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ARGONNE CANCER RESEARCH HOSPITAL
950 EAST FIFTY-NINTH STREET • CHICAGO 37 • ILLINOIS

Semiannual Report to

THE ATOMIC ENERGY COMMISSION

MARCH 1961

LEON O. JACOBSON, M.D.
Editor

MARGOT DOYLE, Ph.D.
Associate Editor

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COMPARISON OF THE EFFECTS OF ISOLOGOUS, HOMOLOGOUS, AND HETEROLOGOUS HEMATOPOIETIC TISSUES ON POST-IRRADIATION SURVIVAL*

By

L. O. Jacobson and E. L. Simmons

The dream of replacing injured or diseased members of the body by a transplant of healthy tissue of human or other animal origin is of some antiquity. One of the earliest authenticated reports of such an attempt dates from 1682, and is described by Gibson.[1] History does not tell us how successful this substitution of a dog's cranium for part of a human skull may have been considered, since the Church disapproved of the operation, and in consequence the offending bone was removed.

In more recent times, successful transplantation of tissues from one animal to another has become a reality, made possible by the development of the atomic bomb and the subsequent increase in our knowledge of radiation and its biologic effects. Research in the fields of radiation biology and medicine has brought new understanding of the fundamental principles of the immune mechanism involved in tissue and organ transplantation, with the possibility of effecting cures.

Even ten years ago, despite the fact that Roentgen discovered x-rays in 1895 and that they have been in use ever since, no treatment for post-irradiation damage was known. It was recognized, to be sure, that the biologic action of irradiation could be modified by pre-irradiation treatment. For example, Treadwell and Gardner[2] had shown in 1938 that mice given estrogens ten days before irradiation were able to survive dosages well above the lethal range. Similarly, in the late 1940's Barron,[3] Patt[4] and Cronkite[5] demonstrated that such sulfhydryl compounds as glutathione and cysteine would protect, provided that they were present at the time of irradiation.

At about the same time, we ourselves were exploring the effect of lead protection of the spleen on regeneration of the blood-forming tissues following total-body irradiation. We realized that this procedure prevented radiation mortality even after lethal dosages. Furthermore, not only did the spleen itself hypertrophy and so prevent the fatal depression of the blood elements, but the spleen shielding was accompanied by a rapid regeneration of the bone marrow which had previously been depopulated. Other protective treatments such as transplantation of spleens of new-born mice, and intravenous injection of homogenates of spleen and embryo liver were also found to protect the mice from dosages well above the lethal range,[6-8] and Lorenz[9] expanded the list of protective hematopoietic tissues to include bone marrow cells. Thus, it became apparent that 28-day survival following administration of a lethal dose of radiation could

*Summary of the address presented as part of a Panel Discussion on Bone Marrow Transplants and the General Immunological Problems, at the Forty-Fifth Annual Meeting of the Radiological Society of North America, Chicago, Ill., November 15-20, 1959, and appearing in Radiology, 75:6, 1960.

be enhanced by injection of a variety of hematopoietic tissues (even from such genetically distinct species as the guinea pig and rat), into the irradiated mouse.

Many explanations were put forward to account for the mechanism responsible for this recovery. Among others, it was suggested that the transplanted cells detoxified a poison supposedly formed in the irradiated tissues, that the healthy cells were self-seeding, or that they released some humoral substance which hastened the recovery of the damaged tissues. At this time we favored the humoral hypothesis because it seemed inconceivable that rat or guinea pig cells would not be rejected by the body of a mouse.

From the outset it was obvious that isologous tissue, that is, tissue from the same inbred strain as the host, was capable of providing long-term survival against irradiation death from dosages often as high as twice the normal LD_{50}. In 1954 however, a new phenomenon was observed which suggested that the injected cells actually colonized the weakened host. Evidence for this was presented by Barnes and Loutit[10] who discovered that although hematopoietic cells of a different strain or species would give excellent survival following the acute radiation crisis, the animals so treated worsened and slowly succumbed by 120 days to what came to be known as "secondary disease" or "homologous disease." This suggested that a severe immune reaction was involved in which the grafted cells were, so to speak, attacking their host, or vice versa.

It is now generally recognized that an immune reaction is going on, and three independent groups of workers have demonstrated that the transplanted cells persist and recolonize the irradiation-weakened host. Lindsley et al.[11] showed by the appropriate use of antisera, that the circulating erythrocytes present in the surviving irradiated injected animal had the same immunological pattern as did those of the animal whose bone marrow was used for the injection, and that therefore these cells must be descendants of the injected bone marrow cells. Nowell, Cole et al.[12] showed by histochemical methods that if a mouse were given rat marrow after lethal irradiation, the marrow in the mouse's body would, following its recovery, stain with alkaline phosphatase in a manner characteristic of the rat. Finally, Ford, Hamerton, Barnes and Loutit[13] removed any doubts remaining that the injected cells were transferring their behavior pattern to their new host, and proved conclusively that the colonizing cells were the descendants of the injected stem cells. They transplanted marrow from other mice, and, using a chromosomal abnormality as a genetic marker, found that the regenerating tissues in the irradiated mouse contained these abnormal chromosomes.

Looking back it seems strange that we did not realize earlier that radiation survival was due to repopulation. A great deal of work on the effect of radiation on the immune response had been published, some of it, like Hektoen's,[14] as early as 1915. It was known, of course, that in the early stages following a low dose of irradiation, an animal lost the ability to respond when challenged with injections of a foreign antigen, but that, as it recovered, its immune production mechanism also recovered. At this time, however, there was no way to investigate challenge with a foreign antigen following a supra-lethal dose of irradiation, since pre-irradiation protection had not yet been discovered, and the animals were dead of the treatment.

Thus, changes in the immune response became the key to an understanding of factors involved in post-irradiation survival, and many experiments performed about this time developed these concepts. A review of the irradiation studies of the period would be incomplete without mention of the transplantation experiments in which Main and Prehn[15] were able to circumvent

the homograft reaction. After giving 800 r to DBA/2 mice followed by supportive injections of BALB/c bone marrow cells, they found that surviving mice retained a skin graft from mice of the strain used as the source of the bone marrow: normally such a transplant between strains is rejected. This host-graft mechanism has since been explored by a great deal of work on experimental tumors.

Realization that supra-lethal doses of irradiation paralyze the immune processes of the body and thus permit the growth of foreign transplanted cells, has been reinforced in recent years by the basic immunologic research of such workers as Billingham, Brent and Medawar[16] and many others. Their work has shown that one can inject an embryo or in some cases the newborn, with foreign antigens during the stage of antibody inactivity. Such an individual will then develop a tolerance to the injected antigens and will not thereafter form antibodies against it. The supra-lethally-irradiated animal is apparently very much like the embryo in the sense that it is not capable of antibody formation at the time when the foreign cells are injected and therefore some degree of tolerance is developed. In the present state of our knowledge, it is difficult to be certain how far we can assume that our research findings in the mouse will be valid for practical application to man. Even studies in mice are not equivocal, for sometimes irradiated mice injected with a homologous bone marrow will live out their life span with a permanent transplant, while at other times the foreign tissues seem to be rejected and the mouse reverts to its original tissues.

Many promising avenues remain to be explored. Experiments with animals suggest that the use of embryonic tissue rather than of adult bone marrow will also lead to the development of tolerance and that fewer secondary immunologic complications may develop when the irradiated animal is colonized by descendants of such embryonic cells. While this has not yet been demonstrated in preliminary experiments using fetal material in man, it will need to be tested in greater detail.

Another method of approach lies in the investigation of the possible action of suppressives, which operate somewhat as follows: bone marrow from mouse B leads to severe secondary disease when it is injected into mouse A. Now, suppose a suppressor substance could be found that would so alter the immune behavior pattern that permanent tolerance would be established, then no secondary immune reaction would occur. Although this approach is as yet only in the introductory experimental stage, some success has been achieved by Beilby and his colleagues,[17] who have reported success in the case of a patient with Hodgkin's disease who suffered from acute bone marrow failure due to chemotherapy. This patient was treated with a bone marrow transfusion from her sister: the bone marrow graft has now survived for more than six months, and is leading to the production of an ever increasing proportion of the patient's erythrocytes.

Finally, it is possible that even the temporary presence of homologous cells may somehow aid the regeneration of the irradiated recipient's own cells, which would thus be restored to normal life. Such an instance may be represented by the case of the accidentally irradiated Yugoslav scientists who were given bone marrow transfusions from French volunteers. Mathé reports[18] that following the recovery of their peripheral blood elements, the donor cells are no longer present. In experiments with rabbits, injections of mouse cells increased survival, but did not lead to colonization, and indeed such non-specific irritants as ground glass have been shown to stimulate the animal's own regeneration and hence aid in radiation survival.

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STUDIES ON THE SURVIVAL OF TRANSFUSED BLOOD IN RATS*

By

J. S. Thompson,† C. W. Gurney,‡ A. Hanel, E. Ford and D. Hofstra

Immunological studies in this laboratory using various strains of rats have led us to reinvestigate the survival of transfused radioactive hexavalent sodium chromate ($Na_2Cr^{51}O_4$)-labeled red cells. Various investigators,[1-5] using this technique have reported normal half survival times ranging from eight to ten days[1,2] to 20.7 ± 2.5 days.[5] Although this discrepancy is generally considered due to a toxic effect of excess chromium ion on the red cell integrity in those studies reporting the lower survival values, at least one criticism is common to all these studies; namely, that measurements were made on genetically close but not identical recipients. Furthermore, the effects of some completely unrelated factors, such as infectious agents, particularly Bartonella muris, were not explicitly taken into consideration.

The present study is concerned with the survival of rat blood in autologous animals and in animals of the same and another strain of rats. One of these commonly employed strains is infected with Bartonella muris, and the survival of the blood of these animals after eradication of the infection will be compared in Bartonella-free normal rats of the other strain, under a variety of conditions which might influence survival of red cells.

MATERIALS AND METHODS

Animals. Male rats of the Long-Evans and Sprague-Dawley strains weighing 230-270 g were used as both donors and recipients.

Chromation. Except in the autologous groups, where approximately 1.5 ml of blood was removed by cardiac puncture from each experimental animal, pooled blood was obtained by aortic exsanguination of at least three rats. The syringes were moistened with heparin and blood was added to A.C.D. solution in the proportion of 1.0 ml of blood to 0.2 ml A.C.D. $Na_2Cr^{51}O_4$ was then added in amounts of approximately 20 μc per 1.0 ml of blood and the resulting mixture incubated for 45 minutes at 37°. The specific activity of the $Na_2Cr^{51}O_4$ used in these experiments varied from 44.5 to 49.5 $\mu c/\mu g$. After incubation 5.0 mg ascorbic acid per ml blood was added[6] and 1.0 ml of the resulting mixture was injected into each recipient rat via the tail vein.

Sampling and analysis of data. Samples of exactly 0.02 ml of blood were withdrawn from the tail into calibrated hemoglobin pipettes and added to 1.0 ml of water in Lusteroid disposable test tubes. Hematocrits were determined by the micro-hematocrit method. All radioactive analyses in an experiment were carried out on the same day in Nancy Wood well-type scintillation counters.

Samples were taken 24 hours after transfusion and thereafter at regular intervals. The values were expressed as a percentage of the 24-hour sample and plotted on semi-logarithmic pa-

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*This report is taken from a paper appearing in Am. J. Physiol., 200:327, 1961.

†United States Public Health Research Fellow of the National Cancer Institute.

‡John and Mary R. Markle Scholar in the Medical Sciences.

per. The time at which 50 per cent of the radioactivity remained (T 1/2) was found from the curve.

Detection and elimination of Bartonella muris. After determining a base line hematocrit, rats of both strains were splenectomized. Subsequently, daily hematocrits and peripheral blood smears were obtained. The smears were stained with Giemsa and examined microscopically for Bartonella muris. If there was no fall in the post-operative hematocrit and the smears did not reveal the organism within 30 days of splenectomy, the animal was considered free of Bartonella muris.

To obtain Bartonella-free blood, rats presumed to be carriers, received 4 mg of oxophenarsine hydrochloride intraperitoneally daily for six days, an arsenical treatment which is an effective method of therapy.[7] The animals were carefully segregated from other possible carriers and were splenectomized one week after completion of therapy. The course of arsenicals was then repeated for a further six days. Hematocrits and Giemsa-stained smears were examined several times for at least two months after the operation. Although the smears were never positive, any animal with a hematocrit below 40 per cent was discarded. To avoid the possibly toxic effect of this large arsenical dosage on donor erythrocytes, a period of 60 days was allowed to elapse before the blood was used in any transfusion experiment. Finally, a group of splenectomized Bartonella-free recipient rats was included in each experiment using this blood. This procedure was a final test to insure freedom from Bartonella in the blood used.[7]

Serological techniques. Agglutinating antibodies to donor red cells were investigated by several methods. Test serum was incubated with a two per cent suspension of washed erythrocytes in saline either for 30 minutes at 37° or for 60 minutes at 5°. After incubation the tubes were centrifuged for three minutes at 500 rpm. Agglutination by means of incomplete antibodies was further sought by the serum-albumin* method of Stratton and Dimond,[8] and by the Coombs' cross-matching procedure using a rabbit anti-rat-serum prepared by the method of Emerson et al.[9]

RESULTS

Identification of Bartonella muris. In a preliminary study, Bartonella muris was identified on smear in 39 of 40 Long-Evans rats following splenectomy. Forty of 40 Sprague-Dawley rats failed to show anemia or any parasitic infestation after splenectomy. Hereafter, Long-Evans rats infected with Bartonella will be referred to as B.I. Long-Evans, and Long-Evans rats treated and free of infection will be called B.F. Long-Evans.

Autologous transfusions in Long-Evans and Sprague-Dawley rats. Figure 1 shows that the T 1/2 of Cr^{51}-labeled autologous blood is approximately 18 days in both the Long-Evans and Sprague-Dawley strains of rats. There is no significant change in the hematocrit in either of the recipient rats.

Reciprocal transfusions between Long-Evans and Sprague-Dawley rats. Figure 2 reveals that B.I. Long-Evans blood normally survives in the Sprague-Dawley rat for approximately four days after transfusion, after which it is rapidly destroyed until less than ten per cent of the activity remains by the tenth day: This pattern of delayed but definitely accelerated destruction corresponds very closely with the survival of Cr^{51}-labeled autologous blood in Sprague-Dawley rats transfused with unlabeled B.I. Long-Evans blood. Figure 2 shows that in both these groups,

*Thirty per cent albumin supplied by Armour and Co.

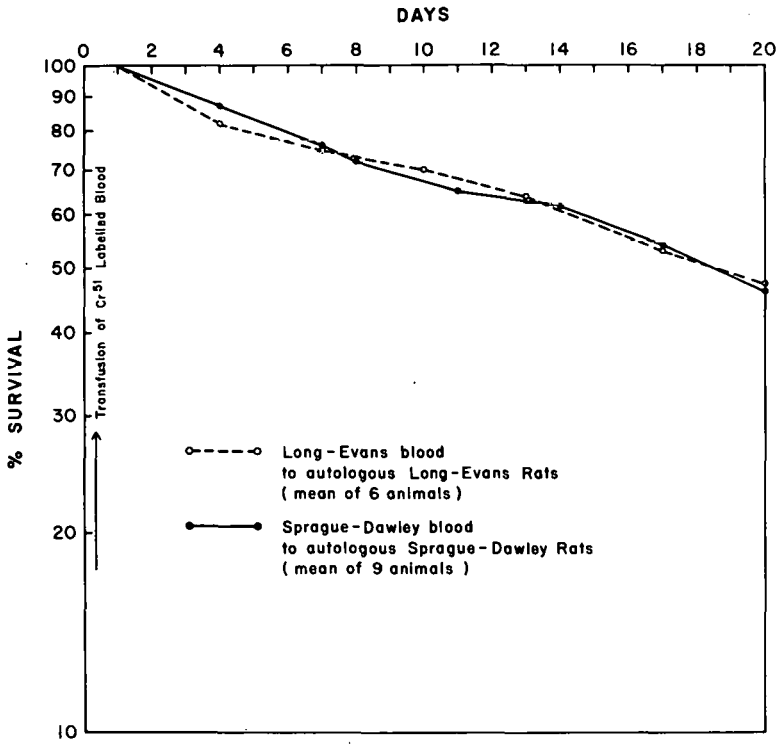

Figure 1. Survival of autologous blood in Sprague-Dawley and Long-Evans rats.

the recipients' hematocrit begins to fall at the same time as accelerated destruction of the labeled cells becomes evident.

Following the reciprocal transfusion of labeled Sprague-Dawley blood to B.I. Long-Evans rats, only a moderate shortening of the survival (T 1/2 - eight days) is noted and there is no appreciable change in the hematocrit (Figure 2).

Homologous transfusions of Sprague-Dawley blood in normal and splenectomized Sprague-Dawley rats. When aliquots of the same pool of labeled blood are injected into four normal and one splenectomized homologous Sprague-Dawley rats on six separate occasions, two different patterns of survival emerge (Figure 3). The T 1/2 of pooled blood in normal recipients is reduced to 12 days, yet this same blood in splenectomized recipients survives even longer than autologous blood in this strain. The hematocrit of the recipient rats remains unaltered.

Homologous transfusions of B.F. Long-Evans blood in normal, splenectomized "hyperimmune," and hyperimmune-splenectomized Sprague-Dawley rats. Prolonged survival of transfused blood after splenectomy is again demonstrated when B.F. Long-Evans blood is transfused to both normal and splenectomized Sprague-Dawley rats (Figure 4). When labeled B.F. Long-Evans blood was injected intravenously into albino rats which had received six weekly intraperitoneal inoculations of 1.0 ml blood from the same donors, the Cr^{51}-tagged cells were virtually

SOME EFFECTS OF RECIPROCAL TRANSFUSIONS
BETWEEN LONG-EVANS AND SPRAGUE-DAWLEY RATS

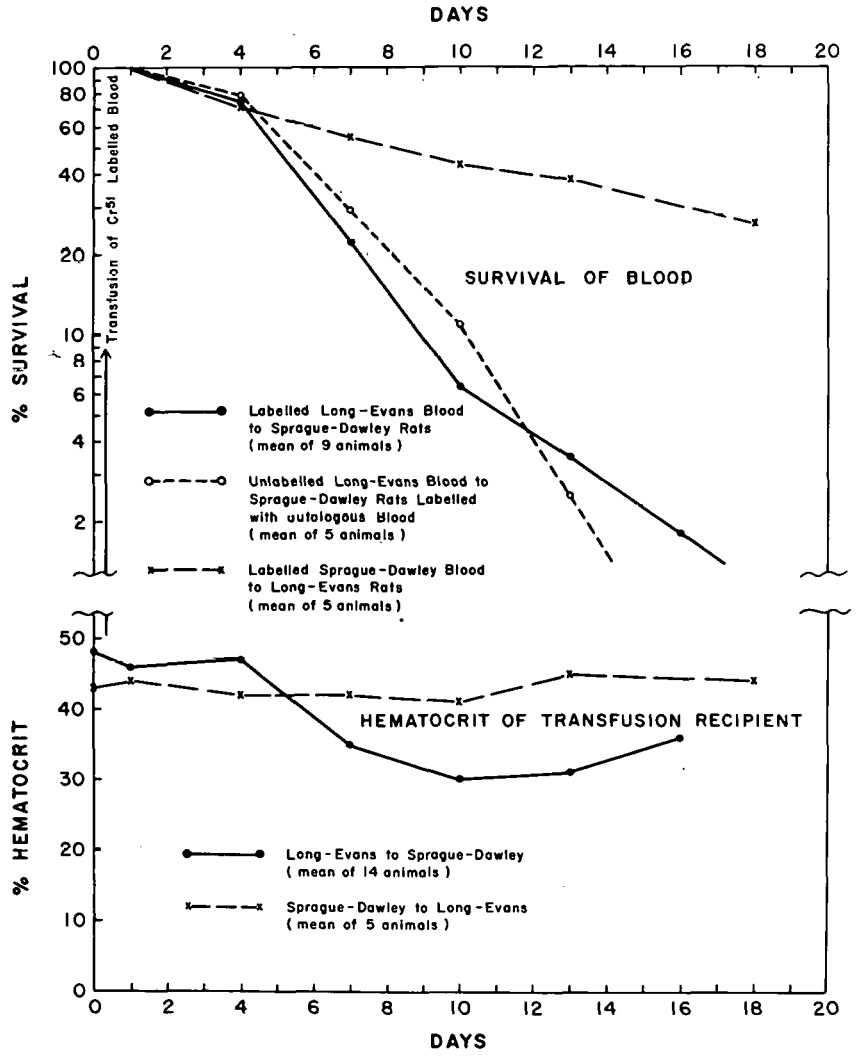

Figure 2. Effect of reciprocal transfusions between Long-Evans and Sprague-Dawley rats.

removed within four days of transfusion. Seven days later these 12 animals were splenectomized. One rat died of the surgical procedure, but when the 11 survivors were given a second Cr^{51}-labeled transfusion seven days later, survival was noticeably prolonged. That is, after splenectomy, the T 1/2 of an eighth inoculation of blood from the same pool as the seven previous injections was now 12 days. No consistent change in the recipients' hematocrit was noted.

Serum from these and other "hyperimmune" Sprague-Dawley rats collected before and after splenectomy has failed to reveal detectable agglutinating antibodies to B.F. Long-Evans washed red cells.

THE SURVIVAL OF SPRAGUE-DAWLEY BLOOD IN AUTOLOGOUS, HOMOLOGOUS AND SPLEENECTOMIZED HOMOLOGOUS SPRAGUE-DAWLEY RATS

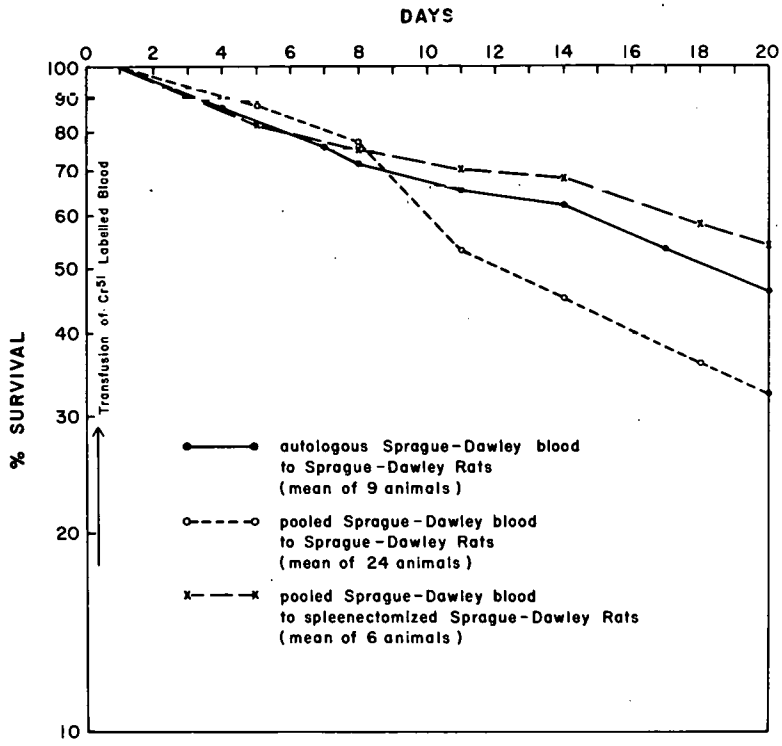

Figure 3. Survival of Sprague-Dawley blood in autologous, homologous and splenectomized homologous Sprague-Dawley rats.

DISCUSSION

Although splenectomy has been shown to produce a striking reduction in the survival of Cr^{51}-labeled blood in autologous carrier rats,[10] our results indicate that the survival of autologous blood in Bartonella-infected rats with intact spleens is within a normal range of 18-20 days.

When blood is transfused from an infected rat to a homologous rat free of Bartonella muris a characteristic pattern of hemolysis sets in after four days and both labeled autologous and transfused homologous blood rapidly disappear from the circulation.

Although Belcher and Harriss[5] and Giblett et al.[4] using the Cr^{51}-technique have previously indicated a T 1/2 of 18-20 days in two different strains of rats, the data in these studies are not completely uniform. In one part of their study Giblett et al.[4] report a normal T 1/2 of 18 days, while another table illustrates an apparent T 1/2 of 11-12 days in control rats. They correlate the use of Cr^{51} source with a low specific activity of 1-3 $\mu c/\mu g$ Cr with the shorter survival and a Cr^{51} source having a higher specific activity of 13-25 $\mu c/\mu g$ Cr with the T 1/2 of 18 days.

THE SURVIVAL OF BARTONELLA-FREE LONG-EVANS BLOOD IN NORMAL AND "HYPERIMMUNE" SPRAGUE-DAWLEY RATS BEFORE AND AFTER SPLEENECTOMY

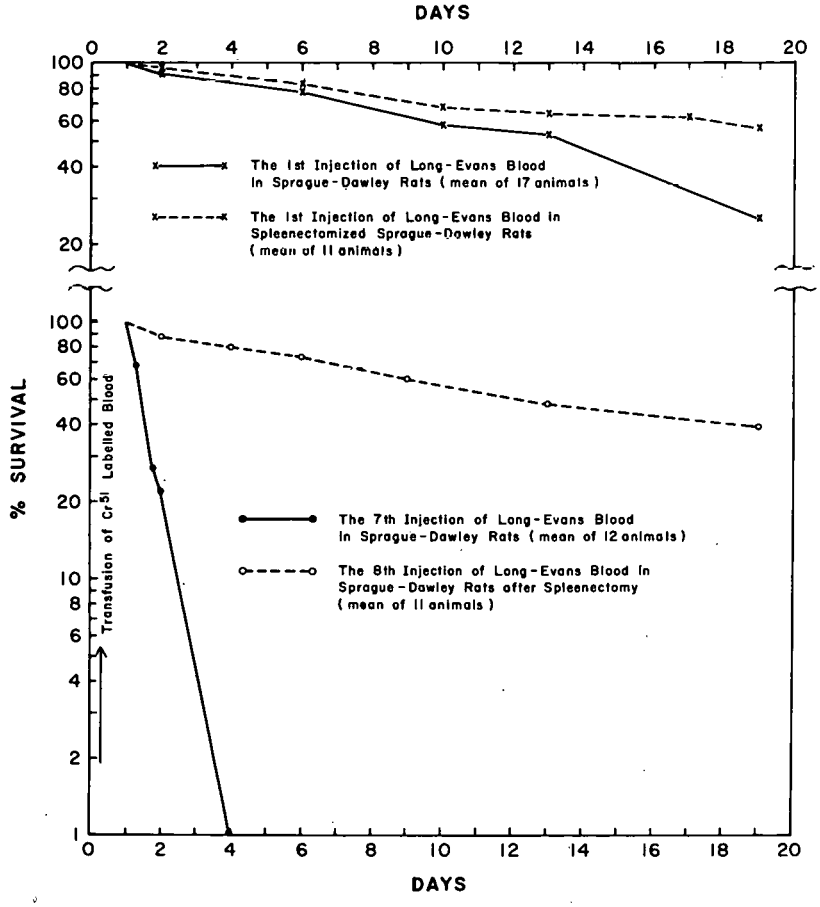

Figure 4. Survival of Bartonella-free Long-Evans blood in normal and hyperimmune Sprague-Dawley rats before and after splenectomy.

They also believe that the toxic effect of excess chromium ion accounts for the lower values reported of eight to ten days.[1,2] Belcher and Harriss[5] report discarding some animals from their normal survival data because "after an interval of eight to fifteen days the remaining labeled cells rapidly disappeared from the circulation and the hemoglobin concentration fell precipitously."

When we studied the survival of pooled blood in the Sprague-Dawley strain the T 1/2 was less than 18 days, yet the concentration of chromium ion was nearly the same, 44.4 to 49.4 $\mu c/\mu g$ Cr, in all these studies. The possibly toxic effect of chromium seems therefore to be excluded. Neither can Bartonella muris infestation account for the decreased survival of pooled Sprague-Dawley blood in Sprague-Dawley rats, since the organism is absent from this strain. The rapid elimination of circulating activity associated with a fall in the recipients' hematocrit,

so characteristic of the pattern when Bartonella-infected blood was transfused into albino rats of this strain, did not occur. Furthermore, splenectomy increased the survival of pooled blood.

Mollison, while stating[11] that the nearly ideal method of assaying the compatibility or incompatibility of transfused blood in the human is offered by labeling with $Na_2Cr^{51}O_4$, cautions strongly against the use of "supposedly compatible" labeled blood in measuring the survival of red blood cells in individuals suspected of having a hemolytic component. We feel that the same reservations should be observed in interpreting data in the rat since we believe that immunological characteristics are not uniform within the strains used in any of the investigations referred to.[1-5] Recently McQuarrie et al.[12] have demonstrated that skin grafts interchanged between members of the albino Sprague-Dawley (Holtzman) strain are usually unsuccessful. Finally, Jäämeri,[13] Owen[14] and Burhoe[15] have described blood types in the rat. The types "C" and "D" reported by Owen[14] do not appear to account for our results, since Smith[3] has used these cell types as the basis of a differential agglutinin technique of erythrocytic survival in the rat. Burhoe[15] was unable to distinguish different blood groups within the same strain, but Jäämeri[13] described a factor which was positive in 50 per cent of the albino rats examined. The relationship of the blood groups described by Burhoe and Jäämeri to transfusion compatibility or incompatibility is unknown.

Our data reveal that the survival of a homologous transfusion, either pooled Sprague-Dawley or B.F. Long-Evans to Sprague-Dawley, is augmented when the recipient has been splenectomized. The effect of splenectomy is much more dramatic when studied in rats previously made "hyperimmune" to homologous B.F. Long-Evans blood. These results differ from those of Swisher[16] who reports that splenectomy does not alter the rapid removal of an incompatible transfusion in previously sensitized dogs.

Rowley[17] has clearly indicated that splenectomized rats fail to produce demonstrable antibody to intravenously administered washed sheep red cells. He additionally demonstrated that splenectomized humans do not produce significant hemolysins to this same antigen administered intravenously.[18] We were unable to detect agglutinins to B.F. Long-Evans red cells either before or after splenectomy. These negative results, however, do not exclude an antibody factor, since they may represent the limitations of the techniques. In fact, the survival of labeled erythrocytes under various circumstances may be compared to the antigen disappearance method of detecting immunological phenomena reported by Talmadge et al.[20]

Although this phenomenon of prolongation of survival of homologous erythrocytes in sensitized recipients is reminiscent of that encountered when albino rats with hypersplenism induced by repeated injections of methylcellulose are subsequently splenectomized,[4,21] one discrepancy is remarkable. Both studies[4,21] report massive splenomegaly, whereas only moderate splenic enlargement was present in Sprague-Dawley rats receiving multiple injections of B.F. Long-Evans blood.

Nevertheless, our data demonstrate that the rat spleen plays an important if not cardinal role in determining the fate of an intravenously administered homologous transfusion. Whether this role is mediated by its function in producing antibodies (perhaps detectable by other techniques[20]), in acting as a trap and phagocytic organ of sequestered red cells, or is a combination of these factors remains for further investigators to answer.

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STUDIES ON ERYTHROPOIESIS. XVII. SOME QUANTITATIVE ASPECTS OF THE ERYTHROPOIETIC RESPONSE TO ERYTHROPOIETIN*

By

C. W. Gurney,† N. Wackman and E. Filmanowicz

Recently we reported a predictable and reproducible response in red cell formation following a single injection of a highly purified preparation of erythropoietin in the transfusion-induced polycythemic mouse,[1] a subject in which erythropoiesis is virtually eliminated by a method which does not exert any recognizable deleterious effects on the hematopoietic system.[2] It is therefore possible to distinguish between experimentally-induced red cell formation and natural erythropoiesis by suppressing the latter. An interesting observation made during our early investigations[1] was that a maximum reticulocyte count under 2 per cent was obtained when a single dose of six units of erythropoietin (as defined by Goldwasser and White[3]) was administered to the polycythemic mouse; larger single doses failed to produce a greater response. This was surprising because the mouse normally has the ability to produce new red cells far in excess of this rate, in response to hypoxia or anemia, even when allowance is made for the elevated red counts in polycythemic animals.

There are several conflicting theories concerning the role played by erythropoietin in the regulation of erythropoiesis. Some authors[4] consider that the rate of red cell production is controlled by erythropoietin delivered to the site of red cell production by the plasma, while others[5,6] envisage more complex schemes, according to which at least two distinct erythropoietins play different roles in promoting this process and are both normally required to sustain it.

Since the response to a single injection of the most highly purified preparation of erythropoietin yet available for intensive investigation, has failed to initiate the production of new red cells in the polycythemic mouse at a rate comparable to that obtained in normal animals under experimental conditions of hypoxic anoxia or anemic anoxia,[1] it appeared reasonable to measure the response in the polycythemic mouse to multiple injections of erythropoietin. This investigation was undertaken in the hope of clarifying the extent to which the humoral factor contributes to the regulation of erythropoiesis.

METHODS AND MATERIALS

In all the studies reported herein, experimental animals were CF No. 1 virgin female mice, 6 to 8 weeks of age at the beginning of each experiment. Red cell donors were CF No. 1 mice chosen without regard for age, but in most instances donors were old retired breeder mice.

Mice were made polycythemic by two intraperitoneal injections of washed homologous red cells as previously described.[1] Studies were begun 6 days after the last injection of red cells, when the hematocrit of the test animals was above 70 per cent, and erythropoiesis was suppressed as evidenced by the absence of reticulocytes in the peripheral blood.

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†John and Mary R. Markle Scholar in the Medical Sciences.

Hematocrit and reticulocyte counts were estimated on blood drawn from a tail vein. Blood for hematocrit determinations was collected in heparinized capillary tubes and spun in micro-hematocrit chambers. Reticulocytes were counted by the direct smear method using brilliant cresyl blue without counterstain. Determinations were made on the basis of numbers of reticulocytes per thousand red cells counted, except where the values were below 0.1 per cent, in which case two thousand red cells were examined.

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

A highly purified preparation of erythropoietin* derived from sheep plasma was used. (The preparation and characterization of this material, glycoprotein in nature, have recently been described.[7]) The erythropoietin was dissolved in 0.9 per cent NaCl and its potency† as used in these experiments is expressed as designated by Goldwasser and White.[3] In one experiment, 1 μc of radioactive iron was administered as ferric citrate via the tail vein, and blood was later obtained from the jugular vein, for estimation of the rate of red cell synthesis as measured by red cell radioiron incorporation. Radioactivity of known volumes of blood was determined in a scintillation well counter.

Normal mice selected for chronic erythropoietin administration were pre-treated with intramuscular injection of 2 mg of iron dextran one week before beginning erythropoietin injections. Blood volumes were performed with red cells of homologous donors, previously labeled in vivo by the intravenous injection of 1 μc of Fe^{59} citrate. After two days, the red cell Fe^{59} content of donor mice was sufficiently great to permit blood volume determinations in the test mice by the intravenous injection of 0.2 ml of washed labeled cells per mouse. Sampling of blood of polycythemic mice by cardiac puncture for determination of blood volumes was delayed for 30 minutes after intravenous injection of labeled cells, because of the possibility of slow equilibration between injected erythrocytes and red cells which might be sequestered in the liver, spleen, etc. of plethoric animals.

RESULTS

Morphological evidence of erythropoiesis following erythropoietin administration. Forty-eight hours after injection of a single dose of erythropoietin, a few reticulocytes can be seen in the peripheral blood of plethoric mice. Prior to this time, although the peripheral blood fails to disclose evidence of erythropoiesis, differentiation and maturation in the marrow and spleen have begun and are progressing in an orderly manner. Figure 1a shows a representative section of the red pulp of the spleen 24 hours after the administration of a single subcutaneous injection of six units of erythropoietin. Clumps of proerythroblasts are now abundant, whereas only rarely can cells presenting this morphological appearance be found in control animals prior to the injection. By 48 hours, differentiation has reached the normoblast stage, and further maturation is evident at 72 hours (Figure 1b). This is also the time of peak peripheral reticulocytosis in

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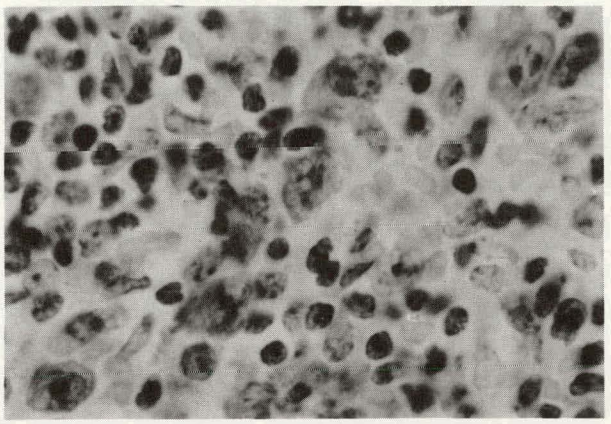
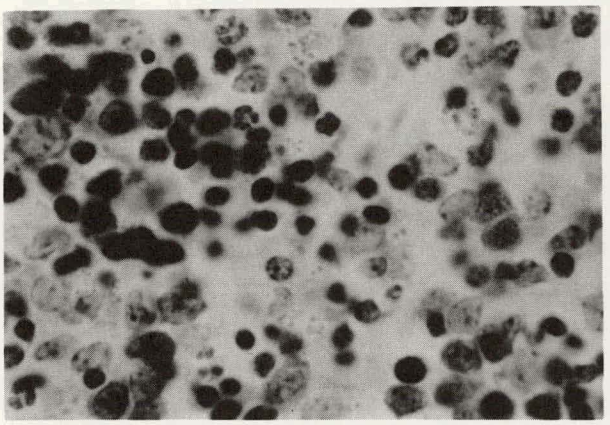
† 2.2 cobalt units/mg.

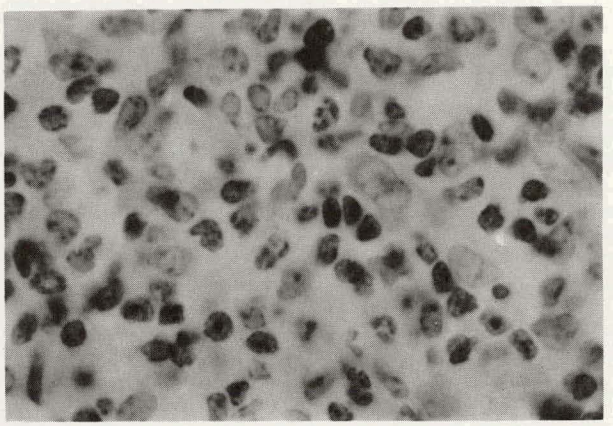
these mice (Figure 2). At 96 hours, erythropoietic activity has decreased markedly (Figure 1c), and by 120 hours no normoblasts remain.



(a)



(b)



(c)

Figure 1. Sections of polycythemic mouse spleen after erythropoietin administration. Hematoxylin-eosine-azure stain. Power 2000 X.

a) 24 hours. Three large cells in center, one at lower left, are proerythroblasts.

b) 72 hours. Most of the cells seen here are normoblasts.

c) 96 hours. Only a few late normoblasts remain.

Effect of multiple injection. Figure 2 illustrates the peripheral reticulocyte response to one and two subcutaneous injections of erythropoietin. In this and subsequent experiments, each group contains a minimum of 5 animals. Reticulocyte counts are made on each animal daily, and the curves plotted are obtained from the average values in each group. A phantom curve (dotted line), shows the result anticipated from a second injection assuming a response equal in magnitude, but occurring 24 hours later, than that produced by the first injection. The sum of the two curves, each representing the anticipated result of a single injection, yields the predicted result

from two such consecutive injections, plotted as a dashed line. It will be seen that the observed result is slightly in excess of the result predicted on the basis of the observed response to a single injection. Although this difference is small, a similar result, again greater in magnitude

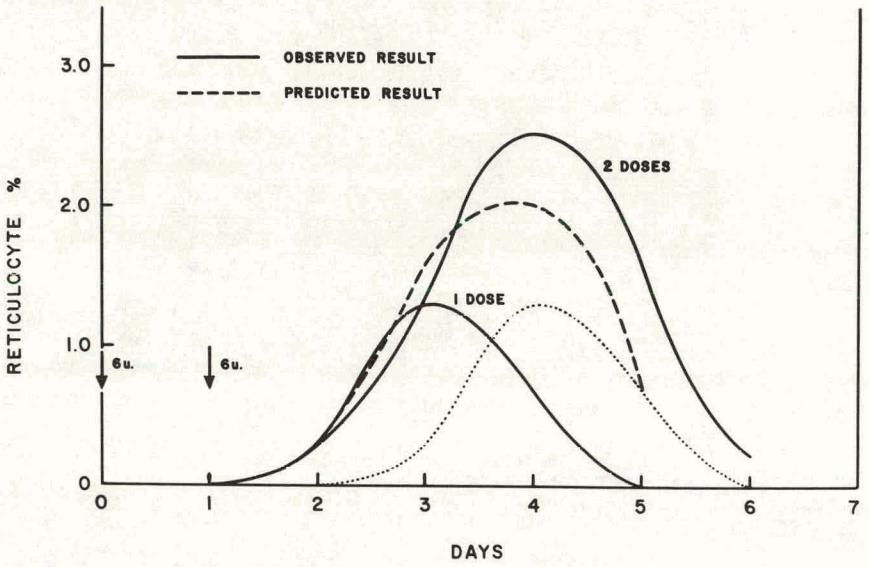


Figure 2. Reticulocyte count in polycythemic mice following one and two subcutaneous injections of erythropoietin.

than predicted, is observed following three and four (Figure 3) consecutive daily injections of erythropoietin. With 3 and 4 injections, the discrepancy between observed and predicted results is greater than it is following only two injections. The data from which Figures 2 and 3 were plotted are recorded in Table 1.

Fractionation of a single dose of erythropoietin. In this experiment, 50 polycythemic mice (5 groups of 10 animals) were used. Each mouse was given 1 μc of radioiron intravenously in 0.25 ml of saline 5 days after the second blood transfusion, at a time when erythropoiesis had ceased. The animals' iron stores were thus labeled with Fe^{59}.

Twenty-four hours later each mouse in group 1 received a single subcutaneous dose of 3 units erythropoietin in 0.5 ml saline; those in group 2 received a first injection of 1.5 units in 0.5 ml saline at the same time, followed by a second similar dose 24 hours later. Mice of group 3 received 2 injections of 1.5 units, the second being given 48 hours later than the first. In group 4, the dose was 0.75 units in 0.5 ml of saline at 12-hour intervals for a total of 4 injections. The fifth group of mice constituted the control group, and these animals received injections of 0.5 ml saline on the 8th and 10th days.

These dose schedules are illustrated in Figure 4: the figures represent the mean values for each group ± one standard deviation. If the radioiron in the peripheral blood on day 15, expressed as net counts per ml per minute, is considered to be proportional to total red cell production in response to the erythropoietin injected, then Figure 4 shows that there is a greater response when a total dose of 3 units is fractionated, than when it is given as a single dose. Furthermore, a given dose of erythropoietin is approximately twice as effective when adminis-

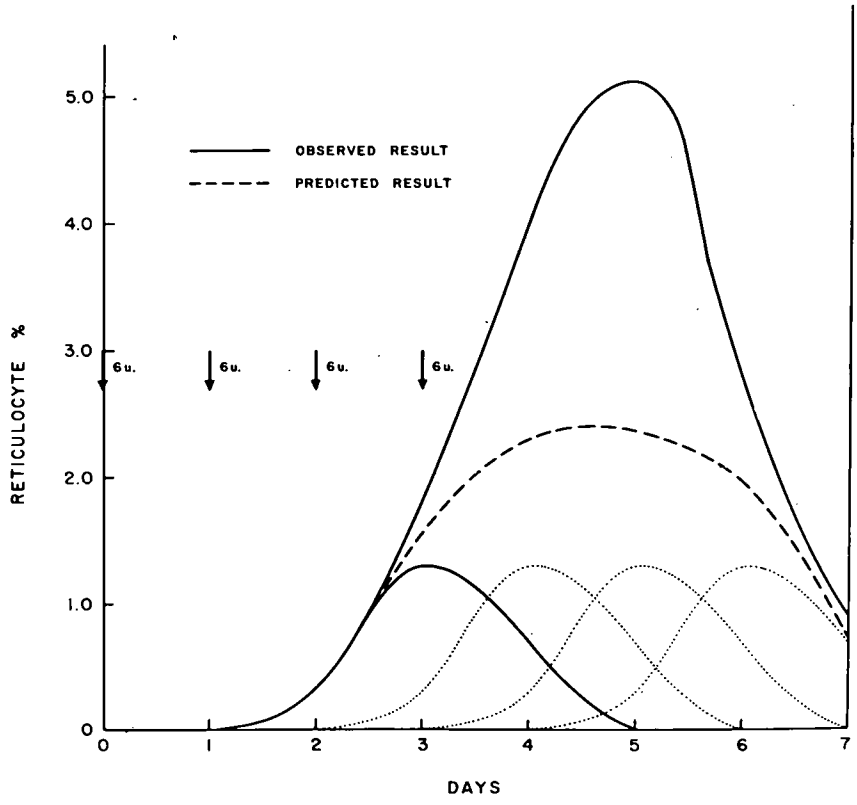


Figure 3. Reticulocyte count in polycythemic mice following four subcutaneous injections of erythropoietin.

Table 1

AVERAGE RETICULOCYTE COUNT (PLUS OR MINUS ONE STANDARD DEVIATION) FOLLOWING SINGLE AND MULTIPLE SUBCUTANEOUS INJECTIONS OF SIX UNITS OF ERYTHROPOIETIN

	0	1	2	3	4	5	6	7
Day 0	.0 ± .0	.0 ± .0	.3 ± .1	1.3 ± .4	.7 ± .3	.0 ± .0	.0 ± .0	.0 ± .0
0,1	.0 ± .0	.0 ± .0	.2 ± .1	1.2 ± .5	2.5 ± .7	1.6 ± .3	.2 ± .2	.0 ± .0
0,1,2	.0 ± .0	.0 ± .0	.4 ± .2	1.6 ± .4	3.8 ± 1.1	4.1 ± 1.1	.7 ± .4	.2 ± .1
0,1,2,3	.0 ± .0	.0 ± .0	.3 ± .0	1.8 ± .4	4.0 ± .7	5.1 ± .9	2.6 ± 1.2	.9 ± .3
Saline control	.0 ± .0	.0 ± .0	.0 ± .0	.0 ± .0	.0 ± .0	.0 ± .0	.0 ± .0	.0 ± .0

tered in two fractionated injections separated by 24 or 48 hours, as when it is administered in a single injection, and almost three times as effective when administered in four small injections given at 12-hour intervals. While the significance of differences between 2 injections of 1.5 units and 4 injections of .75 units is not established, the difference between the response to a single injection of 3 units and the response to 4 injections of .75 units is highly significant (0.01 p 0.001).

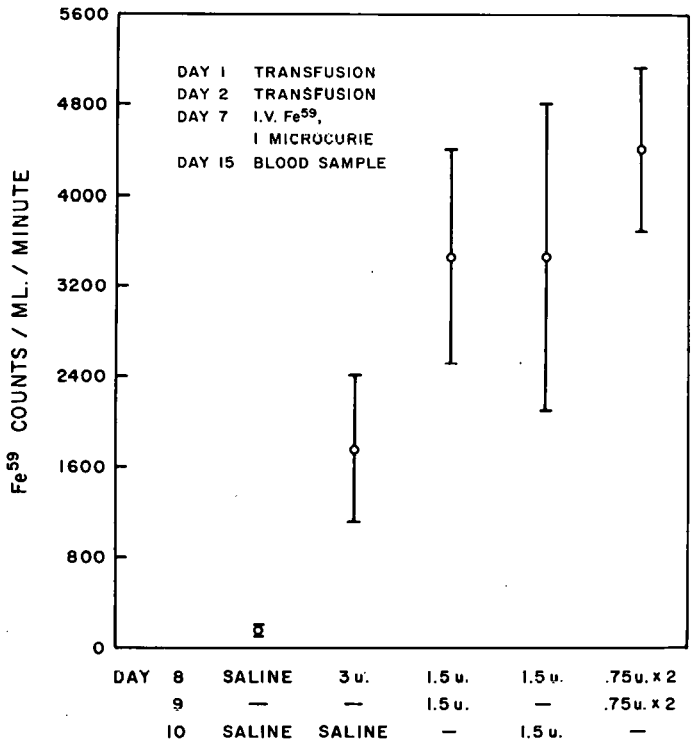


Figure 4. Fe^{59} in peripheral blood following subcutaneous erythropoietin. The figures represent the mean value for each group ± 1 standard deviation.

Maintenance of polycythemia in the transfusion-induced polycythemic mouse. An effort was made to maintain polycythemia in 8 transfused mice by a long-term course of injections of erythropoietin administered at a dose rate of 6 units per day. At the same time, 5 control animals received saline injections. The injections of erythropoietin were begun 6 days after the last transfusion. Figure 5 shows that there was a fall in hematocrit in control animals which was almost linear, and that reticulocytes were absent from the peripheral blood for 16 days. Reticulocytes were first observed again in the blood of the saline control animals on the 18th day, at a time when their hematocrits averaged about 50 per cent. The reticulocyte count rapidly rose to a level of approximately 5 per cent in the animals receiving erythropoietin. Once this rate of reticulocytosis was established, the hematocrit, which had fallen from 80.9 per cent to 73.7 per cent, remained level at a value of approximately 75 per cent for two weeks. When, on the 21st day, it was apparent that erythropoiesis had already begun again in the control animals, the ex-

periment was terminated, at which time red counts and hemoglobins were also estimated. These results are summarized in Table 2, in which it will be noted that although the mean corpuscular hemoglobin concentration was identical in the two groups, the animals receiving erythropoietin had a higher mean corpuscular volume, probably a manifestation of a preponderance of small old cells in the saline-injected animals, and the larger number of newer cells in the animals receiving erythropoietin.

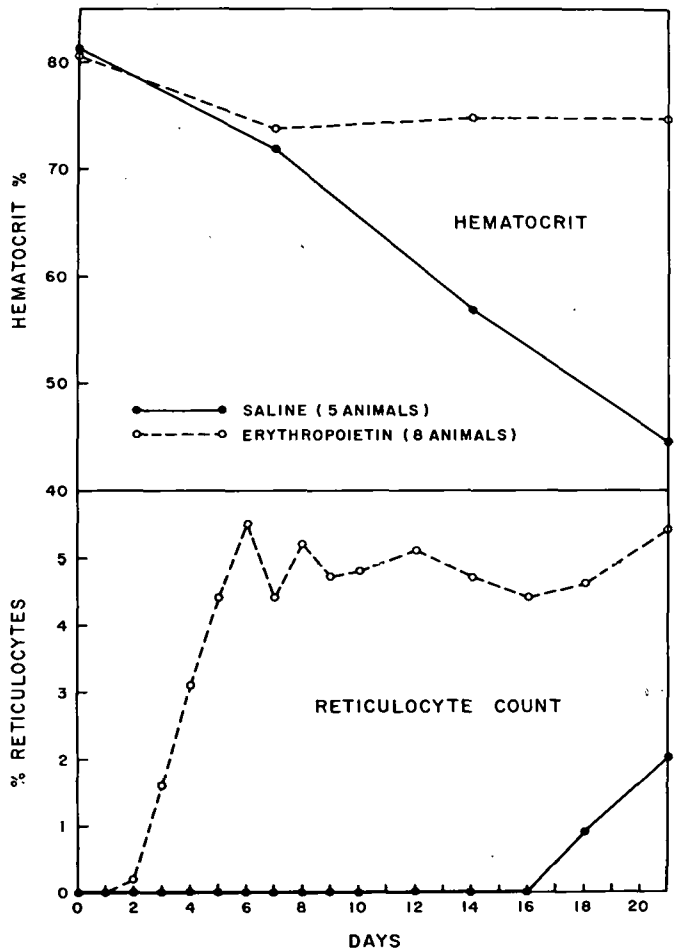


Figure 5. Effect of daily injections of 6 units of erythropoietin in the transfusion-induced polycythemic mouse.

Multiple injections of erythropoietin in the normal mouse. Two groups of 8 normal CF No. 1 mice were given injections of erythropoietin for 27 days, the first group receiving 2 units of erythropoietin twice daily, and the second group 1 unit twice daily. A single group of 8 controls received injections of saline. A steady rise in hemoglobin and hematocrit occurred in both groups receiving erythropoietin. That this was the consequence of red cell production rather than progressive hemoconcentration is apparent from a reticulocytosis in the animals receiving erythropoietin (Figure 6), and a rise in the red cell mass as measured by homologous Fe^{59}-

Table 2

ERYTHROCYTE VALUES (PLUS OR MINUS ONE STANDARD DEVIATION) IN POLYCYTHEMIC MICE FOLLOWING DAILY INJECTIONS OF 6 UNITS OF ERYTHROPOIETIN

	Number of animals	Average				
		Hemoglobin (g %)	Red count (million)	Hematocrit (%)	M.C.V. (cu micra)	M.C.H.C. (%)
Erythropoietin	8	24.0 ± 1.4	14.4 ± .9	74.7 ± 3.5	51.9 ± 2.6	32.1 ± 1.1
Saline	5	14.2 ± .8	10.5 ± .8	44.3 ± 2.7	42.5 ± 3.5	32.1 ± 2.2

labeled red cells (Table 3). At the end of the experiment, on the 27th day, red counts were also made. The differences in average mean corpuscular volume and mean corpuscular hemoglobin concentration between experimental and control groups (Table 3) were small, and of no statistical significance.

DISCUSSION

We believe that these results constitute further confirmation of the role played by the glycoprotein, erythropoietin, in regulating erythropoiesis. This hypothesis, first suggested by Carnot and Deflandre in 1906[8] has been tested repeatedly by numerous investigators, particularly in the last ten years, and a great deal of evidence, summarized in recent reviews,[4,5,9,10] has accumulated. Some investigators[11] however, consider that because our understanding of the role played by the erythropoietins (whether one or more factors is present) is still incomplete, the hypothesis that the volume of red cells is regulated by such substances can only be regarded as tentative. The present study was undertaken to re-examine our beliefs[4] and to determine the degree to which the results of further experiments using a standardized preparation of erythropoietin[3] and a new model[2] would support or contradict them.

Although the sheep erythropoietin used in these experiments is not completely purified, it is a highly concentrated glycoprotein fraction representing approximately 50,000-fold purification of the original plasma.[7] Unless there are two or more such factors of almost identical chemical structure, we must conclude that one factor, glycoprotein in nature, is sufficient to stimulate erythropoiesis both in the transfusion-induced polycythemic mouse and in the normal mouse.

As yet, we do not consider that the existence of more than one kind of erythropoietin has been demonstrated conclusively. Gley and Delor[12] have presented evidence which they interpret as favoring the existence of two stimulating factors. Linman and Bethell,[5] reviewing their own numerous investigations, also arrive at conclusions in support of the existence of two humoral regulators. It is reasonable to believe that many essential constituents, and possibly stimulators or inhibitors of synthesis of heme, globin, and other red cell constituents may be uncovered as formation of red cells is studied in greater detail. We cannot exclude the possibility that more than a single regulatory factor exists. However in the systems employed, namely, the transfusion-induced polycythemic mouse and the normal mouse, we believe that the over-all process of normal red cell formation is initiated and sustained by the administration of a single factor as

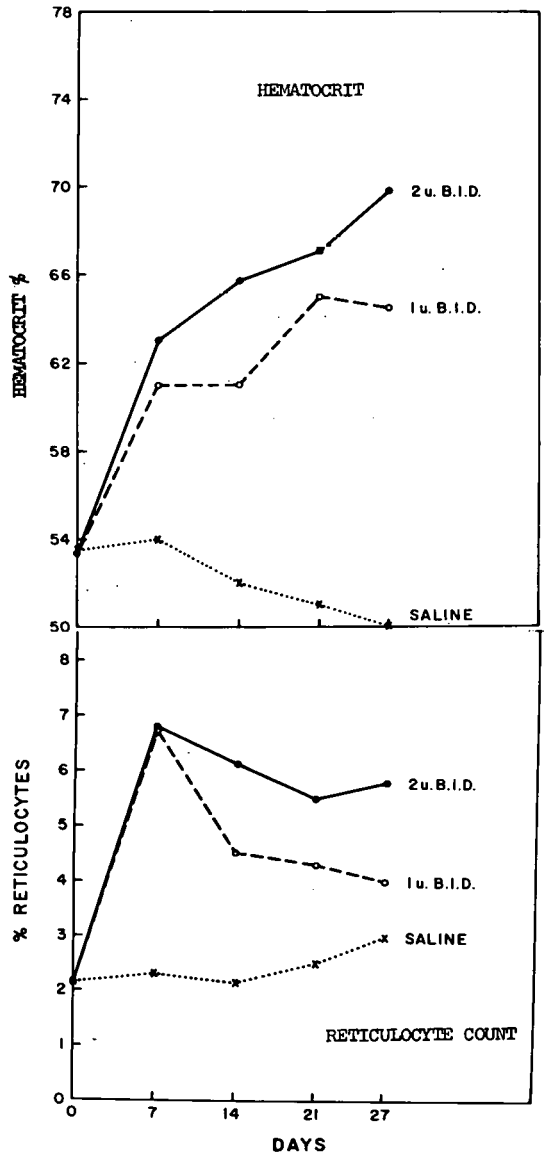


Figure 6. Changes in hematocrit and reticulocyte count associated with prolonged administration of erythropoietin subcutaneously twice daily.

described. Clearly this factor is capable of initiating erythropoiesis in the hypertransfused mouse, and of increasing the rate of erythropoiesis in the normal mouse. That such a factor regulates the normal daily production of red cells in animals and man in the steady state, has not been proved conclusively but is suggested by the erythropoietic response in polycythemic mice and also by reports that concentrates of normal human plasma are erythropoietically active.[13,14] Recently Reichlin and Harrington[15] demonstrated erythropoietin in the plasma of normal rats, although plasma of hypertransfused rats was virtually devoid of activity. Evidence is therefore accumulating to support the concept of continuous regulation of erythropoiesis by erythropoietin. Stohlman et al.,[6] however, have raised objections to this concept which cannot be

Table 3

BLOOD VOLUME AND INDICES (PLUS OR MINUS ONE STANDARD DEVIATION) OF NORMAL ANIMALS MADE POLYCYTHEMIC BY MULTIPLE INJECTIONS OF ERYTHROPOIETIN

Dose	Blood volume (ml)	Red count (million)	Hemoglobin (g %)	Hematocrit (%)	M.C.V. (cu micra)	M.C.H.C. (%)
2 units Twice daily	1.90 ± .18	14.2 ± .8	20.8 ± .9	69.9 ± 3.1	49.3 ± 3.4	29.7 ± .7
1 unit Twice daily	1.86 ± .11	13.0 ± .7	19.6 ± 1.2	64.5 ± 3.5	50.0 ± 2.8	30.2 ± .6
Saline	1.56 ± .20	10.3 ± .5	15.4 ± 1.1	50.1 ± 2.0	48.6 ± 1.1	30.8 ± 1.8

answered completely at this time. As a consequence of these objections, they have postulated a dual regulation of erythropoiesis, the details of which remain to be elucidated.

According to Linman and Bethell,[16] a relatively thermolabile, ether-insoluble agent, mucoprotein in nature, is most probably responsible for initiating erythrocytic differentiation of the stem cell, and also for enhancing hemoglobin synthesis; a second factor, probably lipid in nature, stimulates division of the erythrocytic precursors. If the lipid factor serves as a regulator of division of the erythrocytic precursors, we must conclude either that this factor (itself incapable of inducing differentiation of the stem cells), was already present in excess quantities in our animals; or that it was somehow produced or activated, directly or indirectly, in response to the erythropoietin we injected.

The results of our study differ from those of investigators,[17,18] who have observed increased erythrocyte counts following injections of erythropoietin, but not a concomitant parallel rise in the blood hemoglobin fraction. Because the state of iron balance in mice is precarious[19] and because hypochromia and microcytosis are the expected consequences of iron deficiency, our animals were pretreated with iron dextran prior to the administration of erythropoietin. Rises in hemoglobin and hematocrit, in addition to new red cell formation as measured by reticulocytes, were observed in our study. Red cell counts were made only after long courses of erythropoietin injection, and at those times no consistent hypochromia or microcytosis were observed.

It has been concluded that erythropoietin acts by stimulating differentiation of primitive undifferentiated mesenchymal cells[20,21] and we have tentatively accepted this conclusion since Jacobson et al.[2] reported that erythropoiesis can be reinstituted in polycythemic mice by the injection of plasma containing a high titer of erythropoietin. Stohlman[22] considers that other mechanisms may also be involved in the regulation of erythropoiesis, and although our results do not give a conclusive answer to this question we do not reject the possibility that erythropoietin exerts a more complicated effect on red cell formation, in addition to a direct effect on the stem cell. Although we have not been able to obtain a reticulocyte count as high as 2 per cent with a single injection of erythropoietin,[1] this value is clearly exceeded in the present study by use of multiple injections. A plateau of approximately 5 per cent is reached when multiple daily injections of 6 units are administered. If, in addition to inducing differentiation, erythropoietin were

to stimulate mitosis, a second injection, given at a time when red cell precursors were present, might be expected to produce a greater effect than the first injection. This seems unlikely, however, since any appreciable stimulation of mitosis would probably have resulted in a greater reticulocyte response after 2 injections than was observed. We believe that the present investigations, although employing techniques different from those of Alpen and Cranmore[20] and Erslev,[21] are best explained by their conclusion that the primary action of erythropoietin is on the stem cell, directing its differentiation into the red cell series.

The progressively greater response to repeated doses of erythropoietin was unexpected, and several explanations are possible. Perhaps some of the presumably large number of stem cells which do not undergo differentiation following a single dose of erythropoietin are, nevertheless, influenced in such a manner as to facilitate response to subsequent injections. It is also possible that part of the initial response in the polycythemic mouse takes the form of ineffective red cell formation. Ineffective erythropoiesis has been described in pernicious anemia by Finch et al.[23] and is considered to be present to some degree in normal erythropoiesis by Stohlman[24] and Lajtha and Oliver.[25] If erythrocyte production were to become more efficient as the time interval between stimulations was decreased, then the number of stem cells activated by the third or fourth injection of erythropoietin, although no greater than the number responding to the first injection, would lead to greater numbers of reticulocytes. Again, if repeated doses of erythropoietin were to cause the release of progressively younger reticulocytes into the circulation (Gordon et al.[26]), then one might expect a reticulocyte rise greater than that induced by a single dose, due to the progressive increase in mean life-span of the reticulocytes in the peripheral blood.

Although a single injection of 6 units of erythropoietin appears to produce a maximum erythropoietic response in the polycythemic mouse,[1] it should be emphasized that it does not follow that such a dose is maximal when administered repeatedly. Recently it has been found that the injection of 10 units twice daily for five days produces a reticulocytosis of 20-25 per cent in young Swiss mice.[27] This further directed our attention to the problem of the manner in which the stem cell pool responds to stimulation under varying conditions.

Because it is not technically possible to perform serial kinetic studies of iron metabolism in small animals, we chose to measure erythropoietic responses in some of the present experiments by degrees of reticulocytosis. Limitations of and objections to the use of this parameter as an index of erythropoiesis should therefore be considered since they may account for some of our results. It has already been shown that red cell precursors do not always follow precisely the same pattern in the course of their maturation.[28] It is not essential that all new red cells entering the peripheral blood do so as reticulocytes. It is, however, necessary that the age distribution of any crop of reticulocytes, and the ratio of reticulocytes to total number of new red cells formed in one span of time, should approximate these values at any other time if reticulocytes are to be employed as a reliable index of red cell production. There are at present no data known to us which indicate how constant or variable these relationships might be in experimental conditions similar to those employed here. Another explanation is therefore suggested which could explain the progressive elevation of the reticulocyte level following multiple injections of erythropoietin. If, of the first wave of reticulocytes produced in response to the first injection of erythropoietin, a larger fraction were to be held up in the marrow or spleen for completion of maturation than was held up after subsequent injections, a result similar to

the one observed would be expected.

Still another problem in the interpretation of changes in reticulocyte counts after multiple injections of erythropoietin is posed by our decision not to correct reticulocyte counts for falling red counts. Since the hematocrits of polycythemic mice fell at a rate of approximately 1.7 per cent per day (Figure 5), presumably the red counts also fell at approximately this rate. Ideally, reticulocyte counts should be recorded as an absolute number per cubic millimeter of blood or as a per cent of normal red cells,[29] but the required daily red counts were not done because it was essential to minimize blood loss. Although only small amounts of blood are needed to fill a red cell pipette, the repeated free flow of blood necessary to insure reliable red counts cannot be hazarded without jeopardizing the polycythemic state in the small animals employed. It seems unlikely, however, that more than a small part of the discrepancy between the expected and observed maximum values in Figures 2 and 3 could be attributed to a cumulated fall of 8 to 10 per cent in the red count in the five days preceding the peak reticulocyte level attained in animals receiving multiple doses of erythropoietin.

The demonstration that there is a smaller erythropoietic response to a single submaximal dose of erythropoietin than is obtained when this dose is fractionated into several smaller doses, warrants some consideration. Although 3 units was chosen for this experiment because we have previously demonstrated that the response to this dose is well below the response to a larger dose,[1] further extensive investigations of dose-response relationships will be required before the response to fractionation of a given amount of erythropoietin can be explained. If, as has been suggested,[7,30] the dose-response relationship is an exponential one, it is reasonable to expect that a submaximal but substantial amount of erythropoietin will produce a greater response when divided into several doses than when given as a single injection. Whatever the explanation, the observations recorded here suggest that a slow sustained release of erythropoietin might be even more effective in promoting erythropoiesis. When changes in the erythropoietic stimulus are induced endogenously, as contrasted to the administration of injected erythropoietin, the response is probably evenly modulated and slowly changing. If this is so, a more effective yield of red cells during physiological erythropoiesis may be expected in response to endogenously produced erythropoietin than has yet been described following administration of erythropoietin under experimental conditions.

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INHIBITION OF ERYTHROPOIESIS BY ESTROGENS*

By

P. P. Dukes and E. Goldwasser

The administration of estrogens over rather long periods of time is known to impair erythropoiesis in different animal species. While large doses of estrogens cause severe anemia in dogs[1,2] and mice[3] the effect of similar doses on the monkey is only slight.[4] In studies of the regeneration of red cells in the rat[5,6] it was found that males recover from bleeding more rapidly than females: When bled female rats are given estradiol continuously they completely fail to restore their original red cell levels.

The conclusion drawn from these experiments, which utilized the usual indices of the peripheral blood (red cell count, hemoglobin concentration and hematocrit) in addition to studies of the morphology of the marrow, was that the anemia resulting from estrogen administration is caused by the replacement of erythropoietic tissue in the marrow by edema fluid or by bone.

By using a short method to determine the rate of erythropoiesis[7,8] we were able to study the mechanism of the inhibition of red cell formation by estrogens in more detail than had previously been possible. The results reported here indicate that the estrogens exert a rapid effect on the marrow, and we suggest that this effect acts in opposition to the effect exerted by erythropoietin in the maintenance of the steady state of erythropoiesis.[9]

MATERIALS AND METHODS

Groups of five or more male, Sprague-Dawley rats, six to eight weeks old, weighing 160 to 200 g were used in all experiments. The animals received food (Purina rat chow) and water ad libitum. Test estrogens were injected subcutaneously, dissolved in peanut oil containing three mg of benzyl alcohol per ml. Erythropoietin and inorganic compounds to be injected were dissolved in saline. Erythropoiesis was measured by injecting one μc of Fe^{59} Cl_3 into the tail vein, and 17 to 18 hours later removing a one-ml sample of blood by cardiac puncture. Radioiron was counted in a well-type scintillation counter. Results are expressed either as per cent of the injected iron incorporated into the total red cell mass, or as per cent depression of erythropoiesis compared to the control group injected with only the vehicle.

Cell counts were done in a Coulter counter. Hemoglobin was determined colorimetrically as cyanmethemoglobin.[11] Reticulocyte counts were made from wet mounts of whole blood stained with brilliant cresyl blue, and hematocrits were determined by the micro method.

We are indebted for the following gifts: Allylestrenol from Organon Inc., 4,4'-sec-butylidenediphenol, 4,4'-isopropylidenedicyclohexanol and p,p'-biphenol from the Dow Chemical Company. All other compounds were purchased from commercial sources.

RESULTS

Having observed that estradiol-17β in small amounts could depress the incorporation of Fe^{59} into red cells, we examined the effect of a number of other compounds, steroid and non-

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steroid, with the results seen in Table 1. Both naturally occurring and synthetic estrogens were found to be inhibitory. Allylestrenol, which was found to be active, is one exception in that it is not estrogenic but has progestational activity. Progesterone itself, however, was not inhibitory at the dose tested. Two compounds, 4,4'-sec-butylidenediphenol and 4,4'-isopropylidenediphenol showed slight activity at high doses. Thus the structural requirement for erythropoiesis inhibition by this class of compounds appears to involve one phenolic ring separated from an alicyclic or aromatic ring by a one- or more carbon atom bridge bearing short aliphatic side chains.

With the exception of estriol glucuronidate, conjugated forms of estrogens were at least equally as active as the parent compounds. Estrogens were much more active, on a molar basis, than NaCNS which is known to direct the differentiation of amphibian embryonic tissue away from blood formation.[12]

The dose-response relationships for diethylstilbestrol and estradiol are shown in Figure 1.

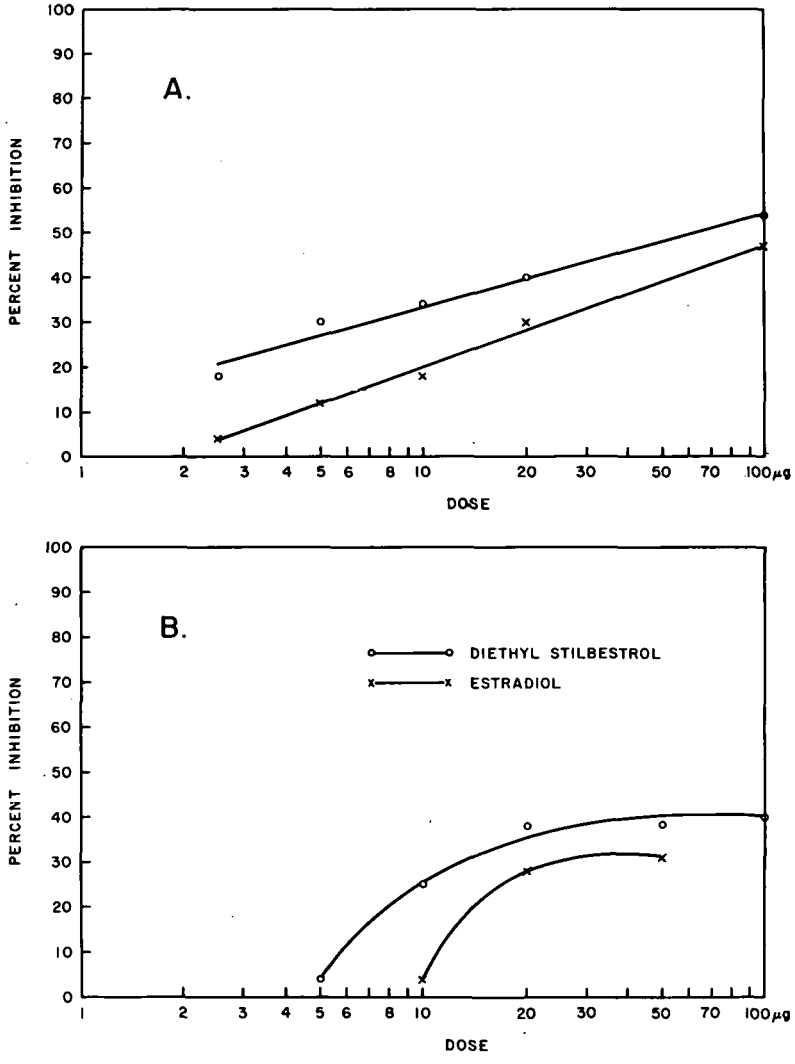

Figure 1. Dose response relationship for estradiol and diethylstilbestrol effect on erythropoiesis. A compounds administered in four equal parts of the indicated dose on four consecutive days. B compounds administered in a single dose three days before Fe^{59} injection.

Table 1

EFFECT OF ESTROGENS AND RELATED COMPOUNDS ON ERYTHROPOIESIS[1]

Compound	Dose/rat μg	Control % Fe^{59} uptake	Experimental % Fe^{59} uptake	Standard error of difference[2]	% Depression of Fe^{59} uptake
Estradiol-17β	20	46.1	34.0***	1.55	26
Estradiol-17β	20	42.5	29.3***	2.48	31
Estradiol-17β	20	43.7	32.5**	2.88	26
Estradiol Dipropionate	20	43.7	32.5*	4.26	26
Estradiol Dipropionate	20	30.6	23.5**	1.64	23
Ethinyl Estradiol	20	35.9	21.5***	2.13	40
Ethinyl Estradiol	20	43.7	21.5***	2.24	51
Estriol	20	46.1	34.9**	2.39	24
Estriol	20	43.7	33.3**	3.08	24
Estriol	20	35.9	32.0	2.17	11
Estriol	20	52.6	48.1	2.24	9
Estriol Glucuronidate	20	52.6	49.9	4.70	5
Estriol Glucuronidate	20	35.9	33.7*	0.95	6
Estriol Glucuronidate	20	43.7	39.8	2.48	9
Estrone	20	46.1	41.9	2.66	9
Estrone	20	42.5	39.2	1.71	8
Diethylstilbestrol	20	49.7	23.3***	5.59	53
Diethylstilbestrol	20	53.4	31.8***	3.01	40
Diethylstilbestrol	20	41.0	26.6**	3.30	35
Diethylstilbestrol	20	38.6	28.2***	1.26	27
Diethylstilbestrol	20	46.1	28.6**	4.02	38
Diethylstilbestrol	20	61.7	36.8***	2.35	40
Diethylstilbestrol	20	43.2	32.7**	2.69	24
Diethylstilbestrol	20	43.9	26.5***	1.67	40
Diethylstilbestrol	20	36.8	27.0*	3.05	27
Diethylstilbestrol	20	39.7	20.6***	2.39	48
Diethylstilbestrol	20	40.2	18.8***	1.94	53
Diethylstilbestrol	20	40.3	24.6***	1.91	39
Diethylstilbestrol	20	36.7	22.7***	1.41	38
Diethylstilbestrol	20	38.3	20.7***	3.50	46
Diethylstilbestrol	20	40.8	25.0***	2.07	39
Diethylstilbestrol	20	30.6	16.3***	1.90	47
Diethylstilbestrol Dipropionate	20	38.3	20.5**	4.72	47
	20	40.8	20.6***	2.70	50
Hexestrol	20	61.7	41.8***	3.66	32
Dienestrol	20	61.7	49.9**	2.62	19
Dienestrol	20	38.3	22.9**	4.17	40
Dienestrol Diacetate	20	38.3	17.9***	4.04	53
Testosterone	20	61.7	53.2	4.00	14
Testosterone	20	42.5	43.0	2.10	- 1
Testosterone	20	39.7	43.3	2.92	- 9
Testosterone	20	38.3	31.7	4.89	17
Testosterone	20	36.6	40.7	1.25	-11
Testosterone Propionate	20	38.3	36.2	3.83	2
Progesterone	20	61.7	58.9	2.84	5
Progesterone	20	30.6	29.1	1.41	5
Allylestrenol	20	43.7	39.0	2.60	11
Allylestrenol	20	30.6	26.6*	1.30	13
Pregnenolone	20	38.3	36.4	3.90	5

Table 1 (continued)

Compound	Dose/rat μg	Control % Fe^{59} uptake	Experimental % Fe^{59} uptake	Standard error of difference[2]	% Depression of Fe^{59} uptake
Cortisol	20	46.1	45.7	1.45	1
Soybean Steroids (21% Stigmasterol)	20	42.5	43.7	2.36	- 3
4,4'-sec-Butylidenediphenol	20	38.3	39.7	4.68	- 4
4,4'-sec-Butylidenediphenol	100	40.8	33.2	4.59	19
4,4'-sec-Butylidenediphenol	100	30.6	29.3	1.18	4
4,4'-Isopropylidenediphenol	20	41.5	38.3	2.43	8
4,4'-Isopropylidenediphenol	20	46.8	40.8	1.56	13
4,4'-Isopropylidenediphenol	20	38.3	35.4	5.13	8
4,4'-Isopropylidenediphenol	20	36.6	38.1	1.39	- 4
4,4'-Isopropylidenediphenol	100	40.8	38.3	1.83	6
4,4'-Isopropylidenediphenol	100	30.6	28.6	1.31	7
4,4'-Isopropylidene-dicyclohexanol	20	36.6	40.4	1.50	-10
	100	40.8	39.1	1.82	6
p,p'-Biphenol	20	38.3	35.7	3.50	7
p,p'-Biphenol	100	40.8	39.4	1.76	3
α-Naphtol	100	36.8	40.4	1.61	-10
β-Naphtol	100	36.8	40.7	2.22	-10
Sodium Thiocyanate[3]	87,000	43.9	37.6*	2.01	14
Sodium Thiocyanate[3]	87,000	41.5	34.0*	2.60	18
Sodium Thiocyanate[3]	87,000	46.8	40.0**	1.90	14

[1]Compounds were administered 72 hours before Fe^{59} injection.

[2]S.E. $(\bar{x}_1 - \bar{x}_2) = s\sqrt{1/N_1 + 1/N_2}$. N_1 and N_2 in all cases equals 5.

*$p < 0.05$.

**$p < 0.01$.

***$p < 0.001$.

No asterisk denotes $p > 0.05$.

When the dose of estrogen is administered in four equal parts on consecutive days, the response is directly proportional to the logarithm of the dose (Figure 1A). The lowest dose of estradiol with a detectable response is 2.5 μg per rat and, as will be seen below, the effective dose of estrogen is only 1.9 μg. When both compounds were given in a single dose 72 hours before the radioiron, there was lessened inhibition of erythropoiesis (Figure 1B). In both experiments diethylstilbestrol was a more active inhibitor of erythropoiesis than was estradiol.

The interval which elapses between administration of estrogen and of Fe^{59} has a pronounced effect on the degree of inhibition produced (Figure 2). When 20 μg of diethylstilbestrol is injected 30 to 60 minutes before the assay of erythropoiesis is started, the iron incorporation remains at the control level. This indicates that the last dose used in the experiment illustrated in Figure 1A was without effect, and that only 75 per cent of the injected estrogen was needed to produce the observed inhibition. Maximal inhibition of erythropoiesis occurs when the interval between a

single injection of diethylstilbestrol and the injection of radioiron is three days. This suggests that diethylstilbestrol acts by inhibiting red cell development in the marrow at one of its earliest phases. Similarly, the stimulation of red cell formation by erythropoietin has been shown by several investigators to involve an early step in erythropoiesis.[13-16]

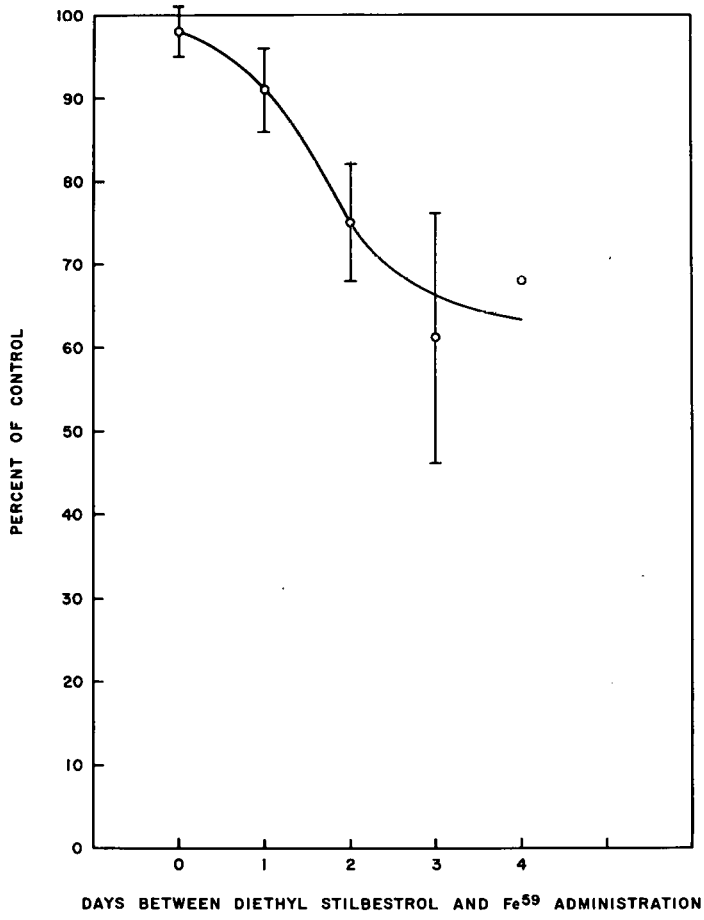

Figure 2. Response to diethylstilbestrol as a function of time between administration of estrogen and injection of radioiron. At each point where applicable, one standard deviation from the mean is indicated.

The stimulation of red cell formation by erythropoietin can be reversed by administration of estrogens, and the inhibitory action of estrogens in its turn can be partly overcome by the amount of erythropoietin used (Table 2). This relationship is noted whether partially purified sheep erythropoietin[9] is administered exogenously or whether the hormone is formed endogenously as a result of cobaltous ion administration.[17] It should be stated that the doses of erythropoietin (five and ten units[18]) used in these experiments are quite large. In the standard, starved rat assay[8] five units of erythropoietin will elevate iron incorporation from the control value of four to five per cent to about 18 per cent of the injected Fe^{59}. Non-starved rats as used in the present study are much less responsive to exogenous erythropoietin and ten units are needed to

increase iron uptake significantly over the control values. On the other hand, ten units of erythropoietin can only partially reverse the depression caused by 20 μg of diethylstilbestrol.

Table 2

EFFECT OF DIETHYLSTILBESTROL ON RESPONSE TO ERYTHROPOIETIN[1]

	% Fe^{59} uptake	p
Control	37 ± 3	
5 Units Sheep Erythropoietin	37 ± 9	-
10 Units Sheep Erythropoietin	42 ± 2	0.02 < 0.01
5 Units Sheep Erythropoietin plus 20 μg Diethylstilbestrol	30 ± 4	0.01 < 0.001
10 Units Sheep Erythropoietin plus 20 μg Diethylstilbestrol	30 ± 4	0.01 < 0.001
20 μg Diethylstilbestrol	23 ± 2	< 0.001

[1]Diethylstilbestrol was injected on the third day and erythropoietin in two equal doses on the second and first day before Fe^{59} administration.

Results expressed as mean ± standard deviation.

Table 3 compares the effect of a series of increasing amounts of cobalt given alone and in combination with a constant amount of diethylstilbestrol. While response to cobalt alone is slight, the rats treated with diethylstilbestrol show a more pronounced response to an equal dosage of cobalt. If these differences could be increased another modification of the Fe^{59} assay method for erythropoietin might be developed which would use these sturdier animals instead of hypophysectomized[19] or starved[8] rats.

Table 3

ANTAGONISM BETWEEN $CoCl_2$ AND DIETHYLSTILBESTROL[1]

μMoles Co^{++}	% Fe^{59} uptake after administration of	
	No diethylstilbestrol	20 ug diethylstilbestrol
0	41 ± 3	27 ± 3†
5	45 ± 4	34 ± 5†
10	46 ± 2	36 ± 6†
15	47 ± 4	40 ± 4*
20	49 ± 4	39 ± 4†

[1]Diethylstilbestrol was injected on the third day and $CoCl_2$ in two equal doses on the second and first day before Fe^{59} administration.

*$p < 0.05$.

†$p < 0.01$.

Results expressed as mean ± standard deviation.

Thiocyanate has a similar effect to diethylstilbestrol in reversing the action of cobaltous ion on Fe^{59} incorporation. For instance, 15 μMoles of $CoCl_2$ injected in two parts, one and two days before Fe^{59} injection, increased Fe^{59} uptake from 40 ± 3 to 47 ± 3 per cent (one standard deviation indicated) ($p < 0.01$). When in addition to this schedule of $CoCl_2$ injection, 87 mg NaCNS, was administered distributed over the three days prior to Fe^{59} injection, the incorporation changed from 47 ± 3 per cent to 37 ± 4 per cent ($p < 0.01$).

A study carried out over a longer period of time (28 days) suggests that estradiol can appreciably effect a lowering of the peripheral blood indices of red cell production. Each treated rat was given a daily injection of ten μg of estradiol in one ml of peanut oil while control rats were given peanut oil alone. The results of this experiment (Figure 3) indicate a rapid interference with erythropoiesis as seen by the sharp fall in reticulocytes while the red cell count, hemoglobin and hematocrit levels fell more slowly. The control group also shows a decrease in the number of reticulocytes; this may perhaps be explained (aside from a normal decrease with age) by the recently reported finding[20] that peanut oil itself has a slight estrogen-like activity. At the end of the 28 days the Fe^{59} incorporation in the treated groups was 21 ± 6, while that for the control group was 34 ± 8 (mean ± standard deviation indicated; $p < 0.02$). The body weight of the estradiol group averaged 263 g, that of the control group 318 g. This slower increase in body weight exactly balanced the fall in red cell mass as measured by dilution of Fe^{59}-labeled red cells, and the red cell volume computed as per cent of body weight remained the same for both groups, 4.8 - 4.9 per cent. The absolute red cell mass of the estradiol-treated group was about 12 g in contrast to 16 g in the control group.

DISCUSSION

The normal rate of erythropoiesis is maintained by a constant discharge of erythropoietin from the kidney[9] and possible other sources[21,22] into the circulation. Superimposed upon this control mechanism there is, we suggest, a counterbalancing effect exerted by estrogenic substances acting on the marrow: the lower red cell levels of females would support this contention. In order to keep the system which produces erythropoietin active and in a state capable of responding to hypoxia by producing increased quantities of the stimulatory substance and yet not produce an excess of red cells the organism would require a method of neutralizing the effect of erythropoietin. Estrogens might serve this purpose by acting to repress the differentiation of the multipotent stem cells toward the red cell line. Estradiol and thiocyanate have been shown by Runnström and Kriszal[23] to suppress differentiation of sea urchin egg endodermal tissue and we have seen that they also depress red cell formation. Similarly, Ken-ichi Ôgi[12] has shown that thiocyanate-treated medio-ventral marginal zone explants of Triturus embryo showed a definite reduction in formation of blood islands after nine days of cultivation.

The decreased weight gain observed in the estradiol-treated group of the 28-day experiment suggests that the adverse influence of the estrogens is not exerted upon the erythopoietic system alone. Attention is called to experiments by Priest and co-workers[24] showing that estradiol reduces the incorporation of radioactive sulfate into cartilage and aortas of rats.

While we are aware that the problem is still far from a final solution, we feel that further study of red cell formation will lead to new insight into the molecular mechanism of differentiation.

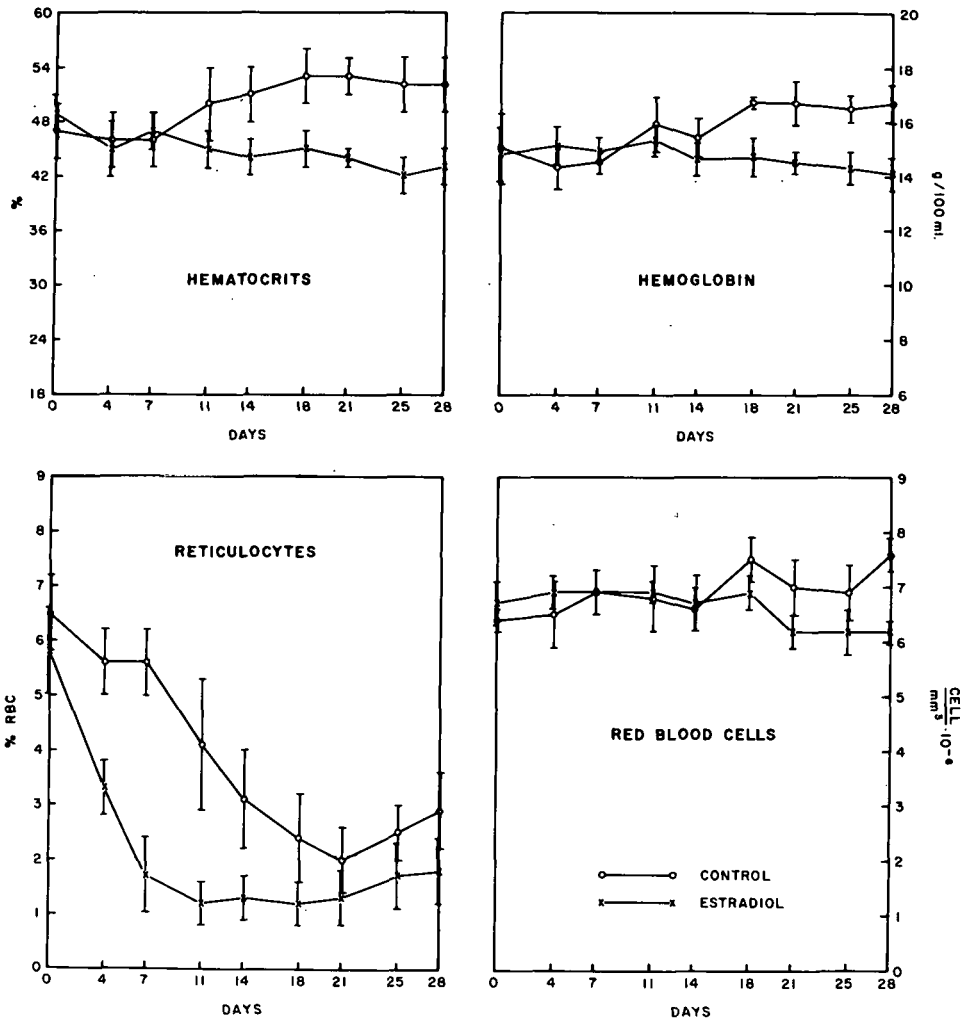

Figure 3. Effect of continued administration of estradiol on red cell indices. Groups of 12 rats were used. One standard deviation from the mean is indicated. On day 28 the differences between untreated and treated groups were: hematocrits, -9 (per cent hematocrit); hemoglobin, -2.6 (g/100 ml); red blood cells, -1.4 (cell/mm^3 10^{-6}); p in all cases < 0.01. Difference in reticulocyte count on day 7 (the day of greatest difference) was -3.9 (per cent red blood count) p < 0.001.

ACKNOWLEDGMENT

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STUDIES ON TRYPTOPHAN METABOLISM
I. URINARY TRYPTOPHAN METABOLITES IN HYPOPLASTIC ANEMIAS AND OTHER HEMATOLOGIC DISORDERS*

By

H. S. Marver†

The principal oxidative pathway "in vivo" of the essential amino acid tryptophan—the tryptophan-kynurenine-niacine pathway[1] has been extensively studied and its stepwise progression appears to be well established (Figure 1).[2,3] A variety of important factors has been implicated in the proper functioning of this pathway among which are four of the B-vitamins (B_1, B_2, B_6, and niacin).[4-8] Insulin and the corticosteroids are also known to influence it,[9,10] and the ferrous ion and a sulfhydryl enzyme appear to be essential.[11] Of paramount importance is pyridoxal phosphate, which is involved at a number of steps.[12] With the development of improved analytical methods, this pathway offers an opportunity for the evaluation of a number of factors of general biological significance in addition to the stepwise study of the oxidation of the indole nucleus of tryptophan.

During recent years a number of studies has demonstrated the elevated excretion of certain urinary tryptophan metabolites in various conditions. For example, the elevated urinary excretion of anthranilic acid in a number of children with an unusual congenital anemia referred to as "erythrogenesis imperfecta,"[13,14] "congenital hypoplastic anemia,"[15] "pure red cell anemia,"[16] and "chronic congenital aregenerative anemia."[17]

The purpose of our investigations was the study of the tryptophan-niacin pathway in patients with aplastic anemia, particularly those with erythrogenesis imperfecta, and the evaluation of these results in the light of studies of other hematologic disorders. Also included was an investigation of such genetic relationships occurring in erythrogenesis imperfecta as might be reflected by urinary tryptophan metabolites.

MATERIALS AND METHODS

This study was carried out on 39 apparently normal subjects having no history of gastrointestinal or renal disease. They ranged in age from five years to 62 years and included 11 females and 28 males. Patients with a variety of confirmed hematologic disorders were also studied, and the results reported are limited to those with oral temperatures below 37.5°C.

Twenty-four-hour urine specimens were collected in bottles containing 20-30 ml of glacial acetic acid and ten ml of toluene and the urine refrigerated after collection. Whenever possible two consecutive 24-hour collections were made, the first being a baseline collection and the second following the ingestion of two g of L-tryptophan. When it was possible to collect only one 24-hour specimen, the post tryptophan-loading collection was selected. No dietary restrictions were placed on the subjects except that the excessive intake of fat, liver, or nuts was avoided.

*Summary of a paper which will appear in J. Lab. Clin. Med., August, 1961.

†Present address: University Hospital, Ann Arbor, Michigan.

OUTLINE OF THE TRYPTOPHAN - NIACIN PATHWAY

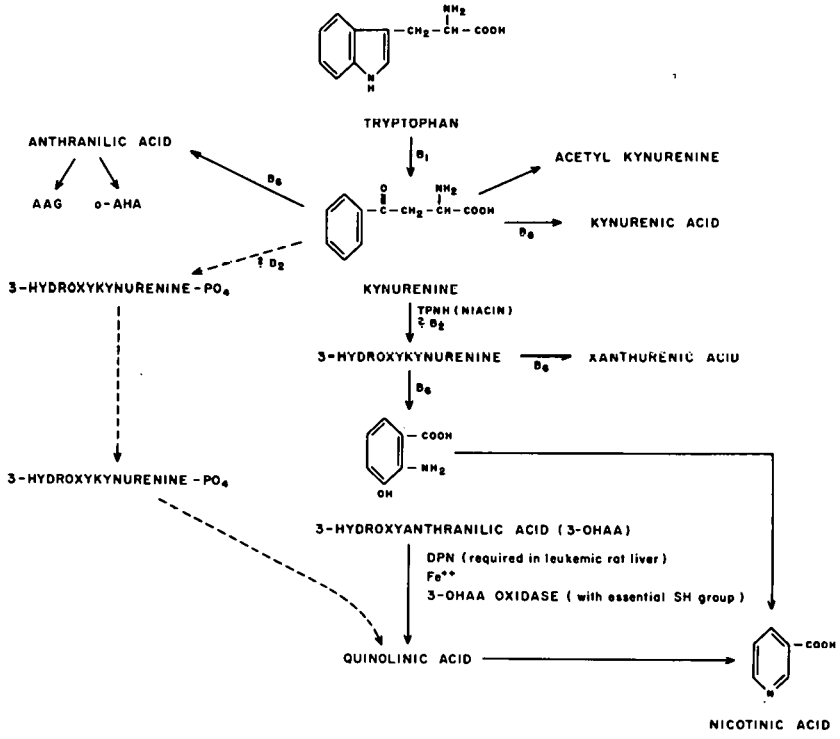

Figure 1. The proposed sites of action of the various B-vitamins are indicated. The alternative oxidative-phosphorylation pathway postulated by Dalgliesh is shown by the dashed arrows. Abbreviations: AAG = anthranilic acid glucuronide; o-AHA = ortho-aminohippuric acid.

Column chromatographic determinations. Modified methods of Brown and Price were used for the determination of anthranilic acid (AA) and its conjugates anthranilic acid glucuronide (AAG), and ortho-aminohippuric acid (o-AHA), acetylkynurenine (Ac.KYN), kynurenine (KYN), and hydroxykynurenine (OH-KYN).[18,19] Because the OH-KYN standard was not available at the beginning of our experiments, a number of the earlier specimens were not analyzed for this metabolite.

The fractions were collected using controlled negative pressure in a series of filter flasks attached to a vacuum pump by way of a manifold. The chromatographic columns were attached to these flasks with tygon tubing and the flow controlled by screw clamps to a rate of 1 to 1.5 ml per minute. Dowex 50 W resin was employed in most of our work. The Bratton-Marshall[20] diazotization of the various metabolites separated was found to give more consistent results when higher concentrations of reagents were used; namely, 0.50 per cent sodium nitrite, 20 per cent ammonium sulfamate, and 0.50 per cent N-(1-naphthyl)-ethylenediamine dihydrochloride. The determinations were carried out in duplicate using a Beckman DU Spectrophotometer.

AA, AAG, o-AHA, and Ac.KYN were determined as AA; KYN was determined as KYN sulfate; OH-KYN was measured as the standard supplied through the courtesy of Dr. L. M. Hender-

son. All determinations were expressed as mg of metabolite excreted per 24-hour urine. An elevation of tryptophan metabolite excretion to a level greater than three times one S.D. above the mean was considered significant.[21]

Paper chromatographic determinations. The results of column chromatography were confirmed both qualitatively and semi-quantitatively by the one dimensional paper chromatographic method of Mason and Berg.[22] 20.0 ml samples of the different fractions were evaporated to dryness under vacuum at a temperature of 37°C. The residue was redissolved in 1.0 ml of 1.0 N HCl and 50-100 μl of the resulting solution spotted on Whatman 1 or Whatman 3M paper. The chromatograms were studied under ultraviolet light and after spraying with Ehrlich's reagent (two per cent paradimethylaminobenzaldehyde in five per cent HCl.[7] Comparisons were always made on the same strips with known metabolites.

RESULTS

General. There appeared to be a significant difference between the excretion of urinary tryptophan metabolites by young children weighing less than 35 kg and by normal adults after the ingestion of two g of L-tryptophan (Tables 1 and 2). In general, this difference lay only in an increase in the urinary Ac.KYN and KYN in the children. No significant difference was noted prior to the ingestion of tryptophan.

Table 1

URINARY TRYPTOPHAN METABOLITES EXCRETED BY NORMAL SUBJECTS

Metabolite	Before tryptophan (20 subjects)			After 2 g L-tryptophan (30 subjects)		
	Range	Mean	1 S.D.	Range	Mean	1 S.D.
Anthranilic acid Glucuronide (AAG)	0.4 - 1.3	0.8 ± 0.1	0.3	0.8 - 2.7	1.7 ± 0.2	0.6
o-Aminohippuric acid (o-AHA)	0.5 - 1.3	1.0 ± 0.1	0.2	1.4 - 3.1	2.1 ± 0.1	0.3
Anthranilic acid (AA)	0.5 - 1.8	1.0 ± 0.1	0.3	0.6 - 2.4	1.3 ± 0.2	0.5
Acetylkynurenine (Ac.KYN)	0.2 - 0.4	0.25 ± 0.04	0.1	0.3 - 1.3	1.1 ± 0.15	0.4
Kynurenine (KYN)	2.5 - 5.4	3.5 ± 0.4	0.9	3.9 - 18.4	9.8 ± 1.4	3.8
Hydroxykynurenine (OH-KYN)	1.5 - 3.3			2.0 - 11.2	8.2 ± 0.6	1.6

Normal subjects demonstrated only slight variations in the urinary tryptophan metabolites studied, before and after the ingestion of tryptophan. Little variation due to weight, age, or sex was noted although there was a suggestion of slightly higher values in the women studied.

The nine children exhibited considerable variation in the excretion of Ac.KYN and KYN, after tryptophan loading. These children ranged in age from five to eight and in weight from 20 to 35 kg. There was, however, no clear-cut reciprocal relationship between age, or weight and KYN and Ac.KYN excretion.

Table 2

URINARY TRYPTOPHAN METABOLITES IN NINE CHILDREN*
(after two g of L-tryptophan)

Metabolite	Range	Mean	1 S.D.
AAG	0.2 - 0.9	0.6 ± 0.1	0.1
o-AHA	0.6 - 2.7	1.7 ± 0.2	0.25
AA	0.1 - 0.7	0.3 ± 0.1	0.1
Ac.KYN	0.7 - 9.7	2.6 ± 2.0	3.0
KYN	4.1 - 31.6	19.7 ± 6.4	9.6
OH-KYN	0.4 - 6.2	4.2 ± 1.3	2.0

*Ages = 5 - 8.5 years. Weight = 20 - 35 kg.

Duplicate determinations by column chromatography seldom showed a variation greater than five per cent. Repeat determinations on the same subjects showed variations of two to 20 per cent with a generally greater variation before tryptophan loading. Normal differences of caloric intake and of protein consumption appeared to have no significant influence. Recovery values for known tryptophan metabolites varied from 78 per cent for 200 μg to 96 per cent for 800 μg.

Hematologic disorders. — Hypoplastic anemias. Two patients with erythrogenesis imperfecta were studied before and after tryptophan loading. One of these (L.S.) was an 11-year-old white girl who had received about 100 units of blood prior to the beginning of these studies. She had a past history of two episodes of transfusion reaction, but not of hepatitis. There was marked hepato-splenomegaly, but otherwise no evidence of liver disease or renal disease. Determinations of the total serum protein, albumin-globulin ratio, serum bilirubin, thymol flocculation, and thymol turbidity were within normal limits. The second patient was an eight-year-old boy (M.M.) who had received nearly 100 transfusions and in addition to his anemia had had a congenital accessory thumb and had an undescended testis. He also had hepatomegaly, but normal liver function studies. Both patients had a confirmed diagnosis of pure red cell anemia on the basis of marrow examination. Both had red hair and the characteristic facies of patients with erythrogenesis imperfecta.[13]

There was no evidence of aminoaciduria on the basis of paper chromatographic studies.[23]

After two g of L-tryptophan, both of these patients excreted elevated quantities of AA and its conjugates to a level five to 20 times and KYN four to eight times one S.D. above the mean values for children. OH-KYN was also elevated in one of them (Table 3, Figure 2).

All four parents of these children demonstrated normal excretory patterns before tryptophan loading. The mother of the boy (M.M.), however, repeatedly demonstrated an abnormal excretion of o-AHA, Ac.KYN and OH-KYN after receiving two g of L-tryptophan (Table 4). She was in her middle thirties, in good health, and had no history of hematologic, gastrointestinal, or renal disorders. Her nutritional state was adequate.

Three other patients with aplastic anemia demonstrated a pancytopenia with marrow hypoplasia of both erythroid and myeloid elements. The anemia of one was attributed to chloramphenicol toxicity. Two patients (H.G. and S.H.) had each received transfusions totaling about

Table 3

URINARY TRYPTOPHAN METABOLITES IN PATIENTS WITH HYPOPLASTIC ANEMIA AND IN ONE PATIENT WITH PRIMARY HEMOCHROMATOSIS—AFTER 2 g OF L-TRYPTOPHAN

Patient	Age	Disorder	AAG	o-AHA	AA	Ac.KYN	KYN	OH-KYN
L.S.	11	Erythrogenesis imperfecta	1.6*	8.7*	3.4*	7.0	53.2*	6.8
M.M.	8	Erythrogenesis imperfecta	1.6*	5.0*	4.3*	9.4	90.0*	15.1* ?
I.S.	56	Idiopathic acquired aplastic anemia	1.5	6.5*	0.9	4.4*	79.0*	24.7*
H.G.	13	Idiopathic acquired aplastic anemia	0.8	1.6	1.0	0.8	12.4	+
S.H.	55	Drug-induced aplastic anemia	1.7	1.3	1.0	3.25	21.2* ?	+
N.B.	40	Primary hemochromatosis	2.5	2.5	2.0	3.5*	20.7	2.0

*Indicates significant elevation (mean + 3 S.D.).

OH-KYN standard not available at the time this urine was studied.

TRYPTOPHAN METABOLITES EXCRETED BY PATIENTS WITH HYPOPLASTIC ANEMIA AND BY ONE ASYMPTOMATIC RELATIVE (after 2g. L-TRYPTOPHAN)

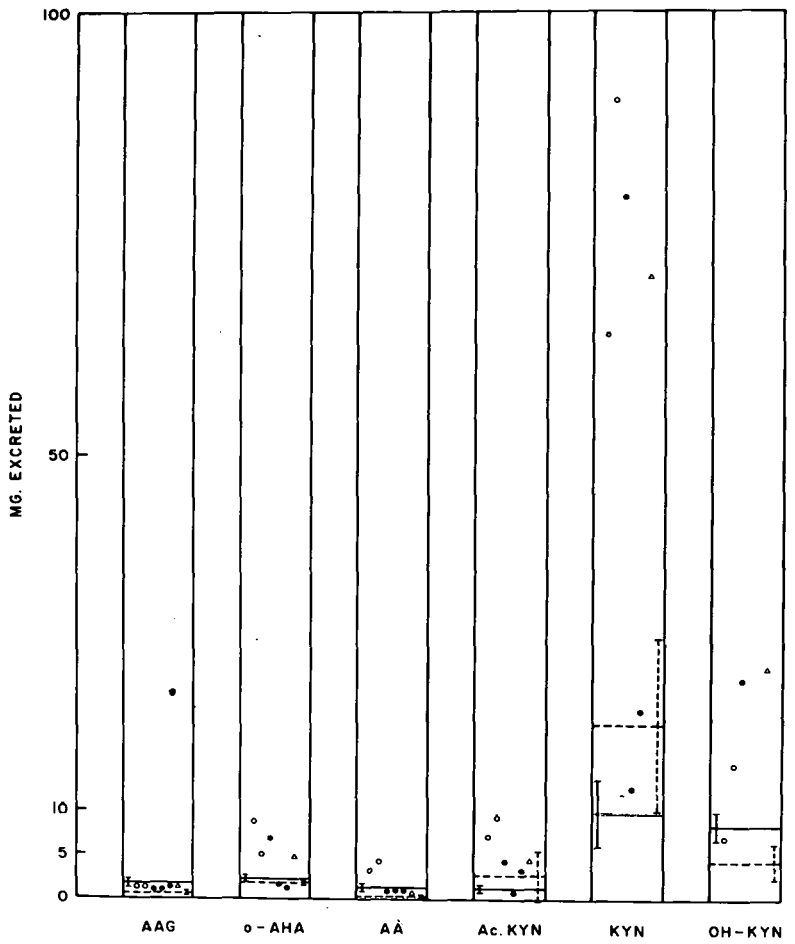

Figure 2. Open circles (o) indicate the urinary tryptophan metabolite excretion of two children with congenital hypoplastic anemia. Closed circles (●) represent the urinary tryptophan metabolite excretion by three adult patients with aplastic anemia. The triangle (△) indicates the urinary tryptophan metabolite excretion by the mother of one patient with congenital hypoplastic anemia. The closed and open horizontal lines represent the mean urinary tryptophan metabolite excretion by normal adults (> 40 kg) and children (< 35 kg) respectively. The closed vertical lines (-) indicate one S.D. above and below the mean normal values for adults; the open vertical lines (----) represent one S.D. above and below the mean normal values for children.

20-30 units of whole blood while the third patient (I.S.) had received about 50 units of blood. Aside from slight hepatomegaly, none of these patients showed any clinical or biochemical evidence of liver disease.

Except for a slight increase in urinary KYN in S.H., no elevation of urinary tryptophan metabolites was noted in two of the patients (S.H. and H.G.). The third patient (I.S.) however, re-

Table 4

URINARY TRYPTOPHAN METABOLITES IN THE MOTHER OF A PATIENT WITH ERYTHROGENESIS IMPERFECTA

Time	AAG	o-AHA	AA	Ac.KYN	KYN	OH-KYN
Before tryptophan	0.3	0.4	0.1	0.9	2.5	<1.5
After 2 g L-tryptophan	1.3	4.6*	0.7	4.2*	70.0*	26.2*

*Indicates significant elevation (> mean + 3 S.D.)

peatedly demonstrated a marked elevation of o-AHA and KYN, with slight or moderate elevations of Ac.KYN and OH-KYN (Table 3, Figure 2).

Other hematologic disorders. After tryptophan loading, sporadic elevations of different metabolites were noted in a variety of other hematologic disorders; namely, lymphosarcoma, multiple myeloma, and chronic lymphatic leukemia (Table 5). There was a questionable increase in the urinary excretion of KYN by a patient with sickle cell anemia. This was a 19-year-old girl who had received a total of approximately 30 units of blood. There was slight hepatomegaly but liver function studies including total serum protein, bilirubin, and flocculation determinations were all within normal limits.

Studies on hemochromatosis. One patient, a 40-year-old woman with hemochromatosis, had marked hepatomegaly, a diabetic glucose tolerance curve, and there were areas of pigmentation over the body surface. Liver biopsy supported this diagnosis. The patient's serum iron was well controlled with periodic phlebotomy. Liver function studies at the time of our investigation included a BSP retention of 20 per cent, an albumin-globulin ratio of 0.80, and a three + cephalin flocculation test. After receiving two g of L-tryptophan, this patient demonstrated a slightly elevated excretion of only Ac.KYN, the excretion of both KYN and OH-KYN being within normal limits (Table 3).

DISCUSSION

Hypoplastic anemias. Using paper chromatography, Altman and Miller demonstrated an elevated excretion of AA in children with erythrogenesis imperfecta.[13] The present investigation indicates that a variety of urinary tryptophan metabolites are elevated in this disorder after tryptophan loading.

Price and his co-workers have shown that the determination of multiple tryptophan metabolites is a more sensitive indicator of the abnormal metabolism of this amino acid than the previously employed technique of only measuring xanthurenic acid excretion.[24] In addition, this method helps to localize the responsible metabolic defect.

Because tryptophan is principally oxidized by way of the kynurenine pathway,[1] an elevation of these metabolites probably represents a metabolic block or deficiency in this route, although no diminished or absent intermediate has yet been demonstrated. An interruption of another pathway with an increased load on the kynurenine route, or a diminished tubular reabsorption of these metabolites are two possibilities which cannot be excluded. Because of the normal to elevated excretion of OH-KYN, a metabolic block would have to occur beyond the formation of this metabolite (Figure 1).[11,12,25,26] In this connection, the development of a reliable method

Table 5

URINARY TRYPTOPHAN METABOLITES IN PATIENTS WITH HEMATOLOGIC DISORDERS AFTER 2 g OF L-TRYPTOPHAN

Patient	Disorder	AAG	o-AHA	AA	Ac.KYN	KYN	OH-KYN
M.N.	Lymphoma	2.4	2.1	2.0	2.0	14.0	+
H.R.	Lymphoma	1.1	1.1	5.3*	9.8*	56.2*	+
H.B.	Lymphoma	1.9	2.1	1.2	1.2	17.1	12.6
M.D.	Multiple myeloma	2.6	1.8	2.3	3.0	56.4*	+
M.E.	Multiple myeloma	1.6	2.0	0.9	1.0	10.8	18.6*
T.E.	Hodgkins	0.7	1.3	0.6	0.4	7.4	+
J.R.	Acute leukemia	1.1	2.9	1.3	0.7	12.2	+
S.V.	Chronic lymphatic leukemia	2.7	3.1*?	1.8	2.0	28.2*	33.8*
F.F.	Erythremic myelosis	1.9	2.6	1.3	0.8	29.2*	29.2*
Z.B.	Sickle cell anemia	1.6	2.9	0.3	2.0	24.3*?	7.8
S.K.	Thallesemia major	0.3	0.7	1.3	0.1	3.0	+

*Indicates significant elevation (mean + 3 S.D.).

OH-KYN standard not available at the time this urine was studied.

for the determination of metabolites beyond OH-KYN in the kynurenine pathway (hydroxyanthranilic acid, and N-methyl-2-pyridone-5-carboxamide) will be of great value.

As can be seen in Figure 1, a multitude of factors must be considered in explaining the elevated excretion of the tryptophan metabolites studied. These include a primary abnormality of pyridoxine function, vitamin B_6 dysfunction secondary to polyvalent cation imbalance, and abnormal riboflavin metabolism. Increased requirements for pyridoxine have been demonstrated in patients with both hypochromic anemias and with convulsive seizures.[27-29] Harris et al.[27] have demonstrated the presence of a tryptophan abnormality in the former condition. Polyvalent cation metabolism may also influence the tryptophan-kynurenine pathway.[26-30] Price and his coworkers have demonstrated that a number of disorders of tryptophan metabolism have been corrected only by the administration of EDTA (ethylenediaminetetraacetic acid), which presumably readjusts a functional pyridoxine deficiency caused by metal ion abnormalities. Dalgliesh has postulated an alternative pathway, from kynurenine, which is dependent on riboflavin and is involved in oxidative phosphorylation.[2] This may be the physiologic, rapid oxidative pathway of tryptophan metabolism, a block in which could result in an elevated excretion of KYN and OH-KYN. It is interesting to note that reticulocytopenias have been recently observed in experimental riboflavin deficiency in man.[31]

Although two g of L-tryptophan probably represents no more than three times the routine daily intake of this amino acid for any of the patients studied,[32] such a dose may stress a number of enzyme-coenzyme systems involved in tryptophan metabolism. Wachstein and Lobel have suggested that a relative pyridoxine deficiency may be made manifest following a tryptophan load test.[33] Thus, a variety of abnormalities may be the result of a moderate increase in ingested amino acid; a more temporary and less easily measurable increase could be expected after a tryptophan-rich meal.

There have been a number of reports of siblings and cousins having erythrogenesis imperfecta as well as other anemias of childhood.[34-36] The repeated elevation of urinary tryptophan metabolites found in the mother of one patient with erythrogenesis imperfecta (M.M.) suggests that the amino acid abnormality noted in at least this case may be genetically determined also. The finding of such abnormally elevated metabolites in an otherwise normal adult probably excludes the possibility that these metabolites are directly responsible for the anemia, although an in utero effect is still conceivable.

One of three adults with acquired aplastic anemia (I.S.) demonstrated an abnormality of tryptophan metabolism resembling that of the patients with erythrogenesis imperfecta. This may then represent a similar defect of tryptophan metabolism with the associated anemia made manifest at a later age.

This patient (I.S.) had received about 50 units of blood at the time of our studies; the children with erythrogenesis imperfecta had received about 100 units each. The resulting increased iron stores may have interfered with tryptophan oxidation sufficiently to account for at least a portion of the elevated metabolites despite the absence of any other proof of liver dysfunction. However, in the other two patients with acquired aplastic anemia and in patients with Cooley's anemia and sickle cell anemia who had received as much as 30 units of blood, normal to only slight elevations of urinary tryptophan metabolites were noted. As has been observed, the one patient with hemochromatosis studied demonstrated only a slight elevation of Ac.KYN with normal KYN and OH-KYN values. This suggests that the marked elevation of tryptophan metabolites

noted in I.S. is probably not entirely the result of increased iron stores. Of course, definitive proof of a basic abnormality of tryptophan-kynurenine metabolism in patients with hypoplastic anemia can only be obtained by the demonstration of the elevated excretion of urinary tryptophan metabolites in untransfused patients.

It has been long suspected that a number of distinct syndromes of aplastic anemia all present with the same clinical picture. The possibility that they could be distinguished on the basis of unique biochemical abnormalities, would therefore seem to warrant further investigation.

Other hematologic disorders. The finding of sporadic elevations of a number of tryptophan metabolites, particularly KYN and OH-KYN, in a number of neoplastic hematologic disorders confirms the observation of Masajo and his co-workers.[37] The fact that these patients were afebrile and did not excrete increased quantities of urinary tryptophan metabolites before tryptophan loading argues against Dalgliesh's hypothesis that increased tissue breakdown associated with fever was responsible for this phenomenon.[38] Wachstein and Lobel have suggested that rapidly growing neoplastic tissue has an increased requirement for pyridoxine. This may result in a relative pyridoxine deficiency made manifest by tryptophan loading and similar to that occurring during pregnancy.[33] A relative niacin deficiency can also be responsible for increased urinary tryptophan metabolites. Auricchio et al.[39] used paper chromatography to demonstrate that the administration of nicotinamide decreased the elevated excretion of such products in one patient with Hodgkin's disease. In this regard, Quagliariello et al.[25] have recently demonstrated that the conversion of 3-OHAA to quinolinic acid is depressed in leukemic rat liver and restored by DPN.

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EFFECT OF DAILY EXPOSURE TO 15 r γ RADIATION ON SUSCEPTIBILITY OF MICE TO EXPERIMENTAL INFECTION*†

By

C. W. Hammond,‡ S. K. Anderle,‡ and C. P. Miller‡

In earlier experiments[1] mice were exposed continuously to gamma radiation at three different levels of intensity, and after various periods of irradiation their susceptibility to bacterial infection was determined by intraperitoneal challenge with graded inocula of Pseudomonas aeruginosa. The results showed that increased susceptibility to this infection was related to dose rate rather than total amount of radiation accumulated. The dose rate in the present experiments was reduced but the challenge inocula (of the same test microorganism) were graded by less than the tenfold dilution used before in an attempt to detect smaller changes in susceptibility than were demonstrable in the previous experiments.

MATERIALS AND METHODS

A total of 895 CF No. 1 female mice was used, approximately half of them irradiated and half controls. In one experiment they were four weeks old, in all others ten weeks old, when irradiation was begun. The mice were irradiated with approximately 15 r/day six days a week. Exposure time was seven hours at first, gradually lengthened to 7 hours 21 minutes as the Co^{60} source decayed. Between exposure periods both irradiated and control mice were kept in the same room, one week in a room reserved for uninoculated mice, the next week in the exposure room with the source shielded. In this way irradiated and control mice lived as much of the time as possible in the same environment. No deaths occurred before challenge. The longest exposure period was 15 weeks because it was necessary to terminate experiments at that time. Cages were placed on a wooden rack with their long sides facing a nominal ten-curie Co^{60} source, their centers all 124 cm from it. The dose rate was based on the assumption that distribution of the mice was random within each cage and disregarded the shielding effect of the mice on each other.

Dose measurements were made with a Victoreen ionization chamber (calibrated by the National Bureau of Standards) placed in the center of one of the cages. The ionization chamber was covered with a close-fitting four-mm Lucite cap of sufficient thickness to provide electronic equilibrium for cobalt-60 gamma rays. Accuracy of these measurements was estimated to be ± three per cent. Each challenge inoculation included unirradiated controls from the same shipment of mice.

The test microorganism was a streptomycin-resistant strain of Pseudomonas aeruginosa

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‡Department of Medicine, University of Chicago.

used in this laboratory for several years to challenge mice exposed to acute x-radiation[2,3] and to continuous gamma radiation.[1] Dilutions were made to provide inocula containing approximately 2.0, 1.2, and 1.0 x 10^8 (and in a few experiments 10^7) which were injected intraperitoneally in 0.5 ml volume into five to 20 mice each. Suspensions of bacteria were prepared and checked for bacterial content as previously described.[1] Total leucocyte counts were made on 242 mice using blood from a tail vein. Their geometric means are shown in Table 1.

Table 1

TOTAL LEUCOCYTE COUNTS (GEOMETRIC MEANS) ON MICE EXPOSED TO 15 r/DAY*

Exposure, week	Radiation accumulated, r	Leucocytes/mm^3
0	0	8150
1	90	6150
2	180	6000
3-4	270-360	5150
5-6	450-540	5100
7	630	4800
9-10	810-900	2700
11	990	2650
12-13	1080-1170	2800
15	1350	2200

*Leucocyte counts were made by Miss Betty Wolfe.

RESULTS

Almost all deaths occurred within 48 hours after challenge. Although 13 mice died later, autopsy cultures of heart's blood and spleen showed only six of them to have died with Ps. aeruginosa bacteremia. The other seven, therefore, were excluded from the mortality data.

From the results of each challenge inoculation, the LD_{50} for irradiated and control mice was determined by probit analysis. The difference between LD_{50} of each irradiated group and its controls is plotted (in tenths of a log) against the duration of their exposure, i.e., accumulated radiation (Figure 1). The mean of these differences (ratios) for each exposure time is also plotted. Points for the regression line were computed from the means by the method of least squares. It is evident that a slight but progressive increase in susceptibility to experimental infection resulted from prolonged daily exposure to 15 r. Between 9th and 15th weeks, this increase was calculated to be .029 log of inoculum per 100 r gamma radiation accumulated.

A comparison of these results with the effect of single exposures to x-radiation is interesting. In earlier experiments,[3] mice were subjected to 300, 400, and 500 r x-radiation in a single acute exposure and challenged five days later by intraperitoneal inoculation with the same strain of Ps. aeruginosa. LD_{50}s were computed by the methods described above. The means of the differences between those of irradiated and control mice are plotted (as whole logs) in Figure 2. The slope shows how much greater was the increase in susceptibility to the

challenge inoculations produced by each increment of 100 r acute x-radiation, approximately .54 log of inoculum per 100 r.

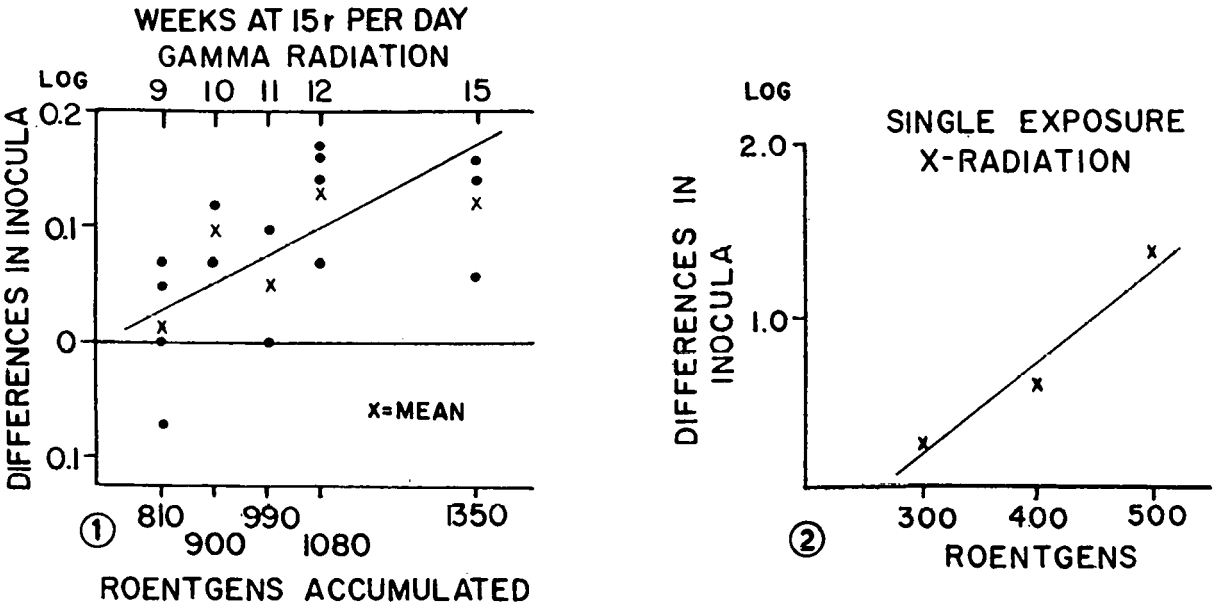

Figure 1. Changes in susceptibility to challenge inoculation plotted as differences in LD_{50}'s of irradiated and control mice.

Figure 2. For comparison: effect of single acute exposures to x-radiation. Challenge five days postirradiation with same strain of Pseudomonas aeruginosa.

DISCUSSION

The differences in LD_{50}s of Ps. aeruginosa for irradiated and control mice in each challenge inoculation indicate a slight but progressive increase in susceptibility to this bacterial infection from the ninth through the 15th weeks of exposure to 15 r/day. The change was too small to have been demonstrable by use of tenfold gradations of inocula as used in our previous experiments on mice exposed to 34 r/day.[1] At nine weeks, the shortest exposure time, the effect on susceptibility was so slight as to raise the question whether any change at all could have been detected earlier.

After 15 weeks' irradiation (total accumulation 1350 r) the increase in susceptibility, while still small, was clearly demonstrable. It is unfortunate that exposures could not have been prolonged beyond that time. Comparison of this effect of chronic γ irradiation with that produced by a single exposure shows that an accumulation of 1350 r (at 15 r a day) increased susceptibility to the challenge inoculations much less than did an acute exposure to 300 r x-radiation.

The effect on total leucocyte counts was about the same. The geometric mean of the white counts on fifth day following exposure to 300 r was 2400.[1] After accumulating 1350 r (at 15 r a day) it was 2200.

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ATTEMPTS TO INCREASE RESISTANCE OF MICE TO BACTERIAL INFECTION BY PROLONGED LOW DOSE γ IRRADIATION*†

By

C. W. Hammond,‡ S. K. Anderle,‡ and C. P. Miller‡

Previous experiments have shown that prolonged daily exposure to γ radiation increased susceptibility of mice to bacterial infection, and that the increase was proportional to dose rate rather than total amount of radiation accumulated.[1,2]

The literature, however, contains some reports[3,4] which suggest that at very low dose rates an opposite effect may result, i.e., that prolonged daily exposure to very small doses may actually increase resistance to bacterial infection. For if one makes the usual assumption that an occasional death among stock laboratory animals is probably caused by intercurrent infection, one may reasonably conclude that any reduction in mortality among irradiated animals below that of their controls, may well be due to a lower incidence of death from intercurrent infection, hence to increased resistance to latent infection. Pertinent to this line of reasoning are the following observations:

Lorenz et al., in an early attempt to establish limits of "permissible, daily doses of ionizing radiation," exposed mature laboratory animals to five levels (8.8 - 0.11 r) of γ radiation daily until death. Although the life span of those receiving the three highest doses was shortened, mortality of those receiving 1.1 r/day was slightly lower and of those receiving 0.11 r considerably lower than that of their controls during the first 23 months of exposure.[3] Carlson, Scheyer, and Jackson found the mean survival time of rats exposed to 0.8 r/day to be longer than that of their unirradiated controls whether their environmental temperatures were maintained at 25° or 5° C.[4]

The present experiments were undertaken in an attempt to detect any increased resistance to an experimental bacterial infection among mice exposed to 0.5, 1.0, or 2.0 r per day for various lengths of time. Preliminary experiments did in fact give evidence of slightly increased resistance but additional experiments failed to confirm earlier results.

MATERIALS AND METHODS

The mice were CF No. 1 females three weeks old on arrival in the laboratory, kept under observation for one week, and then randomly distributed into groups of ten, half of them to be irradiated and half kept as controls.

They were exposed six days a week to γ radiation from a nominal 0.5 curie Co^{60} source at three dose rates: 0.5, 1.0, and 2.0 r/day. The daily exposure time varied from 5 hours 42 minutes to eight hours depending on the activity of the source. Duration of exposure varied from four to 39 weeks.

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‡Department of Medicine, University of Chicago.

Dose measurements were made with a Victoreen ionization chamber (calibrated by the National Bureau of Standards) placed in the center of one of the mouse cages and covered with a close-fitting four-mm Lucite cap of sufficient thickness to provide electronic equilibrium for cobalt-60 γ rays. The accuracy of these measurements was estimated to be ± three per cent.

The time of each challenge inoculation, in terms of duration of exposure, is given in Table 1. Most inoculation groups contained 60 mice from the same shipment, approximately half of them irradiated and half unirradiated controls. Before inoculation, the mice were redistributed in order to separate cage mates. The test microorganism was a streptomycin-resistant strain of Pseudomonas aeruginosa used for a number of years to study resistance and susceptibility in irradiated mice.[1,2,5]

Table 1

COMPARISON OF MORTALITIES OF IRRADIATED AND CONTROL MICE IN EACH CHALLENGE INOCULATION

	Dose rate r/d		
	0.5	1	2
	Weeks of exposure:		
No difference between irradiated and control mice	27	4	14
	30	5	22
	32	9	27
		17	30
		26	
		27	
		30	
Slightly lower among irradiated		26	23
		27	
		30	
		39	
Slightly higher among irradiated		18	
		24	
		27	
		31	

Suspensions of bacteria were prepared and checked for bacterial content as previously described.[1] Since the LD_{100} and LD_0 of Ps. aeruginosa for these mice were known from past experience, inocula were chosen within these limits in an attempt to detect small differences between irradiated and control mice. Approximately 2.0, 1.2, and 1.0 x 10^8 in 0.5 ml were inoculated intraperitoneally into (usually) ten mice each.

At approximately weekly intervals from the third to 26th week of exposure white blood counts were made on ten to 20 mice in each series, a total of 492 counts, about equally distrib-

uted among the three series.* No mouse was used more than once for this purpose.

RESULTS

In 14 of the 23 challenge inoculations the mortality in the irradiated group was practically identical with that of its control group (see Table 1). Slightly lower mortalities occurred among irradiated mice in four challenge inoculations in the 1 r series but were balanced by four which showed equally lower mortalities among controls. Overall, therefore, the experiments (involving 1344 mice) failed to demonstrate any consistent effect of irradiation on host resistance to experimental infection with Ps. aeruginosa. Nor was any correlation found between duration of exposure and increased resistance or susceptibility in individual challenges. Omitted from Table 1 were two additional challenge inoculations on mice exposed 30 and 35 weeks to 0.5 r/day made with a beta hemolytic streptococcus of moderate virulence for mice (strain AD 242).† The results with this test microorganism were inconclusive but suggestive of a very slight reduction in host resistance.

Leucocyte counts fluctuated considerably as did those reported by Lorenz et al.[3] and as we have found in other experiments. Their geometric means indicated the occurrence of mild leucopenia (4800-5800) during prolonged exposure to 1.0 and 2.0 r/d (normal = 8500), but the leucopenia was not progressive as it was in mice exposed to 15 r/d.[2] Individual counts rarely fell below 3000. The geometric means in the 0.5 r series varied from 5100 to 6400 during the 21st to 26th weeks of irradiation.

DISCUSSION

These experiments failed to demonstrate any effect on host resistance to an experimental bacterial infection in mice exposed for many weeks to very low doses of γ radiation. Intraperitoneal inoculation with Pseudomonas aeruginosa, is admittedly a drastic type of challenge. It was used because in previous experiments it had proved satisfactory in demonstrating changes in host resistance of mice exposed to higher doses of x or γ radiation.[1,2,5] Two trials with a β hemolytic streptococcus of medium virulence showed it to be no more satisfactory as a test microorganism.

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RESTORATION OF SERUM BACTERICIDAL ACTIVITY AND PREVENTION OF ITS LOSS IN X-IRRADIATED MICE*†

By

L. Kornfeld**‡ and C. P. Miller‡

Earlier publications from this laboratory have reported that bactericidal activity for a strain of Escherichia coli can be demonstrated in sera of normal rabbits, guinea pigs, and mice and that this bactericidal activity is lost after total-body exposure to x-rays.[1,2] Hook et al.[3] observed a drop in bactericidins for Shigella dysenteriae in sera obtained from guinea pigs three days postirradiation.

Several years ago Jaroslow and Taliaferro[4] found that the hemolysin-forming capacity of x-irradiated rabbits could be restored by treatment with tissue preparations and yeast autolysate. More recently they showed that this could also be achieved with nucleic acid digests and kinetin.[5] These findings suggested that similar substances might also alter the effect of x-irradiation on serum bactericidins of mice.

MATERIALS AND METHODS

CF No. 1 female mice, eight to 16 weeks old, were used in all experiments. They were exposed to six hundred r total-body x-irradiation delivered in a single exposure at a rate of approximately 60 r per minute. Radiation factors were the same as those described previously.[2]

The following reagents were used, all being freshly prepared for each experiment:

Yeast autolysate (Albimi Laboratories, Brooklyn, N. Y., Control #115627) - ten per cent in buffered saline (pH 7.4), autoclaved for 15 minutes at 20 lbs pressure.

Yeast ribonucleic acid (Schwarz Laboratories, Mt. Vernon, N. Y., Lot #NH 5906) - two per cent in sterile saline, neutralized with NaOH.

Spleen homogenate - mouse spleens aseptically removed and homogenized with sterile buffered saline (pH 7.4) in Ten Broeck grinders.

Bacterial endotoxin (Lipopolysaccharide S. typhosa 0901, Difco Laboratories, Detroit, Michigan, Control #110284) - in sterile saline.

Kinetin (Krishell Laboratories, Portland, Oregon)§ - in sterile saline containing 0.02 moles of HCl.

DNA digest - calf thymus DNA¶ incubated one hour at room temperature with DNase¶ in the presence of Mg^{++} (one mg DNA/0.25 mg DNase).

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**National Science Foundation Cooperative Graduate Fellow in the Department of Microbiology during the year 1959-60.

‡Departments of Medicine and Microbiology, University of Chicago.

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Each serum sample for testing was obtained from the pooled bloods of three mice. Details of the procedure and reagents employed for the bactericidal test have been described elsewhere.[1,2] The titers recorded were the reciprocals of the highest serum dilutions showing bactericidal activity for E. coli.

EXPERIMENTAL

Restoration of bactericidins in irradiated mice. CF No. 1 mice were exposed to 600 r. On the following day, when bactericidins were absent, each mouse was injected intraperitoneally with either one ml of ten per cent yeast autolysate or the homogenized spleen of one normal mouse. Sera were collected at intervals thereafter for determination of their bactericidal activity. Table 1 shows that normal levels of bactericidins were reached 18 hours after injection and persisted without further treatment for at least five days. Bleeding 24 hours after treatment was therefore adopted as routine procedure.

Table 1

TIME OF REAPPEARANCE OF BACTERICIDAL ACTIVITY IN IRRADIATED TREATED MICE

Agent (intraperitoneal injection) 24 hours after 600 r		Titer hours after treatment 0	6	12	18	24	48	72	120
Yeast autolysate	1 ml 10 %		<4	<4	16	32	32	64	128
Spleen homogenate	1 spleen		<4	<4	16	64	128		
Buffered saline	1 ml					<2			
None		<2				<2	<2	<2	<2

Single intraperitoneal injections of yeast autolysate, spleen homogenate or yeast ribonucleic acid (one ml two per cent) were given to mice at various intervals after irradiation with 600 r. All animals were bled 24 hours after treatment. As shown in Table 2, bactericidins were present in the sera of mice treated one, two, three or six days postirradiation but remained absent in those of the controls. Administration of bacterial endotoxin (three or ten μg) 24 hours after x-ray exposure also resulted in the appearance of bactericidins on the following day. Kinetin (0.1 mg) and DNA digest (one mg) had no effect.

Prevention of loss of bactericidins in irradiated mice. The loss of bactericidins which regularly followed x-ray exposure could be prevented by treatment with yeast autolysate, RNA, normal mouse spleen homogenate or bacterial endotoxin. Mice were given intraperitoneal injections before and after exposure to 600 r and were bled 24 hours postirradiation. Table 3 indicates that treatment with any of these substances one-half hour before, or one-half, three, and six hours after irradiation prevented the loss of bactericidins which occurred in the controls. However, injections given 18 hours before irradiation had no effect. Although not shown in the Table, bactericidal activity persisted for at least six days as a result of a single injection of yeast autolysate given 30 minutes postirradiation. Treatment with DNA digest or kinetin did not prevent loss of bactericidins after irradiation.

Table 2

RESTORATION OF BACTERICIDAL ACTIVITY AFTER EXPOSURE TO 600 r

Agent (intraperitoneal injection)		Time of treatment days postirradiation			
		1	2	3	6
		Bactericidin titers of sera obtained 24 hours after treatment			
Yeast autolysate	1 ml 10 %	32	32	8	64
Spleen homogenate	1 spleen	32	64	128	
Yeast RNA	1 ml 2 %	64	16	32	
Bacterial endotoxin	10 μg	128			
Bacterial endotoxin	3 μg	128			
DNA digest	1 mg	<2			
Kinetin	0.1 mg	<2			
Buffered saline	1 ml	<2			
None		<2	<2	<2	<2

Table 3

PREVENTION OF LOSS OF BACTERICIDAL ACTIVITY BY TREATMENT SHORTLY BEFORE OR AFTER EXPOSURE TO 600 r

Agent (intraperitoneal injection)		Time of treatment				
		Preirradiation hours		Postirradiation hours		
		18	1/2	1/2	3	6
		Bactericidin titers of sera obtained 24 hours after irradiation				
Yeast autolysate	1 ml 10 %	<2	32	64	32	128
Spleen homogenate	1 spleen	<2	8	64	64	32
Yeast RNA	1 ml 2 %	<2	32	64		
Bacterial endotoxin	10 μg	<2	128	128		
DNA digest	1 mg			<2		
Kinetin	0.1 mg	<2	<2	<2		
Buffered saline	1 ml			<2		
None				<2		

Relative effectiveness of spleens from normal and irradiated mice. Blood and spleens were removed from donor mice before and at intervals after exposure to 600 r. The spleens were homogenized immediately and the equivalent of one spleen was injected intraperitoneally into each recipient mouse, which had been irradiated 24 - 48 hours earlier. Sera were obtained for bactericidal tests 24 hours after treatment. It is evident from Table 4 that spleens removed as early

as one hour after x-ray failed to restore bactericidins in irradiated mice. Furthermore, the effectiveness of a spleen homogenate was not necessarily correlated with the presence of bactericidins in the sera of its donors.

Table 4

RESTORATION OF BACTERICIDINS FOLLOWING INJECTION OF SPLEENS FROM NORMAL AND IRRADIATED DONORS

Spleens obtained:	Bactericidin Titer			
	Experiment I		Experiment II	
	Recipients[1]	Donors[2]	Recipients[1]	Donors[2]
Before 600 r	16	64	32	<2
Hours after 600 r				
1/2	16	64	<2	8
1	<4	128	<2	64
3	<4	128	<2	32
6	<4	128	<2	4
24	<4	<4	<2	<2
Saline treated	<4		<2	
Untreated	<4		<2	

[1]Recipients were injected intraperitoneally on the day after exposure to 600 r. Sera were taken 24 hours after treatment.

[2]Sera of donors were taken at time of sacrifice.

Relative effectiveness of intraperitoneal and subcutaneous routes. It was noted during other experiments that mice injected intraperitoneally with an alkaline solution having a pH above nine showed signs of its irritant action. NaOH produced similar effects. Mice sacrificed 24 hours after intraperitoneal injection of 0.5 ml 0.05 N NaOH were found to have minute masses of white granular necrotic material in their peritoneal cavities. The sera of mice irradiated either 24 hours or 30 minutes before injection of NaOH and bled one day after treatment, were bactericidal. When the same amount of NaOH was administered subcutaneously, restoration of bactericidins and prevention of their loss did not occur. Several experiments comparing the two routes of injection at different times postirradiation are summarized in Table 5. Normal bactericidin levels were found in sera of mice treated with NaOH intraperitoneally either 30 minutes or 24 hours after irradiation. Serum bactericidal activity was absent in mice treated with NaOH by the subcutaneous route and in the controls. In contrast, yeast autolysate and RNA were as effective subcutaneously as intraperitoneally. Bacterial endotoxin was considerably less effective when given by the subcutaneous than by the intraperitoneal route.

DISCUSSION

We have reported previously[2] that normal bactericidins for our test strain of E. coli were lost from mouse serum nine to 12 hours after total-body irradiation with 600 r and remained

Table 5

COMPARISON OF INTRAPERITONEAL AND SUBCUTANEOUS ROUTES OF INJECTION

Agent	Expt. #	Time and route of injection			
		1/2 hour after 600 r		24 hours after 600 r	
		i.p.	s.c.	i.p.	s.c.
		Bactericidin titers of sera obtained 24 hours after treatment			
NaOH 0.5 ml 0.05 N	1	128	<2	128	<2
	2	32	<2	32	<2
	3	4	<2	4	<2
Yeast autolysate 1 ml 10 %	1	16	32	32	32
	2	32	32	64	
	3	16	32	32	4
	4	4		4	
Yeast RNA 1 ml 2 %	1	64	16	32	
	2	32	4	16	16
	3	4	16	16	8
Bacterial endotoxin 10 μg, expts. 1-4 3 μg, expt. 5	1	256		256	
	2	128	32	64	4
	3	64	8		4
	4		2	128	8
	5	128	32	128	16
None	(all)	<2		<2	

i.p. - intraperitoneal
s.c. - subcutaneous

absent for about ten days. In contrast, titers of natural hemagglutinins for chicken cells in mice declined gradually during the first ten days postirradiation[6] as did natural hemolysins for sheep cells in rabbits.[7] The effect of ionizing radiation on bactericidins differs, therefore, from its effect on hemagglutinins or hemolysins. This suggests that the synthesis of bactericidins depends on processes which differ markedly in relative radiosensitivity from those responsible for the synthesis of hemagglutinins and hemolysins.

The absence of bactericidins during the early postirradiation period indicates that their production is promptly interrupted by irradiation. Speculation about the nature of this damage suggests several possible explanations. Since the injection of various cell-free substances maintained or restored the bactericidin level after irradiation, it is unlikely that the bactericidin-producing cells and/or their precursors were destroyed. Again, it is improbable that the absence of bactericidins was due to the inability of the cellular precursors to mature into actively synthesizing cells because of the interruption of cell division. Histological evidence indicates that cellular maturation and repopulation require several days at least,[8] yet bactericidins reappeared as early as 18 hours following injection of yeast autolysate or normal mouse spleen homogenate. A more likely explanation is that the elimination of one or more enzymes or metabolites resulted in a block in the synthesis of bactericidins. As a working hypothesis, it is postulated that such a block in the synthetic process occurs as a result of irradiation.

Bactericidin production in irradiated mice could be restored or maintained in several ways. Once the process had been initiated no further intervention was needed for its continued operation. This is indicated by the presence of serum bactericidal activity in mice on the sixth day postirradiation as a result of a single injection of yeast autolysate 30 minutes or 24 hours after exposure to x-rays.

If it is postulated that parenteral administration of yeast autolysate, RNA or normal spleen homogenate provides the animals with some essential metabolite(s), it might be expected that the intraperitoneal and subcutaneous routes would be equally effective. However, the effect of alkali, presumably due to the release of cell constituents from damaged tissues, did depend on the route of injection. When NaOH was given intraperitoneally, a large surface is subjected to its caustic action, thus making available an abundance of degradation products. The mode of action of bacterial endotoxin is not quite clear but may be similar to that of alkali. However, the slight effect following subcutaneous injection suggests that other factors may also be involved.

Taliaferro and Jaroslow[5] suggested that the presence of an abundant supply of nucleic acid constituents was responsible for the restoration of hemolysin-forming capacity in irradiated rabbits. The experimental evidence presented here concerning restoration of bactericidins and prevention of their loss in irradiated mice is compatible with a similar interpretation. It should be added that the active substances may be of either endogenous or exogenous origin.

Nevertheless, there are certain fundamental differences between the experimental models in the two laboratories. Taliaferro and Jaroslow studied the formation of specific immune antibodies in irradiated rabbits and referred to restoration of antibody-forming capacity as ability to form antibodies comparable in amount to those produced by unirradiated animals. Restoration did not overcome the delay in appearance of antibodies characteristic of irradiated rabbits.[5] Our experiments deal with a natural antibody-like substance. Restoration refers to the resumption of its production, resulting in bactericidal activity at a time when it is absent from the sera of untreated irradiated mice. Prevention of its loss is interpreted as uninterrupted synthesis in spite of exposure to x-rays. These differences between the two systems may explain why not all the substances tested gave comparable results in the two laboratories.

A puzzling observation was the failure of homogenates of spleens from irradiated mice to restore bactericidin production. Trowell[9] has suggested that lymphocytes die one to two hours after irradiation, even before morphological changes are detectable. Thus, as a result of the onset of lymphocyte disintegration shortly after irradiation, spleens removed from donor mice at that time should have been particularly rich in nucleic acid components. Nevertheless, such preparations had no effect. This phenomenon deserves further study.

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IMMUNOHISTOCHEMICAL STUDY OF EHRLICH ASCITES TUMOR*†

By

F. W. Fitch‡

A study of the specific immunotherapy of tumors may be approached either via active immunization of the host with its own tumor or with antigenic materials specific for this tumor; or by the passive transfer of "tumor specific" antiserum prepared in a host of the same or of a different species. Both approaches presume antigenic differences between tumor and normal tissues and that antibodies produced against these "specific" antigens have a deleterious effect on the tumor. The evidence that such antigenic differences exist has been reviewed by Zilber,[1] who concludes that the antigenic structure of tumor cells and their respective normal cells does not appear to be identical.

The localization and cytotoxic effect of antisera prepared against the Ehrlich ascites tumor of the mouse have been widely studied,[2-9] and marked morphological and biochemical alterations have been observed after treatment of tumor cells with antiserum either *in vivo*[3-5] or *in vitro*.[4,6-9]

On the other hand, much of the localizing capacity of "anti-Murphy lymphosarcoma" antisera with the Murphy lymphosarcoma of the rat has been shown to be due to reaction with fibrinogen present in the tumor.[10] Furthermore, rabbit anti-rat fibrin antibodies are found to localize in this tumor as well as in several other rat tumors.[11-13] Immunohistochemical analysis indicates that after *in vivo* administration, anti-Murphy lymphosarcoma antibodies and anti-rat fibrin antibodies localize in the connective tissue stroma of solid tumor masses. *In vitro*, however, the anti-lymphosarcoma serum covers the periphery of the intact cells uniformly while anti-fibrin serum is present only sporadically.[14]

These findings indicate that the specificity of antitumor antibody to various transplantable tumors is a complex subject that requires further investigation using multiple experimental approaches.

The present work was undertaken to study the specificity of antibodies in Ehrlich ascites tumor antiserum, to determine if possible to which components of the tumor cells the antibodies are directed, and to investigate further the cytotoxic effects of antiserum in the presence and absence of complement. To accomplish this, the fluorescence immunohistochemical method of Coons[15,16] was used.

MATERIALS AND METHODS

The Ehrlich ascites tumor, originally obtained from Dr. K. Sugiura of the Sloan-Kettering Institute in 1950,[17] was maintained by weekly intraperitoneal injection of 0.1 ml of ascites fluid

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‡Department of Pathology, University of Chicago.

into young adult CF No. 1 mice. Chromosome analysis indicated that the tumor is, at present, composed mainly of near-tetraploid cells, although some cells have near-octaploid chromosome numbers.

Tumor cells used for immunization of rabbits and for immunohistochemical study were taken from peritoneal fluid removed five to seven days after inoculation, at which time the tumor was rarely bloody. Later, the ascitic fluid became markedly hemorrhagic. Any tumor which contained more than grossly visible traces of blood was not used for antigenic purposes.

The anti-Ehrlich ascites tumor antisera were prepared by repeated injections over a three-week period of washed Ehrlich ascites tumor cells into rabbits using multiple portals as described by Wissler et al.[3] The first day's dose was given as multiple intramuscular depots in Freund's adjuvant. Anti-mouse whole blood sera were prepared by injecting rabbits with fresh heparinized whole mouse blood using a similar injection schedule. A five per cent (V/V) suspension of whole blood was used as antigen. The gamma globulin fraction was separated from these antisera and from normal rabbit serum using a cold ethanol fractionation method.[18]

The complement used was a preparation of lyophilized pooled guinea pig serum from Markham Laboratories, Chicago, Illinois.

The globulin fractions were labeled with fluorescein isothiocyanate using the method of Riggs et al.[19] The labeled proteins were passed through a column of Dowex 2-X 4 exchange resin.[15]

The labeled globulin solutions were heated in a water bath at 56° C for 30 minutes to destroy complement activity. They were then absorbed* with various tissue powders, lyophilized Ehrlich ascites tumor, or fresh whole blood. When indicated, fresh, heparinized, whole mouse blood was added in the proportion of two ml per five ml labeled globulin. Acetone-dried mouse liver powder or beef liver powder or lyophilized Ehrlich ascites tumor was added in the proportion of 160 mg powder per ml of labeled globulin. To compensate for the dilution of the labeled globulin caused by addition of mouse whole blood, two ml of 0.15 M NaCl was added to each five ml of serum absorbed with either tissue powder.

Agglutinin titers for mouse erythrocytes and Ehrlich ascites tumor cells were determined in duplicate for all labeled globulins after absorption. A two per cent suspension of thrice-washed mouse erythrocytes or a two per cent suspension of thrice-washed fresh tumor cells was used as antigen. Double dilution of serum was employed. After addition of antigen, the tubes were left at room temperature for 30 minutes, centrifuged gently for six minutes and the sediment resuspended by shaking each tube. The last tube showing grossly visible agglutination of the cells was the end point.

Smears of Ehrlich ascites tumor cells were prepared using either fresh cells in ascitic fluid or thrice-washed cells. Sections were prepared by centrifuging washed tumor cells in a small conical plastic tube, freezing the cell pellet in the bottom of the tube in a mixture of dry ice and acetone, and sectioning the frozen cell pellet in a cryostat. For histologic staining, the

*Fluorescein-globulin conjugate must be shaken with tissue powder to remove green-fluorescing substances which react non-specifically with normal tissues. This is conventionally referred to as absorption. In addition, fluorescein-labeled globulin may be shaken with particulate materials to remove antibodies. For convenience, both of these procedures will be called absorption, even though the adherence of antibodies to the surface of particles is, more properly speaking, adsorption.

cryostat sections were allowed to thaw and dry on the slides. For immunohistochemical analysis, the sections were floated on a drop of cold 95 per cent ethanol which was then allowed to evaporate. Mouse fibrin was prepared by dialyzing fresh mouse plasma against cold 0.15 M sodium chloride. The resulting clot was quick-frozen and then sectioned in the cryostat. The fibrin sections were allowed to thaw and dry on the slide. Sections of mouse kidney and liver were also prepared in the cryostat. Mouse blood smears prepared from fresh whole blood were fixed for the immunohistochemical study by immersing the slide in 95 per cent ethanol at 37° C for 15 minutes, followed by drying in air at 37° C for 30 minutes.

Slides were treated with labeled globulin for 30 minutes at room temperature, washed for ten minutes in running 0.15 M NaCl buffered at pH 7, and a cover slip was mounted using buffered glycerin.

Representative smears and sections were also fixed in Carnoy's solution and stained with methyl green-pyronin solution, or with hematoxylin and eosin.

The slides were examined using a Leitz microscope equipped with a dark field condenser and an Osram HBO-200 mercury arc lamp as a light source. A Schott UG-2 filter was used as an exciter filter and a Wratten 2-A as a barrier filter. Photographs were made on 35-mm high speed daylight Ectachrome film using an exposure time of one minute. Black and white prints were prepared from negatives made from these color transparencies.* Fields containing comparable numbers of cells were selected for photography, and photographs taken immediately after selection. The dark field condenser was focused as exactly as possible, and the exposure time was held constant. This standardization of photographic and microscopic technique permits semi-quantitative comparison of slides prepared from the same tumor suspension and treated with the same labeled globulin absorbed with comparable amounts of the various materials. The assumption is made that tissues containing comparable quantities of solids absorb "non-specifically" comparable amounts of labeled globulins.

RESULTS

Antibody content of labeled globulins. Antibody potency of each labeled globulin was determined against both Ehrlich ascites tumor cells and mouse erythrocytes before and after absorption with the various materials. The results of the agglutinin titers are summarized in Table 1. The antitumor globulin alone had a very substantial agglutinin titer for the tumor cells. Absorption with mouse or beef liver powder, or with fresh whole mouse blood, had little effect on the antibody content, while absorption with lyophilized tumor cells apparently removed most of this activity. Both antitumor globulin and anti-mouse whole blood globulin had an appreciable agglutinin titer for mouse erythrocytes. Absorption with mouse or beef liver powder had little effect on the agglutination, while absorption with fresh whole mouse blood markedly decreased the amount of mouse erythrocyte agglutinins present. Absorption with lyophilized tumor cells lowered the mouse erythrocyte agglutinin titer of the anti-Ehrlich ascites globulin only.

Immunoelectrophoretic analysis† indicated that the anti-Ehrlich ascites tumor globulin

*Prints of Figures 1 through 7 were prepared by Mr. Jean Crunelle, Chief Photographer of the University of Chicago Clinics, who made every possible effort to retain the intensity differences present in the original color transparencies.

†This analysis was kindly performed by Mr. Bruce Merchant.

reacted with only one component of mouse plasma or serum which had the electrophoretic mobility of a beta globulin. The labeled globulin against mouse whole blood reacted with at least four components of mouse serum or plasma; these components had the mobilities of albumin, alpha-, beta-, and gamma-globulin.

Table 1

AGGLUTININ TITERS FOR MOUSE ERYTHROCYTES AND EHRLICH ASCITES TUMOR CELLS

	Agglutinin titer[a] for:	
	Mouse Erythrocytes	Ehrlich Ascites Cells
Labeled Anti-Ehrlich ascites tumor globulin absorbed with:		
Nothing	1:226	1:2560
Mouse liver powder	1:320	1:1810
Beef liver powder	1:320	1:1280
Mouse whole blood	1:80	1:1810
Ehrlich ascites cells	1:40	1:160
Labeled Anti-Ehrlich whole blood globulin absorbed with:		
Nothing	1:452	1:40
Mouse liver powder	1:320	1:20
Beef liver powder	1:320	1:20
Mouse whole blood	1:20	1:20
Ehrlich ascites cells	1:160	1:20
Normal Rabbit Globulin absorbed with:		
Nothing	1:20	1:20
Mouse liver powder	1:20	1:20
Beef liver powder	1:20	1:20

[a]The highest serum dilution producing grossly visible agglutination. Results are the average of two separate determinations.

Staining of Ehrlich ascites tumor cells with labeled globulin. The main results of the reaction of smears and sections of tumor with labeled globulins are summarized in Table 2. While immunohistochemical methods are difficult to quantitate, semi-quantitative information on the degree of cross reaction of the globulin may be obtained by estimation of intensity of staining of consecutive sections of tissues prepared on the same day and treated with different aliquots of the same labeled globulin absorbed with various substances. These results were preserved by preparing photomicrographs under the standard conditions outlined above.

Antigenic materials must be insoluble to permit their localization by the immunohistochemical method. Since the fixatives used to accomplish this may alter the immunologic properties of the antigens, both fixed and unfixed preparations of tumor cells were examined.

When unwashed, unfixed smears of Ehrlich ascites tumor cells were reacted with labeled anti-Ehrlich ascites tumor globulin absorbed with mouse liver powder, beef liver powder, or mouse whole blood, the results were similar (Figure 1A, B). The cytoplasm of most, but not all, of the tumor cells showed a brilliant yellow-green fluorescence brightest at the surface of

Table 2

FLUORESCENCE SEEN IN EHRLICH ASCITES TUMOR OR NORMAL MOUSE TISSUE PREPARATIONS AFTER REACTION WITH LABELED GLOBULINS ABSORBED WITH VARIOUS MATERIALS

	EA[a] smear (unwashed unfixed)	EA[a] smear (unwashed fixed)	EA[a] smear (washed cells)	EA[a] susp.	EA[a] section (unfixed)	EA[a] section (fixed)	Mouse fibrin	Mouse RBC	Mouse liver[b]	Mouse kidney[b]
Labeled Anti-Ehrlich ascites tumor globulin absorbed with:										
Mouse liver powder	++++	+++	+++	++++	+++	++	±	+++	++	++
Beef liver powder	++++	0	++++	++++	+++	+++	±	+++	+++	+++
Mouse whole blood	++++	++++	+++	++++	++++	+++	±	±	++	++
Ehrlich ascites cells	±	±	±	±	+	±	±	+++	±	+
Labeled anti-mouse whole blood globulin absorbed with:										
Mouse liver powder	+	±	-	±	±	±	++++	+++	++	+
Beef liver powder	++	0	+	±	+	++	++++	+++	+++	++
Mouse whole blood	±	-	-	±	+	±	±	±	++	±
Ehrlich ascites cells	+	±	-	±	±	-	++++	+++	++	+
Labeled normal rabbit globulin absorbed with:										
Beef liver powder	+	0	0	-	+	±	+	+	-	++

[a]EA = Ehrlich Ascites Tumor; ++++ Marked staining; ++ and + Mild staining; - No staining.

[b]= See Text; +++ Moderate staining; ± Slight staining; 0 Test not performed.

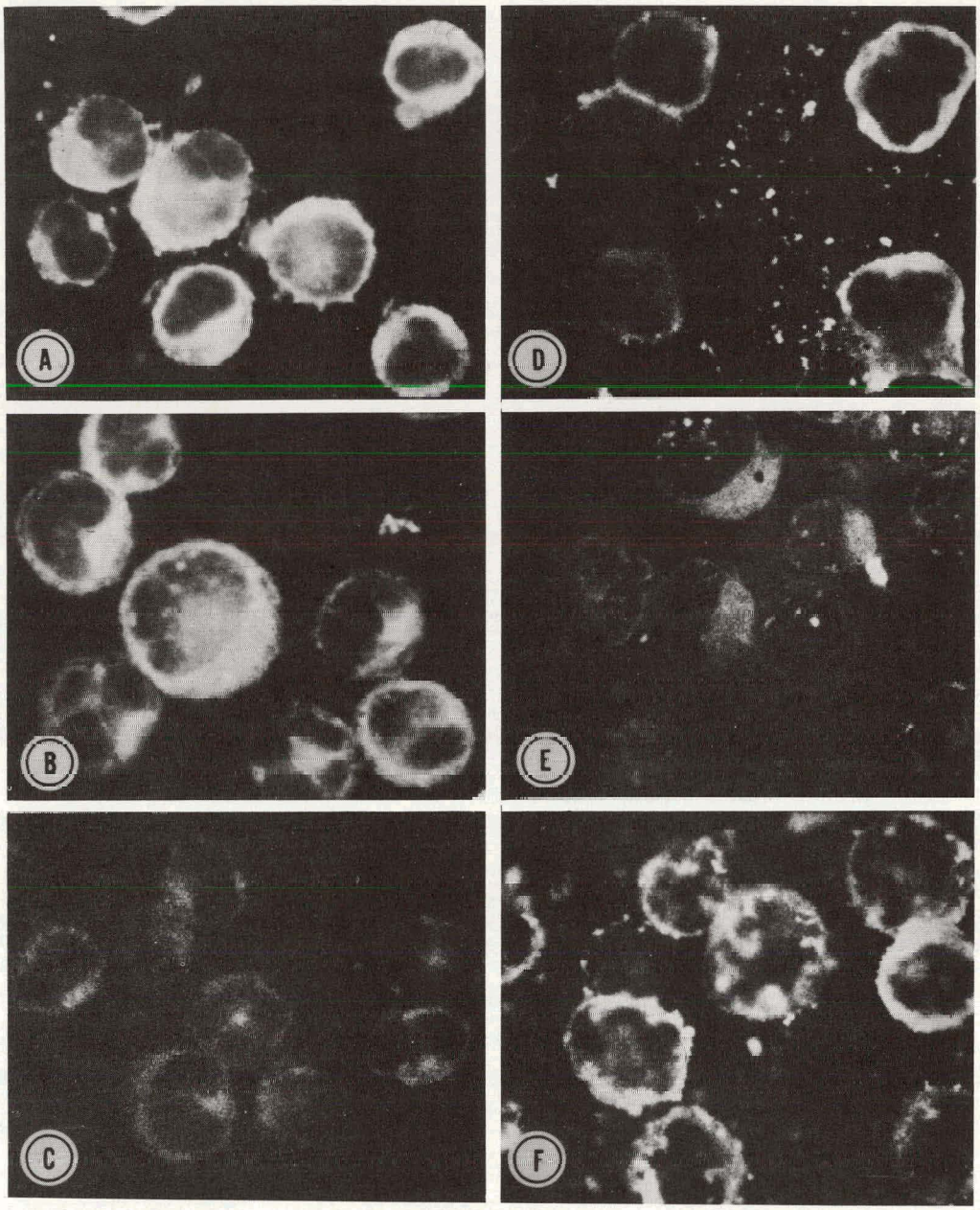

Figure 1. Fluorescence of unwashed, unfixed smears of Ehrlich ascites tumor reacted with labeled globulins absorbed with various materials. X 925.

Labeled anti-Ehrlich ascites tumor globulin absorbed with
- A - mouse liver powder
- B - fresh mouse whole blood
- C - lyophilized Ehrlich ascites tumor cells

Labeled anti-mouse whole blood globulin absorbed with
- D - mouse liver powder
- E - fresh mouse whole blood
- F - lyophilized Ehrlich ascites tumor cells.

the cell. Occasionally, bright granular foci were present on the cell surface, and the stained material extended irregularly beyond the apparent cell surface. The nucleus did not stain appreciably. There was faint fluorescence of the background. Labeled antitumor globulin absorbed with lyophilized tumor cells produced very little staining of the tumor cells (Figure 1C). Following incubation with each of these labeled globulins, leucocytes and platelets in the smears fluoresced brilliantly.

Labeled anti-mouse whole blood globulin absorbed with mouse liver powder, beef liver powder, or Ehrlich ascites tumor cells resulted in much less staining of the tumor cells (Figure 1D, E, F). Most of the fluorescence was localized on the cell surface as fine to coarse granular material. Background fluorescence was appreciable, probably due to reaction with plasma proteins in the smear. Labeled anti-mouse whole blood globulin absorbed with mouse whole blood gave very little staining of tumor cells or background (Figure 1E). Leucocytes and platelets fluoresced brilliantly with these globulins as well as with labeled normal rabbit globulin. The labeled normal rabbit globulin caused only slight fluorescence of the tumor cells, and this was almost entirely limited to the cell surfaces.

In ethanol-fixed smears prepared from unwashed tumor, the cytoplasm of tumor cells had moderate fluorescence after reaction with labeled anti-Ehrlich ascites tumor globulin absorbed with mouse liver powder, beef liver powder, or mouse whole blood (Figure 2A, B). The intensity of the cytoplasmic fluorescence was somewhat less than that seen in comparable unfixed smears. However, background staining was considerably more intense than in unfixed smears, except after reaction with the antitumor globulin absorbed with mouse whole blood. Mouse whole blood absorption almost abolished the background fluorescence (Figure 2B). Absorption with lyophilized tumor cells abolished the fluorescence of the tumor cells, but not of the background (Figure 2C). Under similar conditions, labeled anti-mouse whole blood globulin absorbed with mouse liver powder, beef liver powder, and tumor cells, caused marked staining of the background but slight, if any, fluorescence of the alcohol-fixed tumor cells (Figure 2D, F). The globulin absorbed with mouse whole blood caused almost no fluorescence of either tumor or background (Figure 2E).

Smears were prepared from washed Ehrlich ascites tumor cells and reacted with the labeled antitumor globulin absorbed with the various materials. They appeared to stain in much the same way as the smears prepared from unwashed cells (Figure 3A, B, C). Background fluorescence was almost absent. Moreover, such smears reacted with the labeled anti-mouse whole blood globulin absorbed with the various materials contained very little yellow-green fluorescence (Figure 3D, E, F).

These results were interpreted as indicating that fluorescence seen on the surface of unwashed, unfixed tumor cells treated with labeled anti-mouse whole blood globulin was caused by the staining of mouse serum proteins present in the smear adjacent to the tumor cell. Surface tension would hold greater quantities of ascitic fluid here than in the areas between the cells, and as the smears dried, some of this protein would stick to the slide. Fixation of the smears in ethanol makes the protein less soluble and results in the greater background fluorescence seen when these slides are reacted with labeled anti-mouse whole blood globulin. Washing of the tumor cells with saline removes most of the serum proteins and results in disappearance of the background staining.

Cross reactions of labeled anti-Ehrlich ascites tumor globulin. To investigate further the

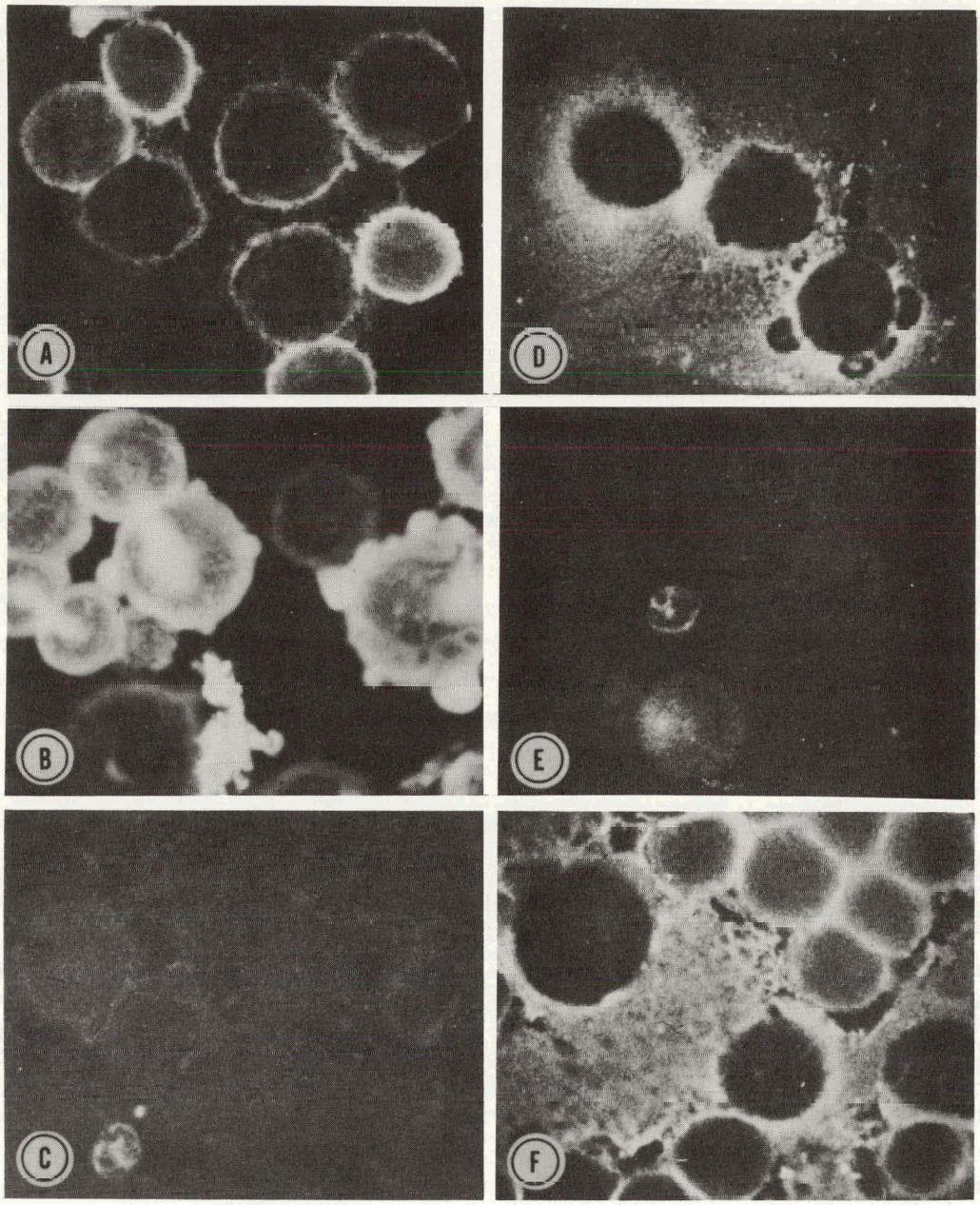

Figure 2. Fluorescence of ethanol-fixed smears of unwashed Ehrlich ascites tumor reacted with labeled globulins absorbed with various materials. X 925.

Labeled anti-Ehrlich ascites tumor globulin absorbed with
- A - mouse liver powder
- B - fresh mouse whole blood
- C - lyophilized Ehrlich ascites tumor cells

Labeled anti-mouse whole blood globulin absorbed with
- D - mouse liver powder
- E - fresh mouse whole blood
- F - lyophilized Ehrlich ascites tumor cells.

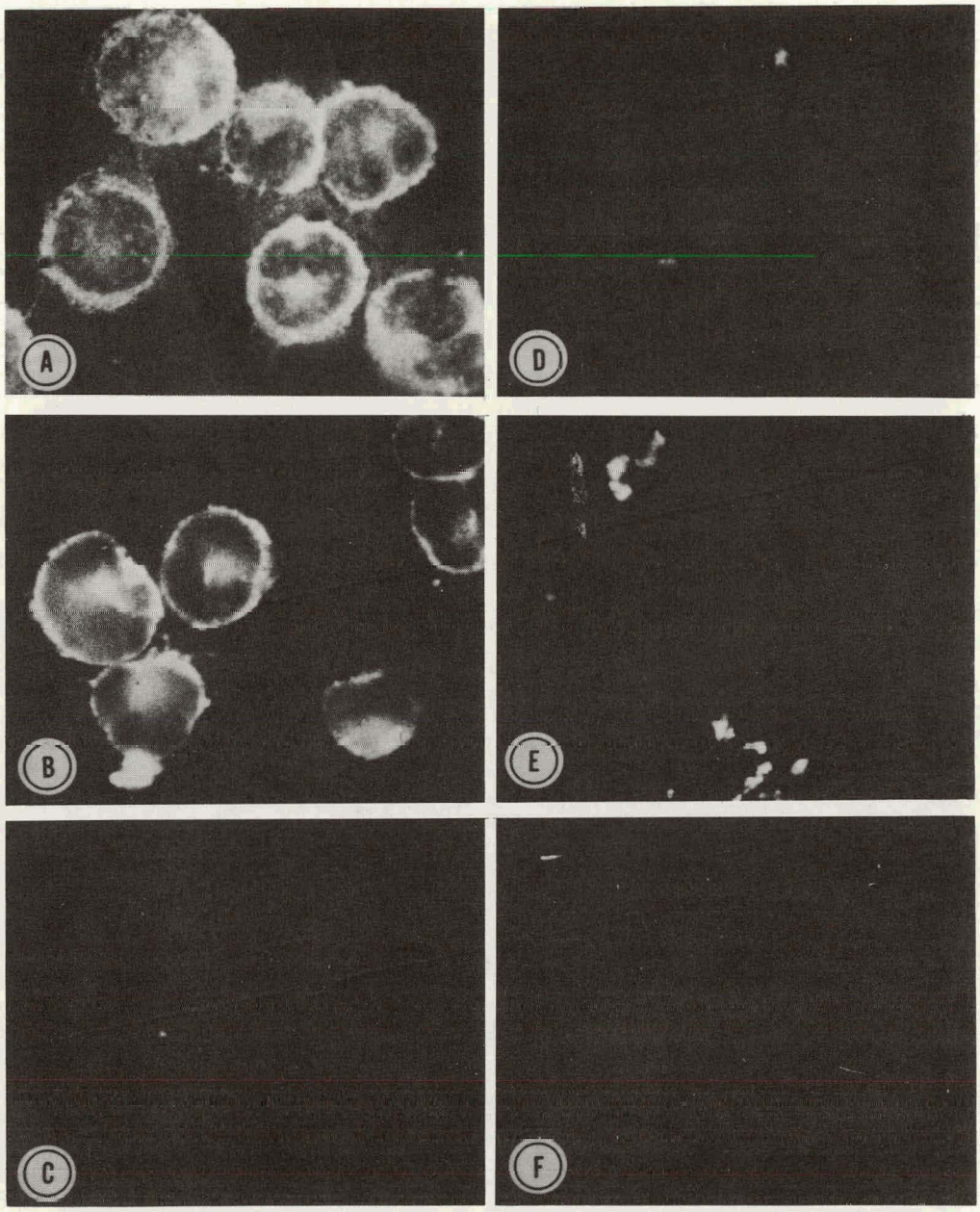

Figure 3. Fluorescence of unfixed smears of washed Ehrlich ascites tumor reacted with labeled globulins absorbed with various materials. X 925.

Labeled anti-Ehrlich ascites tumor globulin absorbed with
- A - mouse liver powder
- B - fresh mouse whole blood
- C - lyophilized Ehrlich ascites tumor cells

Labeled anti-mouse whole blood globulin absorbed with
- D - mouse liver powder
- E - fresh mouse whole blood
- F - lyophilized Ehrlich ascites tumor cells.

sites of staining and the specificity of the antitumor globulins, sections of tumor cells, fibrin clot, mouse liver and kidney, and blood smears, were reacted with the various absorbed globulins.

Fixed and unfixed sections of the tumor cells, after reaction with the labeled globulins absorbed with the various materials, fluoresced in a pattern similar to that seen in the smears. Staining of the tumor cell with labeled anti-Ehrlich ascites globulin was almost entirely limited to the cytoplasm, although occasional coarsely granular areas of fluorescence were seen in the nucleus. Fluorescence was less intense in fixed than in unfixed sections. The decrease in fluorescence of the tumor cells in ethanol-fixed smears, and sections treated with the labeled antitumor globulin, is probably the result of alteration in some of the antigenic sites in the tumor cells.

Sections of fibrin clot fluoresced only slightly after reaction with labeled antitumor globulin absorbed with mouse liver powder, beef liver powder, mouse whole blood, or Ehrlich ascites tumor (Figure 4A, B), but brilliantly after reaction with labeled anti-mouse whole blood absorbed with mouse liver powder, beef liver powder, or tumor (Figure 4C). Absorption with fresh

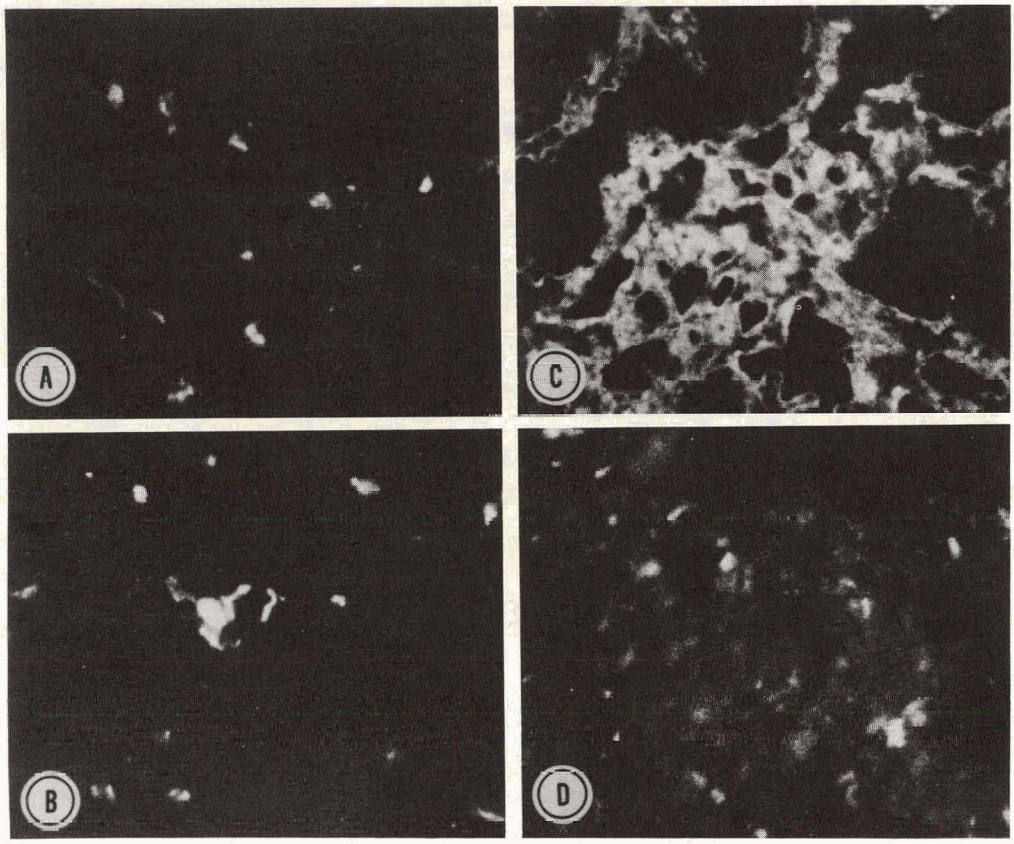

Figure 4. Fluorescence of unfixed sections of mouse fibrin stained with labeled globulins absorbed with various materials. X 925.
Labeled anti-Ehrlich ascites tumor globulin absorbed with
A - mouse liver powder
B - lyophilized Ehrlich ascites tumor cells
Labeled anti-mouse whole blood globulin absorbed with
C - mouse liver powder
D - fresh, whole blood.

whole blood almost completely abolished the staining (Figure 4D). With both labeled antitumor and anti-mouse whole blood globulins, fine granular material within the fibrin fluoresced brilliantly regardless of the material used for absorption. This was thought to be due to non-specific combination of the labeled globulins with platelets trapped within the clot.

Erythrocytes in blood smears fluoresced brightly after reaction with labeled antitumor globulin, or anti-mouse whole blood globulin, absorbed with mouse liver powder, beef liver powder, or tumor cells. Absorption of both globulins with mouse whole blood decreased the staining markedly. With all labeled globulins, platelets and granulocytic leucocytes in the smears fluoresced brilliantly.

Unfixed sections of mouse liver, reacted with labeled antitumor globulin absorbed with mouse liver powder, mouse whole blood, or beef liver powder, showed slight to moderate fluorescence along the surface of the sinuses but no fluorescence of the hepatic parenchymal cells. The walls of blood vessels fluoresced brightly. Blood cells fluoresced moderately, but other blood vessel contents did not. Staining was somewhat more intense with the labeled globulin absorbed with beef liver powder, but absorption with the tumor cells greatly decreased this fluorescence. Labeled anti-mouse whole blood, absorbed with the various materials, caused similar fluorescence of the sinusoids, but not of blood vessel walls. Blood vessel contents fluoresced brightly. Absorption with mouse whole blood decreased the intensity of fluorescence.

Unfixed sections of mouse kidney, exposed to labeled antitumor globulin which had been absorbed with mouse liver powder, or mouse whole blood, showed moderate fluorescence of the interstitial areas, as well as diffuse fluorescence of the glomeruli. The same labeled globulin, absorbed with beef liver powder, caused somewhat more fluorescence in the interstitial areas, and the cytoplasm of the tubular epithelium also stained slightly. The intensity of staining of the tubules was difficult to interpret because of the pale yellowish autofluorescence of the mouse kidney tubules. Absorption with Ehrlich ascites tumor cells resulted in much less fluorescence of the interstitial areas. The labeled anti-mouse whole blood globulin, absorbed with mouse liver powder, or mouse whole blood, caused even less fluorescence than did the labeled antitumor globulin. Staining was completely confined to the interstitial areas and glomeruli. Fluorescence in the glomeruli appeared to be associated with the basement membrane, and was less diffuse than that seen with labeled antitumor globulin. More fluorescence resulted from the globulin absorbed with beef liver powder; less from that absorbed with tumor cells.

Observations on fresh cell suspensions. Intact, freshly removed, living Ehrlich ascites tumor cells were suspended in the various labeled globulins absorbed with the various materials. After 30 minutes incubation at room temperature, the cells were washed in buffered saline, resuspended in a small volume of saline, and examined under the microscope as a wet mount. The tumor cells were clumped by the labeled antitumor globulin absorbed with mouse liver powder, beef liver powder, or mouse whole blood. Only a thin rim of brilliant fluorescence was seen around the tumor cell; there appeared to be little if any staining of the interior of the cell. In some cells, fine to coarse clumps of material on the cell surface also stained. In striking contrast, the globulin absorbed with tumor cells caused almost no staining on the cell surface. Tumor cells suspended in labeled anti-mouse whole blood were not clumped, and there was only very faint fluorescence at the cell surface on scattered cells. This was usually in fine patches on the cell surface and was abolished by prior absorption of the labeled globulin with mouse whole blood.

Fluorescence of the cell cytoplasm was seen in sections and smears of the tumor cells. The selective permeability of the cell membrane however, was destroyed in dried smears of cells, and sectioning allowed equally free access of the antibody to all parts of the cell. When living cells were suspended in labeled antitumor globulin, fluorescence was limited to the cell surface. This is more comparable to in vivo conditions, since antiserum administered to a tumor-bearing animal has contact, at least initially, only with the cell surface. While antibodies to several components of the tumor cell are probably present in this antiserum, it appears that antibodies directed against the cell membrane may be of major importance in producing cytotoxic changes in intact cells.

Flax,[4] Wissler and Flax,[5] Ellem,[8] and others have emphasized the importance of complement for the cytotoxic action of antiserum. To investigate the changes produced in intact cells exposed to antibodies and complement, cell suspensions were incubated with various mixtures of labeled globulins, unlabeled anti-Ehrlich ascites tumor globulin, and reconstituted, lyophilized guinea pig serum as indicated in Table 3. All labeled globulins were absorbed with mouse liver powder. After incubation at 37° C for five, 15, or 30 minutes as well as one or two hours,

Table 3

IN VITRO INCUBATION OF VIABLE EHRLICH ASCITES TUMOR CELLS

Serum fractions:[a]	Effect:
1. Labeled anti-Ehrlich ascites globulin	Agglutination of cells. No cytotoxic effect. Fluorescence apparently only on cell surface.
2. Labeled anti-Ehrlich ascites globulin and complement	Marked cytotoxic effects with cell swelling and cytoplasmic bleb formation. Fluorescence apparently only on cell surface.
3. Labeled normal rabbit globulin	No visible change in cells. A few fluorescent droplets only in scattered cells.
4. Labeled normal rabbit globulin and complement	No visible change in cells. A few fluorescent droplets only in scattered cells.
5. Labeled normal rabbit globulin, unlabeled anti-Ehrlich ascites globulin, and complement	Marked cytotoxic effects with cell swelling and cytoplasmic bleb formation. A few fluorescent droplets only in scattered cells.

[a]All labeled globulins were absorbed with mouse liver powder.

the cells were washed in saline and cell samples were taken for examination. The remaining cells were centrifuged, rapidly frozen, and cryostat sections prepared from the resultant cell pellet. These sections were examined fixed and unfixed, and some were stained with methyl green-pyronin, others with hematoxylin and eosin. Some of the results are summarized in Table 3.

Only the cells incubated with labeled anti-Ehrlich ascites globulin had appreciable fluorescence. In suspensions of cells exposed to labeled globulin alone, the cells were clumped (Figures 5A, 6A). Fluorescence was seen only on the cell surface (Figures 5B, C, 6B). In the presence of both complement and labeled antibody, there was marked cytotoxic change with swelling of the cell, and formation of clear blebs of cytoplasm (Figures 5D, 6C). These changes were

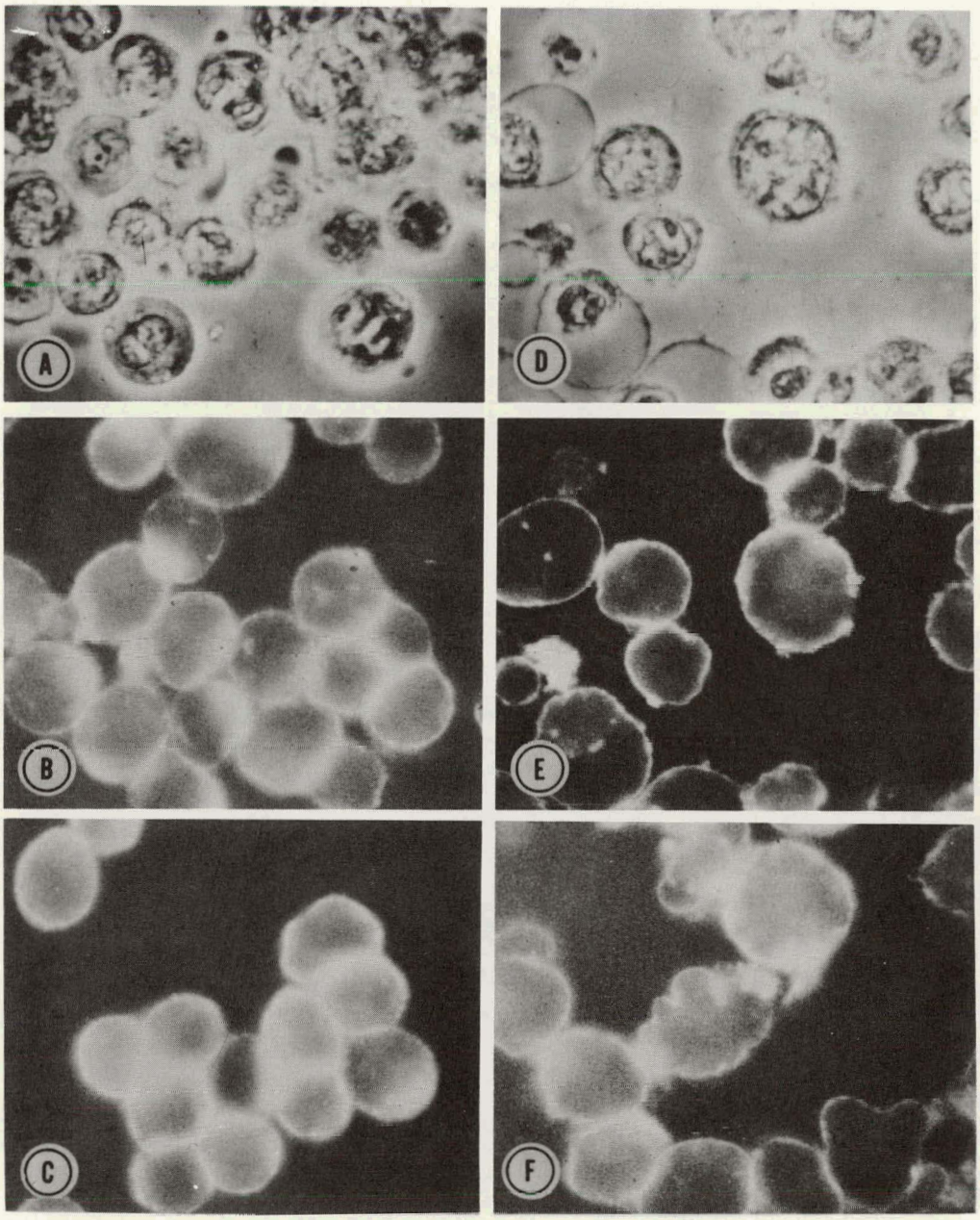

Figure 5. Ehrlich ascites tumor cell suspensions incubated for 15 minutes with labeled anti-Ehrlich ascites tumor globulin absorbed with mouse liver powder. X 875.

Without complement:

A - phase-contrast photomicrograph

B and C - fluorescence photomicrographs

With complement:

D - phase-contrast photomicrograph

E - fluorescence photomicrograph (field D)

F - fluorescence photomicrograph.

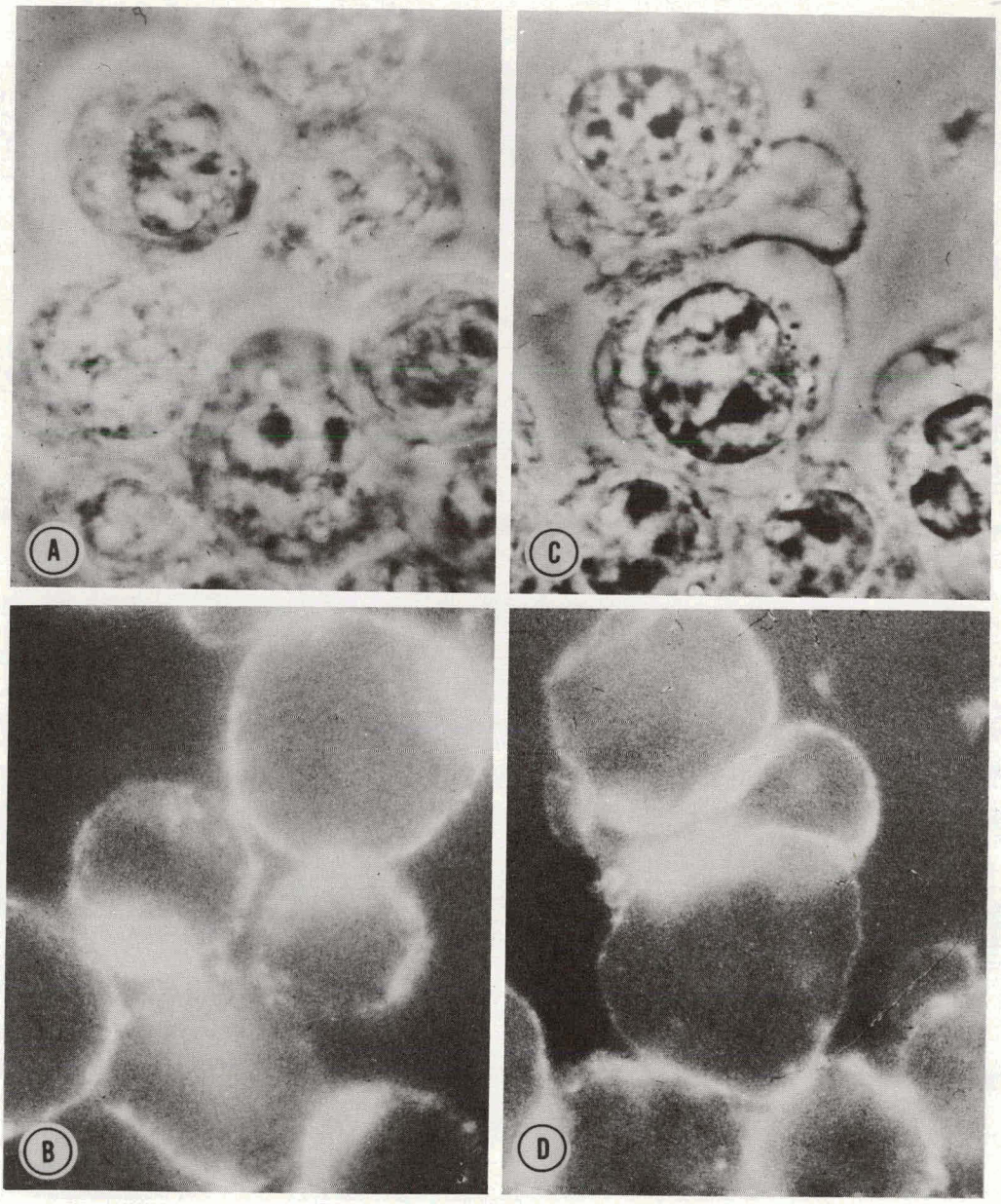

Figure 6. Ehrlich ascites tumor cell suspensions incubated for 15 minutes with labeled anti-Ehrlich ascites tumor globulin absorbed with mouse liver powder. X 1850.

Without complement:

A - phase-contrast photomicrograph

B - fluorescence photomicrograph

With complement:

C - phase-contrast photomicrograph

D - fluorescence photomicrograph (field C).

evident after five minutes incubation and nearly maximal after 15 minutes.

Fluorescence was limited to the cell surface even in these damaged cells (Figures 5E, F, 6D). The globular projections of the cell membrane covering the clear cytoplasmic blebs were brightly fluorescent also. When cell fragmentation occurred after the longer incubation periods, debris and cell fragments fluoresced brightly. Cryostat sections fixed by flotation on cold ethanol confirmed these observations. On the other hand, when unfixed sections were allowed to thaw on the slide, staining was also apparent within the cytoplasm. Antibody from outside the cell presumably combined with intracellular components during the time that the cells and surrounding material were liquid after the section thawed.

Almost all tumor cells suspended in the anti-Ehrlich ascites tumor globulin developed cell surface fluorescence. In the presence of complement occasional tumor cells, although showing fluorescence, did not develop cytotoxic changes during the observation period (Figures 6D, E). Many of these were large, polyploid cells.

Tumor cells suspended in the combination of unlabeled antitumor globulin, complement, and labeled normal rabbit globulin showed the same cytotoxic changes, but there was no fluorescence of either cell wall or cytoplasm. Tumor cells suspended in labeled normal rabbit globulin and complement, and in labeled normal rabbit globulin alone retained their normal appearance. Occasional cells contained a few fluorescent intracytoplasmic droplets, probably as the result of pinocytosis.

Observation of the suspensions by phase microscopy (Figures 5A, D, 6A, C) and of stained, fixed sections (Figure 7A, B) confirmed the presence of cytotoxic changes, and revealed the marked absence of cytoplasmic pyroninophilia presumably due to loss of ribose nucleic acid. The morphological changes were more apparent in sections stained with methyl green-pyronin than in those stained with hematoxylin and eosin.

DISCUSSION

Various techniques have been used to demonstrate the presence in the Ehrlich ascites tumor of antigens not present in normal host tissues. For example, Easty and Ambrose[20] using gel diffusion as well as other in vitro techniques concluded that the Ehrlich ascites cell contained antigens characteristic for the tumor as well as antigens shared with whole blood, kidney, spleen, and liver. Levina[21] used the rather cumbersome but sensitive indirect reaction of anaphylaxis after desensitization, and demonstrated a variety of antigens in Ehrlich ascites tumor cells, some of which appeared to be specific for the Ehrlich ascites carcinoma cell, while others appeared to be shared with various normal mouse organs. Still other antigens were shared with a spontaneous mouse mammary carcinoma of viral etiology, and others with the Crocker sarcoma. Kozlov,[22] using the same technique, obtained similar results.

Using complement fixation, another very sensitive technique, Airapetyan[23] demonstrated specific antigens in tissue homogenates of Ehrlich ascites tumor cells as well as antigens held in common with Crocker sarcoma. Still other antigens were shared with the mouse liver and spleen.

Specific antigens within the Ehrlich ascites tumor also apparently have been shown to be present by Levi et al.[24] These investigators suppressed anti-body formation against normal tissue antigens by injection of newborn rabbits with homogenized normal tissues of the C3H mouse host. Later injection of Ehrlich ascites tumor cells into these rabbits produced an antiserum

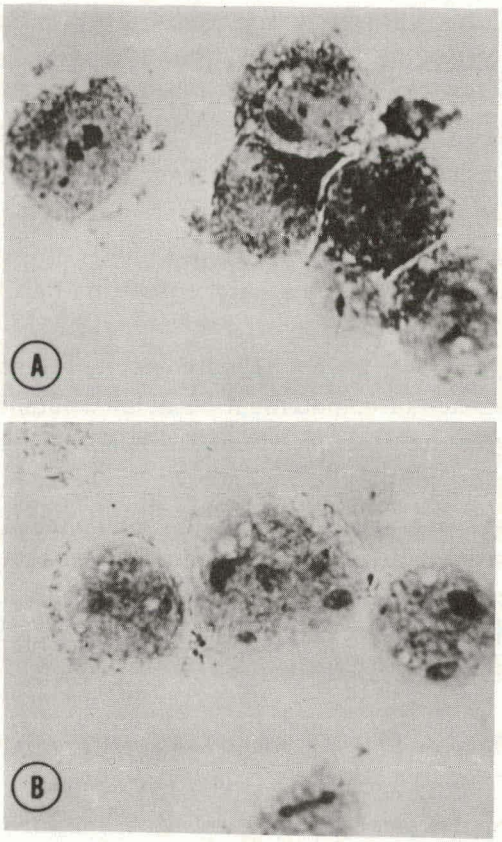

Figure 7. Cryostat sections of Ehrlich ascites tumor cells after incubation for 15 minutes with anti-Ehrlich ascites tumor globulin absorbed with mouse liver powder. X 1325.
Methyl green-pyronin preparation
A - cells incubated without complement
B - cells incubated with complement.

that had striking therapeutic effects on established ascites tumor.

All these studies, however, indicated that Ehrlich ascites tumor cells contained antigens common to various normal tissues.

The results of the present study similarly indicate that antibodies are present in the labeled anti-Ehrlich ascites tumor globulin which are directed toward normal tissue components. However, absorption with normal mouse tissue powders, or whole blood, removes most of the antibodies with avidity for normal tissues.

Hepatic sinusoids and blood vessels as well as renal glomeruli and interstitial areas fluoresced after reaction with labeled anti-Ehrlich ascites globulin. This was true even though the tumor suspensions used to immunize the rabbits contained no mesenchymal cells or fibers. The nature of these cross-reacting antigens is not known. Some antibodies may be directed toward basement membrane material but these are not the only antibodies present, since the hepatic sinusoids do not have a basement membrane.[25] Reticulin fibers are found in some of the areas showing fluorescence after reaction with the labeled anti-Ehrlich ascites globulin. Further studies are necessary to characterize the nature and distribution of antigens in normal mouse tis-

sues that are shared by the tumor. Selective destruction of various types of chemical substances by use of different fixatives and enzyme preparations may give useful information.

It seems unlikely that the cross reactions observed are due to the presence of Forssman antibodies in the antitumor globulin. Hemolysis titrations using the labeled globulins and sheep erythrocytes indicated that there was minimal hemolytic activity in serum diluted 1:15; the labeled globulins were not anti-complementary. It has been reported that antisera prepared against solid Ehrlich tumor contain Forssman antibodies while antisera against Ehrlich ascites tumor do not.[26]

Labeled anti-Ehrlich ascites tumor globulin causes fluorescence of the cell cytoplasm as well as the cell surface, if cells are prepared so that the integrity of the cell membrane is destroyed, but there is minimal staining of the nucleus. If however, the cell membrane is maintained intact and a cell suspension incubated with labeled antiserum, then fluorescence is observed only on the cell surface, and agglutination of the cells occurs. Thus, in this situation, which resembles that of tumor cells within the host, probably the only antibodies that combine with the cell are those directed toward antigenic components on the cell surface.

When living tumor cells are incubated with labeled anti-Ehrlich ascites tumor globulin in the presence of complement, cytotoxic changes develop, of which cell swelling and formation of cytoplasmic blebs are most prominent. Biochemical changes that occur in Ehrlich ascites and Krebs-2 ascites tumor cells suspended in antiserum and complement have been investigated,[5,8,27] and, the ultrastructural changes that take place have been described.[28] These observations indicate that there is marked alteration in permeability of the cell membrane, and that large-sized molecular materials probably diffuse out. Apparently, however, antibody molecules do not diffuse inwards since staining of the cytoplasm does not occur, even when a considerable interval intervenes between administration of labeled normal globulin and antitumor globulin plus complement.

Hiramoto et al.[29] who used the indirect fluorescent antibody method to study the reaction of anti-human connective tissue serum with living and dead HeLa cells in tissue culture, observed cytotoxic action with viable HeLa cells treated with antiserum in the presence of complement. They noted strands of cytoplasmic processes extending beyond the cell, fluorescent antibodies in which indicated that these processes as well as the cell surface, had reacted with the antiserum. The authors did not, however, describe any staining within the cell. Somewhat similar results were obtained with anti-Murphy lymphosarcoma serum and homologous tumor cells.[14]

In several preparations in the present study, it was noted that although occasional tumor cells suspended in labeled anti-Ehrlich ascites tumor globulin and complement were coated by fluorescing material, they did not develop cytotoxic changes. Many of these were large polyploid cells. Neighboring cells not only showed cell surface fluorescence but also developed marked cytotoxic change with cell swelling and cytoplasmic bleb formation. Wissler and Flax[4] examined the ascitic fluid in Ehrlich ascites tumor-bearing animals several days after treatment with antiserum, and found that the few apparently viable tumor cells remaining were unusually large cells with large nuclei. Hauschka has described the immunoselection of polyploid cells in homotransplanted mouse ascites tumors.[30] Feldman and Sachs have reported that isoantibodies are formed during growth of several adapted, homotransplanted mouse tumors. These tumors continued to grow and cause the death of the host despite the antibody response which occurred. They suggested that the tumors had acquired a resistance to the isoimmune response.[31] It is of interest that these resistant tumors had a higher chromosome number than the original tumors.

The present study indicates that antibodies localizing on the cell surface are involved in production of the cytotoxic changes caused by antitumor sera and complement. Antisera produced against cell contents are also cytotoxic. Ultrastructural observations indicate that the endoplasmic reticulum is continuous with the cell membrane and that the microsomes are derived from the endoplasmic reticulum.[32] If cell membrane and endoplasmic reticulum are in structural continuity, then they may have antigenic similarity. However, there is little information other than the physical and chemical studies to indicate the location of the antigenic material within the cell.

It is apparent from the present study that the localization of anti-Ehrlich ascites tumor globulin on tumor cells is not the result of the presence of fibrin antibodies, as has been observed with several transplantable tumors in the rat. Hiramoto et al.[14] were able to demonstrate in vitro that fluorescein-labeled antibodies, formed against the ascites form of the Murphy lymphosarcoma, stain cell contents of the lymphosarcoma cells, while anti-rat fibrin gave only sporadic staining. In vivo however, the localization of both antisera in solid tumor was similar, suggesting that in the solid tumor the anti-fibrin localization is more important. Bale et al.[13] using anti-fibrin antibodies coupled with radioactive iodine have consistently obtained rapid and permanent regression of Murphy lymphosarcoma growing subcutaneously in the rat. Whether anti-fibrin antibodies will localize in the subcutaneous form of the Ehrlich ascites tumor is not known.

There is considerable evidence that tumors contain antigens that differ from those found in normal tissues as well as those shared with normal cells. It is likely that some tumors in man are much more antigenically dissimilar from normal cells than others. Vigorous investigation of antigenic structure of neoplastic cells in man and experimental animals is needed. The present study indicates that specific heterologous antibodies important in producing cytotoxic changes in tumor cells localize on the surface of intact tumor cells. Other studies utilizing cell fractions indicate that these antigens are also found in several other parts of the cell, including the endoplasmic reticulum and microsomes. Further purification of these structures may furnish a more homogeneous and potent antigen, although it may not separate specific tumor antigens. Additional work on the induction of immunologic unresponsiveness to normal antigens before immunization with neoplastic cells, as well as greater emphasis on absorption of antibodies to normal antigens from tumor antisera using normal tissue fractions, should result in antisera with greater tumor specificity. In order to obtain potent, heterologous antisera, a search should be made to find the species for which tumor materials are most highly antigenic. Recent evidence suggests that the horse may be particularly suitable for immunization with human tumor antigens.[33]

There still remains the problem of delivering the antibody to the tumor tissue in vivo in sufficient concentration to be effective. Several possibilities for increasing localization of specific antibodies in solid tumors are being explored by other investigators in this laboratory. Oncolytic virus infection is being used in conjunction with administration of antiserum. Preliminary results[34] indicate that the combined therapy is more effective in causing tumor regression than either method alone. Chemical fractionation of antiserum is being performed in an attempt to decrease the size of the cytotoxic antibody thereby facilitating its passage through the capillary barrier. Attempts will also be made to increase the permeability of the tumor capillary bed by means of local x-irradiation and other measures.[5] Utilization of these techniques coupled with

further investigation of specific tumor antigens appears to offer some hope for immune therapy of tumors.

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COMPARISON OF EFFECTS OF I^{131}-INDUCED HYPOTHYROIDISM AND L-TRIIODOTHYRONINE ON IRRADIATED HAIR ROOTS IN MICE*

By

M. L. Griem, A. Stein,† R. P. Reinertson,‡ R. Reinertson, and R. Brown**

It is well known that after mouse hairs have been plucked, the hair follicles pass from a resting state into one of high mitotic activity. During this period they are more sensitive to ionizing radiation and the morphological changes so produced in the hair root have been described in detail. In the present investigation, we were interested in finding out whether an alteration of the metabolic rate would have any effect on the hair roots of animals irradiated during this sensitive phase. L-triiodothyronine was used to induce the hypermetabolic state in one group of mice while I^{131} was used to produce hypometabolism in another group. Both groups of animals were subsequently irradiated.

METHODS

The animals used were female CF No. 1 strain mice 12 to 16 weeks old weighing approximately 25 g, and maintained throughout on standard Rockland mouse diet containing 0.0012 per cent iodine. Two series of experiments were carried out, Series A in spring and series B in summer.

In both series the mice were divided into three groups: two experimental groups and a control group.

The first experimental group consisted of animals in which hypothyroidism was induced by the administration of radioactive iodine as follows. Four weeks before irradiation, these animals (34 in Series A, 33 in Series B) received single intraperitoneal injections of 300 μc of radioactive iodine, a dose known to be sufficient to lower the metabolic rate in this strain.††

The second experimental group consisted of animals in which L-triiodothyronine‡‡ was used to induce hyperthyroidism. For three days before irradiation these animals (35 in Series A, 37 in Series B) were given single daily subcutaneous injections of 10 μg of L-triiodothyronine, a dose previously determined to be adequate. The triiodothyronine was prepared by dissolving it in N/100 NaOH to a concentration of five mg in 0.5 ml of NaOH and further diluting it with physiologic saline to 50 ml. This solution had a pH of 9. It was immediately chilled and stored at 4° C. There were 34 normal control animals in Series A, 33 in Series B.

Ten days before irradiation, both flanks of the mice in all three groups were plucked, and

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†Present address: Hadassah Medical School, Jerusalem, Israel.

‡Idem.: National Institutes of Health, Bethesda, Maryland.

**Idem.: University of Utah, Salt Lake City.

††D. E. Clark and F. A. Shemwell. Personal communication.

‡‡L-triiodothyronine was supplied as Cytomel® by J. A. Clipson, Smith, Kline and French Laboratories.

those few animals (less than five per cent) from which the hair could not be completely removed were discarded. Since adequate numbers of animals were available, discarded animals did not affect sample size. Only one animal died in this experiment during pretreatment or treatment phases.

On the afternoon of the day of irradiation metabolic rates of individual animals in all three experimental groups were determined using a Walton Kymograph and a respirator measuring oxygen consumption.* The mice injected with I^{131} had an average metabolic rate 15 per cent below the normal rate; those treated with triiodothyronine had an average rate 25 per cent above normal. Since the maximum individual variation of the metabolic rate was only five per cent, metabolic determinations were later carried out on groups of five mice.

Radiation was administered to the left flanks of the animals at 50 kv, 30 ma, with a 2-mm aluminum filter and a focal skin distance of 11 cm. This produces a beam quality of 1.2 cm tissue half-value depth. A 2-cm cone was used in treatment and the dose rate was 387 r/min surface dose. Preliminary trials revealed that the radiation effects were most marked between dosages of 200 and 900 r. Groups of five animals from experimental groups and controls were therefore irradiated at 300, 400, 500, 600, 700, 800 r. Series A also included groups at 900 r and Series B groups at 200 r.

Preliminary studies showed that the third day after exposure was the optimal time for evaluation of radiation effects. On this day, therefore, hairs in the irradiated area on the left flank, and in the non-irradiated symmetrical area on the right flank were plucked and examined under a dissecting microscope using transmitted blue light. The proximal ends of the hairs were cut off and floated on a small quantity of water in a Petri dish (Van Scott and Reinertson[2]). One hundred hairs from each side of the mouse were counted and the number of dysplastic hairs (Figure 1), telogen resting hairs (Figure 2), catagen (involuting) hairs, and anagen (growing) hairs (Figure 3) for the irradiated side were recorded. The non-irradiated side showed growing hair only. There were a very few animals in which both flanks, for unknown reasons, showed a majority of telogen (resting) hairs, and these were discarded from the series, since preliminary experiments had indicated that resting hairs are highly variable in their response to irradiation. These nine discarded animals represented less than five per cent of the total number and did not appreciably affect the sample size.

RESULTS AND DISCUSSION

It is evident from Figure 4 which combines the data of both series of experiments that an increased x-ray dosage results in an increased percentage of dysplastic hairs in all three groups of animals (hypometabolic, hypermetabolic and normal controls). This effect increases sharply between dosages of 200 and 800 r and levels off thereafter.

It is also evident that the percentage of dysplastic hairs counted tended to be highest in animals of the hyperthyroid group and lowest in those of the hypothyroid group. Statistical analysis of the data indicates that these differences between the two groups are significant. In general, the percentage dysplastic hairs appeared to be somewhat higher in Series B than in Series A, possibly representing a seasonal variation. The radiation sensitivity of the hair in all three

*Metabolic rates of nocturnal feeders such as mice are most accurately determined in the afternoon.

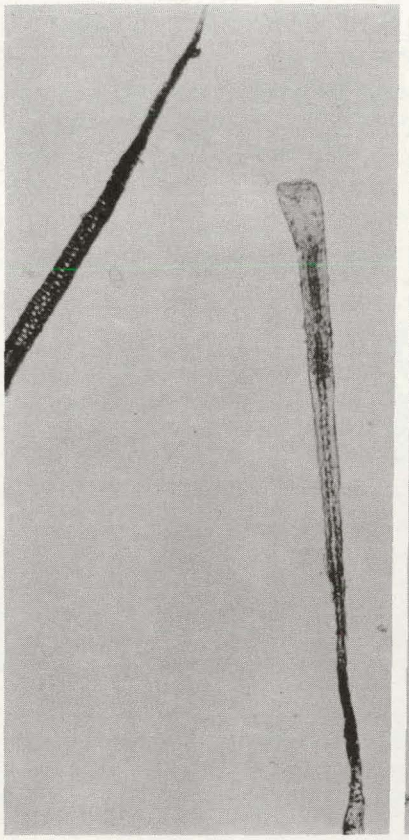

Figure 1.

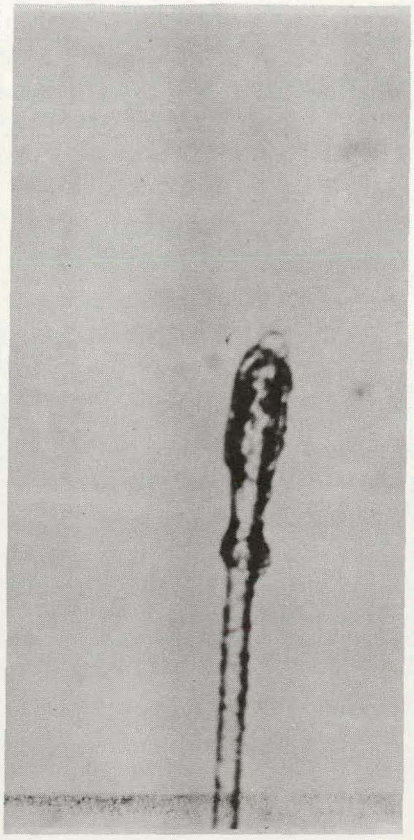

Figure 2.

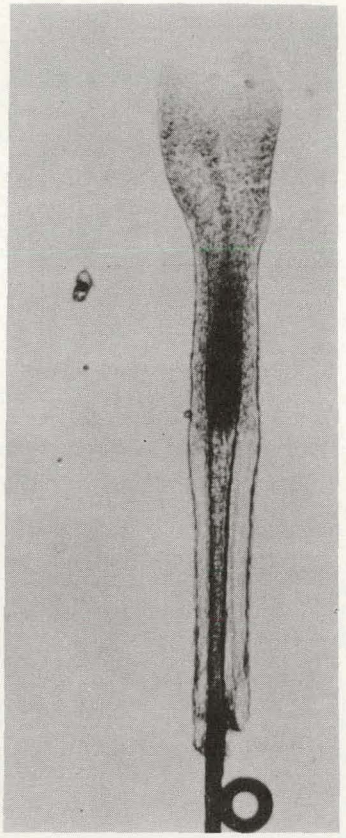

Figure 3.

Figure 1. Irradiated dysplastic hair.

Figure 2. Resting hair (telogen).

Figure 3. Growing hair (anagen).

groups of mice (hypothyroid, hyperthyroid and control) is illustrated by the average per cent dysplastic hairs found at all doses of radiation (Table 1).

At most treatment-radiation dose combinations under the conditions of these experiments, the effect of L-triiodothyronine was to increase radiation-induced dysplasia, while the effect of I^{131} was to reduce it. The excess of dysplastic hairs in normal, as compared with hypothyroid animals, at 700 and 800 r is not statistically significant. This agrees with similar work on effects of thyroxin[4] and L-triiodothyronine[5] in conjunction with total-body irradiation of mice. Smith,[6] on the other hand, working on the epidermis of mouse ear, found that the administration of thyroid did not affect the changes in mitotic activity produced in that tissue by sublethal doses of whole-body radiation. The potentiating effect of hyperthyroidism does not appear to be as great in the experiments reported above as that observed in chloroleukemia-bearing rats subjected to triiodothyronine treatment.[7]

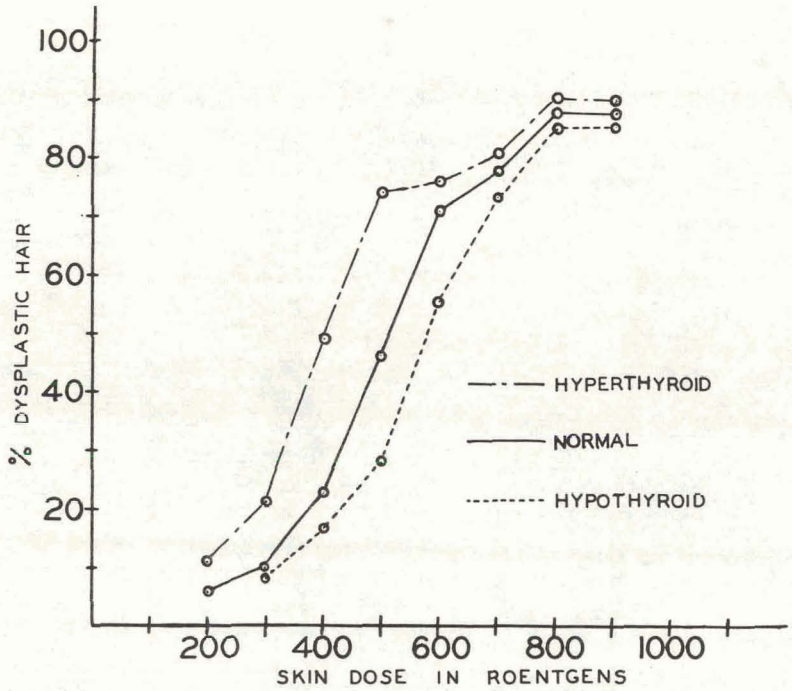

Figure 4. Comparison of the effects of hypothyroidism and hyperthyroidism on production of dysplastic hair in irradiated mice.

Table 1

EFFECT OF VARIATION IN METABOLIC RATE ON AVERAGE PER CENT DYSPLASIA

Experiment	Metabolic condition of animals		
	Hyperthyroid	Normal	Hypothyroid
A	56	47	42
B	68	61	46
$\overline{X}$ (mean)	64	54	44
Standard error of $\overline{X}$[a]	1.4	1.4	1.3

[a]Standard errors were calculated from estimates of variability determined for each dose-treatment experiment group, and applying usual formulae for estimating standard error of a linear combination.[8]

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MODIFICATION OF RADIATION RESPONSES OF TISSUE BY COLCHICINE: EFFECTS ON MOUSE HAIR ROOTS*

By

M. L. Griem, D. Malkinson,† and H. Morse†

Several recent reviews have described the effects of various chemical and physical agents in modifying radiation responses in normal tissues and tumors.[1-3] The observation that irradiation induces profound morphologic changes in animal and human hairs[4,5] has provided a useful experimental device for the screening of such agents in rodents.[6]

Experiments in rats and mice have shown that growing hairs also undergo profound atrophic and dysplastic changes following intraperitoneal administration of large doses of colchicine.[7] Since these microscopic changes strongly resemble alterations in human hair induced by x-ray irradiation,[5] the rodent hair indicator system was utilized in the current study to determine whether colchicine plays a synergistic role in increasing these x-ray effects. Our chief interest centered on the application of positive findings to the therapeutic implications of altering the radiosensitivity of certain forms of malignancy by prior administration of colchicine.

METHODS

The studies were carried out on adult female mice of the CF No. 1 strain. In these animals the hair cycle lasts for 17 to 20 days from earliest anagen to the onset of telogen. Hairs were completely plucked from both haunches of all animals at the start of each experiment, one haunch being used for examination of the effects of colchicine and irradiation, and the other for examination of colchicine effects alone.

The mice received x-ray irradiation on the 10th day after initial plucking, and four days later the newly growing hairs in the control and treatment areas were plucked and examined. (For appearance of resting (telogen) growing (anagen) and dysplastic hair follicles see Figure 1 in "Comparison of Effects of Triiodothyronine and I^{131}-induced Hypothyroidism on Radiation Effects in Root Hairs," p. 83.)

Preliminary studies of radiation changes in the hairs produced at different dosage levels of irradiation revealed a reliable and reproducible sigmoid curve for the production of dysplastic hairs. In these experiments radiation was administered using a Machlett OEG-60 tube operated at 50 kv and 30 ma with 2 mm of aluminum added filtration. This produced a dose rate of 387 r per minute surface dose at a focal skin distance of 11 cm, and a beam quality of 1.2 cm half value depth in tissue. Adequately shielded animals were treated individually and without anesthesia. The results are shown in Figure 1. Each point on the graph represents the average percentage of dysplastic hairs in five animals. For each animal 100 hairs were counted from the treated flank and 100 hairs from the control or unirradiated flank. When a surface dose of 400 r was used

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†Department of Medicine, University of Chicago.

we consistently found between 46 and 52 per cent dysplastic hairs among 500 hairs counted. This same radiation dosage was then used in the animals treated with colchicine, so that both protective and synergistic effects on the radiation reaction could be studied.

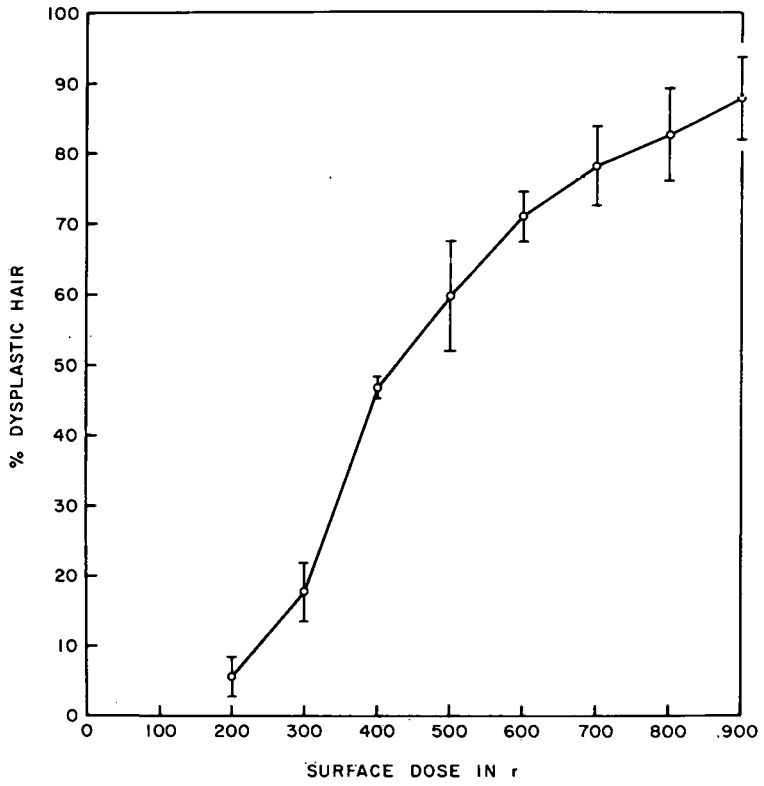

Figure 1. Dose response curve for production of dysplastic hair in mice by a single surface dose.

Colchicine* was administered intraperitoneally at a dosage of 1 mg/kg. (In the CF No. 1 mouse 5 mg/kg was found to be the LD_{50} for this drug.) At successive time intervals after the injection of colchicine a constant dosage of radiation was administered to different groups of animals, each group receiving radiation once only. The percentage of damaged hairs was plotted graphically as a function of the time interval between the administration of colchicine and irradiation.

RESULTS

The results of two separate experiments are shown in Figures 2 and 3, the vertical lines on the graphs representing the standard deviation for each point. As in the preliminary studies with x-ray irradiation alone, each point on a graph represents the average number of dysplastic hairs from a single group of five mice. For every animal a differential hair count was performed on

*Colchicine was kindly supplied by Dr. G. W. Irwin of the Lilly Research Laboratories. The drug was used as provided, each ampoule containing 1 mg of colchicine in 2 ml of aqueous solution.

100 hairs each from the x-ray treated flank and from the control flank. Hairs from the control (unirradiated) flank were counted because colchicine administration alone may produce dysplastic hairs. When 1 mg/kg of colchicine was given intraperitoneally the control flank showed less than two dysplastic hairs/100 hairs in all animals examined, and in over one-half the animals no dysplastic hairs were found on the control flanks.

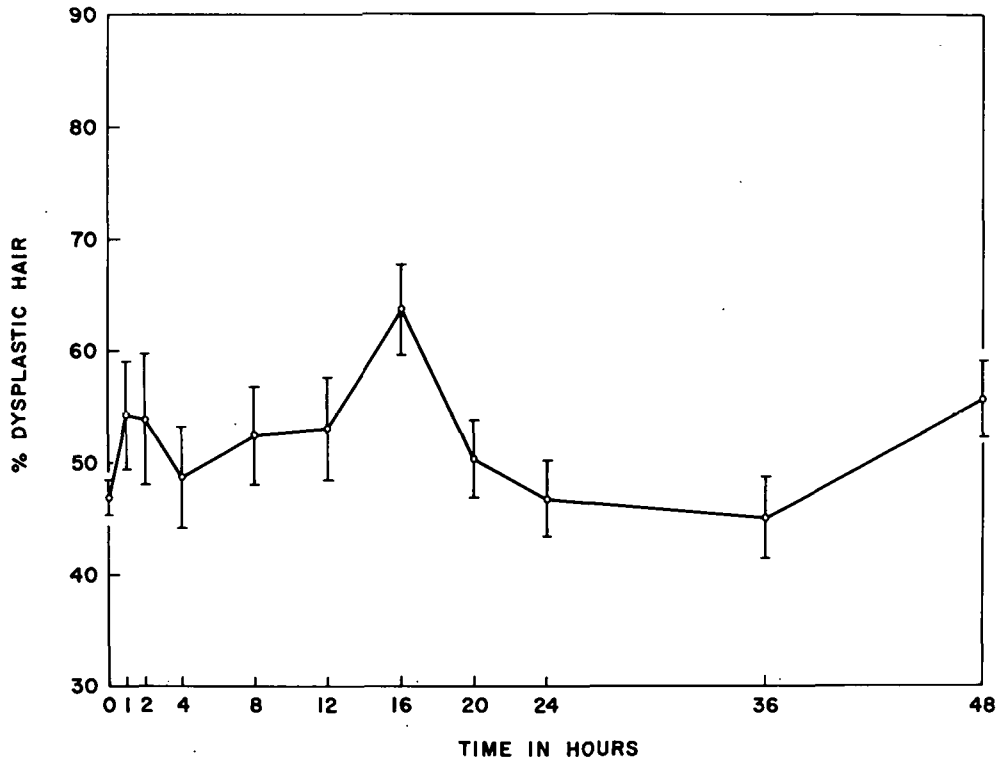

Figure 2. The first experiment showing the effect of varying time interval between injection of colchicine and administration of x-ray on the production of dysplastic hair.

Both graphs reveal a significant synergistic effect when the colchicine injection is followed by irradiation after an interval of 16 hours. Although there was a suggestion of synergistic action by colchicine at two-, four-, and 12-hour intervals in one study, this effect was not confirmed in the other. Both graphs, however, show that there is a decreased response at the end of 24 hours after the earlier 16-hour peak.

No protective effect of colchicine was observed in these studies.

CLINICAL

These preliminary observations having suggested that there might be a cyclic relationship between administration of colchicine and irradiation, a patient with generalized mycosis fungoides in the infiltrative and tumor stages was studied using a treatment method suggested by Friedman.[8] This patient was unresponsive to surface doses of 600 r in a single exposure given at 150 kv and 50 cm focal skin distance with 3 mm aluminum added filtration. Higher doses of

800 and 1000 r seemed similarly ineffectual. Three mg of colchicine was given intravenously and a single surface dose of 600 r was given through successive 10 x 10 cm portals every four hours for 16 hours. The four-hour and eight-hour portals showed no essential change, but there was a definite response at 12 hours, while the most striking response was observed at 16 hours. Combined treatment using the 16-hour interval between colchicine administration and irradiation was repeated a number of times for other involved sites and a good response was noted on each occasion.

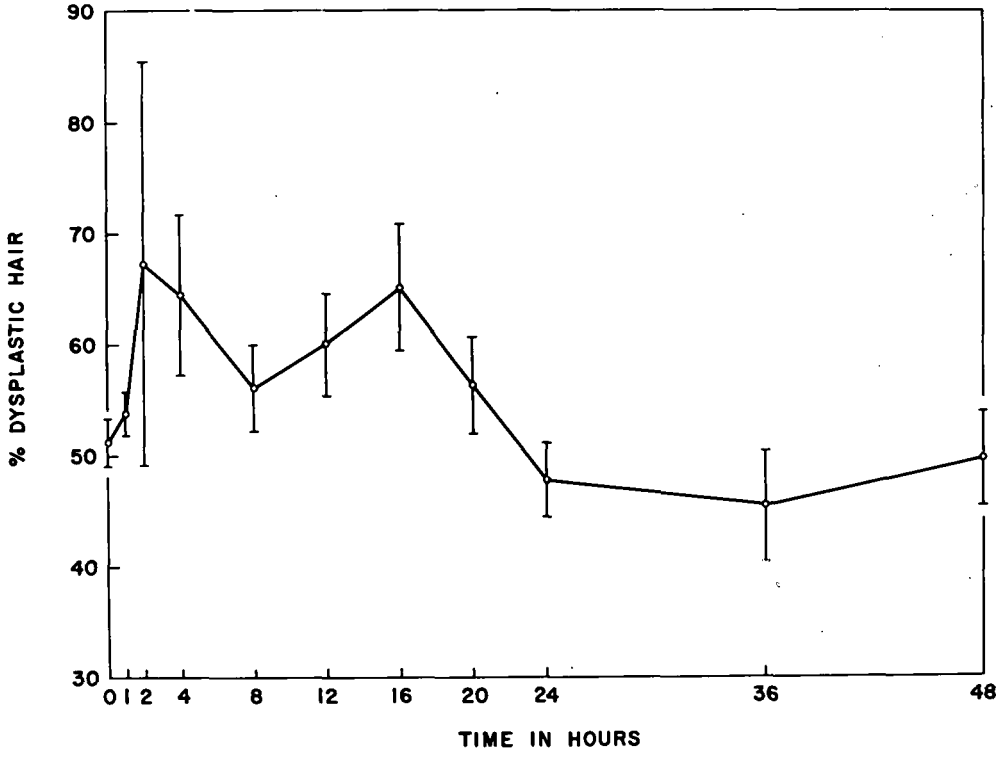

Figure 3. The second experiment showing the effect of varying time interval between injection of colchicine and administration of x-ray on the production of dysplastic hair.

Following these observations we have selected a number of patients with unresponsive tumors for clinical trial. So far it has been found that for these patients a single intravenous dose of four mg of colchicine can be given twice weekly for four weeks without serious difficulty. Occasionally there has been a transitory drop in the leucocyte count with a marked shift to the immature forms of the granulocytic series. In one patient diarrhea was observed, but this subsided when the drug was discontinued. Tumor doses of 400 to 500 rads per treatment are tolerated when combined with colchicine pretreatment.

DISCUSSION

In the past a number of reports has appeared in the literature describing the combined use of colchicine and irradiation for the treatment of tumors.[9-12] Since it was known that colchicine

interrupts cellular mitosis in the stage of metaphase, the earlier studies were based on the supposition that ionizing radiations exerted the most harmful effects on metaphase chromosomes. More recent work, however, indicates that the most radiosensitive stage of mitosis is early prophase, while chromosomes in metaphase are radioresistant.[13] Although several different techniques for investigating the combined effects of colchicine and x-ray therapy have been employed, the results are equivocal. Moreover, no attempt was made to correlate the time of administration of the drug and the subsequent irradiation, since no suitable biological indicator system was known which would lend itself to objective measurements of the synergistic tissue effects of colchicine and x-ray irradiation in contrast to the use of either agent alone. This problem now appears to have been solved satisfactorily by the technique of microscopic examination of newly plucked hairs described above.

Our experimental findings indicate that colchicine exerts a synergistic effect on atrophy and dysplastic changes in the hair matrix and hair shaft induced by x-ray irradiation, and that these alterations are observed only when the administration of colchicine precedes irradiation by 16 hours. No other time interval studied showed consistently significant colchicine effects.

The mechanism of the synergistic action of colchicine on irradiation is presently under study. It is possible that colchicine acts by marshalling a relatively large number of cells into the radiosensitive stage of early prophase at the critical time period for irradiation. This action would be influenced, however, by the prolonged period of metaphase arrest and some degree of cellular degeneration which usually follows colchicine administration. It is perhaps more likely that greater hair damage results from the synergistic effects of two related or unrelated forms of cellular injury separately induced by colchicine and irradiation, and which are independent of colchicine's anti-mitotic properties.

We do not yet know whether other normal or abnormal tissues will respond similarly to the combined use of colchicine and x-ray irradiation. It has been shown, however, that in both tumor-bearing humans and laboratory animals there is diminished urinary excretion of colchicine following the administration of C^{14}-labeled drug.[14] This strongly suggests that the absorption and metabolism of administered colchicine is different in humans and animals afflicted with tumors.

The responsiveness of a variety of experimental tumors as well as a smaller number of advanced malignancies in humans is now under study. The therapeutic results noted in the original patient with mycosis fungoides have already been described, and it is of interest that the best treatment response was obtained only within the same 16-hour period separating colchicine administration and x-ray irradiation that was found to be critical in the experimental work on mice.

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PRODUCTION AND USE OF IODINE^{125}*

By

P. V. Harper, W. D. Siemens, K. A. Lathrop and H. Endlich†

Reports from this[1,2] and other[3] laboratories have suggested that there may be some advantages in substituting iodine^{125} for iodine^{131} in a number of clinical and experimental situations. Among these advantages are the longer half-life of iodine^{125} (60 days), which results in a greatly increased shelf-life of tagged compounds, and the lower energy of its radiations, which reduces shielding requirements and permits more efficient detector design. These factors allow enhanced counting efficiency and scanning resolution, and, together with the absence of β-radiation, lead to decreased radiation dosage and increased tolerable isotope dosage. The availability of the isotope, however, has been greatly limited because production in the cyclotron from tellurium,[3,4] even when enriched target material is used, results in an end product heavily contaminated with the undesirable 13-day I^{126}, and hence, to a considerable extent, unsuitable for human and experimental use. Purification of the I^{125} by allowing the I^{126} to decay results in loss of most of the I^{125}, thus greatly increasing the cost which is at present $15.00 per millicurie with ten per cent contamination with I^{126}. The purpose of the present report is to describe our past year's work on production of I^{125} as a daughter product from the neutron activation of xenon^{124} to xenon^{125}, and to report the preliminary results of several practical applications.

PRODUCTION METHODS

The high, 74 barn, cross section of xenon^{124} reported by Tobin and Sako[5] made the production of relatively pure I^{125} in substantial quantities by the $Xe^{124}(n, \gamma)Xe^{125} \xrightarrow[18\ hr]{EC} I^{125}$ reactions appear quite feasible, even though the natural abundance of Xe^{124} is only 0.094 per cent. However, several samples of xenon irradiated in the Argonne National Laboratory's CP-5 reactor were found to be contaminated with substantial quantities of I^{126}, and this isotope (I^{126}) was also produced when samples of carrier-free I^{125} separated from the irradiated xenon were further exposed to the neutron flux in the reactor. The effective neutron activation cross sections for the $Xe^{124}(n, \gamma)Xe^{125}$ and $I^{125}(n, \gamma)I^{126}$ reactions as calculated from these various experiments are shown in Table 1. The identical cross section value which is obtained by activating I^{125} alone, or in the presence of xenon, indicates that no reaction other than $I^{125}(n, \gamma)I^{126}$ contributes significantly to the production of the I^{126}.

The fact that xenon^{124} is first activated to xenon^{125} and then decays with an 18-hour half-life to I^{125} makes it possible to eliminate the formation of I^{126} to a great extent by activating a large quantity of xenon for a short period of time. Under these circumstances, little I^{125} is formed during the irradiation, and so the production of I^{126} may be kept down to trace levels. In a typical run using this method with 16.5 g of xenon, a seven-day irradiation at a thermal

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*Text of a paper to be presented at the meetings of the Society for Nuclear Medicine in Pittsburgh, June 14-17, 1961.

†Department of Radiology, University of Chicago.

Table 1

PILE NEUTRON ACTIVATION CROSS SECTIONS

Target	σ_a Xe^{124}	σ_a I^{125}	Cobalt Cadmium Ratio
Xe^{124}	169	-	24.2
Xe^{124}	179	-	24.2
Xe^{124}-I^{125}	173	1160	~25
Xe^{124}-I^{125}	175	1138	~25
I^{125}	-	1180	58.9
I^{125}	-	1110	58.9
Xe^{124}-I^{125}	94	980	955.0
Xe^{124}-I^{125}	178	1120	10 (Production Run)
Xe^{124}-I^{125}	167	970	13.8

neutron flux of 6×10^{13} produced 1,350 mc of I^{125} having a five per cent contamination with I^{126}. A two per cent contamination with I^{126} was produced during a similar run for four days at 6×10^{13} $n/cm^2/sec$. The xenon was contained in a thick-walled irradiation vessel of Zircaloy 2 (Figure 1). After evacuation, this container was loaded by freezing the xenon gas (three liters at S.T.P.) into the vessel using liquid nitrogen, which resulted in an internal pressure of approximately 1,500 lbs/in^2 at the temperature of the reactor. Flux depression inside the loaded pressure vessel as measured with cobalt monitors was seven per cent. The activated sample was allowed to sit for six days following removal from the reactor, in order to permit the $xenon^{125}$ to decay to I^{125}. The irradiation vessel was then cooled to the temperature of liquid nitrogen to freeze the xenon, the valve opened, and the vessel warmed in a dry-ice acetone bath to evaporate the xenon into a previously evacuated storage vessel from which it could be re-introduced into the irradiation vessel for a second irradiation. It is necessary to contain the xenon, because approximately 100 curies of various radioactive xenons are produced. The carrier-free I^{125} remained in the irradiation vessel from which it could be almost completely removed with three washings of 0.1 per cent potassium bisulfite adjusted to pH 7-8 with potassium hydroxide. Each washing was allowed to stand in the vessel for several hours. Small quantities of $cesium^{137}$ produced from $xenon^{136}$ ($Xe^{136}(n, \gamma)Xe^{137} \xrightarrow{\beta} Cs^{137}$) were removed by pouring the bisulfite solution containing the iodine activity through a small acid phase Dowex 50 column.

Because all these experiments were carried out in the high flux region of the reactor where the epithermal flux is high, and because the observed neutron activation cross section of the $xenon^{124}$ was approximately twice that of the reported value, a sample was irradiated in the isotope tray where the flux is well moderated (cobalt cadmium ratio $\sim$ 1,000, see Table 1). The observed activation cross section of $xenon^{124}$ under these conditions was 94 barns, which more nearly approximates that of the reported value. The large neutron resonance absorption of $xenon^{124}$ at 5.16 ev[6] should account for this difference. The cadmium ratios of $xenon^{124}$ and

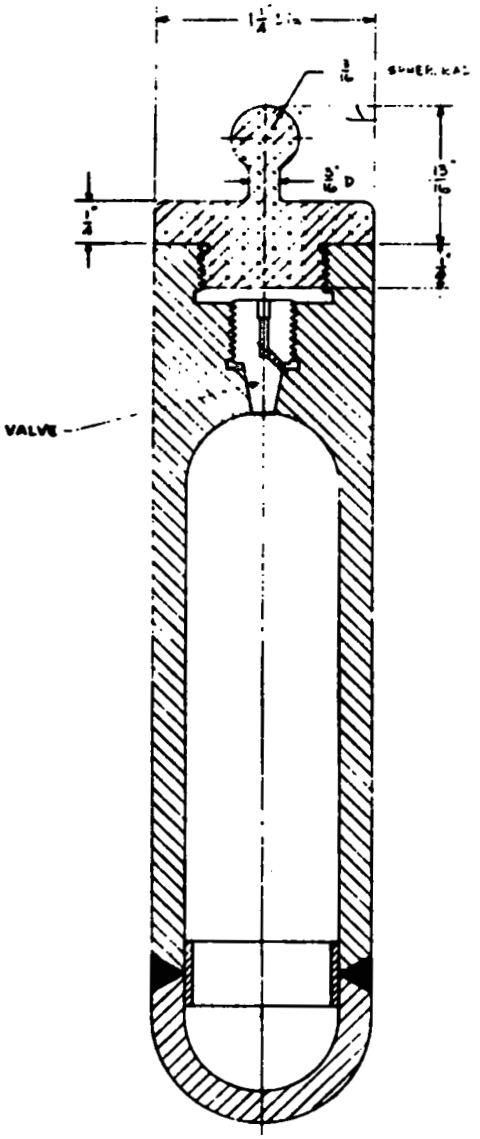

Figure 1. Zircaloy 2 irradiation vessel for xenon. Volume 35 ml, contains 16 g xenon at 1500 lbs/in^2, 90° C.

I^{125} were measured in the hope that I^{125} might be produced in significant quantity without production of I^{126} by activation of cadmium shielded xenon. The results of one experiment are shown in Table 2. Unfortunately, however, the I^{125} also appears to have a high resonance absorption so that this approach results in little improvement. The thermal neutron activation cross sections calculated[7] from these data are 115 barns for Xe^{124} and 890 barns for I^{125}

I^{125} decays with a 60-day half-life by electron capture to the 0.0354 MeV excited state of tellurium^{125}, which then decays by emission of a gamma which is largely converted in the K shell. The significant physical parameters of the decay scheme are shown in Table 3. Since the net result of the decay by these modes is the production of two coincident photons with energies of 27.3 to 35.4 KEV, it is possible by conventional reasoning to develop a formulation for a co-

Table 2

CADMIUM RATIOS (40 mil)

$Xe^{124} \rightarrow Xe^{125}$	$I^{125} \rightarrow I^{126}$	$Co^{59} \rightarrow Co^{60}$
3.05	4.22	13.8

Table 3

I^{125} DECAY SCHEME PARAMETERS

Quantity	Symbol	Value
Physical Half Life	T_p	60 days
Fraction of Disintegration by K-Capture[8]	K	0.813 ± .02
Fraction of Disintegration by L-Capture[8]	L	0.187 ± .02
Fraction of γ's Unconverted[9]	γ	0.07 ± .02
Fraction of γ's Converted in K Shell[9]	C_k	0.80 ± .05
K Fluorescence yield[10]	ω_k	0.855
Total K and γ photons/disintegration	$K\omega_k + C_k\omega_k + \gamma$	1.45 ± .08

incidence method assay in terms of the calculated abundances of the various photons involved (equation 1).

Equation 1: $$A = \frac{N_t^2}{N_c} \frac{K\omega_k(\gamma + C_k\omega_k)}{(K\omega_k + \gamma + C_k\omega_k)^2}$$

A = Activity
N_c = Total coincidences detected
N_t = Total photons detected (1 coincidence = 2 photons).

This expression is quite insensitive to uncertainties in the decay scheme parameters and may be greatly simplified by an approximation (equation 2).

Equation 2: $$A = \frac{\left(\frac{N_t}{2}\right)^2}{N_c}$$

which involves an error of less than one per cent. The coincident photons may be detected with great ease by the crystal summing technique,[11] using a multi-channel analyzer, as shown in Figure 2, or integral bias counting, as shown in Figure 3. This assay method was used in all the above-mentioned cross section determinations.

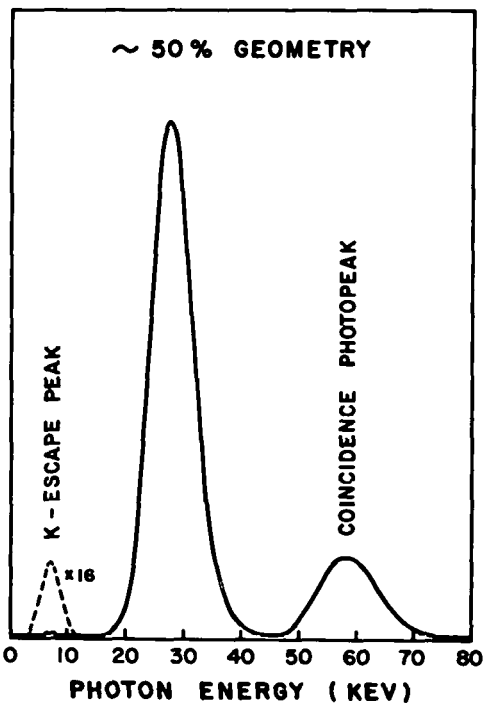

Figure 2. Scintillation spectrogram of I^{125}, using two mm NaI crystal with Be window at ~ 50 per cent geometry. The very small escape peak from the unconverted 35.5 KEV γ ray is visible. The principal photo peak at ~ 30 KEV includes the $K\alpha$, $K\beta$ and γ photons, and the smaller peak at ~ 60 KEV represents coincidence.

DETECTOR DESIGN AND DOSIMETRY

In considering detector design for I^{125}, the low energy of the emitted photon radiation makes very radical differences in the shielding requirements. Virtually complete shielding is obtained with two to three mm of lead so that collimators may be designed with paper-thin septa, thus increasing the counting efficiency by a factor of three or more.[12] A crystal of thallium-activated sodium iodide two mm thick will give virtually complete absorption of the radiation and only a negligible escape peak, since almost all the radiation is below the iodine K edge (see Figure 2). The absorption is almost exclusively photoelectric, so that the interaction ratio and photon fraction both approach 100 per cent. The attenuation of the radiation in the five-mil window of the commercially available Be window crystals is less than one per cent. The use of such a small crystal has the additional advantage of a greatly reduced background from cosmic radiation and other extraneous ambient activity.

In considering the dosimetry of I^{125}, the energy available to produce ionization which is released per disintegration (assuming 81.3 per cent K-capture and 18.7 per cent L-capture), is equal to the weighted sum of the energies of the resultant two excited states of tellurium, plus the 35.4 KEV energy of the gamma transition. Much of the energy appears as conversion electrons, Auger electrons, and the very low energy L, M and N photons, which for practical pur-

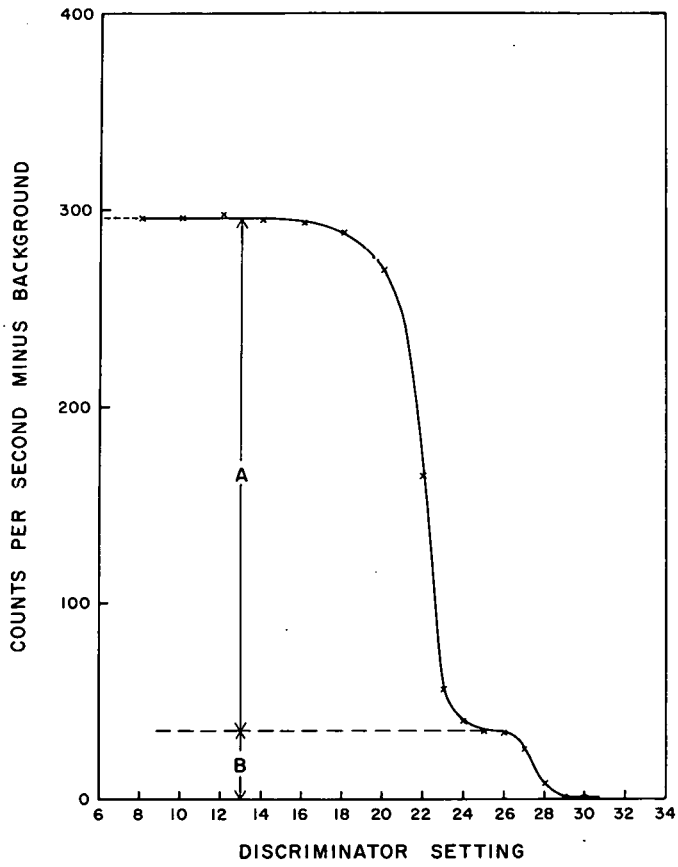

Figure 3. Detector and source as in Figure 2. Observations are displayed as an integral bias curve. A represents area of the principal photo peak and B the area of coincidence peak.

poses in tissue may be treated as β-like radiations in that they are absorbed very close to their point of origin. The other radiations, tellurium Kα and Kβ radiations, and the unconverted gamma radiation make up the gamma-like radiations (see Table 4). The quantity of the β-like energy which is dissipated per disintegration may be obtained by subtracting the energy of the gamma-like radiation from the total available energy. This amounts to 21.0 KEV per disintegration of

Table 4

I^{125} K AND γ PHOTONS

	Photons per disintegration	Fraction of total	KEV/Photon	KEV/Dis-integration	r/mc-hr at 1 cm	Calc. HVL tissue equiv. (cm)
Kα	1.09	0.75	27.3	29.8	1.03	1.82
Kβ	0.29	0.20	31.2	9.0	0.21	2.16
γ	0.07	0.05	35.4	2.5	0.04	2.56
Total	1.45	1.00	-	41.3	1.28	1.92

β-like radiation (Table 5). Comparison of the radiation dosages from I^{125} and I^{131} for three representative dosimetric situations is shown in Table 6.

Table 5

I^{125} ENERGY PER DISINTEGRATION AVAILABLE FOR IONIZATION

γ Transition	35.4 KEV
K-Capture	26.0 KEV
L-Capture	0.9 KEV
Total	62.3 KEV
K and γ Photons (γ-like radiations)	41.3 KEV
Conversion and Auger Electrons L, M and N Photons (β-like radiations)	21.0 KEV

The half-value layer for the I^{125} radiations measured in density 1.00 tissue equivalent solution[13] using narrow beam geometry was 1.99 cm, which is very nearly the calculated value of 1.92 cm. The wide beam half-value layer observed for the same medium was 2.53 cm. This difference is due to the scattered radiation. Considering the use of I^{125} for scanning procedures the narrow beam half-value layer is, of course, the pertinent one, since scattered radiation must be considered as undesirable background in the scan. When the radiation traverses fatty tissue, the HVL is somewhat greater. In Table 7 I^{125} and I^{131} are compared in terms of pertinent scanning parameters.

APPLICATIONS TO SCANNING

In the light of this discussion the substitution of I^{125} for I^{131} [1,2] for thyroid scanning should give under ideal circumstances, approximately ten* times as many detectable photons per unit radiation dosage to the patient. In Rose Bengal liver scanning this factor is increased to about 35. An additional factor is present when one considers the use of radioactive iodine to demonstrate "cold nodules" in either the thyroid or liver. In this situation the normal tissue takes up the iodine tag, while the "cold nodule" does not, and thus appears as a void in the scan. A cold nodule on the surface of the organ appears with greater contrast when I^{125} is used because the soft radiations from the underlying tissue are absorbed in the nodule, whereas the strong γ-radiations from I^{131} pass through. In this situation statistically significant differences in count rates may be observed with much lower over-all count rates, and consequently, much smaller nodules should be detectable. It can be shown that for the same radiation dosage and scanning time, and using optimal collimators,[12] a nodule on the surface of the liver approximately one-half the size detectable with I^{131} should be detectable with I^{125}. Since nodules below the surface of the liver are not readily detectable even with the penetrating radiation of I^{131}, little information is lost.

Using the design features mentioned above, scintillation probes for the detection of I^{125} are under construction in our laboratory. However, it has been found possible to use unmodified commercial scanning equipment,[14] substituting I^{125} for I^{131} μc for μc. About 40 per cent of the

*These values refer to photons originating in the surface layers of the thyroid or liver with one inch of over-lying tissue, and not to over-all count rates.

Table 6

I^{131} AND I^{125} COMPARATIVE RADIATION DOSAGE (APPROXIMATE)

Organ	Average dose rads/μcd/g						Procedure	I^{131}		I^{125}		Dose I^{131}/I^{125}
	I^{131}			I^{125}				Initial Dose Rate	Total Dose	Initial Dose Rate	Total Dose	
	β	γ	Total	β-Like	γ-Like	Total						
30 g Thyroid	110	10	120	92	38	130	Radioiodine Scan 50 μc 60 % uptake T biol = 50 days	Teff = 6.9 d 10.4 rad/d	103 rad	Teff = 27 d 1.5 rad/d	58 rad	1.78
1500 g Liver	110	42	152	92	100	192	Rose Bengal Scan 200 μc T biol = 2 hrs	Teff = 2 hrs 74 m rad/hr	210 m rad	Teff = 2 hrs 12.4 m rad/hr	35 m rad	6.0
70 kg Total Body	110	90	200	92	180	272	Blood Volume Tagged Albumin 5 μc T biol = 17 days	Teff = 5.4 d 1.3 m rad/d	10 m rad	Teff = 43 d .23 m rad/d	14 m rad	0.7

Table 7

I^{131} AND I^{125} COMPARATIVE SCANNING PARAMETERS

	I^{131}	I^{125}
Lead Shielding (10^6 Attenuation)	6.2 cm	3.5 mm
Crystal Thickness 95 % Interaction	6.8 cm	1.1 mm
Photo Fraction	~50 %	~100 %
Photons/Disintegration	0.8	1.45
Relative Background Due to Scatter	~1	~1
Relative Efficiencies of Optimal Collimators	~1	~3
HVL in Tissue (Total Attenuation)	6.3 cm	1.99 cm
Relative Dose per Detectable Photon—Thyroid Scan	~10	1
Relative Dose per Detectable Photon—Liver Scan	~35	1

count rate is lost due to absorption in the aluminum can of the crystal. Using such equipment, comparative scans between I^{125} and I^{131} are shown for the liver and thyroid in Figures 4 and 5. I^{125} and I^{131} scans of the liver show the marked increase in contrast in cold metastatic nodules, and small cold nodules in the thyroid are much more apparent with I^{125}. Thus, the advantages of using I^{125} are sufficiently great that even without special detection equipment, its substitution for I^{131} in routine scanning procedures appears justifiable. Even though the dose per μcd/g of tissue is approximately the same for both isotopes, the fact that I^{131} decays to a considerable extent before a great quantity is excreted, while I^{125} is excreted to a considerable extent

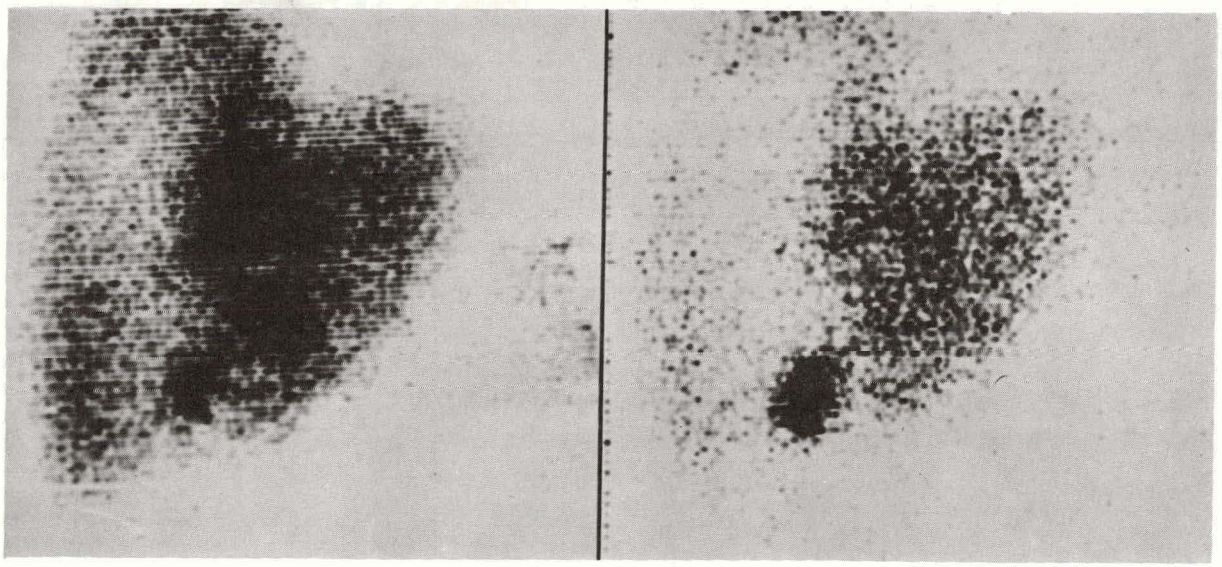

Figure 4. Rose Bengal photoscan of liver using available commercial equipment showing results obtained with 200 μc of I^{131}, on left; and with 200 μc of I^{125}, on right. Note the marked increase in contrast of the metastatic tumor nodules visible with I^{125}. (Picker Magnascanner.)

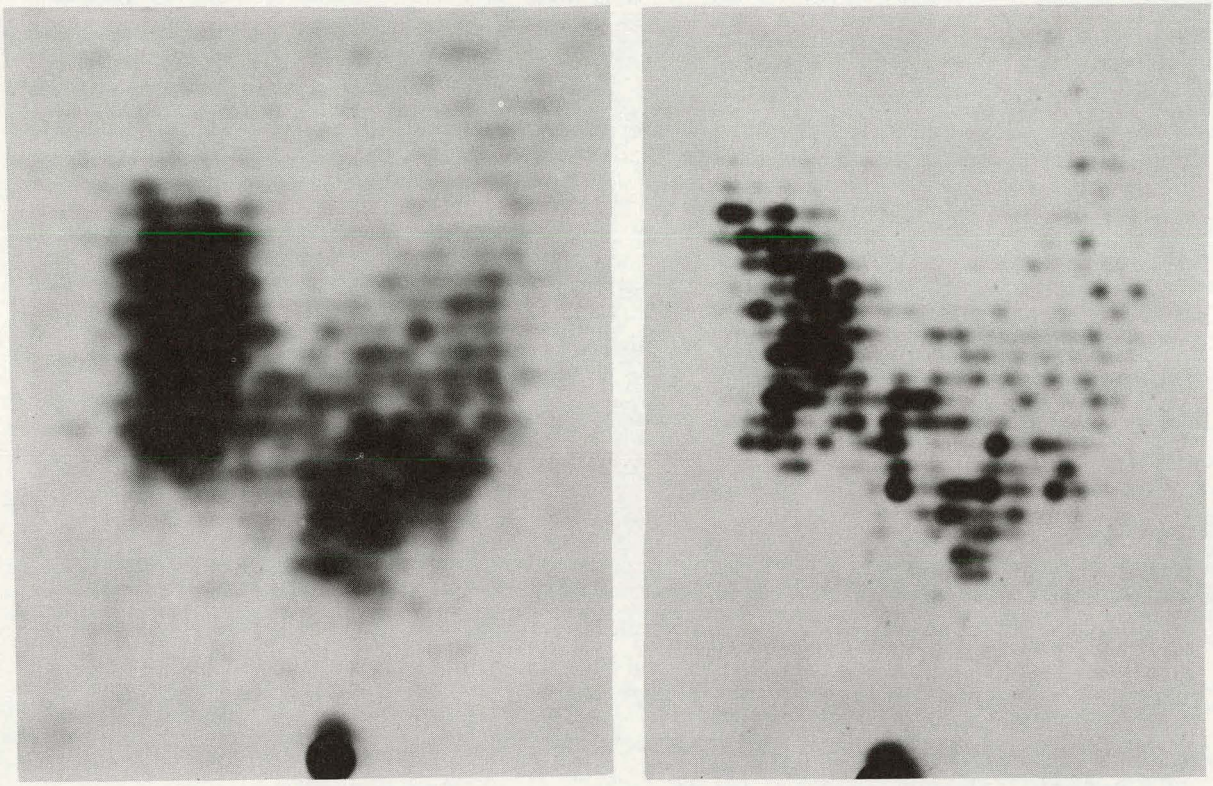

Figure 5. Photoscans of thyroid using same equipment as in Figure 4 with 50 μc of I^{131}, on left; and 50 μc of I^{125}, on right (28 per cent uptake). The cold nodules are much more apparent with I^{125}.

before it has a chance to decay, results inevitably in less of a radiation dosage when I^{125} is substituted for I^{131} μc for μc, except under the most unfavorable circumstances. Stated differently, the energy dissipation rate per μc is much less with I^{125} so that in applications requiring short exposures, such as Rose Bengal liver scans, there is greatly reduced relative exposure from I^{125}.

The fact that I^{125} radiations have a half-value layer of two to two and one-half cm in tissue virtually precludes this material from the measurement of quantitative uptake in the thyroid gland. Variation in the thickness of the overlying tissues and the impossibility of constructing a special phantom for each patient, make necessary the use of an isotope with more penetrating radiation. It is, however, ideally suited for tests in which small in vitro samples are counted, as in blood volume measurements or T3 absorption tests. Such small samples in plastic test tubes may be counted with about 50 per cent efficiency in conventional well counters having the standard 30 mil aluminum lining. In experimental applications, the low energy Auger and conversion electrons of I^{125} may be counted in windowless flow counters, also with about 50 per cent efficiency, in such procedures as counting chromatogram strips. The low energy electrons may also be used to make very high resolution radioautographs, possibly comparable to tritium (Figure 6), and the use of I^{125} for long-term metabolic studies in thyroid physiology is demonstrated in Figure 7.

During the course of this investigation, many hundred millicuries of high purity I^{125} have

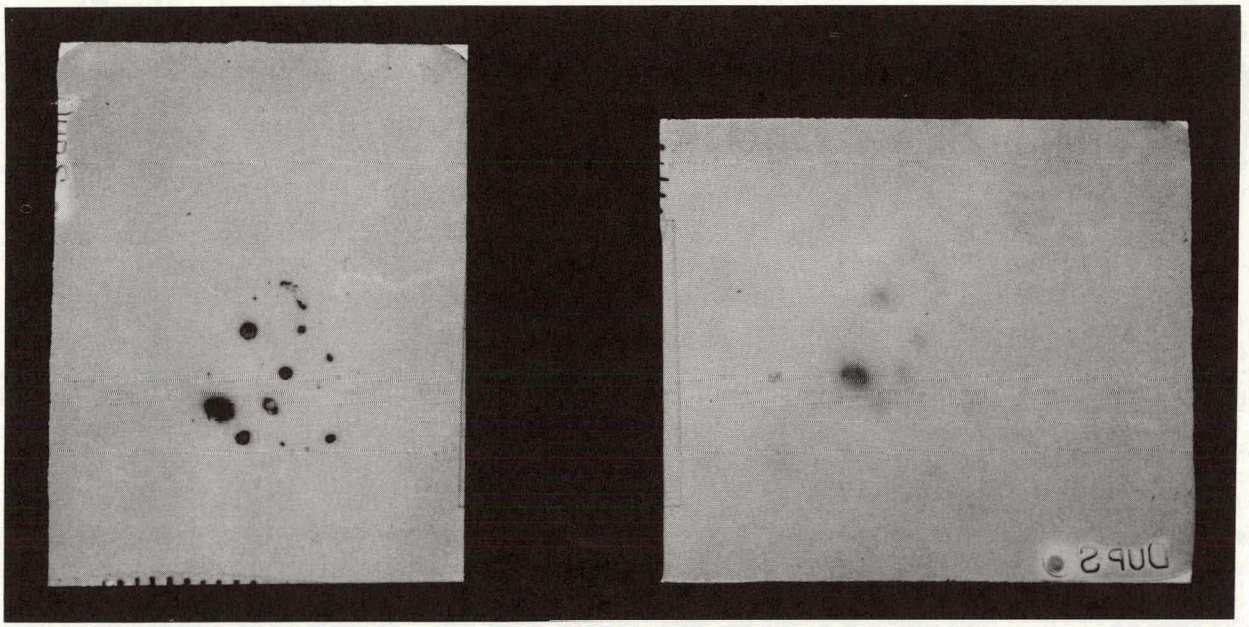

Figure 6. Radioautograph on dental film of I^{125}. On right a sheet of Saran wrap (10 μ thick) was interposed between the source and the film completely cutting out the low energy electrons and allowing only the x-rays to pass.

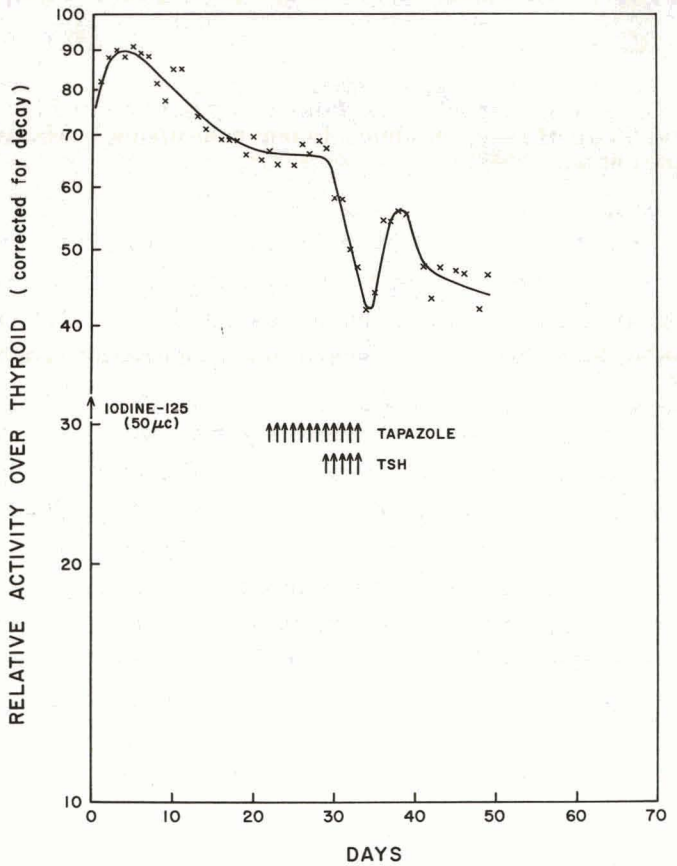

Figure 7. Attempt to influence the excretion of I^{125} by administration of Tapazole and thyroid-stimulating hormone in a euthyroid patient, BMR-2, PBI 6.8.

become available, and rather than let the material decay as radioactive waste, the Chicago Operations Office has granted us permission to furnish limited quantities to qualified investigators in other nonprofit organizations within the United States, and in special cases, to other countries. While it is not the desire of the Argonne Hospital to become an isotope producer, this arrangement seems reasonable in the interim before facilities for the commercial production of I^{125} at reasonable cost are developed at Oak Ridge or elsewhere. To date, 17 such shipments of I^{125}, totaling 375 mc, have been made.

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THE LOCALIZATION OF OCTOIODOFLUORESCEIN-I^{131} IN HUMAN BRAIN TUMORS*

By

E. C. Tocus,† G. T. Okita, J. P. Evans,‡ and S. Mullan‡

Fluorescein labeled with radioiodine (I^{131}) was first used as an adjunct to the diagnosis of tumors of the central nervous system by Moore et al. in 1948.[1] Since then many investigators have tried to improve the procedure using other methods of radiation detection,[2-6] other radioisotopes,[7-9] and other chemical compounds.[10,11] Ideally, the isotope labeled material should attain a concentration in the tumor tissue sufficiently greater than the concentration in normal tissue so that the location and approximate size of the tumor can be determined with some degree of confidence.

In a previous study on mice we examined the potential of octoiodofluorescein labeled with I^{131} (OIF-I^{131}) not only as an agent for localization of brain tumors, but also as a radiographic contrast medium.[12] The acute toxicity of OIF in mice is low, but the amount required to opacify blood for radiographic purposes is too high to permit its use as a contrast medium. In mice the concentration of OIF-I^{131} was found to be ten to 20 times greater in transplantable ependymoma than in the normal brain tissue.

The present report describes the use of OIF-I^{131} in localization of human brain tumors. Since no previous human data are available for OIF, rate of disappearance from blood, rate of excretion, distribution in tissues, and comparisons of concentration in brain tumor and normal brain, were also investigated.

MATERIALS AND METHODS

Preparation and administration of OIF-I^{131}. The synthesis of OIF-I^{131} has been reported previously.[12] For clinical use, 150 mg of the labeled dye was dissolved in five ml of pyrogen-free 1 N sodium hydroxide and buffered at pH 7.6 with pyrogen-free 0.1 M dipotassium phosphate. The volume was adjusted to 30 ml with physiological salt solution in a rubber-capped vial and sterilized. The radioactivity of the solution was determined with a well-type scintillation counter and an end-window G-M counter using appropriate standards. Radioactivity at the time of assay was approximately 300 μc per ml or 60 μc per mg of OIF-I^{131}.

Since OIF is a local irritant and has a deep red color, special precautions were taken while injecting it intravenously. A two-way stopcock was fitted to two syringes, one containing saline plus heparin and the other OIF-I^{131}. Venipuncture was confirmed with the saline syringe. The dye was injected slowly over a one-minute period followed by saline to prevent local irritation

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†Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at the University of Chicago. Present address: Lederle Laboratories, Pearl River, New York.

‡Department of Surgery (Neurosurgery),** University of Chicago.

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from leakage at the site of injection. The dose of radioactivity ranged between 200 and 300 μc.

Prior to the clinical use of OIF-I^{131}, 30 mg of non-radioactive OIF was administered intravenously into a volunteer subject to determine possible toxic effects of the dye. None were noted, and subsequent experience with 102 patients has confirmed this preliminary observation for doses as large as 25 mg. In two patients leakage at the site of intravenous injection resulted in transient local irritation and a pink coloration which disappeared after a few weeks.

Fate of OIF-I^{131}. Patients having, or suspected of having, brain tumors were selected for studies on the fate of OIF-I^{131} and its ability to localize in these tumors. Preliminary observations on the excretion of OIF-I^{131} were carried out on a few selected patients in the radioisotope nursing division of the Argonne Cancer Research Hospital. Permission was subsequently granted by the Health Physics Service to retain patients on the neurosurgical nursing division.

Individual urine and stool samples were collected from six patients over a 48-hour period, and radioactivity was measured by counting an aliquot of each sample in a well-type crystal scintillation counter, using an aliquot of the dose administered as a standard.

Blood concentrations and biological half-life of OIF-I^{131} were determined on samples taken over a 48-hour period from two normal volunteers previously given 5.7 μc of OIF-I^{131} intravenously.

For tissue distribution studies autopsy material was obtained from two cases. Samples of various tissues and organs were taken within 24 hours after injection of OIF-I^{131}, and measured in the well-counter.

Resected brain tissue. Eight selected patients scheduled for brain tumor surgery were injected with OIF-I^{131} from one to 26 hours prior to operation. Samples of brain tumor and tissue immediately adjacent to the tumor were removed and rinsed in saline. Non-necrotic tumor and normal tissues were dissected out and weighed. Radioactivity was measured in a well-type crystal scintillation counter using a dilution of the injected dye as a standard. A concentration ratio of brain tumor to normal brain for equal amounts of wet tissue was calculated.

Tumor localization. The scanning equipment consisted of a Dumont 6292 photomultiplier tube and a 1-1/2 x 2-inch thallium activated sodium iodide crystal shielded with 1/2 inch of lead. A three-cm diameter open-bore non-focusing collimator three inches in length was employed. The counter unit was mounted on a Sieman stand (Model SC-60 from Tracerlab, Inc.) which provided freedom of rotation in three planes for manual scanning. Counts were accumulated utilizing an Atomic scaler with a pulse height selector and timer. All counting was done at preset time. A lead shield 1/2 inch thick was placed over and around the neck perpendicular to the body to shield the counter from other sources of radiation in the body. Thirty-four areas of the head were counted as described by Fields and Seed[13] with additional positions over the eyes. The counter was positioned perpendicular to three intersecting planes to the head so that an imaginary grid was formed. Deviations greater than ten per cent for bilaterally symmetrical areas were confirmed by recounting.

Criteria for diagnosis. All areas and gross counts were listed, differences in symmetrical areas being calculated as percentage increase over the side having the lower value. Counting data were recorded in a grid-form representing the head in order to evaluate adjacent areas. Each area was compared to a normal value for that area expressed in relative percentage values. Areas of suspected tumors were drawn on a diagram of the head with abnormal counts projected according to place and direction. Tumors were tentatively located at the intersection of

the axes when the counting rate was more than 20 per cent greater than the normal value for the position. The scans were interpreted without access to clinical data or other diagnostic procedures.

EXPERIMENTAL RESULTS

Excretion, blood level, and distribution of OIF-I^{131}. The results from excretion studies in six patients are shown in Table 1. The average urinary excretion from four patients with normal urine output was 50.1 ± 3 per cent of the dose during the first 48 hours. The average fecal excretion from five patients was 10.6 ± 3.5 per cent of the dose for a similar period. The total urinary plus fecal excretion was 60.7 ± 4.6 per cent of the dose during the first 48 hours.

Table 1

EXCRETION OF OIF-I^{131}

Case	Urine				Feces			
	24-hr vol ml	% dose	48-hr vol ml	% dose	24-hr g wt	% dose	48-hr g wt	% dose
1	3225	48.22	6355	54.97	0	0.0	34.5	0.38
2	250	5.87	590	* 6.77	0	0.0	27.0	12.37
3	3453	45.62	5010	51.82	150	6.78	224.0	9.23
4	913	45.72	1722	48.05	10	0.03	389.0	16.33
5	372	2.16	856	* 6.79	0	0.0	80.0	11.5
6	978	40.6	3109	46.5	0	0.0	0.0	* 0.0

Average % dose 48-hr urine = 50.1 ± 3
Average % dose 48-hr feces = 10.6 ± 3.5
Total average % dose 48 hr = 60.7 ± 4.6
*Subnormal output not included in average.

The results of blood studies are shown in Figure 1. OIF was found to disappear from the blood at a rate such that the biological half-time was 12 hours. Within a few minutes to one hour after administration of the dye, blood concentration ranged from 0.4 to 0.02 per cent of the injected dose per 100 ml of blood.

The distribution of OIF-I^{131} in tissue was determined in two terminal patients who died 4.5 and 18 hours respectively after injection (Table 2). The radioactivity of the tissues from both cases is in the same relative order of decreasing activity except in the liver.

Resected brain tissue. The concentration of OIF-I^{131} per g of tumor compared to the concentration per g of normal tissue is an index of its ability to localize in tumors and is expressed as a ratio. Concentration ratios from eight patients with various types of brain tumors are shown in Table 3. Since no normal tissue was obtained with the meningioma, an average of the three highest normal values was used to obtain a ratio for this tumor.

Localization of brain tumors. A total of 102 patients was studied. These patients were divided into two categories according to methods of confirmation; surgical confirmation including all patients in whom diagnosis was confirmed by craniectomy, biopsy, or autopsy, clinical confirmation including diagnoses based on some or all of the following: encephalography, ventriculography, angiography, and symptomatic evaluations.

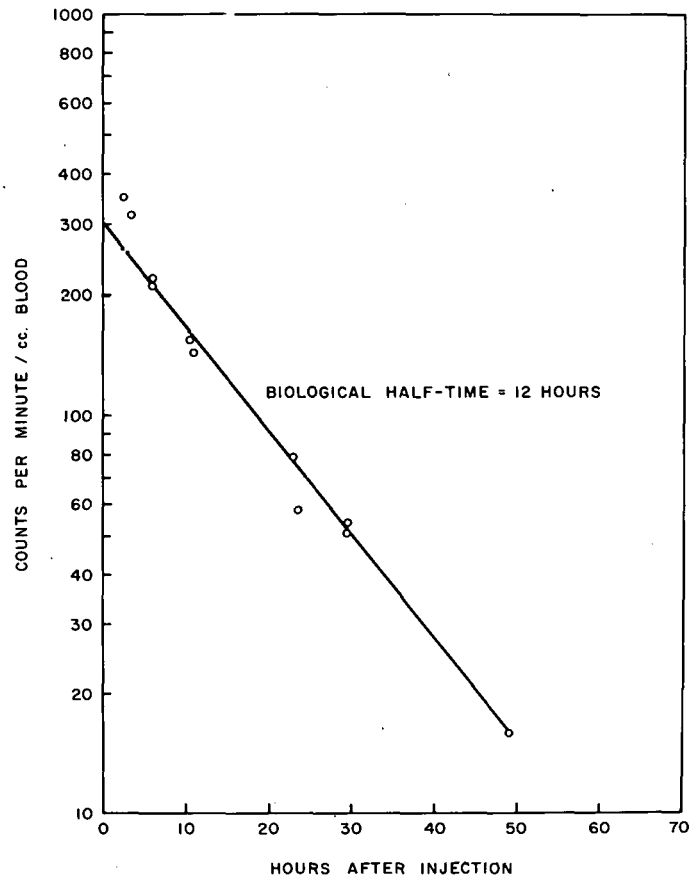

Figure 1. Blood level and biological half-time of OIF-I^{131}.

The 47 patients in whom diagnosis was confirmed at surgery represent the most accurately diagnosed cases. These data are presented in Table 4 in which a correct result implies that both the presence and the location of the tumor were correctly detected by scanning. Where surgery revealed no tumor and prior scanning indicated no area of localization, the result was also listed as correct. All other localizations were scored as incorrect.

Of the 55 patients diagnosed by clinical methods, 32 patients were believed to have no tumors, while the remaining 23 had various types of brain tumors and lesions. Included in this group were patients with symptoms of brain damage in whom the nature of the process was undetermined. The results of this study are shown in Table 5.

DISCUSSION

Although the synthesis of octoiodofluorescein has been known for some time,[14] we are not aware of any previous report on the use of I^{131}-labeled octoiodofluorescein as an agent for localizing human brain tumors.

In the 102 patients studied by us, octoiodofluorescein was found to produce no toxic symptoms in patients when given intravenously at doses below 25 mg, the highest dosage employed. In order to determine the liability of I^{131} on the OIF molecule, thyroids of the two terminal patients

Table 2

DISTRIBUTION OF OIF-I^{131} IN VARIOUS TISSUES

Tissue	4-1/2 hr post injection		18 hr post injection	
	% dose/g	% dose/organ	% dose/g	% dose/organ
Liver	.0226	24.8	.00725	7.33
Thyroid*	.0098	0.423	.0142	0.187
Kidneys	.0097	3.27	.0138	4.00
Spleen	.00715	0.465	.00425	0.038
Lung	.00665	5.45	.00147	1.43
Adrenal	.0047	0.063	-	-
Stomach	.0047	-	-	-
Bone (rib)	.003	-	-	-
Pancreas	.00275	0.236	-	-
Heart muscle	.0017	0.47	-	-
Skin	.00076	-	-	-
Skeletal muscle	.00066	-	.00052	-
Fat	.00023	-	.0003	-
Ileum content	-	-	.102	-
Jejunum content	-	-	.0565	-
Duodenum content	-	-	.034	-
Colon content	-	-	.001	-

*Stable iodine (Lugol's solution) was not given to these patients.

Table 3

ANALYSIS OF RESECTED BRAIN TISSUE

Type of tumor	Dose μc	Hrs after inj	cpm/g		
			Tumor	Normal	Ratio
Astrocytoma	300	26	1,018	105	9.7
Bronchogenic metastatic	300	18	1,026	130	8.0
Adenocarcinoma metastatic	243	8	1,007	131	7.7
Meningioma	300	22	800	(133)*	6.0
Unknown	300	14	675	137	5.0
Oligodendroglioma	125	15	170	43	4.0
Glioblastoma	253	1	232	102	2.3
Astrocytoma	300	22	22	99	0.2

*Average value obtained from other normal brain tissue.

Table 4

LOCALIZATION OF BRAIN TUMORS IN HUMANS USING OCTOIODOFLUORESCEIN-I^{131} (CONFIRMED BY SURGERY)

Type of tumor	Number	Localization using OIF-I^{131}	
		Correct	Incorrect
Glioblastoma	8	7	1
Astrocytoma	5	3	2
Meningioma	8	7	1
Oliogodendroglioma	2	1	1
Metastatic tumor	12	6	6
Acoustic neurinoma	3	2	1
Pituitary	3	3	0
Hemangioma	3	3	0
No tumors	3	2	1
Total	47	34	13

Per cent correctly localized: 72.5.

Table 5

LOCALIZATION OF BRAIN DAMAGE IN HUMANS USING OCTOIODOFLUORESCEIN-I^{131} (CONFIRMED BY CLINICAL METHODS)

Clinical diagnosis	Number	Localization with OIF-I^{131}	
		Correct	Incorrect
Meningioma	2	2	0
Tumor of Calvarium	1	0	1
Hodgkin's disease	1	1	0
Astrocytoma	1	1	0
Ependymoma	1	0	1
Metastatic tumor	5	4	1
Aneurysm	1	1	0
Atrophy of cortex	3	1	2
Chronic Arachnoiditis	1	0	1
Infarct	1	1	0
No diagnosis	6	6	0
No tumor	32	23	9
Total	55	40	15

Per cent correctly localized: 73.

injected with OIF-I^{131} without prior administration of Lugol's solution were analyzed and the amounts of I^{131} found in the total organ were 0.42 and 0.19 per cent of the dose, respectively. Since no attempt was made to separate free I^{131} from OIF-I^{131} in the thyroid tissue, the figures in all probability represent radioactivity from both compounds, and therefore indicate that only an insignificant amount of free I^{131} is released from OIF-I^{131}. Of the other tissues examined for content of OIF-I^{131}, the liver and kidneys contained the greatest amounts of dye indicating the role played by these organs in excretion. The level of OIF-I^{131} in skeletal muscle was low. The dye was found in lowest concentration in fat, which is consistent with the known low fat solubility of the dye.

The best measurement of the uptake of a compound by brain tumors is obtained by comparative analysis of normal and cancerous brain tissue. Results of studies on eight patients showed tumor OIF-I^{131} values to be from 0.2 to 9.7 times that of normal. Two factors which may influence the ratio are time lapse between injection and operation, and tumor type. In this study, tissues were examined between one and 26 hours after injection. The specific activity of normal brain tissue did not differ significantly at any time period. Since the data are limited, no definite conclusions can be made concerning uptake ratio versus time.

Tumor type may influence the ratio in several ways. In a highly vascular tumor, there is a higher concentration of blood and, therefore, of dye in the tumor mass, resulting in an increase in the ratio. However, the degree to which vascularity participates in the success of localization has not been definitively established. From the limited number of resected tumors available it was noted that most vascular tumors had a higher dye activity although there were instances where vascular tumors also had low activity. Avascular tumors, however, invariably had low activity. A compound which localizes by vascularity alone would only be detected as long as the blood level did not fall below a critical value for the detector.

The rate of growth and protein-binding properties of brain tumors may also partially determine the uptake ratio. Differences may occur among tumors of the same type such as the two astrocytomas in our study which gave ratios of 0.2 and 9.7, the extreme values. Such results might be anticipated with astrocytomas due to the frequently cystic character of this type of tumor.

To illustrate cellular uptake of OIF, a section from a metastatic bronchogenic human brain tumor was mounted without staining other than OIF in vivo. A photomicrograph of this section is shown in Figure 2. It is noticeable that individual and groups of cells containing OIF are visible. These cells have an affinity for the dye and thus indicate the role of cellular uptake in tumor localization. A determination of the mechanism of localization is beyond the scope of this report; our studies on protein binding will be reported elsewhere.

For the surgically-confirmed cases (Table 4), external brain tumor scans after intravenous administration of OIF-I^{131} correctly determined the presence or absence of tumors in 34 out of 47 cases or 73 per cent. For the non-surgical group (Table 5), 40 of 55 cases, or 73 per cent, were correctly diagnosed.

When classified according to tumor type the following results were observed. Gliomas (including oligodendroglioma, astrocytoma, glioblastoma, and pinealoma) were correctly localized in 11 out of 15 cases (73 per cent), the glioblastomas being 7 out of 8 cases (87 per cent). Metastatic tumors, as a class, were more difficult, with only 5 of 11 (45 per cent) being correctly located.

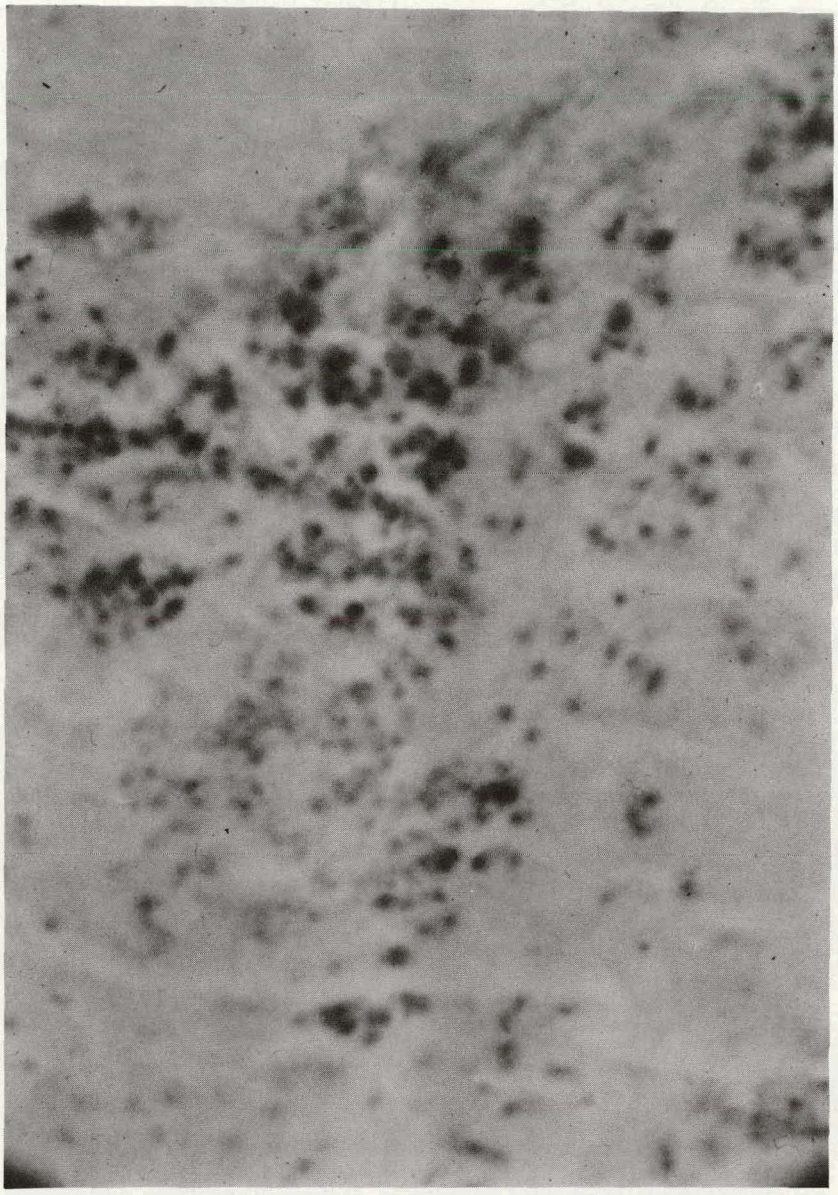

Figure 2. In vivo staining of human brain tumor cells by octoiodofluorescein. Photomicrograph of section of metastatic bronchogenic human brain tumor showing uptake of octoiodofluorescein dye.

The most difficult tumors to detect were those in the midline. In the 64 patients in whom tumor was diagnosed, 42 of 52 (81 per cent) were in one or the other hemisphere, while only 6 of 12 (50 per cent) tumors in the midline region were detected.

With respect to false positive results, out of 35 cases where no tumor was noted either by surgical or clinical confirmatory methods, ten cases, or 29 per cent, were incorrectly reported as having tumor by the scanning technique. However, it is possible that our false positive results may be high since 32 of the non-tumor cases were confirmed by non-surgical methods where the reliability of the clinical diagnosis may not be so definitive.

Optimum scanning time for brain tumors with OIF-I^{131} was found to be from five to 24 hours after injection, although successful scans can be obtained between two to 48 hours after injection. These figures are in good agreement with the fact that the half-time for disappearance of OIF-I^{131} from the blood is 12 hours.

A comparative study was conducted on four patients with each receiving OIF-I^{131} and two of the more widely employed I^{131}-labeled brain tumor localizing agents, radioactive iodinated serum albumin (RISA, Abbott Lab.) and diiodofluorescein-I^{131} (DIF-I^{131}, Abbott Lab.). Utilizing our data as well as the DIF-I^{131} and RISA data of others,[15,3,16,17] it was found that the three agents have comparable accuracy rates for brain tumor localization. However, from the standpoint of "50 per cent reduction time" of vascular radioactivity relative to 100 per cent at zero time, DIF-I^{131} was found to be unfavorable for external scanning purposes with values between 20 to 30 minutes. This necessitates completing a scan within a limited time period and also requires that counting rates be corrected for loss due to rapid elimination of DIF-I^{131}. "Fifty per cent reduction time" values for OIF-I^{131} and RISA were five to seven hours and 20-24 hours, respectively. In comparing "50 per cent retention time" of administered dose between OIF-I^{131} and RISA, it was found that the values for OIF-I^{131} of 20-24 hours resulted in less exposure of patients to I^{131} radiation than did RISA with a longer "50 per cent retention time" of 12-17 days.[18,19]

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A THEORETICAL EVALUATION OF BRAIN SCANNING SYSTEMS*

By

R. N. Beck

The fact that brain lesions and normal brain tissue tend to take up injected radioactive material to different degrees underlies all attempts to locate brain tumors with gamma ray detectors.

Two fundamentally different collimated scintillation detector systems, responding primarily to photons produced in small diameter cylindrical regions through the head, have been successfully developed for this purpose (Figure 1). A systematic scan of the head by these detectors

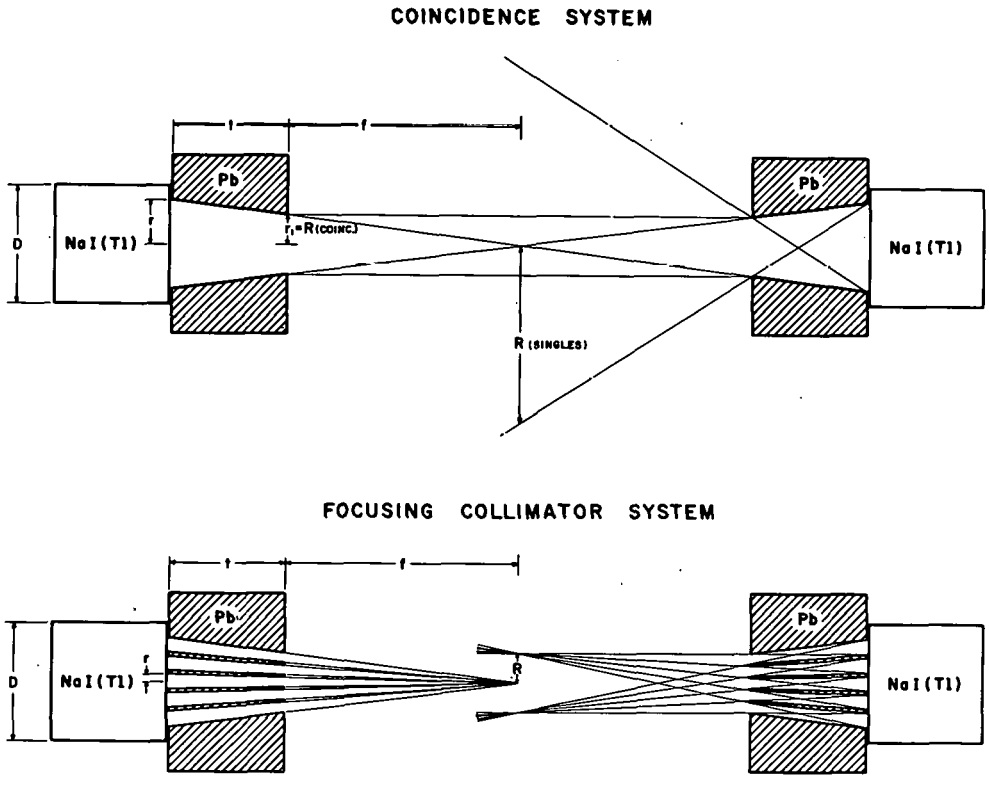

Figure 1. Diagram of coincidence and focusing collimator systems.

produces a two-dimensional mapping of the distribution of radioactivity. The general features of the scanning mechanisms, associated electronic circuitry, display systems, etc., have been amply described in the literature[1] and will not be discussed here. Rather, a comparison is made of the inherent response characteristics of the two systems.

The positron or "coincidence" system responds primarily to simultaneously detected, oppo-

*This paper will appear in J. Nuclear Medicine, 1961.

sitely directed, pairs of .511 MEV photons produced by positron annihilation in the cylindrical region (of radius $R_{(coinc.)}$) between the detectors. In addition, a relatively small spurious response is due to the occasional detection of simultaneous, but unrelated, .511 MEV photons from outside this region.

The focusing collimator or "singles" system responds primarily to single photons which come from the region indicated on the right in Figure 1, the precise shape of which depends on many design parameters. Here we shall deal only with focusing collimators consisting of identical, round, tapered, holes in hexagonal array having a common apex at the focal distance. In such a case, the region of response has a circular cross section of radius R at the focal distance and an hexagonal cross section, somewhat larger, near the collimator. In addition, some gammas from outside this region enter the detector by penetrating the collimator septa.

In order to compare these systems, it has been necessary to develop

1) a criterion based on the statistical nature of count rate information, which depends on both detector sensitivity and the change in count rate over a tumor;
2) a theory of detector response to distributed sources, which, for the focusing collimator system, takes into account gamma ray penetration of the septa.

A very significant consequence of the theory of response for focusing collimators is that it allows the design of optimum collimators (most efficient) for a given gamma energy, crystal diameter, collimator material, focal length, radius of resolution and permitted penetration fraction.

The subject involves a great many independent parameters, in illustration of which, systems are compared for a specific set of conditions (crystal size, collimator material, gamma energies, etc.).

An outline of the theory and its implications in medical isotope scanning are presented here.

Criterion for comparing systems. Using either system, a tumor is detected by observing a more or less abrupt change in count rate over the tumor region. Since count rate information is subject to statistical variations, it is necessary to scan slowly enough that small changes in count rate can be resolved.

If C_o and C_t are the true mean count rates over normal and tumor tissues respectively, then a statistical test of the significance of the difference of observed count rates shows that the time required to detect a tumor is given by

$$\tau = \frac{4Z^2 \left(\frac{C_t}{C_o} + 1\right)}{C_o \left(\frac{C_t}{C_o} - 1\right)^2},$$

where Z is a statistical factor associated with the reliability of tumor detection.

This relation is then used to define the figure of merit of a system, Q, by a function which is inversely proportional to the detection time.

$$\tau = \frac{Z^2}{Q} \quad \text{or} \quad Q = \frac{C_o \left(\frac{C_t}{C_o} - 1\right)^2}{4 \left(\frac{C_t}{C_o} + 1\right)}.$$

Although the figure of merit has been derived from a consideration of detection time, alternative interpretations are possible.

If for the same resolution (R), radiation dosage to the patient, and reliability of tumor detection (Z), the figure of merit Q_A of system A is ten times greater than the figure of merit Q_B of system B then

1) for equal resolution, dosage and reliability, the required scanning time for system A is one-tenth that for system B;

or 2) for equal resolution, dosage and scanning time, system A will be more reliable in detecting tumors than will system B;

or 3) for equal resolution, scanning time, and reliability, the required radiation dosage for system A is one-tenth that for system B;

or 4) for equal dosage, reliability and scanning time, the resolution of system A can be improved over system B.

In order to compute the figure of merit of a system, equations are needed for the normal count rate C_o and contrast ratio, $\frac{C_t}{C_o}$.

Detector response to distributed sources. While a computation of the response of a detector to an arbitrary source distribution is extremely complicated, the response to a uniform sheet distribution can be described very simply.

It has previously been pointed out[2,3] that, as a uniform sheet distribution of radioactive material is moved along the axis of either a focusing collimator system or a coincidence system, the count rate is constant. This is true provided only that the sheet is large enough to cover the entire region viewed by the collimator and that a negligible number of gammas penetrates the collimator and surrounding shielding. This has been adequately demonstrated for a coincidence system.[2] For a focusing collimator system, measurements made in this laboratory with a large sheet distribution of Hg^{197} show that the count rate is constant ± 2 per cent to a distance of 25 cm from the collimator.

The significance of this is twofold. 1) It enables us to find uniform sheet distributions which are equivalent (produce the same count rate) to the cylindrical regions through the head "seen" by the detectors. This can be done for both normal and tumor regions. 2) The response of a detector to a sheet distribution is easily computed for a sheet at zero distance; that is, against the face of the collimator.

For either a focusing collimator or a coincidence system the response to a uniform sheet distribution can be written in the form

$$C = E\,(1 + P)\,\eta\,\sigma,$$

where

$C\ (\frac{\text{counts}}{\text{sec}})$ = count rate

$E(\text{cm}^2)$ = geometrical efficiency of the collimator for a sheet distribution

P = penetration fraction (or the efficiency due to penetration, relative to the geometrical efficiency).

η = over-all crystal efficiency

σ $(\frac{\text{photons}}{\text{cm}^2\text{-sec}})$ = photon density on the sheet (emanating in all directions).

Equations for the detectors. The geometrical efficiency of the collimator for a sheet distribution can be found by integrating over a sheet at zero distance.

$$\text{(F.C.) } E \cong \frac{N \pi R^4}{2\,(8f^2 + R^2)\,(\frac{R}{r} + 2)^2} \quad (\text{cm}^2) = \frac{N \pi r^4}{2\,(2t^2 + r^2)\,(1 + t/f)^2}$$

$$\text{(Coinc.) } E \cong \frac{\pi R^4}{2\,(8f^2 + R^2)} \quad (\text{cm}^2)$$

R = radius of the region of view at the focal distance
f = focal distance
N = number of holes
r = base radius of the holes
t = collimator thickness.

If the source is large enough to cover the entire viewing field of the large hexagonal hole formed by removing all septum material, then the equation for penetration fraction has the form

$$\text{(F.C.) } P = [\frac{E_H}{E} - 1]\, e^{-\lambda t(1 - \frac{b}{b_H})}, \text{ where}$$

E_H = geometrical efficiency of the large hexagonal hole
λ = attenuation coefficient in collimator material
b = total base area of the N small holes
b_H = base area of the large hexagonal hole.

Using for E_H the efficiency of a round tapered hole with equal base area, this equation can be put in a more useful form.

$$\text{(F.C.) } P = \left[\frac{27D^4\,(1 + \frac{R^2}{8f^2})}{64\,\pi^2 r^4 N\,(1 + \frac{3\sqrt{3}\,D^2R^2}{64\,\pi r^2 f^2})} - 1\right] e^{\left[\frac{-2rf\lambda}{R}\,(1 - \frac{8N\pi r^2}{3\sqrt{3}\,D^2})\right]}$$

D = diagonal of the hexagonal hole, which is usually set equal to the crystal diameter
λ = attenuation coefficient in collimator material
(Coinc.) For the coincidence system, it is assumed that the penetration fraction is negligible.

If only "photopeak" pulses are counted, then

(F.C.) η = ip
(Coinc.) $\eta = (ip)^2$
where η = crystal efficiency for the detector system
i = interaction ratio
p = photofraction.

Equations for the source. Assuming that the concentration of isotope is uniform in normal brain tissue, the activity in a cylinder of normal tissue "seen" by the detectors can be reduced to a sheet distribution which produces the same number of counts.

$$\text{(F.C.)} \quad \sigma_o = H'\rho = \left[\frac{1 - e^{-\mu_o H}}{\mu_o}\right]\rho$$

$$\text{(Coinc.)} \quad \sigma_o = H'\rho = [He^{-\mu_o H}]\,\rho$$

$\sigma_o \left(\frac{\text{photons}}{\text{cm}^2\text{-sec}}\right)$ = photon density on the equivalent sheet distribution.

H(cm) = thickness of the head

H'(cm) = equivalent or reduced head thickness when attenuation in the head is taken into account

$\mu_o(\text{cm}^{-1})$ = attenuation coefficient in brain tissue

$\rho \left(\frac{\text{photons produced}}{\text{cm}^3\text{-sec}}\right)$ = photon density in normal tissue

$$\rho \left(\frac{\text{photons produced}}{\text{cm}^3\text{-sec}}\right) = 3.7 \times 10^4 \left(\frac{\text{disint.}}{\text{sec-}\mu\text{c}}\right) \times A \left(\frac{\mu\text{c}}{\text{cm}^3}\right) \times n \left(\frac{\text{photons}}{\text{disint.}}\right).$$

If the concentration of isotope in the tumor is uniform and the tumor is uniformly thick and large enough to cover the cylindrical region of view of the detectors, then the equivalent sheet distribution is given by

$$\text{(F.C.)} \quad \sigma_t = \left[H' + (U - 1)\, e^{-\mu_o d_1} \left(\frac{1 - e^{-\mu_o |d_2 - d_1|}}{\mu_o}\right)\right]\rho$$

$$\text{(Coinc.)} \quad \sigma_t = \left[H' + (U - 1)\, |d_2 - d_1|\, e^{-\mu_o H}\right]\rho$$

$\sigma_t \left(\frac{\text{photons}}{\text{cm}^2\text{-sec}}\right)$ = photon density on the equivalent sheet distribution

U = uptake ratio, or the concentration of isotope in tumor tissue relative to normal tissue

d_1 = depth to the surface of the tumor

$|d_2 - d_1|$ = tumor thickness in the direction of the detector axis.

Since the normal and tumor count rates are proportional to σ_o and σ_t respectively, the intrinsic contrast ratio is given by $\frac{\sigma_t}{\sigma_o}$.

$$\text{(F.C.)} \quad \frac{C_t}{C_o} = \frac{\sigma_t}{\sigma_o} = 1 + (U - 1)\, e^{-\mu_o d_1} \left[\frac{1 - e^{-\mu_o |d_2 - d_1|}}{1 - e^{-\mu_o H}}\right]$$

$$\text{(Coinc.)} \quad \frac{C_t}{C_o} = \frac{\sigma_t}{\sigma_o} = 1 + (U - 1)\, \frac{|d_2 - d_1|}{H}$$

Figure of merit Q. The figure of merit of a system can then be computed for any system and tumor by first computing C_o and $\frac{C_t}{C_o}$.

$$Q = \frac{C_o \left(\frac{C_t}{C_o} - 1\right)^2}{4 \left(\frac{C_t}{C_o} + 1\right)}$$

As an illustration of the theory, a comparison is made between coincidence systems and focusing collimator systems designed for four gamma energies.

For this comparison, we have arbitrarily chosen the following conditions.

1) 2" x 2" NaI(T_1) crystals (D = 2)
2) LEAD, for the collimator material (λ)
3) MIDLINE OF THE HEAD, for the focal distance (f = 4")
4) 0.1, for the permitted penetration fraction (P - .1 is considered negligible)
5) .511 MEV, .364 MEV, .279 MEV, .200 MEV for the focusing collimator systems
6) .25" to 1.25" for the range of diameter of the circle of resolution (2R).

The problem of optimum focusing collimator design then reduces to the problem of finding the hexagonal array (characterized by values of N and r) for which the collimator efficiency (E) is maximum.

A procedure has been carried out on UNIVAC for some 300 combinations of values of D, f, R, P, λ,[4] yielding, for each combination, the values of N and r for which E is maximum.

For the specific conditions listed above, the results of these computations are shown in Figure 2, where optimum collimator efficiency is plotted against resolution for each gamma energy. The geometrical efficiency of a coincidence system with f = 4" is also shown (dashed line).

In order to minimize the effects of scattered radiation, it has been assumed that only "photopeak" pulses are counted. Using interpolated values from published tables[5] of interaction ratio and photo fraction, the crystal efficiency has been computed for each system. Weighting the curves of Figure 2 by crystal efficiency gives detector efficiency, $E\eta$.

The count rate over normal tissue ($C_o = E\eta\ \sigma_o$) may then be determined if, for equal radiation dosages, the concentration of isotope in normal tissue is specified for each system. Since a degree of arbitrariness is involved in computing concentrations of different isotopes which produce equal radiation dosages, this aspect of the problem is not dealt with here. Instead, the comparison is based on the assumption that equal numbers of photons are produced/cm^3-sec (equal ρ 's), for all systems.

It is possible, however, to take the discussion one step further. Making allowance for the reduction in count rate due to attenuation in the head, a factor which is proportional to the count rate over normal tissue ($\frac{C_o}{\rho} = E\eta H'$) is plotted against resolution in Figure 3.

In Figure 4, the intrinsic contrast ratio is plotted against tumor depth for a relatively large tumor, 2.5 cm thick, having an uptake ratio of 2:1. Assuming that the tumor diameter is at least equal to its thickness, the intrinsic contrast ratio is observed at the midline (f) provided that 2R $\leq$ tumor diameter. This ratio is observed at other tumor depths only if the tumor covers the region of view, which may be larger.

For each system, a factor $\frac{C_o}{\rho}$ (for 2R = 2.5 cm) can be selected from Figure 3, and combined

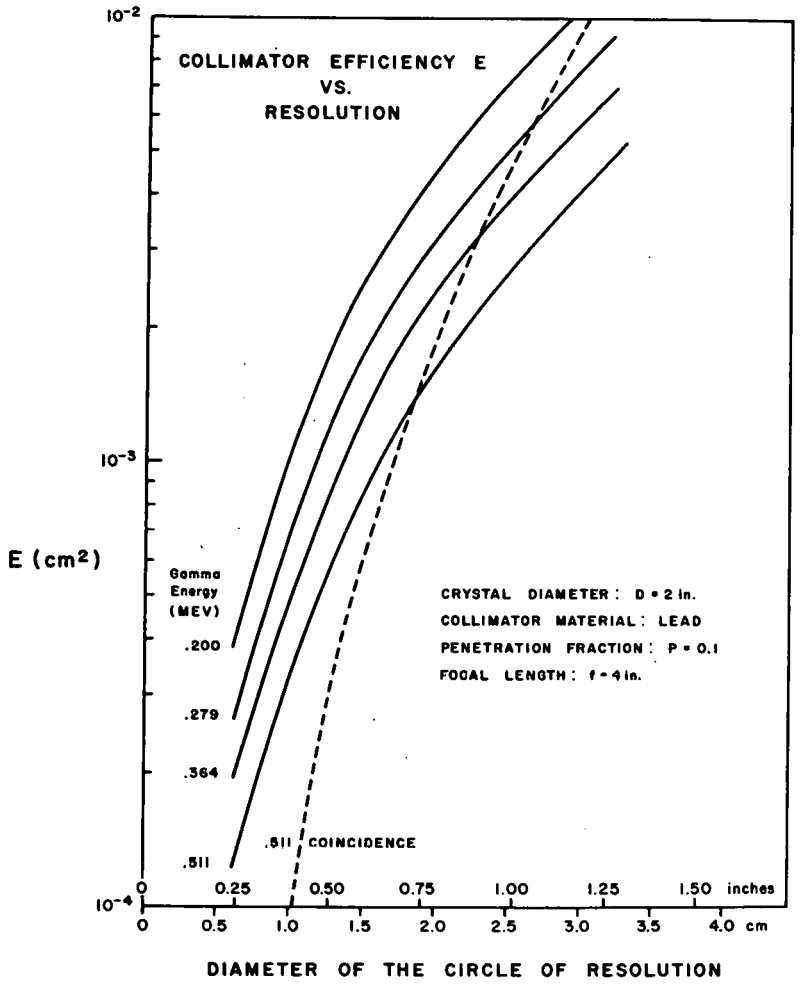

Figure 2. Collimator efficiency vs. resolution.

with corresponding values of $\frac{C_t}{C_o}$ from Figure 4 to determine the normalized figure of merit $\frac{Q}{\rho}$. This is plotted against depth to the center of the tumor in Figure 5. For a midline tumor, the figure of merit of a focusing collimator system designed for .511 MEV, relative to that of a coincidence system with the same resolution, is 2:1. A similar calculation for a 1-cm midline tumor shows this ratio to be 10:1 (when 2R = 1 cm for both systems).

Experimental confirmation of the theory will be presented in a future paper.

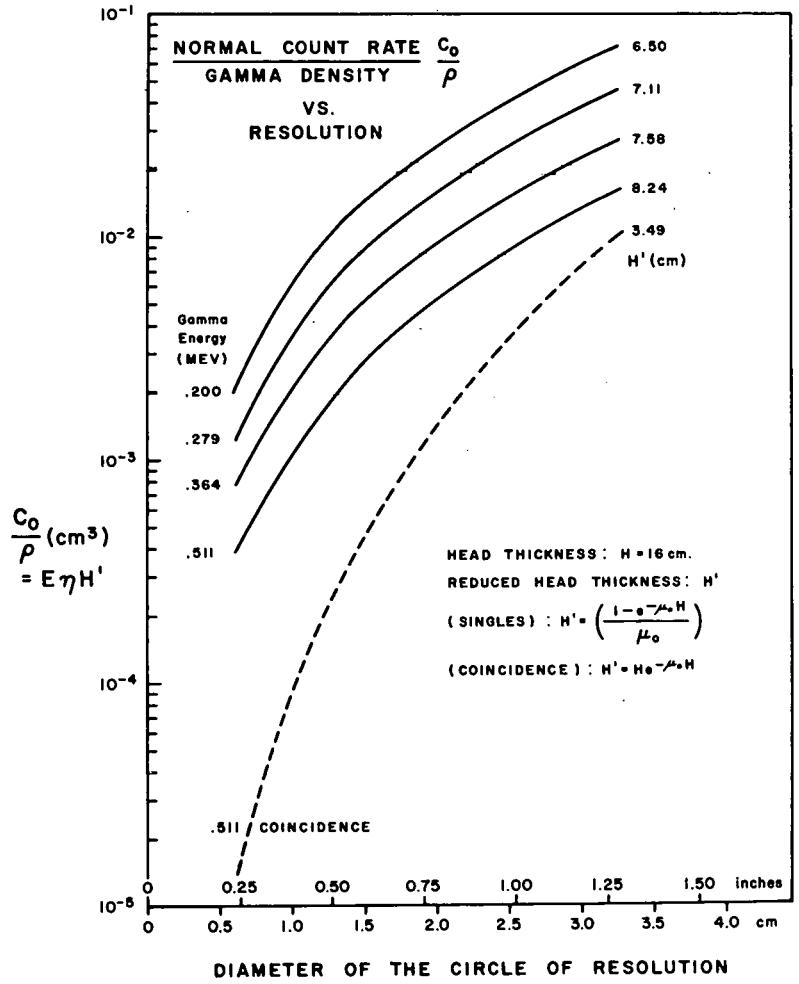

Figure 3. $\frac{\text{Normal count rate}}{\text{Gamma density}}$ $\frac{Co}{\rho}$ vs. resolution.

CONCLUSIONS

Under the conditions listed for the illustration, it appears that focusing collimator systems can be designed for .511 MEV radiation which are superior to coincidence systems by factors of 2 to 10 (depending on resolution) for midline tumors. These factors increase for tumors not on the midline.

Making the further assumption of equal numbers of photons produced/cm^3-sec for all sys-

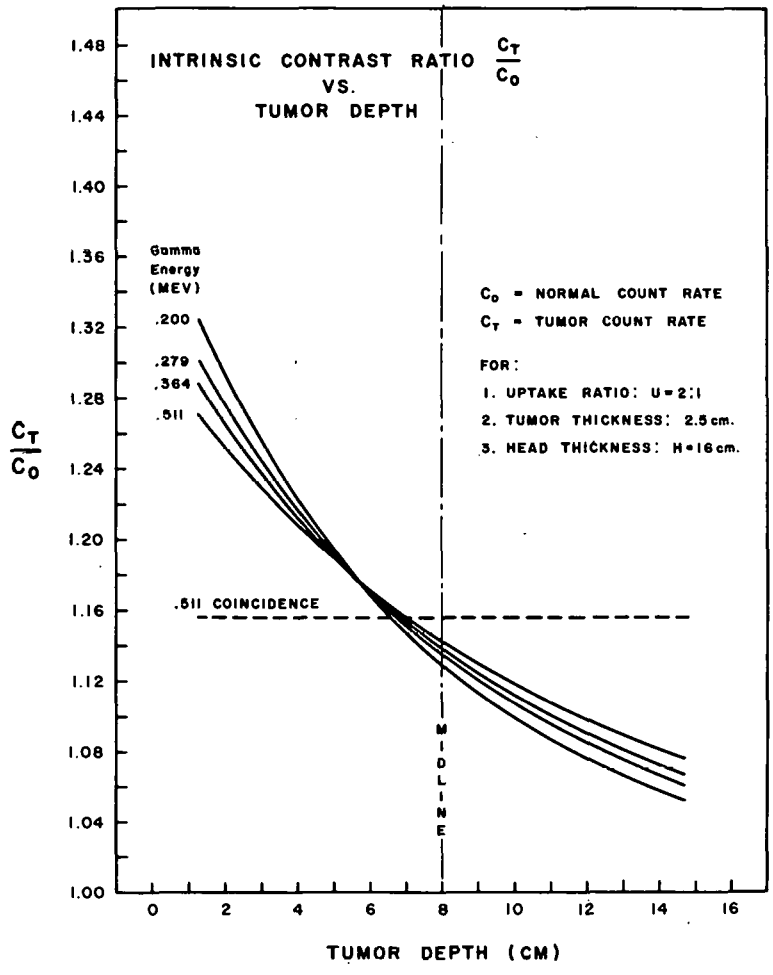

Figure 4. Intrinsic contrast ratio $\frac{C_t}{C_o}$ vs. tumor depth.

tems, the figure of merit for focusing collimator systems increases with decreasing gamma energy down to .200 MEV, the low energy limit of this investigation.* It can be generally concluded therefore that the advantages of low energy gamma radiation for brain scanning have not yet been fully exploited.

ACKNOWLEDGMENT

Among the many people who made this study possible, the author is especially indebted to Mr. Donald B. Charleston and Dr. Paul V. Harper for many suggestions, criticisms and support; to Mr. Paul Eidelberg and Mr. Burton Sandberg for very careful experimental work, and to Mr. John Stupka for enthusiastic cooperation in the construction of focusing collimators.

*The investigation has recently been extended to .088 MEV using extrapolated values of E and η. Under the conditions illustrated in Figure 5, the normalized figure of merit reaches a maximum value of $\frac{Q}{\rho} = 1 + 10^{-4}\text{cm}^3$ at .100 MEV for a midline tumor.

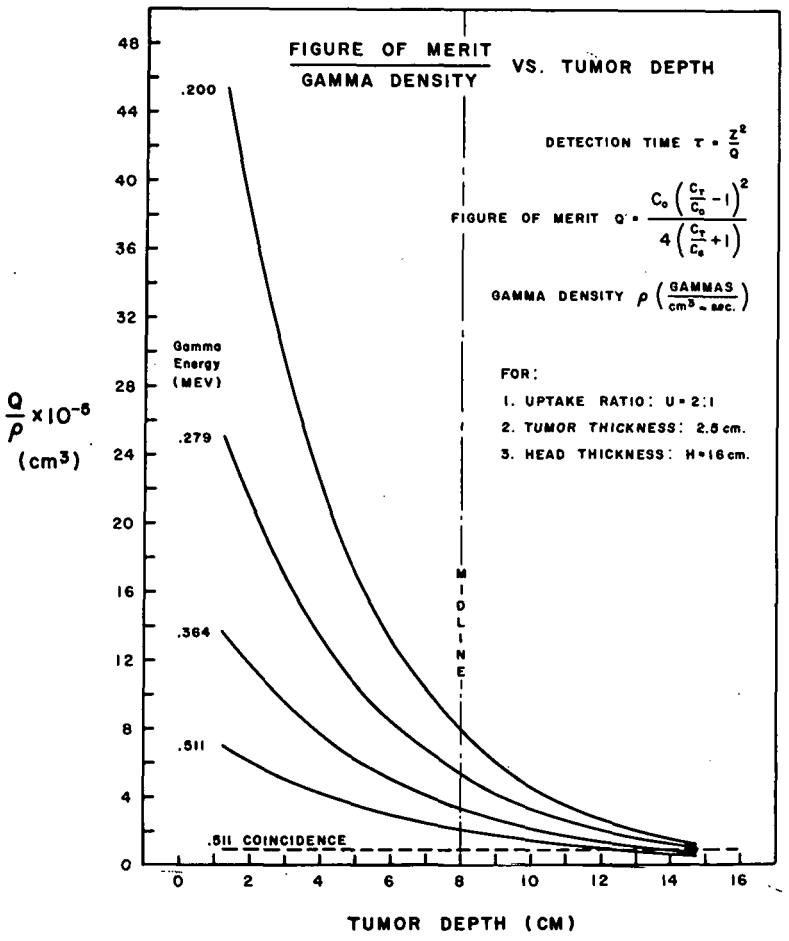

Figure 5. $\frac{\text{Figure of merit}}{\text{Gamma density}}$ vs. tumor depth.

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THE THICK TARGET YIELD AND EXCITATION FUNCTION FOR THE REACTION $Rh^{103}(p,n)Pd^{103}$

By

P. V. Harper and K. Lathrop
and
J. L. Need,* formerly at Oak Ridge National Laboratory,† Oak Ridge, Tennessee

The isotope Pd^{103} has been used with success as a source of interstitial irradiation in the treatment of various advanced tumors.[1] In the early studies the Pd^{103} employed was obtained by neutron bombardment of palladium metal. However, the isotope so produced contains large amounts of inert or carrier-palladium and therefore the specific activity of the end product is low. In an effort to obtain a product of higher specific activity, the ORNL 86-inch cyclotron was used to irradiate rhodium metal. By the $Rh^{103}(p,n)Pd$ reaction, Pd^{103} is produced carrier-free and the precursor isotope is 100 per cent abundant. In addition, neutron activation of impurities such as iridium is avoided.

EXPERIMENTAL PROCEDURES

For the preliminary experiments with the internal beam of the cyclotron, rhodium foils 3-1/2 x 1-5/8 x 0.029 inches were attached to the standard 6 x 5-5/8-inch copper target plate. One foil was soft soldered with a 50-50 lead-tin solder and the other was furnace brazed with silver solder. This foil size was a compromise chosen to minimize the rhodium cost per bombardment while keeping the area large enough to intercept a large fraction of the incident beam. The beam has a width of some 2-3/4 inches but 90 per cent of the total current is contained in the 1-1/2 inch core. The beam strikes the target at a grazing incidence and thus spreads over a length of 4 inches. The maximum angle of incidence of the protons is 7.6° so that the minimum effective thickness of the 2.9-mil rhodium foils is 735 $mg\text{-}cm^{-2}$, which is sufficient to stop an 18.7-Mev proton. The average energy of the beam incident on the target was 16.7 Mev, as determined by simultaneous measurements of the current and power to the target.

The soft-soldered target failed at 900 μa after 10 minutes of bombardment, a hole the size of a half-dollar being melted in the rhodium foil. This target was not processed. The silver-soldered target gave indications of melting at 1100 microamperes (sparking to target and pressure rise) but by adjusting the cyclotron operating conditions it was possible to operate at 1600 microamperes. The total integrated charge was 4444 μa-hr for an average current of 1500 μa in a bombardment of 2.95 hours. A small hole 1/4 x 3/8 inches was melted in the rhodium foil near one corner.

For the external beam measurement, ten numbered foils of rhodium 0.033 inches thick were stacked together in a target cup in the rear of a Faraday Cup assembly 7-1/2 inches long by 3-1/2 inches diameter and bombarded with the 22.4-Mev external proton beam of the cyclotron.

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*Now at the National Aeronautics and Space Administration, Lewis Research Center, Cleveland, Ohio.

†Operated by Union Carbide Corporation for the United States Atomic Energy Commission.

The integrated current was measured with an integrator which produces an output pulse each time a capacitor is charged to the trigger voltage. The integrator was calibrated with an internal current source. The energy of the incident beam was measured by range absorption in aluminum. The range-energy relation for rhodium was constructed by interpolation from the tables of Aron, Hoffman, and Williams.[2] A total of 0.329 μa-hr of protons was collected.

The foil from the internal bombardment was removed from the copper target plate by dripping concentrated nitric acid along its edges. It was then dissolved in $KHSO_4$ at a temperature of 500° C. Aliquots of both the $KHSO_4$ melt dissolved in water and of the HNO_3 solution were counted under conditions of negligible self-absorption (0.2 mg-cm^{-2} rhodium) in a defined geometry. A 2-mm NaI(T1) crystal with a 5-mil Be window was used with a 256-channel analyzer to observe the 20.2-Kev K-X-ray peak of Rh^{103}. A fluorescence yield of 0.80 was assumed. The accompanying 40-Kev X-ray is converted almost completely in the L-shell and does not interfere.

For the external beam target foils, the rhodium outside the window opening of the target cup (shown by radioautographs to have no activity) was trimmed off and the foils dissolved individually in $\sim$ 20 g of hot potassium bisulphate. The melt was dissolved in water and transferred to volumetric flasks after filtering. Any residue was digested further with $KHSO_4$ and added to the rest of the sample. For the total yield, aliquots (1/10) of each sample were combined and made up to volume in a separate flask.

Aliquots (1/10 to 1/25) were transferred to a separatory funnel and diluted to $\sim$ 30 ml with water. Palladium carrier in the amount of 200 μg was added in 0.5 ml concentrated H_2SO_4. Three ml of saturated dimethyl glyoxime in 50 per cent ethanol was then added and, after mixing, KOH was added dropwise until the palladium glyoxime precipitate appeared. This was extracted in two 15-ml portions of chloroform. Repetition of the precipitation and extraction steps yielded 1 per cent additional activity. After evaporation of the chloroform the palladium glyoxime was digested to dryness with a few drops of concentrated HNO_3 and H_2SO_4 and then dissolved in 4.00 ml of 5N HNO_3. Three ml of this was transferred to a 10-ml Lusteroid tube for counting in a NaI well crystal.

The well crystal was calibrated with Pd^{103} sources of known activity as determined by calbration with the thin NaI(T1) crystal. Duplicate samples were prepared by pipetting with the same pipette onto the paper disc for the standardization count, and into 3.00 ml of 5N HNO_3 in a 10-ml Lusteroid tube for the well counter. Using two counts on each of two samples for both counters, the efficiency of the well counter was determined to be 0.300 ± 0.007.

RESULTS

The yield for the internal run corrected to date of bombardment was 430 mc or 96.8 mc/ma-hr. The yield for 22-Mev protons calculated by Martin et al.[3] is 520 mc/ma-hr.

Figure 1 shows the cross section plotted as a function of energy as measured in the external beam experiment. The relative precision can be judged from the scatter in the duplicate points. The absolute values are good to ± 15 per cent considering errors in beam monitoring, chemical yields, and counting normalization. The energy is the proton energy at the center of the foil.

The total yield, determined from four aliquots of the combined sample, was 77.5 μc. This gives 236 mc/ma-hr for 22.4-Mev protons in contrast to a calculated value of 520 mc/ma-hr.

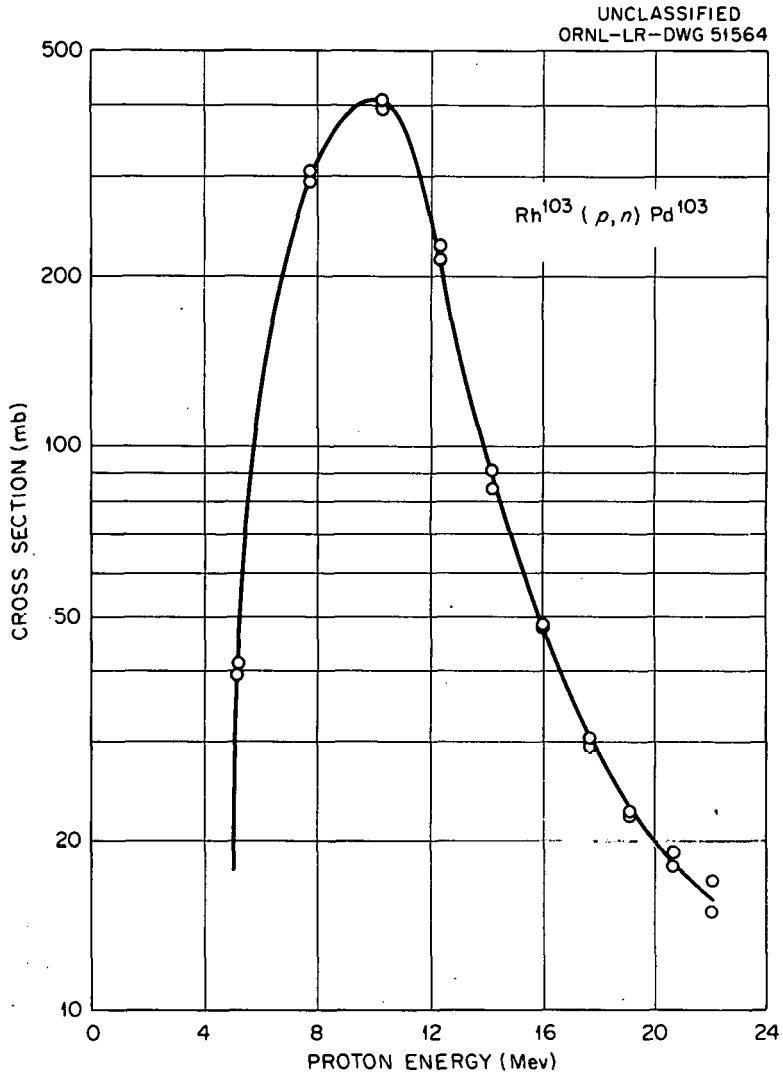

Figure 1. Excitation function for the reaction $Rh^{103}(p,n)Pd^{103}$.

From the excitation function the yield is 219 mc/ma-hr for 16.7-Mev protons (the average energy for the internal bombardment). This value is to be compared with the 96.8 mc/ma-hr obtained from the internal bombardment.

The low yield obtained by bombardment with the internal beam may be accounted for in two ways: 1) Active material may have evaporated when part of the target melted, 2) The change in cyclotron operating conditions noted may have moved the beam core off the rhodium foil for most of the bombardment. It is difficult to explain this discrepancy between the full energy yield and the calculated value; better agreement is usually obtained.

The limit of the current that can be safely put on a target is determined by the power dissipation of the target. Thus, 200 μa at 20 Mev, or 400 μa at 10 Mev could both be handled safely by a given target since the depth distribution of the input power is not altered drastically by the change in energy. Figure 2 shows the relative yield as a function of the energy for conditions of

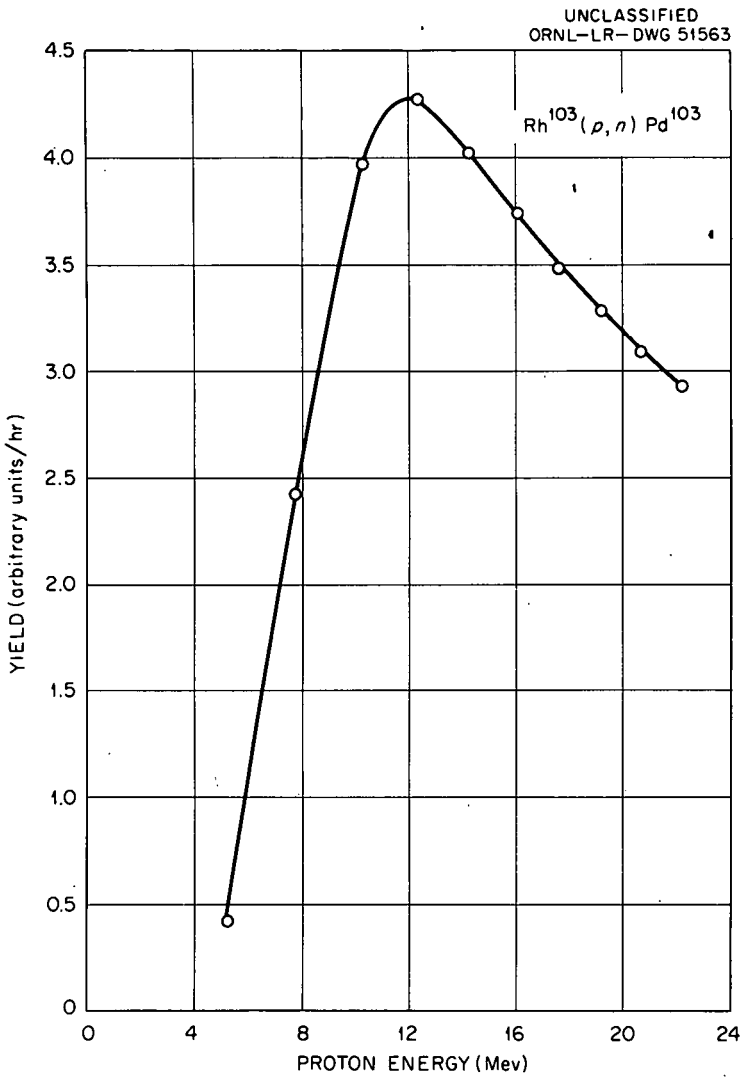

Figure 2. Yield of Pd^{103} in arbitrary units per hour vs beam energy for constant power input to the target.

constant input power. It can be seen that 11.5 Mev is the optimum choice of bombarding energy for this reaction, being a 22 per cent improvement over the yield at 16.7 Mev. Unfortunately, it is unlikely that the modifications of the probe system for the 86-inch cyclotron necessary to permit bombardment at energies below 16.7 Mev will be made; however, the value of 219 mc/ma-hr for the production of Pd^{103} by 16.7-Mev protons is large enough to warrant further work on improved target construction.

ACKNOWLEDGMENTS

We wish to thank J. H. Cupp for the fabrication of the targets and C. L. Viar and F. DiCarlo for the operation of the cyclotron during the bombardments. The encouragement of Dr. R. S. Livingston and J. A. Martin during the investigation is gratefully acknowledged.

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BRAIN CHOLESTEROL: INCORPORATION OF SELECT CARBON-14 AND TRITIUM PERCURSORS INTO BRAIN*

By

J. J. Kabara† and G. T. Okita

Our previous studies [1,2] on the biosynthesis and metabolism of cholesterol revealed that certain precursors were incorporated into the brain cholesterol of adult mice. Although earlier workers reported low or almost undetectable rates of lipid metabolism in the brain of adult animals,[3,4] more recent studies have suggested that the adult mouse brain can synthesize cholesterol in vivo when the route of precursor administration is either intracisternal[5] or intracerebral.[6] On the other hand, only a small amount of incorporation was detected when precursors were administered intraperitoneally. In our initial studies on the in vivo incorporation of precursors into brain sterol we employed the intraperitoneal route of injection, and since our data[7] appeared to differ from those obtained by others, a more detailed investigation was carried out.

MATERIAL AND METHODS

Radioactive precursors. With the exception of DL-mevalonate-2-C^{14} which was generously supplied by Merck, Sharp and Dohme Company, all radioactive precursors were purchased from either Tracerlabs, Inc. or from New England Nuclear Corporation. The metabolites were dissolved in physiological saline to which benzyl alcohol (0.9 per cent) was added as a preservative and the resulting solutions were injected intraperitoneally.

Experimental design. In the first series of experiments, normal female CF. No. 1 mice (9-12 weeks old) and normal female DBA/2 mice (10-12 weeks old) were injected with both a tritium and a carbon-14 labeled precursor of cholesterol. In all cases the tritium-tagged molecule (10 mc/mM) was acetate-2-H^3 (400 μC/kg). The carbon-14 precursor (40 μC/kg) was one of the following: acetate-1-C^{14} (2.8 mC/mM); DL-leucine-3-C^{14} (0.6 mC/mM); D-glucose-U-C^{14} (0.23 mC/mM); and DL-mevalonate-2-C^{14} (5 mC/mM).

Two further series of experiments were carried out using mice which had been starved for 24 hours prior to isotope injection. In the first of these, animals were injected simultaneously with hydrogen-3 and carbon-14 labeled precursors and sacrificed by decapitation and exsanguination 15 minutes later. The organs were removed, washed twice in isotonic saline, and quick-frozen in a mixture of dry-ice and acetone. Samples were kept in the frozen state until analyzed.

In the second of these series, the time course for the in vivo labeling of cholesterol following administration of mevalonate-2-C^{14} was determined. Four groups of 10 mice were injected with both acetate-2-H^3 and mevalonate-2-C^{14} and sacrificed at 10-, 20-, 40- and 80-minute intervals. The tissues were removed and prepared for analysis as before.

Isolation and assay of radioactive cholesterol. The colorimetric determination for cholesterol content and radioassay of isotope incorporation were made on the same tissue sample. The

*Summary of a paper that will appear in the Journal of Neurochemistry.

†Present address: Department of Chemistry, University of Detroit, Detroit, Michigan.

procedure for isolation and assay of the labeled cholesterol was a modification of earlier methods.[9] Briefly, plasma, red blood cell, or tissue cholesterol was extracted with acetone-alcohol (1:1), after which digitonin in 50 per cent alcohol was added to precipitate the free sterol. (The resulting filtrates were not analyzed for esterified cholesterol.) The digitonides thus precipitated were then dissolved in dioxane. One aliquot was used for a quantitative colorimetric determination while the rest of the sample was simultaneously assayed for carbon-14 and hydrogen-3 in a liquid scintillation counter.[10] The resulting data are reported in terms of activity (DPM) per gram wet tissue.

Radiochemical purity. To ascertain radiochemical purity of the cholesterol isolated three groups of five mice each were injected with acetate-1-C^{14} (80 μC/kg) and sacrificed after fifteen minutes. Brain and liver free cholesterol were isolated and counted in the usual manner while the rest of the sample was repurified via the dibromide derivative. The initial specific activity of brain cholesterol before dibromide repurification was 1285 ± 42 DPM/mg and for liver cholesterol 12,918 ± 276 DPM/mg. After bromination and dibromination the specific activities were 1092 ± 95 and 11,686 ± 100 DPM/mg, respectively. Similar experiments were carried out using glucose-U-C^{14} (40 μC/kg). The initial specific activity of brain cholesterol was 940 ± 53 DPM/mg and liver cholesterol 3309 ± 120 DPM/mg. After purification the specific activities were 1106 ± 68 DPM/mg and 3103 ± 180 DPM/mg.

The differences noted for both acetate-1-C^{14} and glucose-U-C^{14} were well within the experimental deviation.

RESULTS

Labeling with selected precursors. The first series of experiments provided a comparison of the efficiency of the different precursor incorporation into cholesterol. Normal adult mice were sacrificed fifteen minutes after intraperitoneal injection of labeled precursors since this was at or before the time of maximum incorporation for lipid labeling for these substances. Individual values for acetate-1-C^{14}, acetate-2-H^3, DL-leucine-2-C^{14}, and DL-glucose-U-C^{14} are given in Tables 1 and 2 and represent the weighted means of five mice.

Both normal CF No. 1 mice and normal DBA/2 animals incorporate the same amount of acetate-1-C^{14} into brain cholesterol. The incorporation of radioactivity after acetate-2-H^3 administration was one-third that of acetate-1-C^{14}.

The use of DL-leucine-2-C^{14} was prompted by increasing interest in the use of this amino acid in the biosynthesis of sterols. The conversion of leucine into the cholesterol nucleus may proceed via one of two pathways.[11] Evidence from our respiratory carbon-dioxide studies[12] as well as work of other investigators seems to indicate a breakdown of the amino acid labeled in the carbon-2 position into acetate-1-C^{14} and its subsequent reutilization as a two-carbon fragment. Using this proposed metabolic scheme as a basis for comparison, the amount of radioactivity that appears in brain cholesterol from leucine-2-C^{14} represents only about 20 per cent of the expected radioactivity derived as an acetate-1-C^{14} fragment. An even greater difference was found in the DBA/2 animals.

Because of its importance as an energy source for nerve tissue, glucose was also investigated as a possible precursor for brain cholesterol. Its incorporation into brain cholesterol was found to be greater than that of mevalonate, acetate or leucine. This is in contrast to the more efficient incorporation of mevalonate into cholesterol of other tissues. The incorporation of

Table 1

INCORPORATION OF VARIOUS PRECURSORS
INTO BRAIN FREE CHOLESTEROL

Precursor 40 μC/kg	CF No. 1 Female Mice		
	Experiment 1	Experiment 2	Experiment 3
Acetate-1-C^{14}	198*	770	1319
Acetate -2-H^3	124	310	453
DL-leucine-2-C^{14}	217	453	596
Acetate-2-H^3	120	710	293
D-glucose-U-C^{14}	2591	2797	3367
Acetate-2-H^3	354	288	503

*Each number represents weighted mean of five animals, DPM/g tissue (wet).

Table 2

INCORPORATION OF VARIOUS PRECURSORS
INTO BRAIN FREE CHOLESTEROL

Precursor 40 μC/kg	DBA/2 Female Mice		
	Experiment 1	Experiment 2	Experiment 3
Acetate-1-C^{14}	644*	685	828
Acetate-2-H^3	371	341	426
DL-leucine-2-C^{14}	69	84	223
Acetate-2-H^3	247	582	-
D-glucose-U-C^{14}	3750	5020	5218
Acetate-2-H^3	528	1136	1238

*Each number represents weighted mean of five animals, DPM/g tissue (wet).

glucose-U-C^{14} into brain cholesterol was four to ten times greater than that of acetate.

In vivo time course of cholesterol labeling with mevalonate-2-C^{14}. A proper interpretation of our incorporation data of mevalonate-2-C^{14} as a precursor for brain cholesterol necessitated a knowledge of the time of maximum incorporation after precursor injection. While mevalonic acid has been carefully studied in a variety of experimental situations[13] the authors were not aware of any data on the time course of in vivo labeling of cholesterol by mevalonate in mice. In consequence, the time-course of incorporation of mevalonate-2-C^{14} into free cholesterol of various tissues was determined.

Compared with acetate the peak time of liver sterol labeling from mevalonate was slower but more complete (Figure 1). The activity from mevalonate-2-C^{14} reaches its highest values

between 40-80 minutes while maximum labeling of tissue sterol from acetate-2-H^3 (Figure 2) occurs approximately at 20 minutes. The amount of radioactivity of liver cholesterol derived from mevalonate however, was greater by a factor of 25 to 30 when compared to acetate labeling. (For comparative purposes, radioactivity values in Figures 1 and 2 were adjusted on basis of equal H^3 and C^{14} radioactivity administered.) In contrast to the high incorporation found with radioactive mevalonate in non-brain tissues, labeling with acetate was more efficient for brain cholesterol.

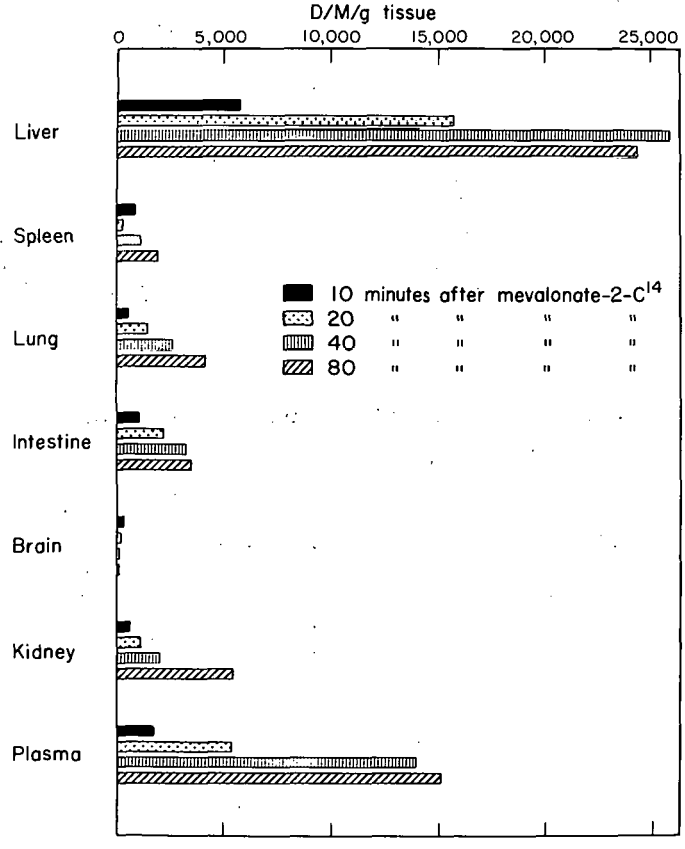

Figure 1. Time-course of incorporation of mevalonate-2-C^{14} into free cholesterol of various tissues after I.P. injection. For comparative purposes radioactivity values in Figures 1 and 2 were adjusted on basis of equal H^3 and C^{14} radioactivity administered.

The difference in lipid labeling between acetate and mevalonate seems to indicate that an unusual metabolic situation exists in the brain. In this tissue the greatest degree of labeling with either precursor was observed in the animals killed 10 minutes after injection. The "apparent turnover" of brain free-cholesterol appears to be so rapid that only trace activity could be detected at the 80 minute interval. Although the biological half-time of this active brain free-cholesterol component was extremely fast, it should be emphasized that this component probably represents a small compartment and does not necessarily reflect the turnover of total brain cholesterol.

Whatever the ultimate mechanism of cholesterol biosynthesis in the central nervous system, the labeled sterol found in the brain was the result of local tissue synthesis and not due to transported radioactive cholesterol to this region. This statement is based on evidence that the values for plasma free-cholesterol were rising at a time when the specific activity of brain cholesterol was decreasing.

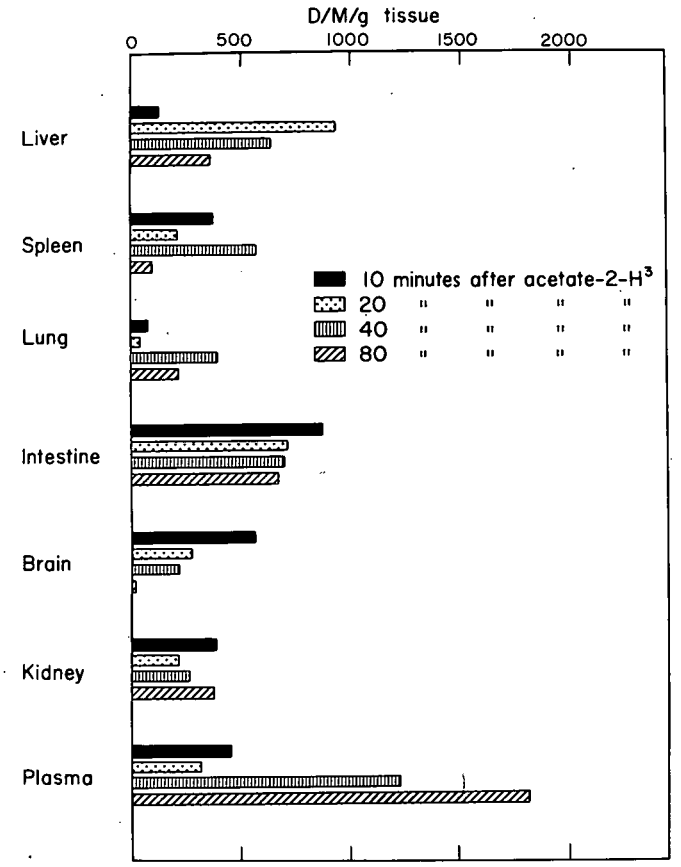

Figure 2. Time-course of incorporation of acetate-2-H^3 into free cholesterol of various tissues after I.P. injection. For comparative purposes radioactivity values in Figures 1 and 2 were adjusted on basis of equal H^3 and C^{14} radioactivity administered.

DISCUSSION

The increase in the cholesterol content of the human brain from 2 g in infancy to 258 g in adulthood[14] may be a result of utilization of plasma cholesterol and/or local synthesis. Early experiments on brain lipids, however, indicated that the cholesterol of the adult brain is relatively inert metabolically, there being apparently no exchange between brain cholesterol and plasma lipoprotein.[15] Moreover, Waelsch et al.[16] found no activity in the unsaponifiable fraction of brain lipids in adult rats given deuterium oxide (D_2O). In agreement with these in vivo findings, in vitro techniques showed that carbon-14 acetate can be readily converted into brain

sterol in very young animals while surviving brain tissue slices of adult rats and rabbits cannot accomplish this conversion.[17,18] The lipids of adult human brain tissue have been described as relatively inert in terms of in vitro acetate-1-C^{14} incorporation studies.[19]

During the past few years an active metabolism for brain cholesterol has been demonstrated where special experimental techniques are utilized. E. Grossi et al.[19] using several different radioactive metabolites showed activity in brain slices for both young (10-day) and old (60-day) rats. In vivo data on brain lipid synthesis in intact mature animals (6 months) have also been provided by Moser and Karnovsky.[20] Additional evidence for biogenesis of brain cholesterol was presented through data obtained after intracisternal[5] and intracerebral injections.[6] Nevertheless there is a general feeling that intraperitoneal injection of a precursor leads to little if any brain lipid labeling[6] and that this resultant negligible incorporation reflects a lack of metabolite penetration into the brain rather than an inherent decrease in the synthetic capacity of the organ.

Our data indicate an active rather than static metabolism for adult brain free-cholesterol, a possible explanation for which may be due to 1) our experimental techniques for isolating tissue cholesterol; 2) the fact that our animals were sacrificed so soon after labeled precursor injection; and 3) most important, our analysis of the data. The high concentration of cholesterol in brain tissue renders expression of experimental results in terms of specific activity (disintegration per minute per mg) misleading. The initial radioactive cholesterol which forms in the brain shortly after isotope administration is diluted by a factor of from 5 to 8 times greater than in other non-neural tissue. Moser and Karnovsky[20] also noted that whereas the specific activity of brain lipids was low, the total activity incorporated into mature animals was significant.

In our experiments glucose proved to be a more efficient precursor than acetate, leucine or even mevalonic acid. It should be emphasized that while mevalonate is a more immediate precursor to cholesterol than the other metabolites studied it was less efficient in the in vivo labeling of brain sterol than acetate. This apparent discrepancy may be due to difficulty in penetrating the blood-brain barrier or to the failure of cell penetration, since Garattini[21] found that the efficiency of labeling of acetate-1-C^{14} more nearly resembled that of mevalonate-2-C^{14} when brain homogenates were used. From these and our own data, it is evident that the efficiency of labeling cholesterol depends partly on the precursor used, partly on the tissue under study, and partly on the experimental approach employed, i.e., in vivo or in vitro methods, and (in the former method) in the length of time between administration of the precursor and sacrifice of the animal.

Labeled cholesterol has been shown to persist in the mammalian central nervous system for over a year by Davison[22,23] and others.[5,6] The rapid half-life obtained for brain free-cholesterol in our time-course experiments with acetate-2-H^3 and mevalonate-2-C^{14} acid may reflect the turnover of an active, and probably a small, free-cholesterol compartment in the brain. While the biological half-time for total brain cholesterol may be much longer, it could not be measured after the dose of isotope employed in our experiments due to the rapid loss of labeled cholesterol from the active compartment. The available data seem to suggest that there is a series of sterol compartments in tissues each having its individual turnover rate, and that the present experiments only emphasize the controversial nature of studies on adult brain cholesterol metabolism. Obviously more data will be necessary before a clearer biochemical picture emerges.

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NET SYNTHESIS OF RNA WITH A MICROBIAL ENZYME REQUIRING DNA AND FOUR RIBONUCLEOSIDE TRIPHOSPHATES*

By

S. B. Weiss and T. Nakamoto

Recent reports from this laboratory have described an enzyme system from rat liver which catalyzes the incorporation of ribonucleotides into the framework of RNA.[1,2] This incorporation was shown to require the participation of all four ribonucleoside triphosphates. Inactivation of the enzyme system with small quantities of DNase pointed to a possible dependence of the reaction on intact DNA. Indeed, partial reactivation of the DNase-treated mammalian preparations could be achieved by the addition of heated or unheated purified rat liver DNA (unpublished results). This communication describes some of the properties of a similar partially purified enzyme from Micrococcus lysodeikticus which carries out the net incorporation of ribonucleotides into a polyribonucleotide fraction.

A 2 per cent suspension of M. lysodeikticus, in 0.02 M phosphate buffer of pH 7.5, was incubated with crystalline lysozyme for 45 minutes at 30°. The viscous, lysed preparation incorporated labeled nucleotides into an acid-insoluble fraction. This incorporation was stimulated fourfold when all four ribonucleoside triphosphates were also present in the incubation medium. Exposure of the lysed bacterial preparation to sonic treatment or to the action of DNase, resulted in a disappearance of this stimulatory effect, as well as in a marked decrease in the viscosity of the extract.

While this work was in progress, Stevens[3] and Hurwitz et al.[4] presented evidence that extracts from E. coli B catalyzed the incorporation of labeled ribonucleotides into RNA. This incorporation also required the presence of all four ribonucleoside triphosphates. Hurwitz and co-workers further showed that their preparations were dependent on DNA for optimal activity.

The partial purification of the M. lysodeikticus enzyme was effected by exposing the viscous lysate to sonic oscillation in a Raytheon, 10-Kc sonic oscillator. The enzyme was precipitated by the addition of protamine sulfate and the precipitate was extracted with 0.05 M phosphate, pH 7.5. This first extract was discarded. A second extraction was made with 0.20 M phosphate of the same pH, and this solution contained most of the enzyme. The pH of this extract was lowered to 5.4 and the precipitate which formed was collected and suspended in 0.02 M phosphate, pH 7.5. The enzyme was adsorbed onto calcium phosphate gel and removed again by washing the gel with 0.20 M phosphate of the same pH. Extracts so prepared had a ratio of absorbancy at 280 mμ to 260 mμ of about 1.4, and were used as the source of enzyme in the work reported here.

The partially purified bacterial preparation catalyzes the incorporation of radioisotope from CTP^{32}, labeled in the ester phosphate only, into a fraction containing RNA (Table 1). This incorporation shows nearly an absolute requirement for the presence of ATP, GTP, and UTP as well as for DNA. The reaction is specific for the ribonucleoside triphosphates. Substitution by the corresponding diphosphates or the deoxynucleoside triphosphates results in a marked reduction of

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Table 1

COFACTOR REQUIREMENTS FOR P^{32}-CYTIDYLATE INCORPORATION

Nucleotides added	Labeled CMP incorporated into RNA (mμmoles)
Complete	1.28
Omit ATP	0.02
Omit GTP	0.03
Omit UTP	0.02
Omit DNA	0.03
Complete: RNA in place of DNA	0.03
Complete: ADP, UDP, GDP in place of ATP, UTP, GTP	0.02
Complete: dATP, dGTP, dTTP in place of ATP, UTP, GTP	0.01

The complete system contained 3 μmoles of $MnCl_2$, 100 μmoles phosphate buffer of pH 7.5, 0.10 μmoles of CTP^{32} (5.3×10^6 cpm per μmole), 1 μmole each of ATP, GTP and UTP, 200 μg of calf thymus DNA and 0.12 mg of enzyme. The final volume of the system was 1.0 ml. 200 μg of rat liver RNA and 1 μmole each of the deoxyribonucleoside triphosphates and ribonucleoside diphosphates were used as described above. The reaction was stopped after 20 minutes at 37° by the addition of 0.30 ml of 3 N $HClO_4$. 2 mg of carrier yeast RNA was added to each vessel. After 3 minutes at 0°, 2 ml of H_2O was added and the precipitate was collected by centrifugation. The acid-insoluble residue was washed three times by solution in 0.10 N NaOH and reprecipitation with 0.5 N $HClO_4$. The final residue was suspended in 3 ml of 0.02 N NaOH and 1 ml was dried for counting.

isotope incorporated. Table 1 also illustrates that equivalent amounts of RNA cannot replace the DNA requirement. The system is completely dependent upon the addition of certain divalent metal ions: manganese and cobalt can activate the enzyme; magnesium is approximately one-third as effective. The product of the bacterial enzyme reaction resembles RNA in that it is (1) acid-insoluble, (2) non-dialyzable, (3) sensitive to RNase but not DNase, and (4) hydrolyzed by alkali. Alkaline hydrolysis yields 2'-and 3'-nucleoside monophosphates, of which all four mononucleotides are labeled.

In addition, the M. lysodeikticus enzyme, incubated with the four ribonucleoside triphosphates and DNA, catalyzes the net incorporation of ribonucleotides into the acid-insoluble and alkali-sensitive material. During the course of incubation with the complete system, the amount of alkali-sensitive material (Table 2) located in the acid-insoluble fraction is approximately doubled. Omission of one nucleotide (UTP) or DNA prevents a net increase. In another experiment, a similar inhibition was obtained when GTP alone was omitted. Exposure of DNA to sonic oscillation, prior to its addition to the reaction mixture, reduces by nearly 50 per cent the amount of ribonucleotide incorporated. This evidence strongly suggests that net RNA synthesis was achieved.

Table 2

THE NET INCORPORATION OF RIBONUCLEOTIDES INTO A RNA FRACTION CATALYZED BY M. LYSODEIKTICUS ENZYME

Additions	Total counts incorporated	Total optical* density units	Δ optical density units	Total† P_i (μ moles)	Δ P_i (μ moles)
Complete (zero time)	0	4.67	0.00	0.53	0.00
Omit UTP	1,800	4.98	+0.31	0.62	+0.09
Omit DNA	510	4.25	-0.42	0.64	+0.11
Complete: DNA sonicated	19,300	8.36	+3.69	0.94	+0.41
Complete	34,400	11.50	+6.83	1.26	+0.73

The complete system contained 60 μ moles of $MnCl_2$, 2 mmoles of phosphate buffer of pH 7.5, 20 μ moles of CTP^{32} (2.26×10^5 cpm per μ mole), 20 μ moles each of ATP, UTP and GTP, 4 mg of calf thymus DNA and 9.8 mg of M. lysodeikticus enzyme in a final volume of 20 ml. UTP and DNA were omitted where indicated. Both of these components were added back to the appropriate vessels after the reaction was stopped. DNA was exposed to 10 minutes of sonication in a Raytheon 10-Kc sonic oscillator. 4 mg of this material replaced the untreated DNA as indicated. After 25 minutes at 37° the reaction was stopped with 4 ml of cold 3 N $HClO_4$ and placed in ice. The residue was collected and washed four times in a manner similar to that described in Table 1. The final washed residue was suspended in 2 ml of 1 N KOH and incubated at 37° for 20 hours. Each vessel was neutralized with 70% $HClO_4$ at 0°, and sufficient excess was added to lower the pH below 1. The precipitate was centrifuged and the supernatant fluid was saved. The precipitate was washed twice with 0.50 ml of 0.10 N $HClO_4$. The combined supernatant and wash fluids were analyzed for radioactivity, optical density, and total phosphate.

*Total optical density measurements were made at $\lambda 260$ in a Zeiss spectrophotometer.

†Total phosphate was determined by the method of Fiske and SubbaRow.[5]

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A NEW SYNTHESIS OF (P^{32}) URIDINE 5'-PHOSPHATE*

By

D. B. Straus† and E. Goldwasser

Smith, Mofatt, and Khorana[1] have reported the quantitative esterification of phosphoric acid with methanol and with benzyl alcohol by reaction with DCC using the esterifying alcohol as solvent. We have adapted this reaction for the synthesis of (P^{32})UMP.

A solution containing 21.8 μmoles $H_3P^{32}O_4$,‡ 43 μmoles tri-n-butylamine, and 6.2 mmoles 2',3'-O-isopropylidene uridine[2] in 3.3 ml p-dioxane was obtained by heating the mixture at 100° for 2 minutes. After cooling, 125 μmoles DCC were added and the homogeneous solution kept 100 h at 55°. Neutral triester in the reaction mixture was then hydrolyzed with LiOH by the method of Moffatt and Khorana[3] for tri-p-nitrophenyl phosphate. Dowex-50W H^+ was added until the pH was 6.6, and the mixture filtered. The residue was washed with small volumes of water until radioactivity was low, filtrate and washes were extracted with ether to remove p-dioxane and tri-n-butylamine. After removal of ether, the product mixture was treated with 300 μmoles magnesium (acetate)$_2$, 500 μmoles Tris-acetate buffer (pH 9.2) and 8 mg lyophilized Crotalus adamanteus venom** and incubated 10 h at 37° (refs. 4 and 5). The isopropylidene group was then removed by heating the solution 60 minutes at 100° after addition of 3.0 ml glacial acetic acid and 0.1 ml 12 M HCl. The added acids were removed by repeated concentration-dilution cycles in a flash evaporator and finally the mixture was dried to a syrup which was dissolved in 60 ml water, neutralized with NH_4OH, and passed through a 0.9 x 16.5 cm column of Dowex-1 Cl^-. Water and then 0.002 M HCl were used to wash the column until all uridine was eluted. Radioactive orthophosphate was eluted by approximately 50 resin-bed volumes 0.003 M HCl with only a small loss of (P^{32})UMP most of which was then eluted with 0.004 M HCl. In some experiments, another u.v.-absorbing and radioactive peak was eluted by 0.004 M HCl after (P^{32})UMP. The specific radioactivity of this material, assuming an A_M of $9.9 \cdot 10^3$, was about one-half that of the (P^{32})UMP and, though full characterization has not been attempted, is thought to be diuridine 5'-phosphate. In an experiment where this substance was found, the yields, based on radioactivity, were: orthophosphate, 40 per cent; (P^{32})UMP, 34 per cent; presumed diester, 27 per cent. The (P^{32})UMP was characterized by its u.v.-absorption spectrum,[6] by co-chromatography with authentic UMP in two solvent systems, and by enzymic conversion to uridine diphosphate and triphosphate, both labeled in the ester phosphate, with a nucleotide kinase mixture isolated from a brewer's-yeast autolysate[7] with subsequent ion-exchange and paper-chromatographic characterization of the uridine polyphosphates; the latter had the same specific radioactivity as the (P^{32})UMP (130 mC/mmole, after character-

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†Present address: Department of Chemistry, Princeton University, Princeton, N. J.

‡A p-dioxane solution (120 mg/ml) of 99% H_3PO_4 (City Chemical Co., New York, N. Y.) plus 5 mC $H_3P^{32}O_4$ (100C/gP) obtained from Oak Ridge National Laboratory.

**Ross Allen Reptile Farm, Silver Springs, Florida.

Abbreviations: DCC, N,N'-dicyclohexylcarbodiimide; UMP, uridine monophosphate; GMP, guanosine monophosphate; Tris, tris (hydroxymethyl) aminomethane.

ization). In the paper chromatograms, radioactivity was always associated exclusively with the single, appropriate, nucleotide standard.

The yield of (P^{32})UMP is markedly lowered by reducing the ratio of nucleoside to $H_3P^{32}O_4$, and slightly lowered by using less DCC, and by using 85 per cent $H_3P^{32}O_4$. There is no requirement for absolutely anhydrous conditions. Yield is not affected by omitting tri-n-butylamine thus confirming the findings and supporting the proposed reaction mechanism of Smith, Moffatt and Khorana.[1] When the alkaline hydrolysis step is omitted, approximately 5 per cent of the total radioactivity is eluted with uridine; this is probably due to the neutral triuridine 5'-phosphate.

The use of a protecting group has three advantages: a) the protected nucleoside is more soluble in p-dioxane thus allowing a higher ratio of alcohol to $H_3P^{32}O_4$; b) the formation of nucleoside cyclic phosphates[1] and nucleoside 2'- or 3'-phosphates is prevented, and c) crude snake venom can be used since venom 5'-nucleotidase does not attack 3'-substituted nucleotides.[4]

Elmore and Todd[8] have reported that adenosine-5'-uridine-5'-phosphate, analogous to the diuridine 5'-phosphate expected in this esterification reaction, is hydrolyzed to adenosine and uridine by Russell's viper venom. It is probable that a similar hydrolysis occurs, since the presumed diester was absent in several experiments. The lack of complete hydrolysis in some experiments is not understood at present.

In addition to 2',3'-O-isopropylidene uridine, p-nitrophenol, and the 2',3'-O-isopropylidene derivatives of adenosine and guanosine have been used in this synthesis. Esterification of H_3PO_4 was 57 per cent, 40 per cent, and 10 per cent respectively, under conditions similar to those described. The low yield of GMP was probably due to the relative insolubility of the protected nucleoside in p-dioxane. In view of these results, this esterification reaction appears to be one of general application for the synthesis of phosphate esters provided that a suitable solvent and protecting group(s) are found for each alcohol. The method is particularly good for the synthesis of P^{32}-labeled esters since chemical yields are high and the product ester has the same specific radioactivity as the $H_3P^{32}O_4$ used.

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METABOLISM OF 17 α -HYDROXYPROGESTERONE-4-C^{14}-17 α -CAPROATE BY HOMOGENATES OF RAT LIVER AND HUMAN PLACENTA*

By

M. Wiener, C. I. Lupu† and E. J. Plotz‡

Esterification with caproic acid of the hydroxyl group at carbon 17 of 17 α -hydroxyprogesterone changes this weakly progestational compound into one which has greater and more prolonged activity than has progesterone itself (Junkmann[1]). The metabolism of 17α -hydroxyproterone-17 α -caproate has been the subject of investigations in this laboratory. Partition of urinary extracts from pregnant patients given C^{14}-labeled HPC into neutral, acidic, and phenolic fractions has consistently shown the major portion of the radioactivity to be present in the acidic and phenolic fractions, with only minor amounts of C^{14} being found in the neutral fraction (Lupu et al.,[2] Davis et al.[3]). Langecker and Prescher[4] and Langecker[5] have found no increase in urinary pregnanediol or pregnanetriol in humans after the administration of HPC. While these experiments indicate that HPC does not follow the ordinary metabolic pathways of progesterone or 17 α -hydroxyprogesterone, they have thus far failed to demonstrate what metabolism this compound does undergo.

The following in vitro studies were undertaken in the hope that it would be possible to isolate specific transformation products of HPC and that these would suggest what happens to the compound in vivo.

METHODS

Radioassay. The course of extraction and fractionation of the incubation mixtures was followed by the performance of crude radioassays in a windowless gas flow counter. Final exact measurements were done in a Tri-Carb liquid scintillation counter (Packard Instrument Company, Model #314), using as counting medium, toluene containing 2 mg per ml of 2,5-diphenyloxazole.

Steroid purification. 60 μ c of HPC-4-C^{14}** prepared according to the method of Schulze[6] was added to 1.5 g of non-radioactive HPC, and the steroid recrystallized from methanol-water until a constant specific activity was found. Five 280 μ g aliquots of the recrystallized steroid were taken from a standard solution. Three of these aliquots were radioassayed by liquid scintillation counting in order to determine the specific activity. The two remaining aliquots were chromatographed for four hours on Whatman #1 paper in a ligroin-propylene glycol paper system. The steroids were located on paper by scanning with ultraviolet light, and the U.V. absorbing areas were eluted with methanol. The total steroid present in the eluates was determined by; a) measurement of the ultraviolet absorption at 240 m μ and b) assay of the total radioactivity present. The recoveries of the steroid from paper calculated from the two sets of data proved to

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† Present address: University of Virginia School of Medicine, Charlottesville, Virginia.

‡ Department of Obstetrics and Gynecology, University of Chicago.

** This steroid was donated by Dr. Horst Witzel of Schering AG, Berlin.

be identical (90.15 per cent and 89.16 per cent respectively). The steroid was assumed to be radiochemically pure, and was further diluted with nonradioactive HPC to give a final specific activity of 11,255 DPM/mg. (DPM = disintegrations per minute)

Tissue preparation. Adult male and female rats were stunned by a blow on the head, and their throats were cut. The livers were removed immediately and kept on ice until used (within 30 minutes). The tissue was homogenized in a Waring Blendor with 1 ml/g of tissue of a solution of 0.01 M magnesium chloride; 0.1 M fumaric acid, adjusted to pH 7.4; 0.1 per cent glucose (W/V); and 0.03 M sodium phosphate buffer, pH 7.4. The homogenate was strained through gauze.

Placental tissue was obtained from a healthy patient who delivered normally at term. The membranes were dissected away from the placenta, the blood washed off with normal saline, and the tissue ground in a meat grinder. The brei was weighed, and homogenized in a Waring Blendor with 1 ml/3 g tissue of a solution of 0.25 M sucrose, 0.04 M nicotinamide and 0.1 M sodium phosphate buffer, pH 6.0.

All tissue preparations were done in a cold room at 4°C using chilled solutions.

Incubation. The homogenate was pipetted into Erlenmeyer flasks which contained steroid dissolved in propylene glycol (20 mg/ml). One mg of steroid was used for each gram of tissue. The flasks were swirled rapidly to mix the tissue and steroid, and incubated for 2 hours with shaking in a water bath at 37.5 ± 1°C.

Extraction. The homogenate was poured into 10 volumes of acetone. The flasks were rinsed with a small quantity of distilled water, which was added to the acetone. This was stirred for one hour and filtered through a Büchner funnel. The tissue was re-extracted with an additional five volumes of acetone, and filtered. The combined extracts were distilled under vacuum until all organic solvent was removed. The aqueous residue was extracted with chloroform, which was then dried over anyhdrous sodium sulfate, filtered, and removed under vacuum. Further purification and fractionation of the resulting oily residues was done chromatographically, using the ligroinpropylene glycol paper system of Savard,[7] and silica gel column absorption chromatography. Steroids were located on paper by scanning with short wave U.V. light; by the phosphomolybdic acid reaction (5 per cent W/V in ethanol) and/or by 2,4-dinitrophenyl hydrazine (DNPH), (saturated solution in 1 N HCl).

RESULTS

The results of two initial small-scale incubations of HPC with rat liver indicated that the steroid was transformed in a nitrogen atmosphere to two or more steroids of greater polarity. Incubation in air did not appear to result in any transformations.

HPC-4-C^{14} (300 mg, 3,376,500 DPM) was incubated under nitrogen with rat liver homogenate, and extracted as described above. The chloroform extract was dissolved in ether and partitioned into neutral, acidic and phenolic fractions according to the procedure of Engel.[8] Small amounts of radioactivity were present in the acidic and phenolic fractions, but further purification of these fractions showed only neutral steroids to be present. It was assumed that there had been incomplete separation of the organic and aqueous phases during the partition.

The neutral fraction contained approximately 80 per cent of the initial radioactivity. Chromatography on a silica gel column, using increasing concentrations of ethyl acetate in benzene, divided this activity into four major fractions, of which two proved to be the same substance.

Fraction A (1,241,500 DPM): Ultraviolet, sulfuric acid and infra red spectra, mixed chro-

matography and mixed melting points showed this compound to be starting material.

Fraction B (502,740 DPM) and Fraction F (296,800 DPM): these two fractions were worked up separately but were found to be the same substance. Only Fraction B will be described.

Fraction B was eluted from a silica gel column with 10 per cent ethyl acetate in benzene, along with Fraction D (see below). The two fractions were separated by paper chromatography in ligroinpropylene glycol. Fraction B moved 2.25 cm/hour in this system. The substance was eluted from paper with methanol-benzene and was crystallized from methanol-water. The crystals had a melting point of 133.5-136°C (uncorrected). The ultraviolet spectrum showed no maximum near 240 m μ. The sulfuric acid spectrum is shown in Figure 1. The infra red spectrum was identical with that of allopregnane-3β, 17α-diol-20-one-17α-caproate (Figure 2).*

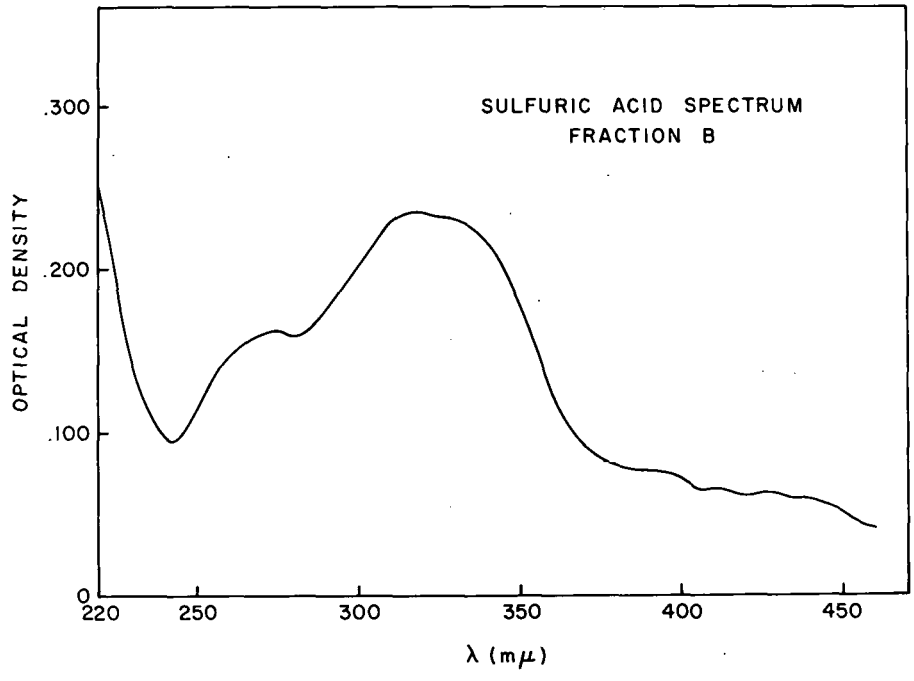

Figure 1. Sulfuric acid spectrum of Fraction B (allopregnane-3β,17α-diol-20-one-17α-caproate).

Fraction D (56,200 DPM) moved 2 cm/hour in the ligroin paper system. It was eluted from paper with methanol, and recrystallized from methanol-water. The crystals had a melting point of 111-112.5-115°C. The ultraviolet spectrum had no 240 mμ maximum. The sulfuric acid spectrum is shown in Figure 3. Infra red spectroscopy (Figure 4) showed the substance to be either allopregnane-3α, 17α-diol-20-one-17α-caproate or pregnane-3β, 17α-diol-20-one-17α-caproate.

In order to decide between these two isomers, the mobility in paper chromatography was considered. According to Savard[7] pregnane compounds move less rapidly in paper partition chromatography than do allopregnane compounds, and compounds containing hydroxyl substituents with an equatorial configuration move less rapidly than isomers containing an axial hydroxyl. The 3-hydroxyls in both possible formulae for D are axial, while the 3β-hydroxyl in B is equatorial.

*Infra red spectra were obtained by the courtesy of Dr. Horst Witzel.

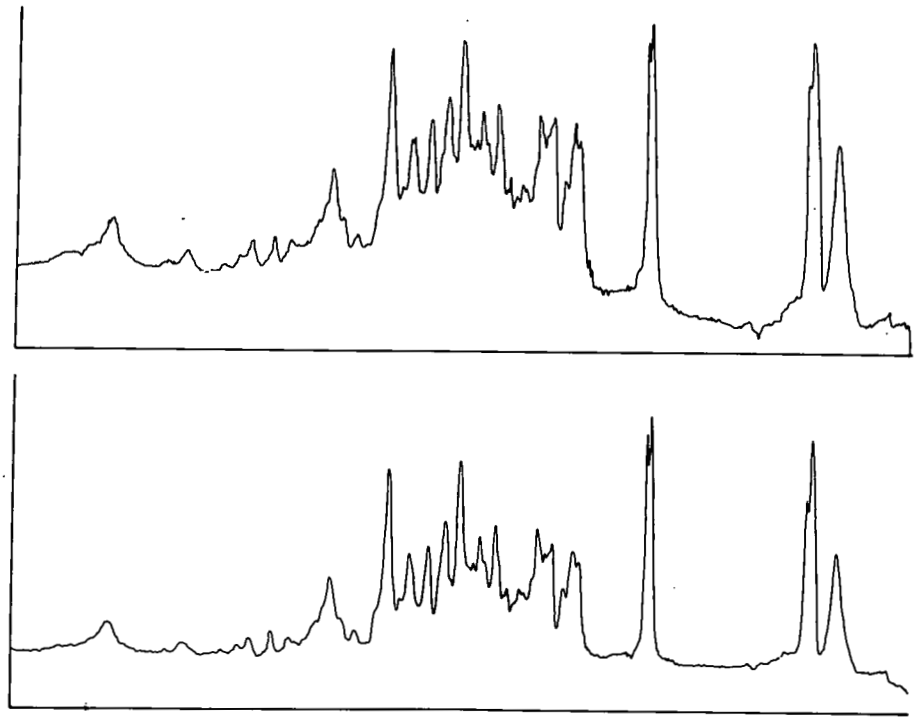

Figure 2. Infra red spectra of Fraction B (upper) and allopregnane-3 β,17 α -diol-20-one-17α-caproate (lower).

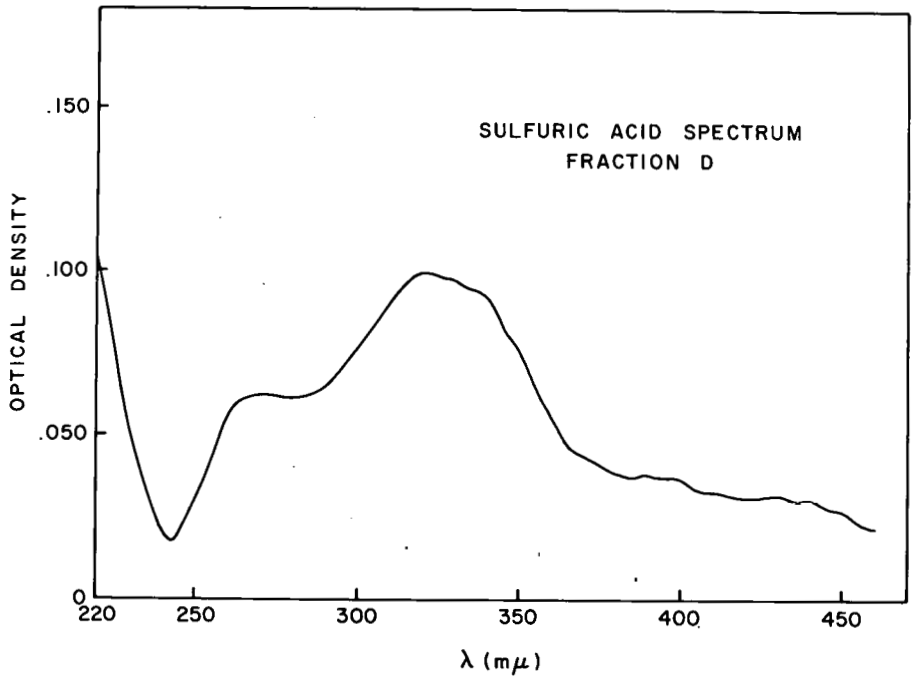

Figure 3. Sulfuric acid spectrum of Fraction D.

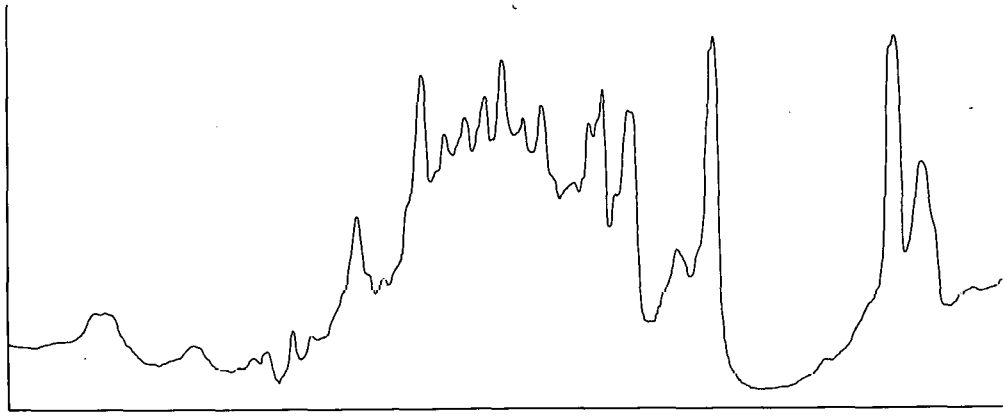

Figure 4. Infra red spectrum of Fraction D.

Since D moves less rapidly than B in the same paper system, D is probably pregnane-3β, 17α-diol-20-one-17α-caproate.

Little et al.[9] have shown progesterone to be converted to Δ^4-pregnene-20α-01-3-one by human placental tissue, and it was considered of interest to see whether this tissue could also attack HPC. The system used was that found optimal by Little for the C-20 reduction of progesterone, except that whole homogenate was used instead of the supernatant obtained by centrifugation at 105,000 x g.

Progesterone (10 mg) and HPC (10 mg) were each incubated for two hours with the homogenate from 30 g of placenta. The incubation was started within 30 minutes of the time of delivery of the placenta. Reduced triphosphopyridinenucleotide (13.8 μM) was added to each flask 10 minutes after the start of the incubation. The homogenate was extracted with acetone and the extract analyzed by paper chromatography.

The progesterone yielded a single more polar product which moved in paper chromatography at a rate similar to that of Δ^4-pregnene-20α-ol-3-one. This substance gave an orange DNPH reaction, showed an ultraviolet absorption maximum of 242 mμ, and was assumed to be 20-reduced progesterone. Both product and starting material were eluted from paper by shaking for one hour with each of three 50 ml portions of methanol, and quantitative measurements were done in the Beckman Model DU Spectrophotometer. The yield of Δ^4-pregnene-20α-ol-3-one was calculated to be 8 per cent.

A single steroid was isolated from the HPC incubation. A paper chromatogram of this substance showed a single ultraviolet absorbing area, and a single radioactive area in the same location. All (100 per cent) of the radioactivity was recovered in this substance, which was shown to be identical with starting material.

Thus, under the conditions of this experiment, HPC was not transformed by placenta.

DISCUSSION

Rat liver homogenate has been shown to reduce Ring A of 17α-hydroxyprogesterone-17α-caproate to yield allopregnane-3β, 17α-diol-20-one-17α-caproate and pregnane-3β, 17α-diol-20-one-17α-caproate. The allopregnane compound is the major product, there being at least a 25 per cent transformation to this steroid; while the pregnane isomer appears in a yield of about

2 per cent. It is of interest that the caproic acid ester on C-17 is not removed or altered. Kimbel et al.[10] also have noted that the ester is not removed during the metabolism of this compound, and we have found other evidence that HPC is excreted in the urine by the human with the ester still present. Ring-labeled HPC and carboxy-labeled HPC were administered to different patients (Lupu,[11]). The urinary excretion of radioactivity in both cases followed the same pattern for total, free and conjugated radioactivity (Figure 5).

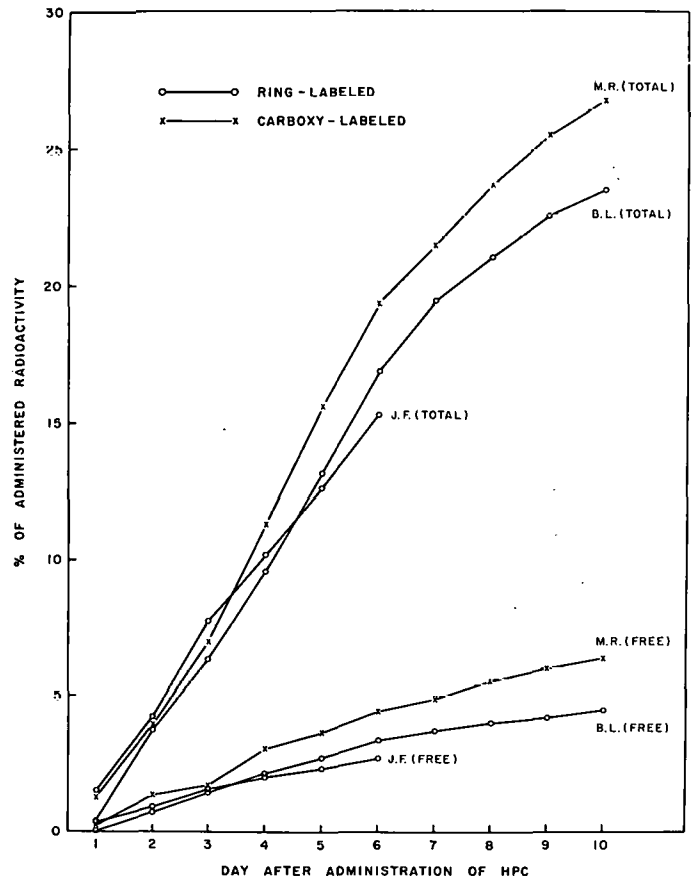

Figure 5. Cumulative excretion of free and total radioactivity in the urine of patients given ring-labeled and carboxy-labeled 17α-hydroxyprogesterone-17α-caproate-C^{14}.

There is one other instance of a synthetic steroid ester being metabolized without alteration of the ester. This is the steroid anesthetic, Viadril (pregnane-21-ol-3,20-dione-21-hemosuccinate), which is transformed by enzymes isolated from rat liver to pregnane-3α,21-diol-20-one-21-hemisuccinate (Jakoby and Tomkins,[12]). However, another fraction of rat liver hydrolyzed the reduced ester to produce succinate and pregnane-21-ol-3,20-dione.

Placental homogenate, in a system which reduced the 20-ketone of progesterone, did not reduce HPC. A similar enzyme in ovaries (Wiest,[13]) has been found to reduce both progesterone and, to a lesser extent, 17α-hydroxyprogesterone at C-20. However, there are systems in bovine corpus luteum (Hayano et al.,[14]) human liver (Atherden,[15]) and cultured uterine fibroblasts (Sweat et al.,[16])

which will also accomplish this reduction. The steric hindrance of the 20 position in HPC resulting from the presence of the caproic acid at C-17 may provide an explanation for the absence of attack on the ketone. This C-20 reduction has been suggested as a deactivating mechanism for progesterone, and its absence with HPC may provide a partial explanation for the increased progestational activity of this compound.

In his report on an enzyme in rat ovaries similar to the placental 20α-reductase, Wiest[13] found the reduction to be reversible, and suggested (skeptically) the involvement of progesterone in a transhydrogenating system as a possible mechanism of hormone action. The results of this study make one question this possibility, as we have here a progestational agent which does not appear to take part in such a transhydrogenating system.

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