate	39.3	227	7.3
Estriol benzoate	1.25	9.7	8.8

[a]Corrected for quenching.

at carbon atom 4. The amounts excreted by way of the urinary or the gastrointestinal tract were not influenced by the presence or absence of active sources of progesterone (corpus luteum, placenta) or the viability of an early fetus. At present, there is no evidence that the ring structure of the steroid molecule is split into small fragments during its metabolism, but a considerable amount of radioactivity was found in the expired air of 1 patient to whom progesterone labeled with carbon 14 at carbon position 21 of the side chain had been administered. This finding indicates a cleavage of the side chain of the steroid ring which is oxidized to such small molecules as carbon dioxide and water. The excretion of radioactive metabolites by way of the skin appears to be minimal. Thus, three important pathways for the excretion of progesterone metabolites have been established: the urinary, gastrointestinal, and respiratory tracts.

Determinations of radioactivity in the blood plasma following intravenous and intramuscular administration of labeled progesterone demonstrated an extremely fast disappearance of the free steroid from the circulation due to (1) a speedy conjugation and inactivation, presumably in the liver and kidneys, and (2) a rapid diffusion into body tissues, mainly into the fat compartment. Although the urinary excretion of metabolites begins soon after the intravenous administration of the hormone, this fact does not account significantly for the disappearance of almost 92 per cent of an administered dose within 25 minutes following the injection.

The rapid diffusion of the hormone and/or its metabolites into the fat compartment of the body could be regarded as a mechanism by which a more prolonged hormonal supply is provided for the target cells from a storage place. Indeed, Kaufmann and Zander[25] have found progesterone in relatively high concentration in the fat tissue of pregnant women. However, as shown in numerous clinical and animal experiments, the rate and degree of progestational response to a single intramuscular or intravenous dose of the hormone is minimal and cannot be accelerated to a significant degree by excessive doses. These observations lead to the concept that only a limited amount of the administered hormone is being utilized by the target cells. The portion that is in excess cannot contribute to the biologic response and is wasted. This principle has been recognized as "overflow."[26] If this concept is correct, the rapid diffusion and subsequent retention of steroid substance in the fat compartment would constitute an effective mechanism by which excessive amounts of hormones from endogenous or exogenous sources are diverted from the target organs, thus preventing the accumulation of larger amounts of active steroid hormones in the target cells.

The results of our studies on the metabolism of tagged progesterone were compared with

that of 17(α)-hydroxyprogesterone caproate labeled with carbon 14. Distinct differences between the two progestational compounds became evident: (1) There is a distinct tendency toward an increased excretion of metabolic end products into the feces of patients who had received the esterified compound. (2) The daily urinary excretion curve reaches a plateau within 36 to 48 hours following a single intramuscular injection of the caproate. This plateau is maintained for a period of from 5 to 6 days in most patients, followed by a rather slow decline in the excretion curve thereafter. (3) The determination of radioactivity in the blood plasma following the administration of 17(α)-hydroxyprogesterone-4-C^{14} caproate demonstrates a slow rise of the amounts present in the total plasma volume until the fifth day, followed by a slow decline thereafter. (4) The highest concentration of radioactivity in tissues is found in maternal fat, but the total radioactivity retained here is less in comparison with the amounts present following the administration of tagged progesterone. (5) A relatively low concentration of activity is found in the endometrium and myometrium following the administration of both compounds. However, when the amounts present in the target area at various time intervals following an equal hypothetical dose are compared on a weight basis, larger amounts of steroids derived from the caproate are present as compared with the amounts derived from progesterone.

On the basis of these findings we are inclined to ascribe the greater and more prolonged activity of the steroid ester to a retarded absorption from the injection site rather than to temporary storage in the fat compartment. Thus, a more economical supply of the active hormonal principle would be available in the target cells. The mechanism by which such an accumulation of the active steroid hormone in the target area is achieved is presently unknown.

The experiments of Csapo[27] have indicated that a high concentration of progestational substance in the myometrium is desirable and may be achieved by a direct diffusion from the most active source of progesterone, the placenta, to the myometrium. It is entirely possible that the effectiveness of progesterone on the myometrium depends largely on such a direct diffusion in order to provide a sufficient concentration of hormonal substance. Then, the generalized effect of progesterone via the blood stream would play a less important role. This concept, together with the principle of "overflow," would explain the ineffectiveness of exogenous progesterone often observed under pathologic conditions in human pregnancy. Thus, any hormonal therapy that could provide an increased accumulation of active hormonal substance in the target area should constitute a significant advance in our therapeutic efforts.

The preliminary investigation of the urinary and fecal metabolites of 17(α)-hydroxyprogesterone caproate revealed a distinct difference between the mode of conjugation of its metabolites and that observed in the end products of progesterone. Furthermore, the known metabolites of the free steroid, 17(α)-hydroxyprogesterone, do not constitute the principal metabolites of its ester. Only relatively small amounts of radioactivity were found in the neutral steroid fraction of the urine and feces following the administration of the tagged caproate, whereas considerable activity was present in the phenolic and acidic fraction of the crude lipid extracts. These findings indicate that the metabolism of 17(α)-hydroxyprogesterone caproate differs considerably from that observed in progesterone, 17(α)-hydroxyprogesterone, and other steroid hormones. It is hoped that the identification of these substances will help to shed more light on the mechanism involved in the marked and prolonged action of 17(α)-hydroxyprogesterone caproate.

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FEVER-PRODUCING STEROIDS OF ENDOGENOUS ORIGIN IN MAN*

By

A. Kappas, W. Soybel,† P. Glickman,† and D. K. Fukushima‡

This paper is a report of studies on the pyrogenic action of a series of steroid metabolites in man. The chemical prototype for this form of biological activity is the hormonal derivative, 3α-hydroxyetiocholane-17-one. This compound, whose capacity to provoke fever in humans was first reported in 1956,[1-3] represents the first pure substance of known chemical structure and of physiologic origin having consistent pyrogenic action in man. The present studies indicate that this thermogenic activity extends to a number of steroid metabolites derived from adrenocortical and gonadal hormones. Certain aspects of the structural basis of this action are described and the effects of in vivo metabolic transformations on the fever-producing activity of these hormone derivatives are noted.

METHODS

The following steroids were tested for pyrogenic activity in man: 3α-hydroxyetiocholane-17-one; 3α-acetoxyetiocholane-17-one; etiocholane-3,17-dione; 3α,11β-dihydroxyetiocholane-17-one; 3α-hydroxypregnane-20-one; 3α-acetoxypregnane-20-one; pregnane-3,20-dione; pregnane-3α,20α-diol; 21-hydroxypregnane-3,20-dione; 3α-hydroxypregnane-11,20-dione; 3α,17α-dihydroxypregnane-20-one; and 3α,17α,21-trihydroxypregnane-11,20-dione. Steroids were prepared by partial synthesis and were purified until the physical constants were the same as the best samples described. All subjects studied were adult volunteer hospital patients who had been afebrile for at least 7 days preceding steroid administration. All temperatures were measured rectally at 1- or 2-hour intervals for periods of 24 to 48 hours following steroid injections. For the intramuscular studies, steroids were dissolved in sesame oil containing a small amount of benzyl alcohol, rigid precautions being taken to exclude pyrogens.[2] For the intravenous studies, steroids were dissolved in small amounts of 95 per cent ethyl alcohol and administered over varying periods of time by means of a constant infusion pump (Harvard Apparatus Co., Model 600-900). All injection syringes, needles and glassware were heated at 300° C for 3 or more hours before use. Control studies utilizing intramuscular and intravenous solvent vehicles were made and no pyrogenic reactions were observed.

RESULTS

3α-HYDROXYETIOCHOLANE-17-ONE: Intravenous administration. The pyrogenic response which follows the intramuscular injection of this steroid in man has been described previously.[1-3] Its failure to provoke fever in rabbits has also been noted.[2] For this study, the steroid was administered intravenously to 4 subjects. One received 5 mg over a 2-1/2-hour period and 3 re-

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‡Sloan-Kettering Institute for Cancer Research, New York.

ceived 18 mg over 2-1/2- to 7-hour periods. No temperature elevation was produced by the 5 mg dose, but all 3 subjects receiving 18 mg developed fevers comparable to those following the intramuscular administration of this metabolite.[2,3] The subject receiving 5 mg during 2-1/2 hours, and one subject receiving 18 mg over 7 hours, developed thrombophlebitis during the 24- to 48-hour period following infusion, and evidence of this phlebitis persisted for 2 weeks in both. During this continuing phlebitis no significant temperature elevation was observed. Infusion of the solvent vehicle (ethyl alcohol) without steroid in both subjects did not produce any inflammatory reaction, and the other two subjects receiving 18 mg of this steroid intravenously had no significant local reactions. Two examples of the fever produced by the intravenous injection of this steroid are shown in Figure 1.

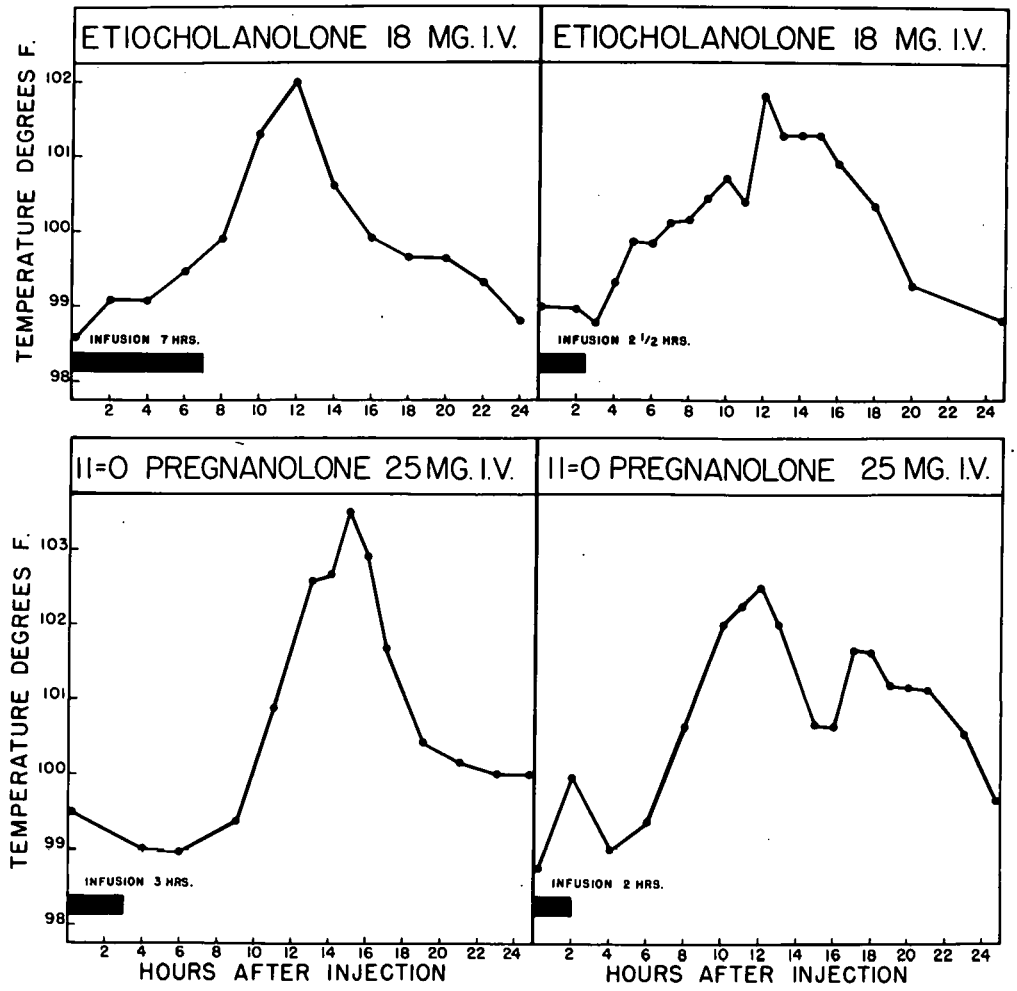

Figure 1. Temperature response to the intravenous administration of steroid pyrogens in man.

3α-ACETOXYETIOCHOLANE-17-ONE: Intramuscular administration. Single doses of 25 mg each were given to 2 subjects, neither of whom developed temperature elevation over a succeeding period of 48 hours. In view of the consistency of pyrogenic reactions to unesterified etiocholanolone at this dose level, as well as the failure of 3α-acetoxyprenane-20-one to pro-

voke fever (see below), these two observations were considered significant.

ETIOCHOLANE-3,17-DIONE: Intramuscular administration. Single doses of 25 mg each were given to 4 subjects. In one subject a maximum temperature elevation to 100.9° F ten hours after injection was noted. No temperature elevation exceeding 99.9° F was observed in the other 3 patients.

3α,11β-DIHYDROXYETIOCHOLANE-17-ONE: Intramuscular administration. Single injections of 25 mg each were administered to 4 subjects. Temperature elevations to 100.5° F or greater were recorded in all 4 patients. In 2 subjects, temperatures reached 102° F.

3α-HYDROXYPREGNANE-20-ONE: Intramuscular administration. Single doses of 25 mg each were given to 5 subjects. It was intensely pyrogenic in each as indicated in Figure 2.

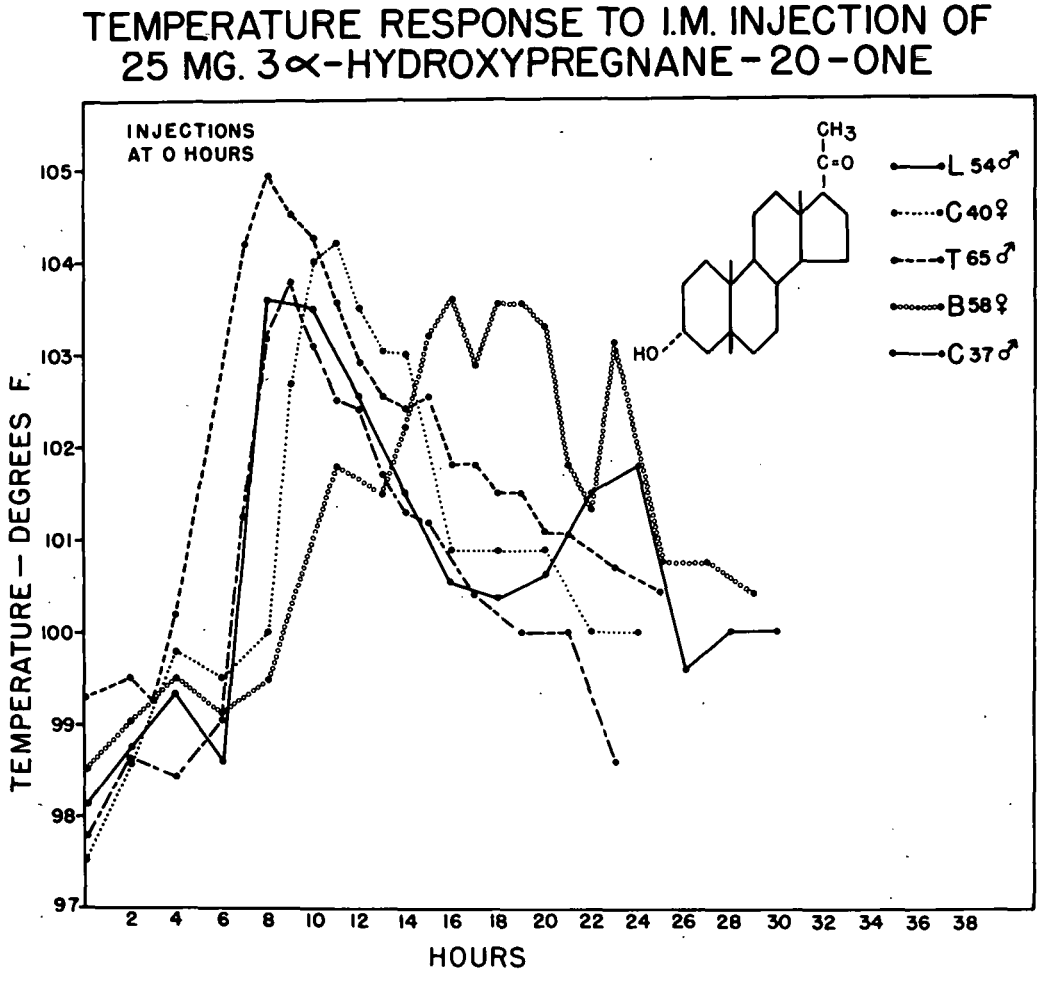

Figure 2.

3α-ACETOXYPREGNANE-20-ONE: Intramuscular administration. Single doses of 25 mg each were given to 3 subjects. This compound proved to be devoid of pyrogenic activity, as noted previously, for 3α-acetoxyetiocholane-17-one.

PREGNANE-3,20-DIONE: Intramuscular administration. This steroid was given in single doses of 25 mg each to 4 subjects. In one subject a temperature peak of 103° F was reached 16

hours after injection. A decline in temperature to 99.5° F twenty-two hours after the injection was followed by a second temperature rise to 101° F during the succeeding 24-hour period. In 2 other subjects, peak temperatures of 101° F were noted 10 to 12 hours after steroid injection. Temperature of the remaining subject did not exceed 100° F.

PREGNANE-3α,20α-DIOL: Intramuscular administration. This compound was given in single doses of 25 mg each to 4 subjects. It was intensely pyrogenic in each, as shown in Figure 3. The response of subject M27 is of interest. No temperature elevation was noted for the first 24 hours. During this period of time a small localized indurated area was felt in the fatty tissue at the site of injection. This disappeared during the subsequent 24 hours, at the time when the elevation in temperature was noted. This delayed temperature response, therefore, was attributed to a delay in absorption of injected steroid.

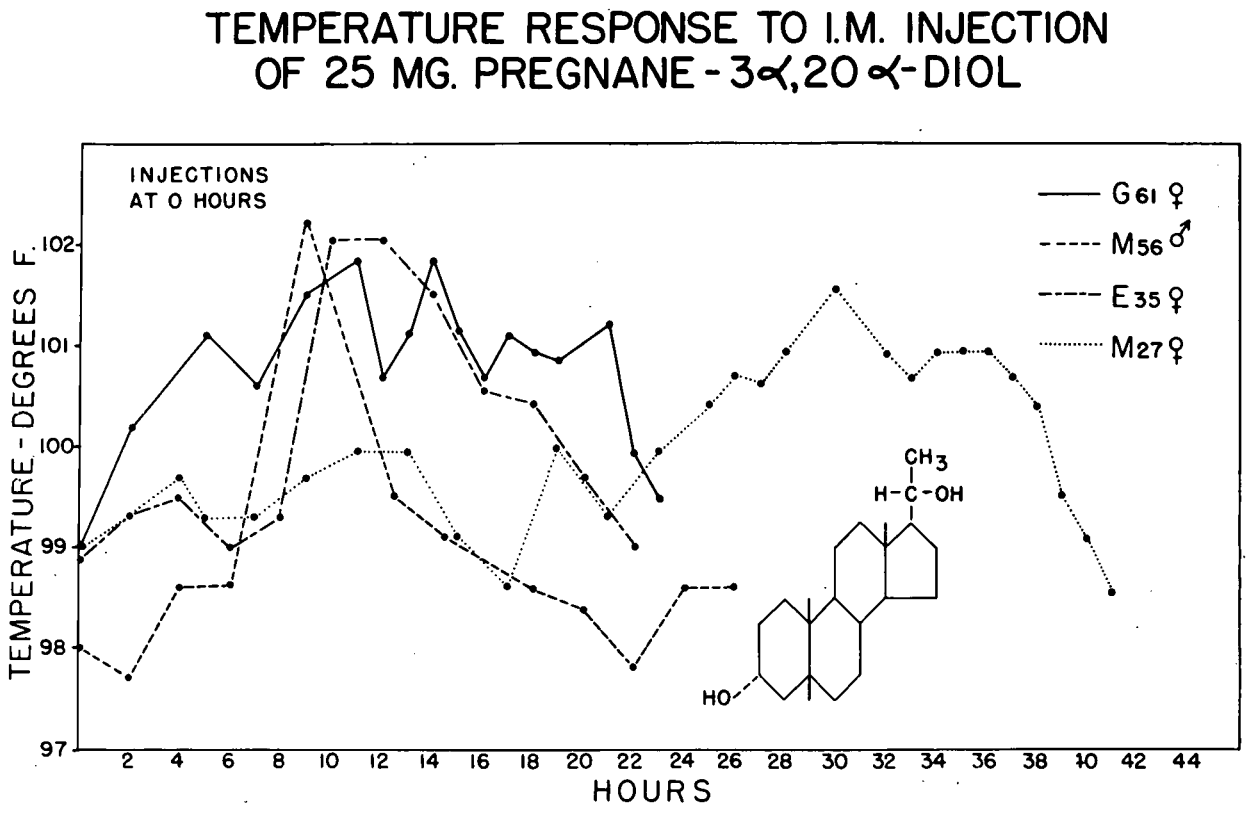

Figure 3.

21-HYDROXYPREGNANE-3,20-DIONE: Intramuscular and intravenous administration. This steroid was given by intramuscular injection in single doses of 25 mg each to 7 subjects, 50 mg each to 2 subjects and 75 mg each to 2 subjects. In 4 of the subjects who received 25 mg intramuscularly, temperature elevations from 101° F to 104.5° F were noted (Figure 4). The remainder who received 25 mg and all 4 receiving 75 mg each showed no elevation in temperature. The subjects showing no thermogenic responses to intramuscular injections of this steroid received doses ranging from 25 to 75 mg intravenously. Moderate to severe phlebitis was noted in each, but no pyrogenic reactions were observed.

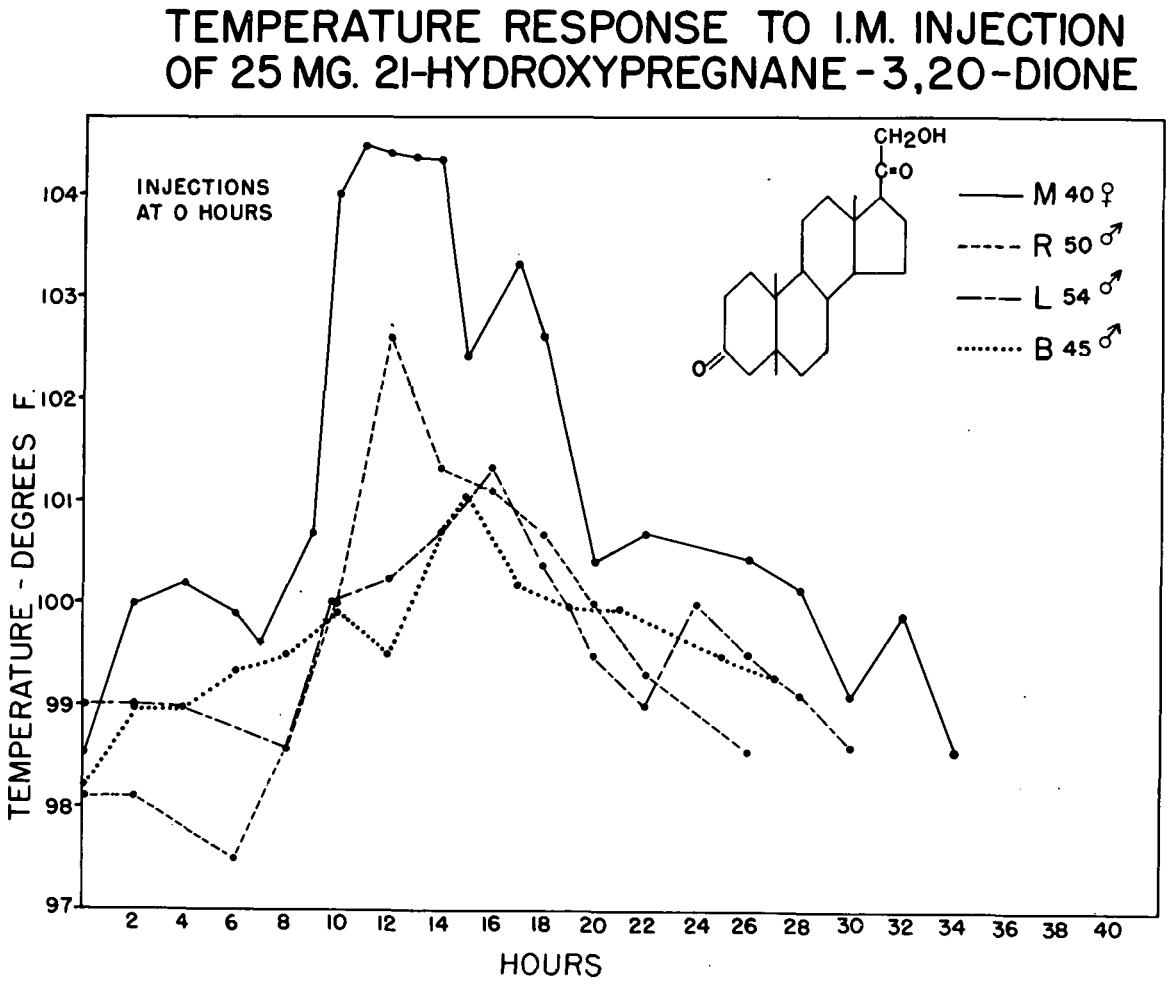

Figure 4.

3α-HYDROXYPREGNANE-11,20-DIONE: Intramuscular and intravenous administration. This compound was given by intramuscular injection in single doses of 25 to 75 mg each, a total of 17 times to 12 subjects. In each instance, a significant temperature elevation was noted. The temperature response of 5 of these subjects to intramuscular doses of 25 mg each is shown in Figure 5. Intravenous doses of 25 to 75 mg were given 13 times to 7 subjects and pyrogenic reactions were observed in all cases. In 4 subjects who developed fever following the intravenous administration of this steroid in 50 mg doses (a total of 5 injections), no evidence of local inflammation or phlebitis was noted. In the remaining 3 subjects receiving a total of 8 infusions, mild to moderate degrees of phlebitis developed 1 to 2 days after injection and persisted for 2 to 3 weeks.

Another group of 3 subjects received a total of 6 intravenous infusions of 50 mg of this steroid without developing fever. Four out of 6 of the infusions which did not provoke fever, were followed by signs of phlebitis. In each of these cases, infusions had been given rapidly (under 30 minutes) and subsequent studies indicated that pyrogenic responses could be provoked in each subject by reinfusion of the same amount of steroid over a minimum period of 2 to 3 hours. Local signs of phlebitis were noted following the latter infusions. In these cases, it was further

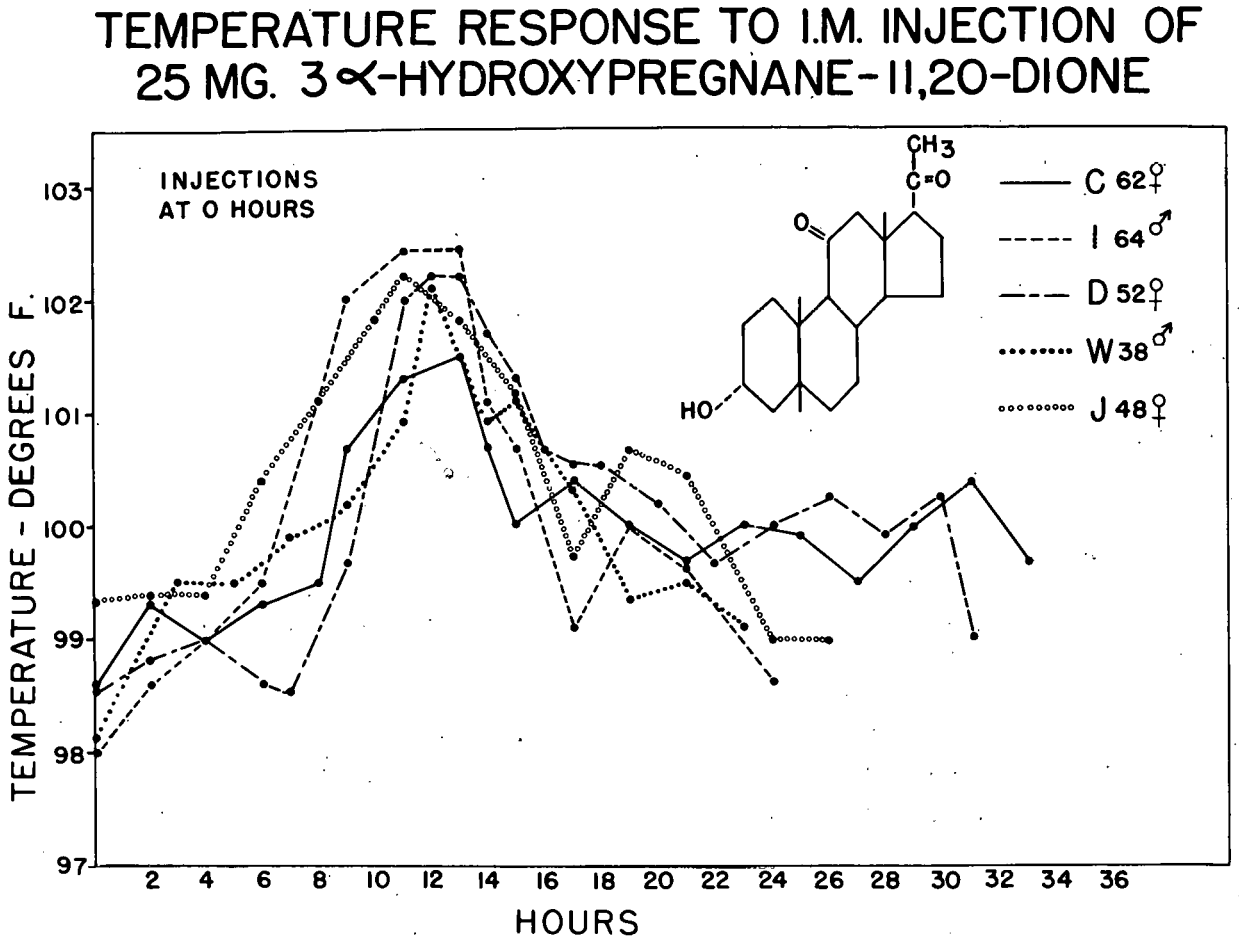

Figure 5.

demonstrated that high fevers could be provoked by intramuscular injections of only 25 mg of the steroid. Two examples of the fever produced by the intravenous injection of this steroid are shown in Figure 1.

3α,17α-DIHYDROXYPREGNANE-20-ONE: Intramuscular and intravenous administration. This steroid was administered by intramuscular injection in single doses of 25 mg each to 4 subjects, and no significant temperature elevation was noted in any. An additional 3 subjects received intravenous infusions of 25 mg each over 3- to 4-hour periods and no pyrogenic responses were evoked. Intravenous injection of this steroid did not produce phlebitis.

3α,17α,20-TRIHYDROXYPREGNANE-11,20-DIONE: Intramuscular administration. Single doses of 25 mg each were administered to 4 subjects. This compound proved to be devoid of pyrogenic activity.

DISCUSSION

These studies demonstrate that the capacity to provoke fever in man extends to a variety of adrenocortical and gonadal hormone derivatives of the pregnane and etiocholane (5β-H) type. The prevalent nature of the pyrogenic action of 3α-hydroxyetiocholane-17-one is thus indicated and a new form of biologic activity established for this class of compounds. The observations

relating to the structural basis of steroid pyrogen action are of interest. Previous studies of 4 isomeric 17-ketosteroids[2] indicate that only the 3α-hydroxy-5β-H derivative (i.e., 3α-hydroxyetiocholane-17-one) provokes fever in man and this report, as well as other studies,[4,5] confirms the potent thermogenic action of steroids of the 5β-H series. The moderate fever produced in some subjects by the repeated administration of the 5α-H steroid androsterone,[6] however, indicates that structural specificity of this action is not complete and that susceptibility to fever may vary from individual to individual with certain metabolites. The intense fever-producing activity of the progesterone metabolite, 3α-hydroxypregnane-20-one is indicated in Figure 2. Single injections of this steroid may provoke fevers lasting 24 to 36 hours or longer in some subjects and it is our impression that this compound is the most potent steroid pyrogen we have studied. Replacement of the alcohol group at carbon 3 of 3α-hydroxyetiocholane-17-one and 3α-hydroxypregnane-20-one by a ketone, significantly lessens the fever-producing activity of both compounds. The residual pyrogenic capacity of pregnane-3,20-dione, however, is greater than that of etiocholane-3,17-dione.[4] The related compound, 17β-hydroxyetiocholane-3-one, appears to have significant, although inconsistent, pyrogenic activity in man as well.[7] The acetate derivatives of the very potent 3α-hydroxyetiocholane-17-one and 3α-hydroxypregnane-20-one, are devoid of pyrogenic activity in man. The formation of sulfate and glucosiduronate conjugates, which is the physiologic counterpart of this esterification, therefore, serves not only to solubilize these substances for ultimate urinary excretion, but undoubtedly deprives them of significant pyrogenic action in man under normal circumstances.

The 11-oxygenated derivative of 3α-hydroxyetiocholane-17-one retains a lesser degree of pyrogenic activity than the related derivative of 3α-hydroxypregnane-20-one, which is in keeping with our impression[4] that among the steroids we have studied, the pregnane compounds are in general more potent fever-producing agents than the comparable etiocholane derivatives. It is of interest that an oxygen atom at carbon 11 of these steroid pyrogens does not completely eliminate their thermogenic activity, as might be expected from the particular association of this functional group with the anti-pyretic adrenocortical hormones. It may be noted that the two 11-oxygenated steroid pyrogens studied are related to antipyretic hormones either in the form of a chemical precursor[8] or a transformation product.[9] The fact that these substances possess biologic activity directly antagonistic to that manifested by the hormone is of considerable interest and offers the theoretical possibility of a novel physiologic means for modification of the expressions of hormone action in man.

Replacement of the ketone group at carbon 20 of 3α-hydroxypregnane-20-one with an alcohol function to form another progesterone metabolite, pregnane-3α,20α-diol does not eliminate pyrogenic activity, although the latter compound does not appear to be as potent in this regard as the former.[4] Pregnane-3α,20α-diol does, however, retain significant capacity to provoke a variety of constitutional symptoms following its parenteral administration to man.[10] Incorporation of an alcohol group at carbon 17 of the pregnane steroids appears to deprive them of significant capacity to provoke fever in man. That this failure to provoke fever is not an artifact of defective absorption is confirmed by the intravenous studies with 3α,17α-dihydroxypregnane-20-one.

The studies with 21-hydroxypregnane-3,20-dione are not sufficient to indicate the general effect of 21-hydroxylation on steroid pyrogen action, but the fact that this compound is pyrogenic in a few subjects (Figure 4) is, nevertheless, of interest. The hemi-succinate ester of this ster-

oid has been used clinically as an intravenous anesthetic agent (Viadril, Pfizer) and, although phlebitis may result from this use,[11] pyrogenic reactions have not been described, despite the large doses (usually exceeding 500 mg per subject) employed. There may be several reasons for this. Esterification may eliminate pyrogenic activity, as was noted with the acetate esters of 3α-hydroxypregnane-20-one and 3α-hydroxyetiocholane-17-one. The very rapid infusion of steroid required for anesthetic purposes may be ineffective in provoking fever, as we have noted in our experience with the highly potent steroid pyrogen, 3α-hydroxypregnane-11,20-dione (see Methods section above and reference 4). Large doses of the 21-hydroxypregnane-3,20-dione ester may elicit pharmacologic effects which counteract those produced by small doses in a manner comparable to the contrasting effects on body temperature which may be induced by small and large doses of bacterial pyrogen.[12] Finally, it is emphasized that a significant pyrogenic effect of 21-hydroxypregnane-3,20-dione administered by the intramuscular route is noted only in a few subjects. The demonstrated central nervous system depressant action of the 21-ester of this compound may, in these susceptible individuals, block the effect of pyrogens on cerebral thermoregulatory centers.

The intramuscular injection of 3α-hydroxyetiocholane-17-one was frequently followed by a significant degree of local inflammation as well as by a variety of systemic disturbances,[3] similar to those observed by Heckel[10] following the administration of pregnane-3α,20α-diol. In the present study, local reactions were also noted, but were frequently limited to a transient period of discomfort in the area of injection. Objective evidence of significant inflammation at intramuscular sites of injection was uncommon. Nevertheless, the potent inflammatory activity of these compounds is indicated by the frequent development of phlebitis following their administration by the intravenous route in man. It is probable that the capacity of these steroids to provoke inflammation represents a biological property distinct from their fever-promoting action, for the following considerations. Pyrogenic and inflammatory responses have been observed to occur independently of each other in a number of subjects and the separate occurrence of these reactions has been observed following intravenous, as well as intramuscular, administration of these compounds. Moreover, there is a considerable disparity in time of occurrence of these reactions, and in most subjects objective evidence of inflammation does not become evident until long after the febrile response has subsided. This dissociation of inflammatory and pyrogenic activities is most clearly demonstrated following the intravenous administration of these steroids when evidence of phlebitis does not become manifest until 24 or 48 hours after lysis of the fever. This phlebitis, moreover, may persist or become clinically more pronounced without significant associated temperature elevation, as noted above (see Methods). Intravenous injection of the steroid anesthetic Viadryl has also been noted to provoke thrombophlebitis in some patients, although this activity appears related, in part, to rate of administration of the compound.[11] It is highly probable, moreover, that esterification of this steroid anesthetic considerably lessens its inflammatory activity since injection of free steroid pyrogens in amounts approximating only 2 to 5 per cent of the anesthetic dose of Viadryl produces frequent venous inflammation. The failure of solvent infusions to provoke these local reactions presumably indicates that this vascular inflammation represents a direct effect of the steroid on the vessel wall. The pathologic characteristics of this inflammatory reaction are of interest and will be reported at a later date.

Studies on the pathogenesis of fever have led to interest regarding the existence of pyrogenic substances of endogenous origin as well as attempts to characterize such materials.[13] The ster-

oids described in this and previous reports,[1-5] represent the first pure compounds of established chemical structure and of physiologic origin having consistent fever-promoting activity in man. The variety of steroids shown to have thermogenic action indicates the diverse nature of possible endogenous pyrogens, and the fact that these compounds can be produced in vivo from adrenocortical and gonadal hormones naturally stimulates interest regarding their possible role in the pathogenesis of fever in man. Indeed, the study of the relationship of etiocholanolone (3α-hydroxyetiocholane-17-one) to Periodic Fever reported by Bondy et al.,[14] implicates one of these compounds in a febrile disease. It is reasonable to suggest that a similar role in certain forms of clinical fever may ultimately be established for other endogenous steroid pyrogens. The possible relationship of thermogenic progesterone metabolites to the physiologic temperature elevations of the menstrual cycle and pregnancy has already been indicated.[14,15]

SUMMARY

A number of endogenous steroids of the 5β-H series (pregnane and etiocholane) have been examined for pyrogenic action in man. 3α-hydroxyetiocholane-17-one, 3α,11β-dihydroxyetiocholane-17-one, etiocholane-3,17-dione, 3α-hydroxypregnane-20-one, pregnane-3,20-dione, 3α-hydroxypregnane-11,20-dione, pregnane-3α,20α-diol, and 21-hydroxypregnane-3,20-dione possessed varying degrees of such activity. Replacement of the C3-alcohol group of 3α-hydroxyetiocholane-17-one and 3α-hydroxypregnane-20-one with a ketone resulted in significant suppression of pyrogenic activity. Incorporation of an oxygen atom at C11 of the etiocholane or pregnane nucleus similarly modified pyrogenic activity of the parent steroid but the effect was much less marked for the C21 compound than it was for the 17-ketosteroid. Acetylation of 3α-hydroxyetiocholane-17-one and 3α-hydroxypregnane-20-one completely eliminated fever-provoking activity and a similar effect was noted following incorporation of an alcohol group at C17 of the pregnane compounds.

These studies are of interest in the following respects: they establish the existence of a new form of biologic activity for a class of steroids generally considered physiologically inert; they suggest the possibility of other unrecognized types of biologic activity among the large number of hormonal transformation products in man; they define a new class of pyrogenic substances and represent the first demonstration of the consistent pyrogenic activity of pure compounds of known chemical structure and of endogenous origin in man; and they provide a useful experimental means for studies on the physiologic effects and mechanism of fever in man.

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SPECIES SPECIFICITY OF STEROID-INDUCED FEVER*

By

A. Kappas and B. Ratkovits

Previous studies from these laboratories have demonstrated that a number of steroid metabolites of the 5β-H type (pregnane and etiocholane) derived from adrenocortical and gonadal hormones provoke fever when administered intramuscularly or intravenously to man.[1-4] These pyrogenic hormonal transformation products include C19 and C21 steroids of both the 11-desoxy and 11-oxygenated series such as: 3α-hydroxyetiocholan-17-one; pregnane-3α,20α-diol; 3α-hydroxypregnan-20-one; 3α,11β-dihydroxyetiocholan-17-one; and 3α-hydroxypregnan-11,20-dione. These studies established the existence of a new form of biological activity for a class of compounds previously considered physiologically inert and defined a new category of potent fever-producing substances of endogenous origin in man.

Further studies of the biological properties of these compounds have led to the demonstration of an apparent high degree of species specificity of steroid-induced fever. 3α-hydroxypregnan-11,20-dione, a metabolite of endogenous hormones[5] is intensely pyrogenic when administered by intramuscular or intravenous injection to man in doses of approximately 0.2 to 1.0 mg/Kg body weight.[3,4] This steroid, dissolved in small amounts of sesame oil-benzyl alcohol vehicle[2] was given by intramuscular injection in doses of 1.0 and 5.0 mg/Kg to between 8 and 12 animals of each of the following species: rat, mouse, guinea pig, dog, cat, rabbit, and monkey (M. Rhesus). In addition, 2 animals of each of the 4 latter species were given intravenous injections of the steroid (in small amounts of ethyl alcohol) in doses of 1.0 mg/Kg. In order to demonstrate that the laboratory environment and conditions of mild physical restraint and animal handling used in these experiments did not inhibit fever production, these animals were later given intravenous injections of bacterial pyrogen (Piromen). Temperatures were measured rectally by means of indwelling thermistor probes and were recorded continuously during appropriate control periods and for 18 to 24 hours after injections.

Table 1 summarizes the results of this study. Injection of the steroid pyrogen 3α-hydroxypregnan-11,20-dione did not provoke significant temperature elevation in any of the animal species studied despite the fact that it was administered in doses comparable to or greatly exceeding those demonstrated to consistently provoke intense fever in man. Under the same experimental conditions, bacterial pyrogen administration did provoke fever in susceptible species (dog, cat, rabbit, monkey), as expected.

These results, in conjunction with the previously noted failure of etiocholanolone (3α-hydroxyetiocholan-17-one) to provoke fever in rabbits,[1] indicate that there is a high degree of species specificity to the fever-producing action of steroid hormone metabolites. The physiologic basis of this species specificity is not clear since the mechanism by which these steroids provoke fever in humans is not known. The phenomenon is itself of intrinsic biological interest however and may imply the special significance of these or structurally related endogenous com-

*Accepted for publication in the Journal of Clinical Endocrinology and Metabolism 1960.

Table 1

SPECIES SPECIFICITY OF STEROID-INDUCED FEVER

Species	Fever production following injection of steroid and bacterial pyrogen		
	Steroid pyrogen injection I.M.	Steroid pyrogen injection I.V.	Bacterial pyrogen injection I.V.
Rat	None	-	-
Mouse	None	-	-
Guinea pig	None	-	-
Dog	None	None	Fever
Cat	None	None	Fever
Rabbit	None	None	Fever
Monkey	None	None	Fever
Man	Fever	Fever	-*

*Bacterial pyrogen injection is well known to provoke fever in man.

pounds to thermogenic processes under certain circumstances in man. The probable participation of steroid pyrogen in the febrile mechanism of disease has indeed already been indicated[6] and the possible role of pyrogenic progesterone metabolites in the temperature elevation of pregnancy and the luteal phase of the menstrual cycle has been suggested.[3,4]

The species specificity of steroid-induced fever is moreover one of a number of biological properties which distinguish steroid from bacterial pyrogens.[7] The thermogenic activity of the latter substances in susceptible animals has provided the most widely used experimental technique for investigations on the pathogenesis of fever. The endogenous origin of pyrogenic steroids, the species specificity of their thermogenic action and their availability as pure compounds through chemical synthesis however, would seem to offer unique advantages to their use in studies on the mechanism of fever directly in man. Studies along these lines are in progress in these laboratories.

We wish to express our thanks to Dr. Elwood V. Jensen, Department of Biochemistry and Ben May Laboratory for Cancer Research, The University of Chicago and Dr. David K. Fukushima, The Division of Steroid Metabolism and Biochemistry, Sloan-Kettering Institute for Cancer Research for generous gifts of steroids.

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STUDY OF THE GENETIC AND EXTRA-GENETIC DETERMINANTS OF α-KETOSTEROID PRODUCTION IN MAN*

By

A. Kappas and T. F. Gallagher†

The present investigation was undertaken to evaluate the influence of heredity on steroid hormone production of man. The steroid patterns of adult male, dichorionic monozygotic triplets were examined in appropriate periods before, during, and after adrenal stimulation with adrenocorticotrophic hormone (ACTH). The results of this investigation provide evidence for the existence of a significant genetic determinant of the level of steroid hormone production and metabolism, as well as an important extra-genetic influence which can modify this factor. The nature of this extra-genetic influence is suggested from previous studies of physiologic differences in twins.

METHODS AND SUBJECTS OF STUDY

The subjects studied (Subjects F, H, and J) were 25-year-old, white male, monozygotic triplets in excellent health. They were hospitalized during the course of the study, the essential details of which were as follows: two consecutive 3-day periods (periods 1 and 2) were followed by three 1-day periods (periods 3-5) during which each subject received intravenously 20 units of ACTH dissolved in 500 ml of normal saline over 8 hours. Care was taken to assure that equal doses of ACTH from a common lot were given over the fixed periods of time. Injections were started after breakfast and the men were kept in bed during the infusions. Following the ACTH periods, five consecutive 1-day control studies were made (periods 6-10). Four years after the first studies, subject H was re-examined during a control period followed by three consecutive 1-day periods during which he received 20 units of ACTH intravenously, each day over an 8-hour interval.

Complete urine collections were made during all periods and were processed generally within 48 hours after collection. Qualitative and quantitative ketosteroid patterns were determined in all periods by the methods of steroid fractionation previously described.[1,2] Briefly, these methods include sequential and separate hydrolysis of steroid conjugates with β-glucuronidase and mild acid treatment, preparation of neutral fractions from ethereal extracts of the hydrolysates, fractionation of neutral extracts into "ketonic" and "non-ketonic," and "α" and "β-ketosteroid" subfractions, and isolation of individual α-ketosteroids by quantitative paper chromatography with steroid identification by infrared spectrometry when necessary.

The proof of monozygosity of these triplets was based on information derived from both fetal membrane examination and the similarity method.[3] These triplets were subjects of long-term study by L. W. Sontag and associates[4,5] at the Fels Research Institute for the Study of Human Development, Antioch College, Yellow Springs, Ohio, and have been carefully followed from

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birth. The birth membranes were carefully preserved following delivery and were examined in detail by L. W. Sontag and V. L. Nelson of the Fels Institute and G. W. Streeter of the Carnegie Institution of Washington.[4] The principal features of their description are summarized: there was a single placenta, the membranous surface of which was divided into three somewhat unequal parts by two membranous septa. On section, one of these membranes consisted of two amnions fused back to back. The other also showed two amnions but between them was another layer, evidently a much reduced chorionic septum containing remnants of blood vessels. There was no indication of fusion of two placentae. The evidence of the membranes thus indicates the monozygotic, dichorionic origin of these triplets. Similarity in a large number of physical and mental traits was also considered in establishing the single egg origin of the triplets, and these have been reviewed by Sontag and Nelson.[4] In addition to the physical traits described above, detailed blood typing was performed (through the courtesy of Dr. A. S. Weiner, Brooklyn, New York) at the time of this study and all three triplets had identical blood reactions as follows: O, M, k, Rh_1, rh, P, and F.

The triplets were born in the following order: F, weight 2.0 Kg, J, weight 1.8 Kg, and H, weight 1.6 Kg. All three were artificially fed after birth. J and F gained well on this schedule but H began to regurgitate his feedings. He gained weight slowly and the nutritional problem persisted with such severity that he was not expected to survive during the greater part of the first six months following birth. At 14 weeks, H weighed 2.8 Kg compared with 4.1 Kg for J and 4.4 Kg for F. At six months however, the gastrointestinal difficulty of subject H ceased and he began to gain weight rapidly. At two years, H weighed 11.3 Kg, F weighed 12.7 Kg and J weighed 12.3 Kg and this weight ratio continued into adulthood. All three were raised together in a moderate-sized community in Ohio, where they continue to live. Except for the precarious postnatal experience of subject H, their health has been and continues to be excellent.

RESULTS

Steroid patterns were studied in each subject during 8 of the periods noted above. These were the two pre-treatment control periods (1 and 2), the three ACTH periods (3, 4, and 5), the post-control period (6) immediately following the last day of ACTH and the post-control periods on the third day (8) and fifth day (10) after ACTH. During the second study on subject H, steroid patterns were determined during one control day and on each of the three days on ACTH. The following abbreviations are used in the figures: A, for androsterone (3α-hydroxyandrostane-17-one); E, for etiocholanolone (3α-hydroxyetiocholane-17-one); OHA, for 11-hydroxyandrosterone (3α,11β-dihydroxyandrostane-17-one); OHE, for 11-hydroxyetiocholanolone (3α,11β-dihydroxyetiocholane-17-one), and 11=OE, for 11-ketoetiocholanolone (3α-hydroxyetiocholane-11,17-dione).

Quantitative values for individual ketosteroids during the two combined pre-treatment control periods (periods 1 and 2) for each triplet are shown in Figure 1. Production of 11-oxygenated and 11-desoxysteroids by F and J was essentially equivalent. Production of all these steroids by H was markedly lower than his brothers.[1]

Figure 2 shows the ketosteroid patterns of these triplets during the three days of ACTH administration and during the various control periods. The effect of ACTH was to increase production of 11-desoxy and 11-oxygenated adrenal steroids by J and F in comparable fashion. The decline in steroid production after cessation of ACTH by these two subjects was essentially identical as well. There was a marked difference in adrenal responsiveness to ACTH demon-

strated by subject H. Total production of 11-desoxysteroids during the ACTH periods was considerably less than that of his brothers. The 11-oxysteroids, 11=OE and OHE, which are metabolites of hydrocortisone, showed slight increase on ACTH but this was in no way comparable with the vigorous rise exhibited by his brothers'. Interestingly, OHA, which is the major metabolite of 11 β-hydroxy Δ^4-androstene-3,17-dione,[6] increased from a control level slightly less than his brothers' to a somewhat higher level than either F's or J's during the third day of ACTH.

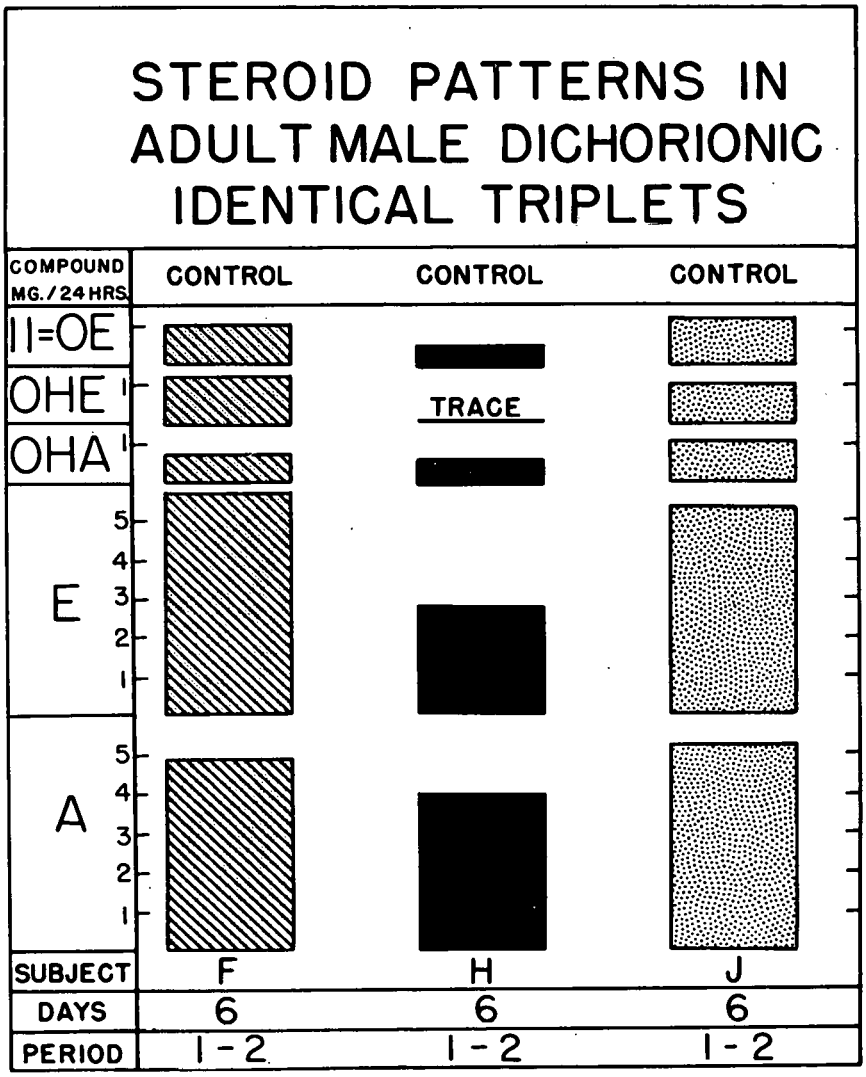

Figure 1.

Figure 3 demonstrates the ketosteroid production of H during control and ACTH periods in an experiment conducted 4 years after that shown in Figure 2. The striking failure of the 11-oxygenated etiocholane derivatives to increase significantly under ACTH stimulation is once more noted and the excellent quantitative agreement with the previous values is impressive evidence for the validity of the methodology employed.

DISCUSSION

The patterns of ketosteroid production have been described in detail in previous reports from these laboratories. These steroid patterns differ quantitatively from person to person[1,2] but are reproducible and constant[7] for a given individual over periods of time. Although subject to alteration by such influences as endocrine[8-10] and non-endocrine disease[11,12] or exogenous hormone administration[2,8,9,13,14] the principal determinants of these patterns under physiologic conditions apparently remain well defined. The results of the present study indicate that both genetic and extra-genetic factors play a significant role in determining the characteristics of

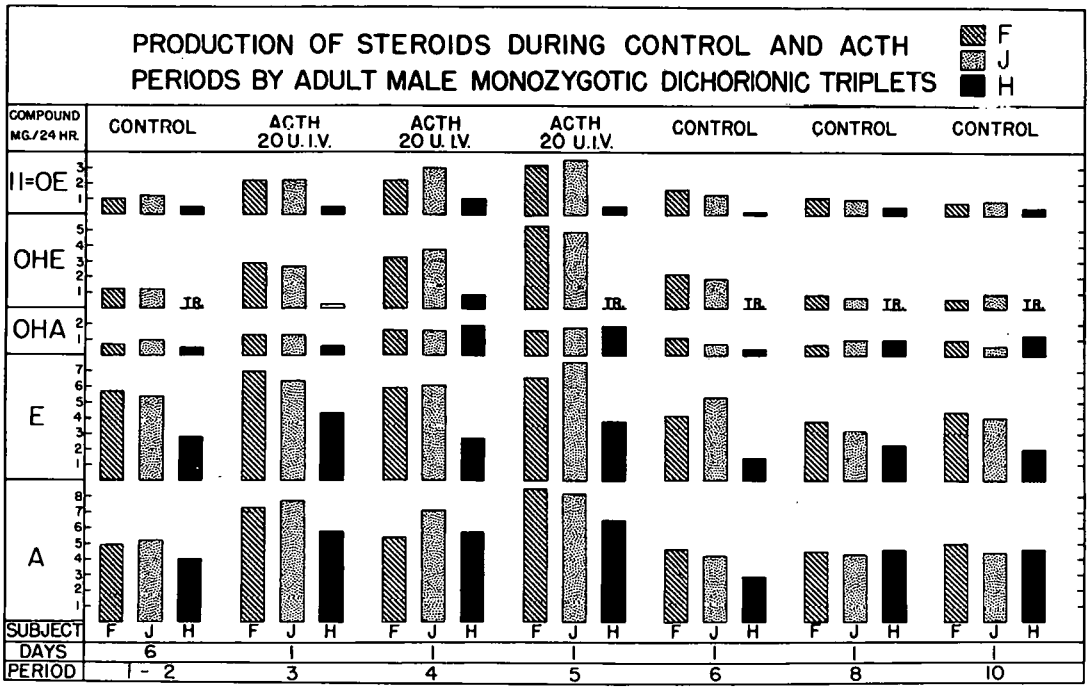

Figure 2.

the steroid pattern in man. The production of 11-desoxy and 11-oxygenated ketosteroids by two of the triplets (F and J) was identical, within experimental error during all control periods and during each day on ACTH. The metabolites examined originate from at least five precursors; i.e., A and E from testosterone dehydroisoandrosterone as well as Δ^4-androstene-3,17-dione,[15] the probable "adrenal androgen"; OHE and 11=OE principally from hydrocortisone;[10] and OHA from 11β-hydroxy-Δ^4-androstene-3,17-dione.[6] It is evident therefore that two of these triplets exhibited a striking similarity, during all of the periods studied, not only in steroid excretion, but also in the rate and pattern of biosynthesis of steroid hormones, the release of these substances into the blood stream, and the enzymatically-determined structural transformations of these hormones to the metabolites isolated and measured. In view of the individuality of these patterns in normal men,[1] the variable adrenal response to ACTH by different men[16] and the established monovular origin of these triplets, the striking similarity of the steroid patterns of F and J in all periods, could not be attributed to chance. These data therefore may be considered to demonstrate the existence of an important genetic determinant of the characteristic pattern

of steroid hormone production and metabolism in man.

There was a significant divergence of the steroid patterns of subject H from his brothers. Subject H produced smaller amounts of 11-desoxy- and 11-oxysteroids during the control period (Figure 1) and during the administration of ACTH his steroid production failed to increase in comparable manner (Figure 2). This difference persisted through the post-ACTH control periods and was likewise demonstrable four years later (Figure 3).

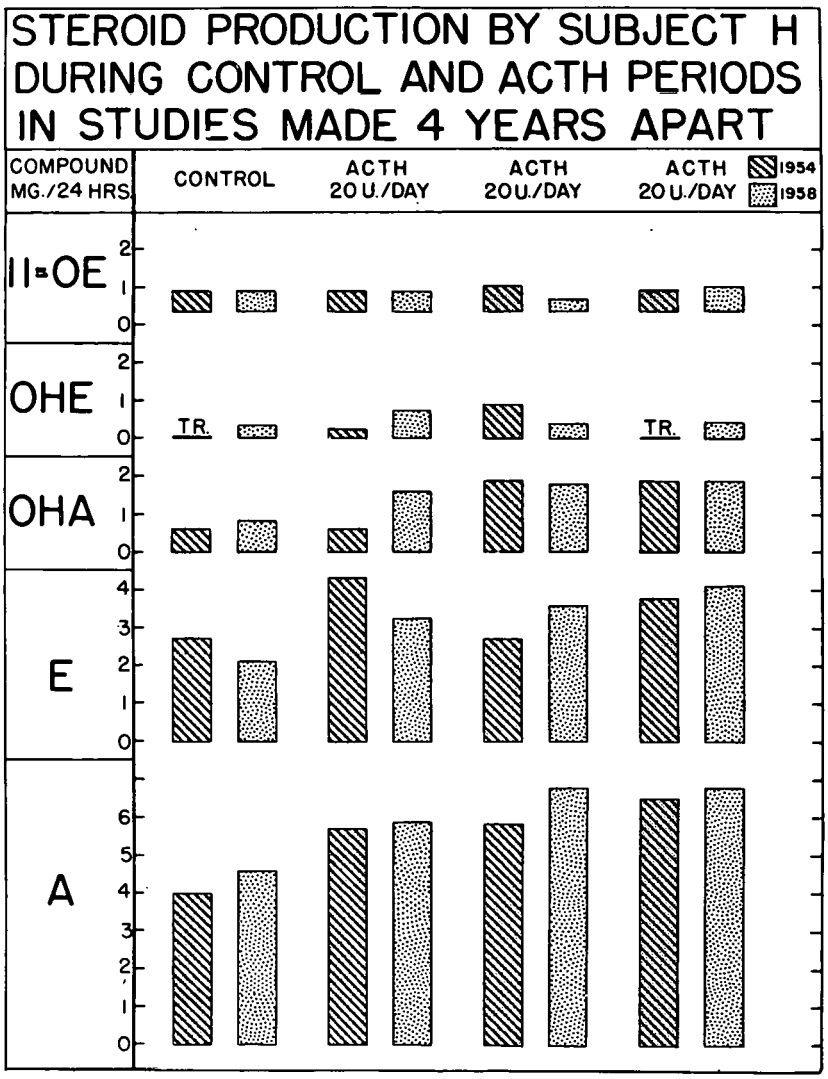

Figure 3.

These results with subject H clearly indicate the existence of an extra-genetic influence operating to modify either the pattern of his steroid hormone production, or steroid hormone metabolism to the compounds isolated, or both. The intra-uterine locus of action of this modifying influence is suggested by the small birth weight and subsequent deficient growth pattern of this triplet. The precise nature of this influence cannot be established with certainty, but its selective effect on subject H, together with information derived from previous twin studies in

man,[16] permit a plausible explanation. Single egg twins may be either dichorial or monochorial with the latter sharing a mutual fetal circulation from early gestation to birth. This common prenatal circulation operates through a variety of intravascular anastomoses, and is consistently associated with a functional vascular asymmetry resulting in a disproportionate distribution of fetal blood supply to one twin partner. This asymmetry of the mutual circulation of monochorial pairs may be lethal, or may result in serious handicap to the intrauterine or neonatal development of the deprived twin. Although there may be considerable postnatal recovery from this early nutritional deficit, the more frequent occurrence of physiologic differences between monochorial as compared with dichorial single egg twins indicates that many of the effects are lasting.[17]

The triplet secundines were dichorial in character[4] indicating a mutual fetal circulation for two of the triplets, comparable to that of monochorial twins. In view of the circulatory imbalance of such a fetal blood supply, it can be presumed that one of this pair of triplets was consistently deprived of a fetal blood flow equivalent to that of his brothers during gestation. The small birth weight and deficient postnatal growth rate of subject H make it likely that he was the handicapped member; it is suggested that his differences in production or metabolism of steroids represent a physiologic aftermath of a relatively deficient fetal blood supply. This same vascular factor may indeed have proved lethal at an early stage to the anticipated fourth embryonic individual.

The nature and physiologic implications of the hormonal discordance shown by subject H are of interest. His steroid pattern is deficient only by comparison with his brothers'. Indeed, subject H compared favorably in terms of steroid production, with a series of normal males of comparable age.[1] However, his response to adrenal stimulation with ACTH was not only different from that of his brothers, but was highly unusual when compared with the response of normal men and women.[2,13,16] Androsterone, etiocholanolone and 11-hydroxyandrosterone increased—clear evidence that a portion of the adrenal secretion was capable of response to a trophic hormone. There was, however, no significant elevation in the two hydrocortisone metabolites, OHE and 11=OE, despite the administration of ACTH in a dose and by a route known to produce maximal adrenal stimulation.[18]

This apparent impairment in response to ACTH may have the following basis. The inability to increase production of 11-oxygenated etiocholane metabolites during adrenal stimulation may denote a failure to enhance adrenal production of hydrocortisone and may therefore indicate a lack of "adrenal reserve" function. This subject would thus have a superficial physiologic resemblance to the patients described by Abu Haydar et al.[19]

In contrast to these patients, however, the capacity of subject H for vigorous physical activity, including war service in Korea, was comparable in all respects to that of his brothers and contradicts the likelihood of latent hypoadrenalism.

Alternatively, the failure to demonstrate increased production of 11-oxygenated etiocholane steroids during ACTH administration may be due to metabolism of hydrocortisone to end products of unusual type, or the consistent transformation of this hormone to C-21 metabolites such as "tetrahydrohydrocortisone" or the "cortols" and "cortolones" described by Fukushima et al.[20] These possibilities could not be investigated at the time of this study. If shown to occur, they would in any case only serve to emphasize the difference between subject H and his brothers, and affirm in effect the interplay of genetic and extra-genetic determinants of the patterns of

steroid production and metabolism in man.

SUMMARY

The steroid patterns of normal, adult male, dichorionic monozygotic triplets were examined in detail during control periods and during adrenocortical stimulation with ACTH. In two triplets these patterns were virtually identical, before, during, and after ACTH administration. In view of the highly individual nature of these patterns, this is impressive evidence of a genetic influence on steroid hormone production and metabolism in man. The third triplet, however, produced significantly smaller amounts of individual ketosteroids as compared with his brothers, during all periods. This finding is interpreted as evidence for the existence of an extragenetic influence by means of which steroid production or metabolism had been modified. Related physiologic differences in monochorionic twins have been attributed to demonstrated vascular asymmetry and unequal distribution of the mutual fetal circulation to one partner of the pair. The birth membranes of these triplets were dichorial in character and it is evidence that a fetal situation comparable to that seen in monochorionic twins existed for two of the brothers. It is presumed, therefore, that the triplet with divergent steroid patterns was the deprived member of this pair, and that his differences in production or metabolism of steroids represent a physiologic aftermath of a relatively deficient fetal circulation.

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