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

Semiannual Report to
THE ATOMIC ENERGY COMMISSION

SEPTEMBER 1960

LEON O. JACOBSON, M.D.
Editor

MARGOT DOYLE, Ph.D.
Associate Editor

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THE KIDNEY AND ERYTHROPOIETIN*†

By

C. W. Gurney‡ and L. O. Jacobson

Ten years of intensive investigation into the fundamental nature of the stimulus for red cell production have uncovered much evidence pointing to the central role played by a humoral stimulating factor, erythropoietin, in the regulation of this process. This substance appears to be a glycoprotein,[1] present in the plasma of normal donors,[2,3] and is markedly increased in the plasma of many anemic patients.[2,4] We have postulated the existence of a dynamic equilibrium of red cell production,[5,6] according to which, the rate of red cell production is continuously regulated by the plasma titer of erythropoietin. The normal production of red cells, just sufficient to compensate for the normal daily destruction of approximately 1 per cent of the circulating red cell mass, represents the action of a small amount of erythropoietin on the stem cells in the bone marrow. Anemia or hypoxia, as well as the cobaltous ion, act to increase red cell production indirectly by stimulating the production, or increasing the liberation into the plasma, of erythropoietin from some distant site. Jacobson et al.[7] first demonstrated that one action of erythropoietin probably was to induce differentiation of primitive cells of the bone marrow. Alpen and Cranmore[8] and Erslev[9] have verified this conclusion by different methods, and have further presented evidence suggesting that this is the only important action of erythropoietin insofar as it pertains to red cell production. The historical aspects of the study of erythropoietin, its chemistry, physiology, and clinical relevance have been reviewed elsewhere.[1,10,11]

In the last three years, interest has been focused upon the kidney as a source of erythropoietin production, and it is the purpose of this review to summarize the evidence and discuss some of its implications.

Interesting case reports by Stohlman, Rath and Rose[12] and Schmid and Gilbertsen[13] were consistent with the thesis that a major source of erythropoietin was located somewhere caudal to the diaphragm. These investigators reported accounts of patients with long-standing patent ductus arteriosus, pulmonary hypertension, and reversal of blood flow through the patent ductus such that inadequately oxygenated blood entered the aorta at its junction with the patent ductus. Presumably organs supplied by blood coming from the aorta above the site of the patent ductus were oxygenated normally, and unsaturated blood, most of which ultimately supplied the body below the level of the diaphragm, was somehow implicated in erythropoietin production.

Numerous experiments by many investigators (reviewed by Gordon[10]), failed to demonstrate the critical importance of any particular organ in erythropoietin production. In 1957 however, Jacobson, Goldwasser, Fried, and Plzak[14] reported that nephrectomized rats lost the ability to respond to phlebotomy or the injection of cobaltous chloride by the production of an increased plasma titer of erythropoietin. It was necessary to exclude the possibility that the uremia coin-

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

cident to nephrectomy acted by suppressing erythropoietin production at another distant site, and rats with bilateral ureter ligation were therefore used as controls. These animals had blood urea nitrogen elevations comparable to those found in nephrectomized rats, but when the kidneys were present, the animals retained the ability to respond to cobalt, phlebotomy, or, as subsequently shown,[15] hypoxia. Although one interpretation of these data is that erythropoietin is produced by the kidneys, other alternatives must be considered. The experiments cited do not exclude the possibility that a toxic substance, coincident to uremia in the nephrectomized animal and suppressing erythropoietin production elsewhere, can be destroyed or inactivated by kidneys in ureter-ligated animals, thereby permitting erythropoietin production. Another possible interpretation to be kept in mind is that an erythropoietin precursor, produced in an organ other than the kidney is activated by the kidney in response to anemia or hypoxia, and this process is not abolished by ureter ligation.

Not all reported results are in agreement with the conclusion that erythropoietin may be produced in the kidney. Erslev[16] presented evidence interpreted as indicating "that the anemia of uremic rabbits is related to the metabolic changes associated with uremia rather than to the presence or absence of kidney tissue." Neither his nephrectomized rabbits nor unilaterally nephrectomized contralaterally ureter-ligated rabbits produced demonstrable erythropoietin elevations after bleeding. On the other hand, Mirand and Prentice[17] reported the demonstration of a high titer of erythropoietin in plasma obtained from nephrectomized rats after exposure to an environment of low oxygen tension.

In further experiments, Jacobson et al.[18] reported evidence for production of a slight amount of erythropoietin in nephrectomized animals. These data suggest that the kidney, if it is the major site of erythropoietin production, is not the only organ in which it can be elaborated.

Naets[19] found rapid depletion of erythroblasts from the marrow of dogs following removal of both kidneys, but although bilateral ureter ligation in other dogs produced comparable elevations of blood urea nitrogen, erythropoiesis was essentially normal in these animals. Further, maintenance of marrow erythroblasts of one bilaterally nephrectomized dog was observed after injection of erythropoietic factor obtained from the urine of an anemic patient. According to Osnes[20] the anemia following intensive irradiation of the kidneys could be prevented if one-quarter to one-third of one kidney was shielded during irradiation, in spite of striking elevations of the blood urea nitrogen in such animals. Finally, Kuratowska et al.[21] have recently described erythropoietic stimulating properties shown by the perfusate obtained from isolated kidneys perfused with hypoxic blood. These experiments all tend to substantiate the thesis of Jacobson et al. that the kidney is a major source of erythropoietin production.

Many clinical observations and investigations, in addition to those already mentioned, suggest the existence of important relationships between renal and bone marrow function. Emerson and Burrows[22] concluded that anemia in four patients with chronic nephritis and azotemia was mainly attributable to a depression of erythropoiesis although excessive hemolytic activity was also demonstrated. Decreased production of red cells has also been reported by others.[23-26] Although Markson and Rennie[27] have found an inhibitory effect exerted by serum from azotemic patients on maturation of normoblasts in suspension culture, it may be that suppression of red cell development as a result of azotemia, is not entirely responsible for the anemia of uremia. We have previously suggested the possibility that the anemia of uremia might be attributable at least partially to inadequate erythropoietin production.[2,28] Gallagher et al.[29] confirmed our

findings of absence of demonstrable erythropoietin in the plasma obtained from most anemic uremic patients although in a more extensive series[30] a few plasma specimens from uremic patients contained some demonstrable erythropoietin. When these investigators added inactive plasma from anemic uremic patients to plasma shown to contain erythropoietin, there was no inhibition of the response on bioassay of the active plasma. This demonstration is noteworthy because it tends to exclude the presence of plasma inhibitors produced in uremia which obscure the demonstration of erythropoietin.

On the basis of bioassay of over 200 plasma specimens obtained from anemic subjects with a wide variety of disease, and employing three different assay techniques[31,32] we have come to expect the demonstration of an increase in erythropoietin in the plasma of most patients with hemoglobin levels under 9 g per cent. In Table 1 are recorded some of the more striking instances of demonstration of erythropoietic activity by the three bioassay procedures we have

Table 1

BIOASSAY OF PLASMA ERYTHROPOIETIN OBTAINED FROM ANEMIC PATIENTS
METHOD: RED CELL Fe^{59} INCORPORATION BY ASSAY ANIMALS

Diagnosis	Assay animal	Result	Saline control
Leukemia	Hypophysectomized rat	28.9 per cent	3.0 per cent
Hodgkin's disease	Hypophysectomized rat	27.1	2.3
Aplastic anemia	Hypophysectomized rat	51.2	3.7
Aplastic anemia	Starved rat	33.8	4.8
Leukemia	Starved rat	26.8	4.8
Gastrointestinal hemorrhage	Starved rat	33.6	5.3
Pernicious anemia	Polycythemic mouse	9.4	0.4
Aplastic anemia	Polycythemic mouse	42.6	0.5
Megaloblastic anemia of pregnancy	Polycythemic mouse	23.3	0.4

employed. In not all instances however, is it possible to demonstrate increased titers of erythropoietin in plasma of anemic patients. Of several exceptions, one occurs when the plasma is obtained from anemic uremic patients. Our experience, summarized in Table 2 and that of Gallagher et al.[29,30] leads us to conclude that the compensatory rise of plasma erythropoietin level anticipated in anemic patients can usually not be demonstrated by currently employed assay techniques in those patients with advanced renal disease, regardless of the level to which the hemoglobin declines.

The importance of this inability to demonstrate erythropoietin in the plasma of uremic patients cannot be evaluated at the present time. The development of more sensitive assay systems may show that the titers of erythropoietin in plasma obtained from many patients whose anemias are secondary to chronic renal disease are normal or even increased. Another approach to a determination of the degree to which erythropoietin deficiency contributes to the anemia present in patients with chronic renal disease would be an evaluation of the response of such patients to purified preparations of erythropoietin administered in amounts sufficient to produce an adequate

response in normal volunteers. At this time it is premature to do more than speculate on the possibility that underproduction of red cells in uremic states is secondary to or in any way related to a state of erythropoietin insufficiency.

Table 2

ERYTHROPOIETIN ASSAY OF PLASMA FROM ANEMIC PATIENTS WITH CHRONIC RENAL DISEASE

	Age	Sex	Diagnosis	BUN*	Hb.†	Animal	Result	Control
1	36	F	Pyelonephritis	115	7.2	Hypoxic rat	1.7 per cent	1.9 per cent
2	16	F	Pyelonephritis	196	5.0	Hypoxic rat	2.6	2.5
3	44	M	Pyelonephritis	136	7.0	Starved rat	8.7	9.8
4	39	F	Pyelonephritis	97	6.2	Starved rat	5.2	5.3
5	37	F	Glomerulonephritis	206	6.1	Hypoxic rat	1.9	4.5
6	34	M	Glomerulonephritis	155	8.5	Hypoxic rat	1.2	2.5
7	16	F	Glomerulonephritis	100	5.7	Hypoxic rat	2.1	4.5
8	57	M	Glomerulonephritis	55	9.3	Starved rat	3.8	3.8
9	58	F	Nephritis, Etio.?	59	8.7	Hypoxic rat	4.4	4.5
10	60	M	Intercapillary glomerulosclerosis	74	7.3	Hypoxic rat	1.3	2.5
11	37	M	Intercapillary glomerulosclerosis	100	6.5	Hypoxic rat	1.7	2.5
12	26	M	Intercapillary glomerulosclerosis	64	7.5	Polycythemic mouse	0.6	0.7

*BUN - Blood urea nitrogen.

†Hb. - Hemoglobin.

On numerous occasions the kidney has also been implicated in excessive production of blood. Well over 50 cases of erythrocytosis have been reported accompanying carcinoma of the kidney, and an additional dozen instances of erythrocytosis in association with benign disease of the kidney are recorded in the literature. These clinical reports have been enumerated elsewhere.[33] Recently we have tested extracts of two such tumors in transfusion-induced polycythemic mice and have found unequivocal evidence of erythropoietin in both. In each case, normal kidney tissue adjacent to the tumor served as control, and was negative for erythropoietin content. This confirms the findings of Hewlett et al.[34] who were the first to demonstrate an elevated titer of erythropoietin in extracts obtained from a renal carcinoma removed from a patient with erythrocytosis.

Benign renal disease has also been associated with excessive production of erythrocytes, and in one such extremely complex case, we found a remarkably high erythropoietin titer in the fluid obtained from superficial renal cysts.[33]

Clinical studies however, do not provide convincing support of the idea that the kidney is sole site of erythropoietin production. Excessive red cell production, or erythrocytosis, has been noted as co-existent with other abnormalities, including uterine myoma and cerebellar hemangioblastoma. Recently Waldman and Levin[35] demonstrated a high titer of erythropoietin in the cystic fluid obtained from a cystic cerebellar hemangioblastoma removed from a patient with erythro-

cytosis. If this erythropoietin is assumed to be produced in the cyst wall rather than removed from the plasma and concentrated within the cyst, it seems necessary to conclude that, at least in some disease states, extrarenal production of erythropoietin exists.

Although Kuratowska et al.[21] have interpreted results of their experiments as showing production of erythropoietin by perfused rabbit kidneys, these authors consider it possible that the vascular endothelium rather than renal cells are responsible for this production. Such a possibility is worthy of serious consideration and further investigation since it would reconcile many of the seemingly conflicting results and conclusions reported.

In the short span of three years, a number of new vistas have been opened. Basic studies and clinical investigations have supported one another in a surprising and gratifying manner. Although much additional research will be needed to demonstrate clearly the manner and extent of kidney participation in erythropoietin production and the significance of such findings in the elucidation of human diseases, it appears reasonable to conclude at this time that the kidney plays more than a passing role in erythropoiesis.

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ERYTHREMIA IN RENAL DISEASE*

By

C. W. Gurney

The coexistence of polycythemia and renal disease is well documented. Bliss briefly mentioned it in one patient in 1929[1] although Medvei is frequently credited with the first description of this syndrome.[2] By 1946 a possible causal relationship was suggested, and since then at least 51 cases of polycythemia and renal carcinoma have been recorded in the literature. Occasionally benign renal disease is associated with polycythemia.

The limitation of the polycythemia to the red cell series has frequently been stressed, and the term erythremia better serves to designate this condition. In our laboratories we have been interested in the regulation of red cell formation by a stimulating factor, or hormone, erythropoietin.[3-5] Much evidence points to the existence of a state of dynamic equilibrium in red cell production, according to which, increased red cell formation occurs only when the marrow is stimulated by increased amounts of erythropoietin. Granulopoiesis and thrombopoiesis do not appear to be influenced by this hormone. If this concept is correct, erythremia should be associated with an increased production of erythropoietin.

The syndrome of erythremia associated with renal disease is especially interesting in light of evidence that the kidney is the major source of erythropoietin production presented by Jacobson, Goldwasser, Fried and Plzak.[6]

The interpretation of this experiment has, however, been challenged, for while ureter-ligated rats, comparably azotemic, retain the capacity to produce increased amounts of erythropoietin, it has been postulated that erythropoietin production in some organ other than the kidney is depressed by nephrectomy.[7] Evidence that at least some erythropoietin is produced by the kidneys is supplied by the work of Naets[8] who was able to restore erythropoiesis in nephrectomized dogs by the administration of erythropoietin. In addition, Kuratowska, Lewartowski and Michalak[9] reported the appearance of an erythropoietic stimulating factor in the perfusate of the isolated rabbit kidney when hypoxic blood was used to perfuse the kidney.

It is the purpose of this presentation to report the extraction of erythropoietin from the renal adenocarcinoma of one erythremic patient and to describe the finding of an unusually high titer of erythropoietin in fluid aspirated from cysts of the kidney of a second erythremic patient.

A 62-year-old male, complaining of pain in the right arm, was seen elsewhere a year prior to the present investigation. After roentgenograms had demonstrated a lesion in the bone, a portion of the humerus was removed, and the specimen was interpreted as metastatic carcinoma. Ten months later the patient experienced right sciatic pain and was referred to the University of Chicago Clinics for further diagnostic studies and therapy.

On physical examination the patient demonstrated a plethoric appearance. The spleen could not be palpated. Except for scars of previous surgery, the physical examination was

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*This paper is based on a report presented at the Meetings of the Association of American Physicians at Atlantic City, May 1960, which will appear in Trans. Assoc. Am. Phycns., 73:, 1960.

normal. The hemoglobin was 20.8 g per cent; red count, 7.5 million; hematocrit, 68 per cent. Other blood counts and urinalysis were normal. On pyelography, distortion of the left inferior calyces was noted.

Despite a solitary metastasis in the ilium, the therapeutic program recommended by the orthopedic surgeons was removal of the kidney presumed to contain the primary neoplasm, followed by intensive x-ray therapy of the metastasis. A left nephrectomy was performed and the tumor found to consist of gray and yellow-orange nodular and infiltrating areas. Figure 1 shows that the tumor was composed of relatively uniform large clear cells with small round basophilic nuclei. The tumor grew in irregular cords through much of its bulk. The hemoglobin was 16.3 g per cent on the day following surgery, and has remained normal during the first two post-operative months.

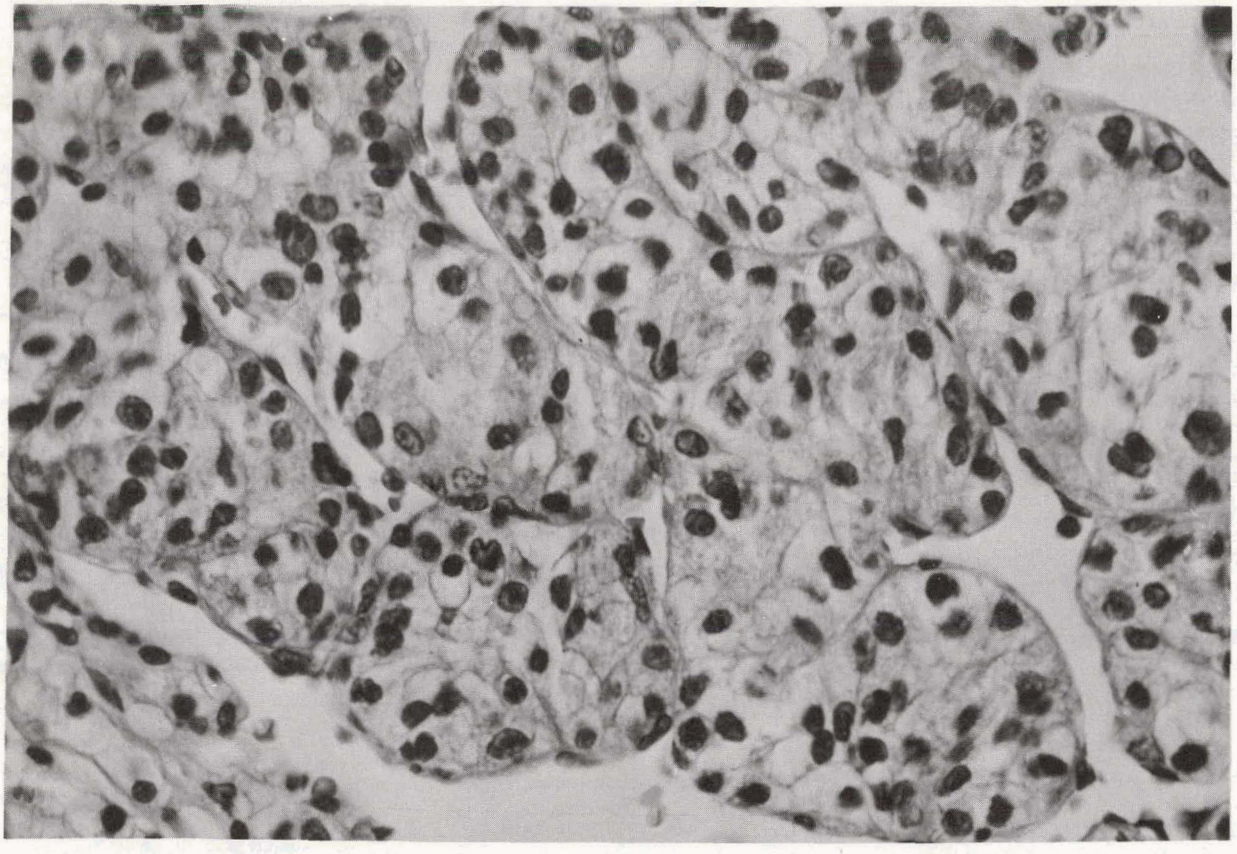

Figure 1. Section through a renal adenocarcinoma associated with erythremia.

Saline extracts of the tumor and of normal kidney tissue from areas adjacent to the tumor were prepared. After weighing, the tissue was cut into small pieces, then was ground into a suspension for 10 minutes. Measured volumes of saline were added during the grinding. After centrifugation, the supernatant fluid was removed and used for assay.

A bioassay not previously described was used in this experiment.[10] It uses CF No. 1 mice rendered polycythemic by the transfusion of homologous red cells, and, except for alteration of time intervals, is essentially the system described in transfusion-induced polycythemic rats by

Fried et al.[11] This is a far more sensitive procedure, particularly in the lower ranges, than that relying on the starved or hypophysectomized rat. Its main advantage appears to lie in the fact that erythropoiesis is virtually eliminated in the polycythemic mouse. The baseline incorporation of radioiron into newly-formed circulating erythrocytes of the polycythemic mouse is in the order of 0.2 per cent compared with 4 per cent in the starved rat.

Table 1 shows the relative erythropoietic stimulating properties of the tumor extract and normal kidney extract. A saline extract prepared from 0.32 g of normal kidney, produced no increase in erythropoiesis over an injection of saline. On the other hand, the extract from 0.14 g of tumor, produced a 15-fold augmentation of erythropoiesis as measured by the incorporation of radioiron by newly-formed red cells, and the extract from 0.72 g of tumor led to a 50-fold increase in red cell formation in polycythemic mice.

Table 1

BIOASSAY OF ERYTHROPOIETIN IN THE EXTRACT OF A RENAL NEOPLASM
ASSAY ANIMAL - POLYCYTHEMIC MOUSE

	Mass of tissue* (g)	No. of assay animals	Fe^{59} incorporation‡ (per cent)	Average hematocrit at end of assay (per cent)
Extract of tumor	0.72	5	10.0 ± 4.2	68
	0.14	5	3.0 ± 1.8	68
Extract of normal kidney	0.32	4	0.2 ± 0	67
Plasma	2.0	4	0.2 ± 0.1	64
Saline	0	4	0.2 ± 0	68

*The mass of tissue represented by 1 ml of extract.

‡± 1 standard deviation.

That the erythropoietin measured is not derived from plasma within the tumor is evident since both normal kidney extract and the patient's plasma failed to stimulate erythropoiesis in the assay animals. Heat and acetone powder extracts of the tumor prepared by Dr. Eugene Goldwasser were comparably active, while similarly prepared extracts of normal kidney tissue were inactive.

Here I would like to mention briefly an investigation undertaken prior to the one just described. Dr. R. Sternheimer of the Michael Reese Hospital sent me the polycystic kidney removed from an erythremic patient. Six ml of cystic fluid was aspirated from superficial cysts. At that time we were routinely using the starved rat for the bioassay of erythropoietin,[11] and the cystic fluid was assayed without prior fractionation. Table 2 shows the result obtained. Erythropoiesis was increased almost 8-fold when compared with the baseline of 3.2 per cent in starved rats receiving saline. This is a striking result since each assay animal received only 0.6 ml of cystic fluid daily for 2 days instead of the total volume of 4 ml usually employed.

The subsequent course of this patient has been unusual. After several months, the red cell values again began to rise and several phlebotomies were required in the following 14 months. Simultaneous albuminuria and renal sediment changes indicated disease of the remaining kidney.

Table 2

BIOASSAY OF ERYTHROPOIETIN IN RENAL CYSTIC FLUID
ASSAY ANIMAL - STARVED RAT

	Fe^{59} incorporation (per cent)	No. of assay animals	Average hemoglobin of assay animals (g)
Cystic fluic	23.5 ± 4.7	5	17.5
Control human plasma	5.9 ± 1.3	5	17.5
Saline	3.2 ± 0.9	6	17.4

Most recently, a rise in the peripheral white and platelet count, and the appearance of a palpable spleen are convincing evidence for the existence of polycythemia vera.

The major question arising from these observations is whether erythropoietin is made in the diseased renal tissue, or whether it is produced elsewhere only to be removed from the blood and concentrated by the diseased renal tissue.

If the second patient has polycythemia vera presenting as erythremia, it may be that erythropoietin was removed from the plasma and concentrated by his renal cysts. Slight concentration of creatinine and electrolytes by superficial renal cysts has been found by Bricker and Patton.[12] If, however, renal neoplasms also concentrate plasma erythropoietin, it is necessary to postulate increased erythropoietin production at a different site in order to account for the erythremia, and in addition to postulate that production at this site is regulated by the tumor, at least in cases where removal of the diseased kidney is followed by a return to normal red cell levels and so, presumably, to normal production of erythropoietin.

As an alternative to the production of erythropoietin at a different site followed by concentration by a renal neoplasm and a renal cyst, a more simple, direct, and therefore attractive possibility is that an excess of erythropoietin is produced in the abnormal renal tissue. The hormone would then be found locally, and if sufficient escaped into the blood, either periodically or continuously, erythremia would result. Under such circumstances, it is easy to understand why removal of the diseased kidney was followed by disappearance of the erythremia.

Finally, the failure to find erythropoietin in the patient's plasma requires comment. It is possible that erythropoietin does not enter the plasma continuously, so that its presence can only be detected by periodic plasma sampling. It is more likely, however, that the assay system is not yet sufficiently sensitive to demonstrate very low titers of plasma erythropoietin. A titer sufficient to sustain normal erythropoiesis is extremely low and can be demonstrated only by extraction and concentration.[13,14] A very slight increase above the normal titer level may be sufficient to produce erythremia if this level is maintained. Clarification of this problem will require extraction and concentration of large volumes of plasma. Unfortunately present extraction techniques do not yield consistent quantitative results. At least one more possibility remains; it may well be that our concept of regulation of erythropoiesis is too simple. It has been suggested that dual mechanisms[15] or multiple factors,[16] are involved, and although the evidence is limited, these possibilities cannot be discounted.

In summary, erythropoietin has been demonstrated both in the cystic fluid and the saline extract of a renal carcinoma obtained from two patients presenting with erythremia. It is probable

that this syndrome is more common than is generally appreciated. Polycythemia limited to the red cell series and not obviously secondary to other disease warrants vigorous investigation of the kidneys. Successful removal of renal carcinoma may be anticipated when this early sign is heeded. Furthermore, the demonstration of erythropoietin in a renal tumor and in renal cystic fluid, while not absolutely conclusive, tends to support the concept that erythropoietin is produced by the kidney.

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A COOPERATIVE ASSAY OF A SAMPLE WITH ERYTHROPOIETIC STIMULATING ACTIVITY[†]

By

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Under the stimulus of severe anemia or hypoxia an erythropoietic stimulating factor (ESF)* appears in the plasma in sufficient concentration that injection of relatively small amounts of such plasma into normal animals results in stimulation of erythropoiesis.[1] With the realization that erythropoiesis is under hormonal control have come developments in methods for detecting ESF, in methods for producing high titers in animals and for extracting and concentrating the activity. The chemical natures of ESF, its tissue of origin, and its importance as an etiologic and possible therapeutic agent in blood diseases are unsolved problems which are now being investigated in laboratories throughout the world.

The lack of a common basis for comparing ESF from different sources, prepared in different ways, and assayed by different methods is recognized by workers in this field as a deterrent to the solution of the many unsolved problems. The preparation of a uniform standard which can be made available to all interested investigators through some central agency such as the National Institutes of Health or the Medical Research Council of Great Britain would be an ideal solution.[2] However, lack of adequate sources of ESF makes this impossible at present. As a step in this direction the group at the California Institute of Technology prepared a batch of partly purified ESF. There was enough for preliminary testing; the remainder was divided up and sent to laboratories in this country where ESF preparations were being assayed. The standard sample was to be assayed by whatever methods were being used by that laboratory and the results were to be compared. A comparison of the results from seven laboratories is the subject of this paper.

MATERIAL

The Standard ESF was prepared from the filtrate of boiled plasma from rabbits made ane-

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[†]This paper appears in Blood, 16:1424, 1960.

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*In a recent review Gordon[1] suggested the term, "erythropoietic stimulating factor(s)" (ESF) rather than hemopoietin or erythropoietin. This term and the abbreviation seem suitable and we propose that they be adopted for general use.

mic by injection with phenylhydrazine. The material which precipitated below 60% by volume ethanol was discarded. The material which precipitated between 60 and 80% ethanol was collected, extracted with water and the water soluble portion dialyzed and lyophilized.[3] From 3100 ml of plasma the yield was 450 mg of soluble white powder. It was not intended to provide a highly purified preparation. The pool was tested by three different assay methods to be sure of its activity. The results are given below (Keighley et al.). Small numbers of animals were used to conserve the limited amount of material. The dry powder was distributed in 50 mg lots, labeled CS-1.

METHODS AND RESULTS

G. Keighley, P. Lowy, and H. Borsook, California Institute of Technology. Increase in hemoglobin concentration in normal rats. Two female rats of the Sprague-Dawley strain weighing 250 gm were injected subcutaneously with 5.0 mg standard in saline daily for 4 days followed by 2.5 mg daily for 4 more days. A sample of tail vein blood was taken for hemoglobin determination at the beginning, and on the 5th and 8th days of the experiment. See Table 1. Increase in hematocrit in normal mice. Four female mice of the Webster-Swiss strain with an average weight of

Table 1

INCREASE OF HEMOGLOBIN IN NORMAL RATS

Treatment	Daily dose		Hb. g. per 100 ml. blood		
	Days 1-4	Days 5-8	Day 1	Day 5	Day 8
ESF standard	5.0 mg	2.5 mg	14.9	15.8	17.6
ESF standard	5.0 mg	2.5 mg	14.5	16.0	17.9

31 gm were injected subcutaneously with 1.0 mg of standard in saline daily for 5 days. Microhematocrit measurements were made on samples of blood from the orbital sinus on the 1st and 7th days. See Table 2. Fe^{59} red cell incorporation in fasted rats. Six female rats of the Long-Evans strain weighing 150-170 gm were used. Fasting started at 0 hours. Three were injected subcutaneously with 0.5 mg of standard in saline at 24, 48, and 72 hours, and three were injected

Table 2

INCREASE IN HEMATOCRIT IN NORMAL MICE

Treatment	Daily dose (5 days)	Hematocrit per cent	
		Day 1	Day 7
ESF standard	1 mg	44.9	48.8
ESF standard	1 mg	45.1	48.0
ESF standard	1 mg	44.8	52.5
ESF standard	1 mg	43.9	51.8
	Average	44.7	50.3

with normal plasma. At 76 hours approximately 1 μc Fe^{59} as ferric chloride (in sterile buffer; 0.005 M KH_2 PO_4, NaCl 0.9%, pH adjusted to 3.5) was given intravenously and at 94 hours blood was taken by heart puncture for counting. The uptake was calculated by assuming a blood volume of 5% body weight at time of bleeding. The results are presented in Table 3.

Table 3

FE^{59} RED CELL INCORPORATION ASSAY IN STARVED RATS

Treatment	Daily dose (3 days)	Fe^{59} red cell incorporation Per cent injected dose
ESF standard	0.5 mg	22.6
ESF standard	0.5 mg	21.2
ESF standard	0.5 mg	27.8
		Average 23.9
Normal rabbit plasma	1 ml	6.3
Normal rabbit plasma	1 ml	4.1
Normal rabbit plasma	1 ml	3.3
Normal rabbit plasma	1 ml	3.1
Normal rabbit plasma	1 ml	4.2
		Average 4.2

E. Goldwasser, University of Chicago: Fe^{59} red cell incorporation assay in fasted rats. Male rats (100-150 gm) were deprived of food at 0 hours, 0.5 mg of the standard in 2 ml of saline was injected subcutaneously at 19 hours and at 43 hours. At 72 hours 1 μc of Fe^{59} (as ferric citrate) was injected intravenously. At 89 hours blood was drawn by cardiac puncture and counted in a well-type scintillation counter. The blood volume was assumed to be 5% of the body weight.[4] The results are summarized in Table 4. Fe^{59} red cell incorporation in normal rats. This assay was conducted in exactly the same manner as the previous assay except that the animals were not deprived of food. The results are presented in Table 5. Increase in reticulocyte count in polycythemic mice. Twelve female CF No. 1 mice weighing 22-24 g were injected with 0.5 ml of packed, washed red cells intraperitoneally on days 1, 2, 3, and 5. Six were injected with 0.5 mg of the standard in 0.5 ml saline and six with saline alone on days 8, 9, 10, and 11. Reticulocyte counts were made on day 12. The results are summarized in Table 6.

A. S. Gordon, New York University: Increase in hematocrit and reticulocyte count in normal rats. Groups of 3 normal adult female rats of the Long-Evans strain were given doses of 0.5 and 2.0 mg of the standard in 3 ml of saline subcutaneously daily for 5 days. On the 6th day reticulocyte counts and hematocrit determinations were made on peripheral blood. The results are summarized in Table 7.

T. C. Prentice, Roswell Park Memorial Institute. Fe^{59} red cell incorporation assay in normal and hypophysectomized rats. Rats of the Sprague-Dawley strain fed ad libitum were used. One group was intact normal rats and the other group had been hypophysectomized 14 days previously. Half of each group was given 0.5 mg of the standard in 1 ml saline subcutaneously daily for 3 days. The controls received no treatment. On the 4th day 1 μc of Fe^{59} was given intrave-

Table 4

FE^{59} RED CELL INCORPORATION ASSAY IN STARVED RATS

Treatment	Daily dose (2 days)	Fe^{59} red cell incorporation Per cent injected dose
ESF standard	0.5 mg	14.5*
ESF standard	0.5 mg	26.4
ESF standard	0.5 mg	17.1
ESF standard	0.5 mg	22.8
		Average 20.4
Saline	2 ml	5.8†
Saline	2 ml	4.7
Saline	2 ml	4.9
Saline	2 ml	3.2
Saline	2 ml	3.2
Saline	2 ml	4.8
Saline	2 ml	5.0
Saline	2 ml	5.7
Saline	2 ml	5.5
Saline	2 ml	5.7
		Average 4.8

*Mean weight at time of sampling, 90 g.

†Mean weight at time of sampling, 100 g.

Table 5

FE^{59} RED CELL INCORPORATION ASSAY IN NORMAL RATS

Treatment	Daily dose (2 days)	Fe^{59} red cell incorporation Per cent injected dose
ESF standard	0.5 mg	56.0*
ESF standard	0.5 mg	50.6
ESF standard	0.5 mg	49.8
ESF standard	0.5 mg	47.0
		Average 50.8
Saline	2 ml	45.4
Saline	2 ml	42.4
Saline	2 ml	46.0
Saline	2 ml	49.1
Saline	2 ml	41.2
		Average 44.8

*Mean weight at time of sampling, 170 g.

Table 6

POLYCYTHEMIC MOUSE ASSAY

Treatment	Daily dose (4 days)	Final Hct. per cent	Reticulocytes per cent
ESF standard	0.5 mg	73	9.2
ESF standard	0.5 mg	66	0.8
ESF standard	0.5 mg	72	10.8
ESF standard	0.5 mg	72	5.2
ESF standard	0.5 mg	66	6.1
ESF standard	0.5 mg	74	5.2
			Average 6.2
Saline	1 ml	70	0.00
Saline	1 ml	69	0.00
Saline	1 ml	69	0.00
Saline	1 ml	66	0.00
Saline	1 ml	65	0.00
Saline	1 ml	69	0.00

nously. Twenty-four hours later blood was drawn for counting. The results are presented in Table 8.

W. A. Rambach, Northwestern University: Fe^{59} red cell incorporation assay in normal and dehydrated rats. The standard material was assayed by two methods. The first is a method which has been published.[5] Female, albino rats (180-190 gm) of a Sprague-Dawley strain were injected subcutaneously daily for seven days with 0.5 mg of the test material in 1.0 ml of 0.9% NaCl. On the seventh day the animals were given an I.V. injection of approximately 0.5 μc Fe^{59} as iron citrate contained in 0.5 ml of solution. They were sacrificed 24 hours later and the total per cent of the administered iron appearing in the red cell mass was calculated, Table 9.

The second method constitutes a modification, the details of which are in preparation for publication, in which a dehydrated animal was used. In the dehydrated animal a relative polycythemia has developed. All water was withheld from the animals for 72 hours. Food was allowed ad libitum. At the end of this period the animals were injected subcutaneously daily for four days with 0.5 mg of the test material in 1.0 ml of 5% dextrose in 0.9% NaCl. On the fourth day Fe^{59} was given as described above and the animals were sacrificed in twenty-four hours. During the period of injection of test substance no water was allowed the animals, but food was not restricted, Table 10.

F. Stohlman, Jr., National Institutes of Health: Fe^{59} red cell incorporation and increase in reticulocyte assay in starved rats. Six groups of 7 rats each of the Sprague-Dawley strain weighing 150-170 gm were used. Group one was given 2% by body weight of a solution of 5 mg of the standard in 25 ml of normal saline intravenously daily (average of 0.64 mg/rat/day). The second group was given 0.3 ml of a solution of 4 mg in 2.4 ml of normal saline subcutaneously daily. Groups 3 and 4 were given plasma obtained from 150-170 gm Sprague-Dawley rats following 18 hours exposure at 310 mm Hg barometric pressure and group 5 was given plasma from normal donors. All plasma samples were given intravenously. The recipients were starved at 0 hr; first

Table 7

ASSAY OF STANDARD USING NORMAL RATS

Treatment	Daily dose (5 days)	Reticulocytes per cent		Hematocrits per cent		Body weight g.	
		Before	After	Before	After	Before	After
Untreated control	0	2.6 ± 0.5*	2.1 ± 0.4	47.5 ± 1.7	47.8 ± 1.1	217	217
		N.S†		N.S.			
ESF standard	0.5 mg	2.5 ± 0.6	4.8 ± 0.1	47.5 ± 0.9	50.3 ± 1.1	220	230
		P < 0.05‡		N.S.			
ESF standard	2.0 mg	2.4 ± 0.7	8.7 ± 0.7	48.4 ± 0.9	53.9 ± 1.6	222	226
		P < 0.01		P < 0.05			

*Mean value ± standard error of the mean.

†N.S. - not significant.

‡Probability values determined from the distribution of Fisher's t (Snedecor, '46).

Table 8

FE^{59} RED CELL INCORPORATION ASSAY IN HYPOPHYSECTOMIZED AND NORMAL RATS

Recipient	No. of rats	Daily dose (3 days) mg. of ESF standard	Final Hct. per cent	Fe^{59} red cell incorporation per cent injected dose
Hypophysectomized	5	0.5	51.3	51.9 ± 5.5*
Hypophysectomized	5	0.0	49.8	6.8 ± 0.7
Normal	6	0.5	51.0	64.3 + 3.9
Normal	4	0.0	45.8	51.1 ± 7.6

*Mean value ± standard error of the mean.

Table 9

FE^{59} RED CELL INCORPORATION ASSAY USING NORMAL RATS

Treatment	Body weight		Hct.		Hgb.		Retic.		Fe^{59} red cell incorporation
	Init.	Final	Init.	Final	Init.	Final	Init.	Final	
	g.		per cent		g/100 ml.		per cent		per cent
ESF standard*	184	195	46	50	15.8	15.4	1.7	7.1	52.0
ESF standard	183	189	47	51	15.7	16.2	1.9	6.6	54.9
ESF standard	182	190	47	48	14.1	15.8	2.1	7.5	55.5
ESF standard	188	194	49	50	15.4	16.5	2.0	6.6	54.8
Saline									
1 ml.	194	202	52	46	16.0	15.2	1.5	2.4	32.8
1 ml.	188	194	46	41	14.6	13.0	1.6	2.5	34.9
1 ml.	185	190	47	44	14.8	14.3	2.2	2.5	22.7
1 ml.	184	187	48	44	14.4	14.1	2.3	2.5	29.7

*0.5 mg. daily for 7 days.

Table 10

FE^{59} RED CELL INCORPORATION ASSAY USING DEHYDRATED RATS

Treatment	Daily dose (4 days)	Body weight Initial	Body weight Final	Retic. per cent	Fe^{59} red cell incorporation per cent injected dose
		g.			
ESF standard	0.5 mg.	140	110	3.1	25.7
ESF standard	0.5 mg.	143	115	3.8	37.4
ESF standard	0.5 mg.	150	112	5.4	53.4
ESF standard	0.5 mg.	140	126	5.9	57.2
Saline	1 ml.	154	131	0.0	3.35
Saline	1 ml.	137	105	0.1	5.5
Saline	1 ml.	144	120	0.1	3.2

injection given at 24 hrs; 2nd injection at 48 hrs; blood drawn for counting at 90 hrs. The results are summarized in Table 11.

Table 11

FE^{59} RED CELL INCORPORATION ASSAY IN STARVED RATS

Treatment	Daily dose (2 days)	Route of administration	Hct. per cent	Retic. per cent	Fe^{59} red cell incorporation per cent injected dose
ESF standard	0.64 mg	I.V.	50 ± 0.73*	1.88 ± 0.23	28 ± 2.3
ESF standard	0.50 mg	S.Q.	49 ± 1.9	2.24 ± 0.27	32 ± 1.3
Alt. plasma†	2 per cent‡	I.V.	47 ± 0.73	2.17 ± 0.25	26 ± 2
Alt. plasma	1 per cent	I.V.	49 ± 0.69	1.16 ± 0.1	19 ± 1.2
Nor. plasma	2 per cent	I.V.	48 ± 1.05	0.64 ± 0.1	5.5 ± 0.6
None			49 ± 0.75	0.5 ± 0.1	5.6 ± 0.41

*Standard error of the mean (7 rats/group).

†Plasma obtained from 150-170 g Sprague-Dawley rats following 18 hours exposure at 310 mm Hg barometric pressure.

‡Per cent figures indicate volume of material given as per cent of body weight on each of 2 days.

D. C. Van Dyke, University of California: Increase in total circulating red cell volume in normal rats. The 50 mg of standard were dissolved in 84 ml of physiological saline, of which 6 ml were put in each of 14 injection vials and the entire lot immediately frozen. This material was given to 5 normal female rats of the Long-Evans strain weighing 200 gm for 14 days. Each day one of the vials was thawed and each of 5 rats received 1 ml subcutaneously which represented a dose of 595 micrograms per day. On the 15th day the total circulating red cell volume was determined by the Fe^{59} labeled red cell dilution method.[6] Five uninjected control rats of the same weight were alternated with the injected group during the blood volume determination. The results are presented in Table 12.

DISCUSSION

The standard material was assayed in 7 different laboratories by 10 different methods or major variations, 8 using rats and 2 using mice. By whatever method the material was assayed a positive result was obtained and the response obtained in different laboratories using similar assays was comparable. Most of the assay methods now being employed were represented so that other laboratories should be able to compare their results to those obtained in this study, thus providing some basis for comparison between laboratories.

A unit for ESF has been proposed by Schlueter, Norgello and White[7] based on the response obtained from injection of 5 micromoles of cobalt using the radioiron starved rat assay developed by Fried et al.[4] This proposal has the advantage that it employs a standard preparation which is readily available to all investigators as well as a standardized assay procedure, but has the disadvantage of the small response which the injection of cobalt elicits in the Fe^{59} red cell incorporation assay at any dose at which it is given.

Table 12

INCREASE IN TOTAL CIRCULATING RED CELL VOLUME OF NORMAL RATS

Treatment	Hct. per cent	Hgb. g	Body weight Initial g	Body weight Final g	Total blood volume ml	Total circulating red cell volume ml
ESF standard*	49.0	14.4	199	224	13.7	6.7
ESF standard	45.6	14.2	214	236	15.3	7.0
ESF standard	57.5	17.2	216	220	12.7	7.3
ESF standard	54.7	16.5	200	212	12.7	6.9
ESF standard	52.8	16.0	194	210	13.2	7.0
Average	51.9	15.7	205	220	13.5	7.0 ± .97†
None	38.2	12.6	216	221	12.1	4.6
None	42.0	13.5	207	226	13.5	5.7
None	40.1	11.6	198	212	12.4	5.0
None	38.6	12.3	197	210	13.5	5.2
None	42.0	12.4	208	224	12.1	5.1
Average	40.2	12.5	205	219	12.7	5.1 ± .57

*0.59 mg/day for 14 days.

†Standard error of the mean, probability values determined from the distribution of Fisher's t, $P < .001$.

Variations of the same assay procedure (Fe^{59} red cell uptake in whole rats with depressed erythropoiesis) were used by 4 different laboratories in the comparative assay of the standard preparation CS-1 in this study with fairly good agreement between the results. Table 13 compares the results of these assays, two using 2 daily injections, one using 3, and one using 4. It is proposed that until an adequate standard becomes available for widespread distribution, a unit of ESF be defined tentatively as that amount of activity which will produce a net incorporation into red cells of 20% of injected Fe^{59}, in a standardized starved rat assay. Thus if in control rats, uninjected or injected with inactive material, 5% of injected Fe^{59} is incorporated into red

Table 13

COMPARISON OF RESULTS FROM FOUR LABORATORIES USING THE FE^{59} RED CELL INCORPORATION ASSAY

Investigator	Treatment	Daily dose mg. of ESF standard	No. of daily doses	Fe^{59} uptake minus control uptake per cent
Goldwasser	starved	0.5	2	15.6
Keighley	starved	0.5	3	19.7
Stohlman	starved	0.5	2	26.4
Rambach	dehydrated	0.5	4	39.4

cells, and if in experimental rats injected with ESF, 25% of injected Fe^{59} is incorporated in the red cells, there is a net incorporation of 20% in the experimental rats. A slight modification of the assay as originally proposed by Fried et al. is suggested. Rats, 150-170 gm, given water but no food from 0 hours; test material administered subcutaneously; first injection at 24 hours, second injection at 48 hours and Fe^{59} given intravenously at 72 hours; blood drawn at 90 hours for determination of Fe^{59} uptake assuming a blood volume of 5% of the final body weight. The proposed unit, defined in terms of a response, may not be as desirable as one defined in terms of a standard material; it should be useful until standards are generally available.

By defining an ESF unit as above, and judging by the assays done by Goldwasser, Keighley, and Stohlman (Table 13), one unit of the standard preparation CS-1 would be equivalent to a total dose of approximately 1 mg.

It should be noted that comparisons may have to be made by diluting unknown samples to give the same Fe^{59} uptake as the proposed unit. Unless the dose response curves, which may not be linear, are known for the samples to be compared, and for the assays being used, extrapolations are unjustified.

It appears that the unit proposed would be equivalent to approximately 10 of the cobalt units proposed by Goldwasser and White.[7] Here also caution is needed in the use of dose response curves.

SUMMARY AND CONCLUSIONS

A batch of erythropoietically active material made from the plasma of phenylhydrazine-treated rabbits was distributed to 7 laboratories in this country for the purpose of comparing assay methods. The results of the assays using 10 different methods or major variations are presented.

Most of the assay methods being employed were represented so that other laboratories should be able to compare their results to those obtained in this study.

The importance of obtaining some uniform basis for comparison of activity of erythrocyte stimulating factor between laboratories has been emphasized and a tentative unit has been proposed based on a net incorporation into rat red cells of 20% of injected Fe^{59}, using a standardized assay procedure.

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HETEROLOGOUS TRANSPLANTATION OF MOUSE TUMORS INTO THE NEWBORN ALBINO RAT[†]

By

J. S. Thompson[‡*] and C. W. Gurney

Successful heterotransplantation of the mouse lymphoma P1534, indigenous to the DBA/2 strain of mice, into albino rats, appears to be dependent upon the age of this host,[1] progressive growth only occurring when inoculation takes place during the first 24 hours of its life. On the other hand, growth of this lymphoma in the untreated hamster as well as in some homologous strains of mice has been reported.[2]

The present work was undertaken to confirm the observation noted above[1] and also to test whether successful heterotransplantation of other mouse tumors is possible.

MATERIALS AND METHODS

Host animals consisted of newborn and young adult (6 to 8 weeks of age) CFN rats of Carworth-Wistar origin and young adult mice (8 to 14 weeks of age) of the strain of routine tumor passage. Each experimental litter of newborn rats contained animals selected at random from 10 litters born during the same 24 hours.

The following eight mouse tumors were used in the experiments:

1. The Lymphoma P1534, indigenous to the DBA/2 strain,
2. The Melanoma S91, indigenous to the DBA/2 strain,
3. The Myeloma X5563, indigenous to the C3H strain,
4. The Mammary Carcinoma, BW10232, indigenous to the C57BL/6 strain,
5. The Neuroblastoma C1300, indigenous to the A/Jax strain,
6. The Ehrlich Ascites tumor, transplantable in many strains of mice,
7. The Lymphoma L4946, indigenous to the AKR strain,

and

8. The Gardner Lymphosarcoma 6C3HED, indigenous to the C3H strain.

The myeloma was kindly supplied by Dr. Donald Korst of Ann Arbor, Michigan, and the other tumors were obtained from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine. The strains of mice used for routine passage of these tumors are from the same source, with the exception of the CF No. 1 strain (in which the Ehrlich Ascites tumor is passaged) which is from Carworth Farms, New City, New York.

The Ehrlich Ascites, 6C3HED and L4946 tumors, were maintained by intraperitoneal passage, while all other tumors were selected from subcutaneous masses. Using clean, but not sterile technique, the tumors were excised and cut into small pieces with fine dissecting scissors, and gently homogenized in a Tenbroek Tissue Grinder. Tyrodes solution was added and the vol-

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ume adjusted so that approximately 0.1 ml of the mixture contained 20 x 10^6 nucleated cells.

The ascitic fluid from the Ehrlich Ascites, L4946, and 6C3HED tumor-bearing mice was transplanted without dilution.

Subcutaneous and intraperitoneal injections of tumor suspensions of Ehrlich Ascites, Lymphosarcoma 6C3HED, Lymphoma L4946, Lymphoma P1534 and Neuroblastoma C1300, were given to all groups of experimental animals: newborn rats and young adult mice receiving 0.1 ml and young CFN adult rats 1.0 ml. Similar volumes of Melanoma S91, Myeloma X5563, and Mammary Carcinoma BW 10232 were administered by the subcutaneous route only.

All experimental and control animals were routinely inspected, and times of initial tumor appearance, regression, and death were recorded. Surviving animals were kept under observation for at least 28 days from the time of inoculation. If tumor growth in the newborn rats seemed questionably progressive, the tumor-bearing animals were divided into 2 groups. The first was sacrificed for gross and histological verification of the character and health of the transplanted tumor, and the second was maintained so that ultimate time of death and/or regression of the tumor could be recorded.

RESULTS

The results of the experiments fall into 3 groups—the first group comprising the 3 "moderately slow-growing" tumors, namely: the mammary carcinoma BW10232, myeloma X5563, and the melanoma S91; the second group consisting of 2 "rapidly-growing" tumors not adapted to the ascitis form—the neuroblastoma C1300 and the lymphoma P1534; and the third group made up of 3 "rapidly-growing" tumors which are maintainable by the passage of ascitic fluid—the Gardner Lymphosarcoma 6C3HED, the lymphoma L4946, and the Ehrlich Ascites tumors.

Table 1 reveals that there was never any demonstrable growth of the slow-growing mammary carcinoma, myeloma or melanoma in the newborn rat. In all but 1 case the inoculum resulted in progressive tumor growth and ultimate death in the mouse host. This group of neoplasms was

Table 1

GROWTH OF 3 "SLOW-GROWING" MOUSE TUMORS IN MICE AND NEWBORN RATS

Type of tumor	Growth in mice			Growth in newborn rats	
	Strain / no. takes†	T. palpable	T. death	Number inoculated	Number with tumors†
Mammary Ca. BW10232	C57BL/6 10/10	21	52	31	None
Melanoma S91	C3H 10/10	24	56	32	None
Myeloma X5563	DBA/2 9/10	35	77	39	None

T. palpable = average number of days from transplantation to appearance of tumor.
T. death = average number of days from transplantation to death.
† = Animals were regularly inspected for 60 days following transplantation. More than 50 per cent in each group survived this period.

also slow-growing in the indigenous hosts, compared with growth of the other tumors. Transplantation into young adult rats was unsuccessful, none of these tumors exhibiting progressive growth.

Tables 2 and 3 summarize the results of isotransplantation and heterotransplantation of the 5 "rapidly-growing" tumors in their indigenous mouse hosts and in newborn rats. It is at once apparent that for the adult mice the rate of tumor growth was faster and the time of death was much earlier than in the former group. In young adult rats, furthermore, the Ehrlich Ascites tumors grew subcutaneously in 3 of 12 animals and intraperitoneally in 12 of 23 animals before regressing. Rejection was usually complete by the 14th day after transplantation. Regardless of route of inoculation, none of the other mouse tumors achieved detectable growth at any time in the young adult rats.

On the contrary, all 5 of these "rapidly-growing tumors" reached a palpable size by the 7th to 10th day after subcutaneous transplantation into newborn rats, and all but the neuroblastoma grew intraperitoneally in this host.

At approximately the 10th day after heterologous transplantation, a divergent pattern becomes evident distinguishing those rapidly growing tumors which could be maintained by the passage of ascitic fluid in their indigenous mouse hosts (the lymphoma L4946, the lymphosarcoma 6C3HED, and the Ehrlich Ascites tumors) and those which could not be so passaged (the lymphoma P1534 and the neuroblastoma C1300). Table 2 clearly demonstrates that ultimate regression of the neuroblastoma C1300 was the rule. The considerable mortality occurring in those newborn rats injected with the lymphoma P1534 suggests that progressive growth fatal to the heterologous host frequently took place. Some of the animals developed subcutaneous masses measuring as much as 1.5 x 1.5 cm while others had considerably enlarged spleens. In contrast, autopsy of many of these dying rats revealed a very small or absent thymus, a small spleen, and no evidence of the transplant. Attempts at serial passage of this tumor in newborn rats have so far proved unsuccessful.

Table 3 demonstrates that the ascites forming 6C3HED, L4946, and Ehrlich Ascites tumors generally flourished in the newborn rat and if injected intraperitoneally resulted in progressive growth fatal within 28 days. Serial passage of ascitic fluid of each of these 3 tumors by intraperitoneal inoculation of newborn rats was readily accomplished. At this time, the Ehrlich Ascites tumor is in its 6th generation in newborn rats, and successful back-passage fatal to CF No. 1 mice has been accomplished after the 5th generation. The L4946 is in the 5th generation, the 6C3HED in its 3rd generation in newborn rats. Passage back to the indigenous mouse host was accomplished successfully, in each case at the previous generation.

No change in the characteristics or rate of growth have been detected in these serially-transplanted tumors either in the isologous or heterologous hosts. The newborn rats continue to succumb to progressive tumor growth by the 14th day after transplantation of the serially-passaged L4946, 6C3HED, and the Ehrlich Ascites tumor.

Autopsy of young rats dying as a consequence of intraperitoneal inoculation of any of the 3 Ascites-forming mouse tumors at birth usually revealed a bloody fluid associated with frequent tumorous nodules studding the omentum and dorsal peritoneal surfaces, while the spleen and thymus were grossly normal or enlarged. Occasionally after subcutaneous inoculation and much more rarely after intraperitoneal inoculation, considerable growth occurred but regression in size would be evident before death. Autopsy in these cases revealed a shrunken spleen and a

Table 2

GROWTH OF 2 "RAPIDLY-GROWING NON-ASCITIC" TUMORS IN MICE AND NEWBORN RATS

Name of tumor	Route of inoculation	Growth in mice			Growth in newborn rats					
		Strain no. takes†	T. palpable	T. death	Number inoculated	No. +/total no. surviving‡ 7 d	10 d	14 d	18 d	28 d
Neuroblastoma C1300	SQ	A/Jax 10/10	12	22	19	18/19	14/16	0/15	0/15	0/14
	IP	A/Jax 10/10	18	34	23	0/21	0/21	0/20	0/20	0/19
Lymphoma P1534	SQ	DBA/2 19/19	8	19	19	0/19	7/16	6/13	3/12	0/8
	IP	DBA/2 20/20	10	12	20	0/18	2/11	2/8	0/6	0/6

† = Number of animals with progressively growing tumors/total number inoculated.
‡ = Number of animals with palpable or ascitic tumors/total number surviving at this age.
d = Days after transplantation.
T. palpable = average number of days from transplantation to appearance of tumor.
T. death = average number of days from transplantation to death.

Table 3

GROWTH OF 3 "RAPIDLY-GROWING ASCITIC" MOUSE TUMORS IN MICE AND NEWBORN RATS

Name of tumor	Route of inoculation	Growth in mice			Growth in newborn rats					
		Strain / no. takes†	T. palpable	T. death	Number inoculated	No. + /total no. surviving‡ 7 d	10 d	14 d	18 d	28 d
Ehrlich ascites tumor	SQ	CF No. 1 / 10/10	5	31	19	15/17	17/17	7/7	5/2	2/4
	IP	CF No. 1 / 21/21	6	13	54	40/41	21/21	6/6	0/1	0/0
Gardner lymphosarcoma 6C3HED	SQ	C3H / 19/19	6	13	20	0/11	5/11	3/10	0/8	0/4
	IP	C3H / 19/19	7	11	41	26/28	10/13	5/5	2/2	0/0
Lymphoma L4946	SQ	AKR / 22/22	4	8	20	17/17	17/17	6/7	0/1	0/1
	IP	AKR / 21/21	5	8	41	37/37	15/15	1/1	0/1	0/0

† = Number of animals with progressively growing tumors/total number inoculated.
‡ = Number of animals with either palpable or ascitic tumors/total number surviving at this age.
d = Days after transplantation.
T. palpable = average number of days from transplantation to appearance of tumor.
T. death = average number of days from transplantation to death.

small or absent thymus as well as considerable necrosis of the tumor.

An important feature of these experiments, therefore, is the high mortality encountered in nearly all newborn rats inoculated with mouse tumors. Table 4 summarizes the mortality of the newborn rat after inoculation with the 8 transplantable mouse tumors and includes the results from 2 control groups, one of which consists of animals injected with either Tyrodes solution or saline at birth, while the other was identically handled but not injected.

Table 4

MORTALITY OF RATS INOCULATED AT BIRTH WITH 8 TRANSPLANTABLE MOUSE TUMORS

Material inoculated at birth		Number inoculated	Per cent mortality at intervals post-partum		
			7 days	14 days	28 days
Ehrlich Ascites	(IP)	54	24	89	100
Lymphosarcoma 6C3HED	(IP)	41	32	88	100
Lymphoma L4946	(IP)	41	10	98	100
Lymphoma L4946	(SQ)	20	15	65	95
Lymphosarcoma 6C3HED	(SQ)	20	45	50	80
Ehrlich Ascites	(SQ)	19	11	63	79
Lymphoma P1534	(IP)	20	10	60	70
Lymphoma P1534	(SQ)	19	0	32	58
Melanoma S91	(SQ)	32	6	42	42
Mammary Ca BW10232	(SQ)	31	9	35	40
Neuroblastoma C1300	(SQ)	19	5	21	26
Myeloma X5563	(SQ)	39	5	21	21
Neuroblastoma C1300	(IP)	23	9	13	17
Tyrodes	(SQ and IP)	79	3	6	11
Control		139	1	2	5

(IP) Intraperitoneal injection.
(SQ) Subcutaneous injection.

It is immediately evident that an increased mortality generally occurs when any mouse tumor is inoculated into the rat during the first post-partum day. The exceptions appear to be those rats injected at birth with either the myeloma or neuroblastoma. As previously indicated, many of the rats injected at birth with the lymphoma L4946, the lymphosarcoma 6C3HED, and the Ehrlich Ascites tumors died with large expanding subcutaneous masses or ascites, although occasionally the tumors appeared to be regressing at the time of death. In addition, many of those rats injected with the lymphoma P1534 died after complete regression of the tumor, and quite pronounced mortality of the rat host occurred although no heterologous growth of either the mammary carcinoma BW10232 or the melanoma S91 could be observed.

Finally, many of these young rats demonstrated markedly stunted growth, sparse hair, a hunched posture, and sometimes diarrhea until death. Autopsy of these rats has revealed that

the spleen is generally small, the thymus quite reduced, and that the mesenteric as well as other lymph nodes are usually absent. No other gross abnormalities are noticeable and the consistent histological feature is limited to a striking depletion of mature lymphoid elements, resulting in a great decrease of splenic white pulp and virtual absence of the cortical areas of the thymus. Sometimes necrotic residue of the lymphomas P1534 and L4946 could be identified microscopically in the thymus, but more often no recognizable tumor tissue was found.

DISCUSSION

Heterotransplantation of either normal or neoplastic tissue into the untreated adult animal has generally proved to be unsuccessful, although the rejection of the graft may be delayed and sometimes prevented if the heterotransplant is injected into the anterior chamber of the eye of the host,[3] or if the defenses of the host are weakened by irradiation and/or the administration of steroids.[4-8]

There appears to be a growing body of evidence demonstrating the successful homo- and heterologous transplantation of some tumors into some embryonic or newborn hosts.

The classic studies of Billingham, Brent and Medawar[9] demonstrated that when an animal is injected in utero with viable cells of a homologous strain, an inability to react against homografts of this same donor strain persists far into adult life. In apparent confirmation of this principle of acquired tolerance is the reported successful growth of the rat AH130 hepatoma and Yoshida sarcoma tumors injected in utero or intracerebrally into the newborn mouse.[10,11] Growth of the human tumors H Ep #1, H EP #3 and H Sub Rh #1 has been demonstrated in the chick egg, but is not sustained in the newborn chicken.[12] Again, while attempted transplantation of 80 human tumors into newborn mice failed,[13] growth of 9 of 34 human tumors in newborn Wistar rats has been reported[5] although serial passage beyond the second generation was not achieved. The mouse sarcoma 37[14] and the human H Ep #3 tumors[15] have been transferred successfully to embryo rats, but the mouse lymphoma L1210 apparently always regresses if inoculated into newborn rats.[16]

While growth of the Ehrlich Ascites tumor through many serial passages has been reported in adult rats[17] and in adult hamsters[18] it should be stressed that the host was not killed by the heterotransplant. It has also been clearly demonstrated[19] that cell death was complete 10 days after transplantation of this tumor from the mouse to young adult rats. We can confirm the fact that sustained growth does not occur in the adult rat, but progressive growth apparently fatal to the host usually results when it is transplanted into the newborn rat.

The lymphomas P1534, 6C3HED, and L4946 will also grow and kill their newborn rat host, particularly when inoculated intraperitoneally.

In contrast to the Ehrlich Ascites tumor we were unable to detect any palpable growth of these tumors in the young adult rat. Although factors such as the route of inoculation, dosage, genetic disparity, etc., may all prove critical, successful heterotransplantation under the experimental conditions described appears to be limited to those tumors with a more rapid growth potential. While few human tumors can approach the growth rate of these 4 transplantable tumors, the acute leukemias of man may do so. In view of the fact that serial heterotransplantation of a human lymphoma is not yet possible,[3-5,6-8,12,13,14,15] our data seem sufficiently encouraging to warrant further investigation of the newborn animal as a possible heterologous host.

One of the more intriguing aspects of these studies has been the relatively high mortality at

a time when the transplanted tumor may not be palpable. The not infrequent occurrence of animals with sparse hair and stunted growth, many of which died during the course of the study, and exhibited marked depletion of mature lymphoid tissues, is reminiscent of the "runts" encountered in homologous transplantation studies.[20,21] At least 3 possibilities suggest themselves. 1. Mouse lymphocytes, transferred with the tumor, exert an adverse effect on the rat. 2. Mouse tumor cells exert an adverse effect on the rat. 3. Responses in and by the rat to the foreign cells act adversely on the recipient.

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SERUM PROTEIN ABNORMALITIES IN "RUNTED" ALBINO RATS*

By

J. S. Thompson and C. W. Gurney

When newborn albino rats are injected with either normal or neoplastic tissues of mouse origin, occasional animals will develop a wasting disease characterized by roughened scaly skin, sparse hair growth, failure to gain weight normally, and sometimes actual weight loss.[1,2] This condition usually appears at 7 to 14 days of life and while many of the animals so afflicted succumb very shortly, others may survive for at least 35 days. Apart from sparse hair and stunted growth, these survivors are apparently normal.

The same phenomenon is occasionally observed following intraperitoneal inoculation of normal adult DBA/2 mouse spleen cells into newborn albino rats,[1] but it occurs much more frequently after subcutaneous, intraperitoneal,[2] or intracerebral injection of one of several transplantable mouse tumors into this host. The transplantable mouse tumors so far associated with this disease are the Ehrlich Ascites tumor, the lymphoma L4946, the lymphoma P1534, the lymphosarcoma 6C3HED, the mammary carcinoma BW10232, the melanoma S91 and the neuroblastoma C1300. Many of the young rats appeared to expire as a direct result of the growth of the Ehrlich Ascites tumor, the lymphoma P1534, the lymphoma L4946 and the lymphosarcoma 6C3HED, but there were individuals in which the wasting condition became evident during or after tumor regression. Furthermore, this disorder occurred rather frequently after inoculation of the mammary carcinoma and the melanoma, neither of which ever demonstrated palpable growth.

This "runted" rat is interesting because it is grossly similar to the runts described in homologous transplantation studies.[3-5] Autopsied animals dying with the disorder reveal some of the general characteristics noted by Billingham and Brent;[3] the spleen is small, the thymus absent or quite small, the mesenteric lymph node usually absent, and lymphogenous tissue generally reduced. When we exclude from consideration those rats appearing underdeveloped at birth and those which were survivors of litters having a high initial mortality, only 6 animals which might be grossly classified as runts have been encountered in 251 control rats injected with Tyrodes solution or not injected at all. Unfortunately, these were not autopsied, since the significance of the condition had not then been realized. In contrast, 15 of 23 rats inoculated at birth with the mammary carcinoma BW10232 developed the runting disease.

Since other abnormalities of the reticuloendothelial system are probably present in the runted animals, we have begun an intensive investigation of the serum proteins. So far this investigation is limited to those animals surviving the first 5 weeks of life. At this age, normal littermates weigh 75-100 g, whereas the runts weigh from 35 to 55 g. This paper constitutes a preliminary report on the serum protein fractions in these animals.

Tissue suspensions of mouse melanoma S91, mammary carcinoma BW10232, and normal DBA/2 spleens were prepared by mincing with fine dissecting scissors followed by gentle manipulation in a glass homogenizer to which Tyrodes solution had been added. Newborn CFN rats

*Based on a report presented at the Meeting of Radiation Research Society in San Francisco, May 9-11, 1960. Accepted for publication in Nature.

were injected with approximately 20 x 10^6 cells from one of these suspensions. The normal spleen cells were administered intraperitoneally, whereas the tumor cells were injected subcutaneously. Control groups of rats received either 0.1 ml of Tyrodes solution or no injection at all. The animals were inspected at intervals of 2-4 days and were weighed each week.

Following the weighing at 5 weeks of age, blood was drawn by cardiac puncture, and paper electrophoresis of the serum proteins carried out with a Spinco Model RB electrophoresis apparatus. Serum aliquots of 0.008 ml were subjected to electrophoresis at 4.0 ma/cell for 18 hours at room temperature. The strips were dried, stained, and analyzed with a Model RB analytrol.

The serum protein fractions are indicated in Table 1. It can be seen that the beta and particularly the gamma globulins are elevated in those rats with subnormal weight. As Figures 1

Table 1

SERUM PROTEIN FRACTIONS IN 5-WEEK-OLD NORMAL AND RUNTED ALBINO RATS INJECTED AT BIRTH WITH MOUSE TISSUES

Material injected at birth	Number tested	Mean weight (g)	Serum protein fraction			
			Albumin (per cent)	α_1 and α_2 (per cent)	β (per cent)	γ (per cent)
None (untreated control)	15	88	60.6	20.8	14.7	3.9
Tyrodes solution* (control)	9	90	60.2	20.9	14.6	4.3
Adult mouse spleen†	6	84	58.2	21.7	15.8	4.3
Mammary carcinoma‡	3	89	60.5	20.0	14.8	4.7
Melanoma**	4	88	61.9	20.8	14.0	3.3
Adult mouse spleen†	7	45	45.0	20.3	21.0	13.7
Mammary carcinoma‡	3	31	48.1	20.6	18.6	12.7
Melanoma**	3	42	48.7	21.4	16.9	13.0

*Tyrodes solution is used in preparation of the cell suspensions.

†From 12- to 16-week-old DBA/2 female mice.

‡Mammary carcinoma BW10232 indigenous to the C57BL/6 strain of mice.

**Melanoma S91 indigenous to the DBA/2 strain of mice.

and 2 further indicate, the correlation is not with the type of tissue injected at birth, but rather with the fact that a runt was produced by 5 weeks of age. For example, approximately 20 x 10^6 normal adult DBA/2 mouse spleen cells were injected intraperitoneally at birth into two rats, yet as shown in Figure 1, only the runt had an increased percentage of beta and gamma globulins. Furthermore, when melanoma S91 cells were inoculated subcutaneously into another pair as illustrated in Figure 2, the increased globulin percentages were present in the runts but not in the normal animals.

The 6 untreated animals that died with the gross characteristics of wasting prevent us from concluding with certainty that the disease is directly attributable to the injection of normal or neoplastic mouse cells. We share with Billingham and Silvers[4] the opinion that in some instances the effect of this inoculation may rather be to aggravate or enhance another as yet unrecognized

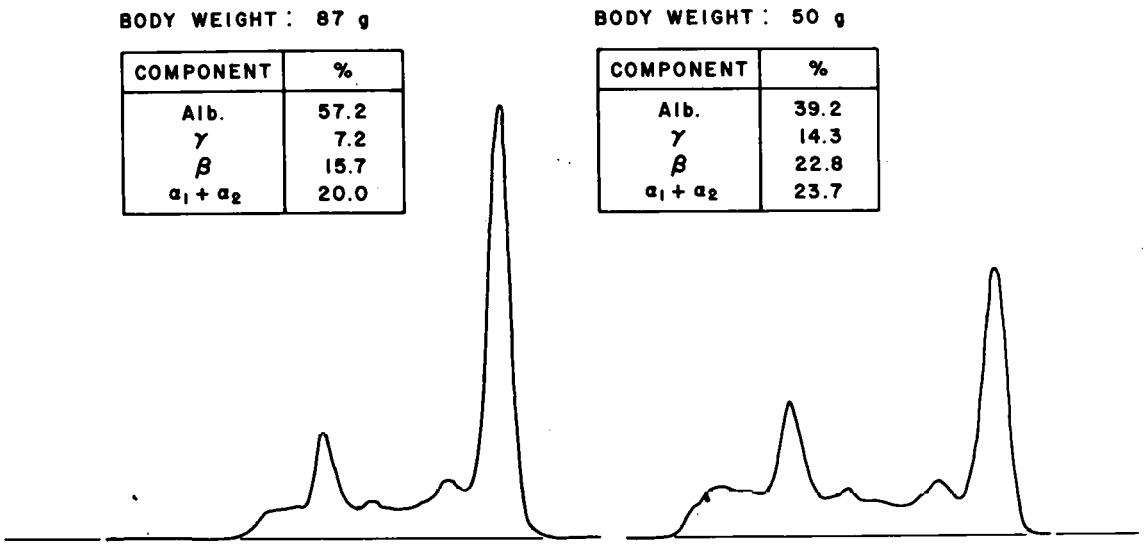

Figure 1. Paper electrophoresis of serum obtained from 5-week-old albino rats inoculated at birth with normal adult mouse spleen cells. Note the decreased albumin and increased beta and gamma globulin percentages in the animal with subnormal weight.

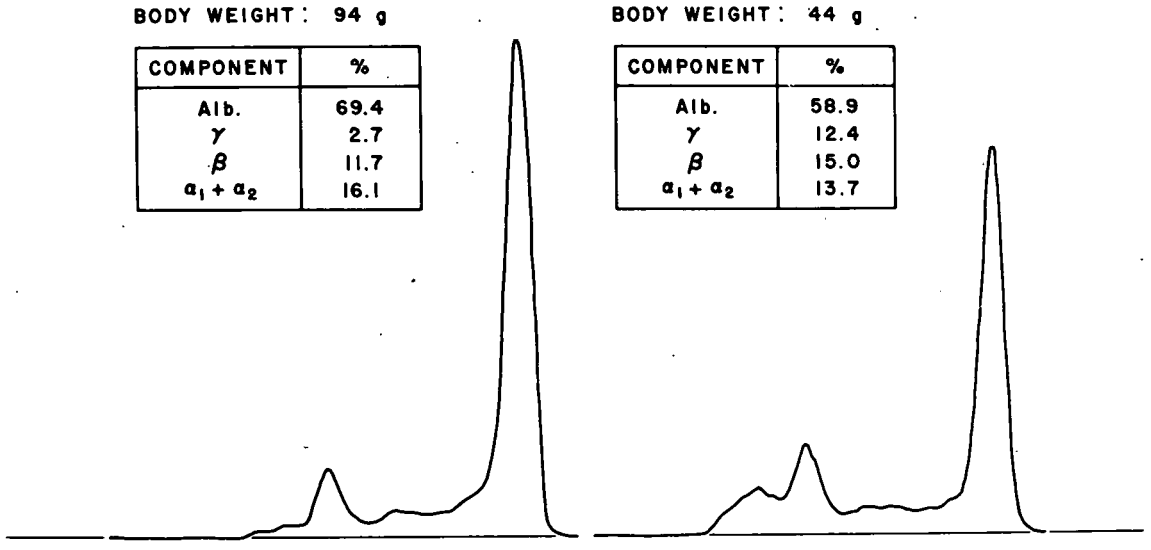

Figure 2. Paper electrophoresis of serum obtained from 5-week-old albino rats inoculated at birth with melanoma S91 cells. Note the decreased albumin and increased beta and gamma globulin percentages in the animal with subnormal weight.

factor. These authors have also described a runted rat which follows the injection of homologous adult rat spleen or bone marrow cells. The most obvious feature of the homologous disease so produced was a severe dermatitis. Anderson and co-workers,[5] however, have described runt disease produced by the intravenous injection of homologous thoracic duct lymphocytes into albino rats without reporting any skin involvement. Neither have we observed severe dermatitis in the runt disease of our albino rats following the inoculation of heterologous mouse tissues. Although these discrepancies may be due to the fact that different strains of rats were used by each group of workers, we must consider the possibility that different etiologic mechanisms are operating in the three experimental systems. Nevertheless, our data indicate that whatever the etiology of the runt encountered by us, the production of an unusually high percentage of gamma and beta globulins results.

Of particular interest is the recent work of Gengozian,[6] who reports a consistent elevation in the lipoproteins (gamma and beta globulins) in the radiation-induced rat-to-mouse chimera. This abnormally high fraction seems to be produced by the mouse host and not by the donor rat tissues. Using similar techniques, we intend to measure quantitative changes in proteins with a view to determining directly whether the higher percentages of beta and gamma globulins observed by us are of host rat or donor mouse origin.

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THE TUMOR-INHIBITORY EFFECTS OF 3-METHYLCHOLANTHRENE ON A TRANSPLANTABLE AND 3-METHYLCHOLANTHRENE-INDUCED TUMORS IN C3H MICE*

By

J. S. Thompson, C. W. Gurney, and W. H. Kirsten†

It has been reported that certain of the carcinogenic hydrocarbons will retard the growth of various transplantable tumors.[1-4] Only a few studies[5-7] have been made on the effect of these agents on the growth of either induced or spontaneous tumors in experimental animals. Huggins and McCarthy[8] have found some regression of human metastatic mammary cancer following the administration of 3-methylcholanthrene, while Bauer et al.[9] have demonstrated regression of cancer of the skin in 7 out of 22 patients treated with 3,4-benzpyrene.

This study is designed to show that a dosage of 3-methylcholanthrene effective in the treatment of a transplantable tumor is also effective in retarding the growth of induced neoplasms and in prolonging life in the same highly inbred strain of mice.

MATERIALS AND METHODS

3-methylcholanthrene and dihydrotestosterone were prepared for injection as follows. Commercially-purchased[a] 3-methylcholanthrene was dissolved in sesame oil by heating to 85° C for 3 to 4 hours, and the final concentration adjusted so that 0.1 ml of mixture contained 0.2 mgm of 3-methylcholanthrene. Dihydrotestosterone[b] was dissolved in this solution or in sesame oil alone so that its concentration was 0.2 mgm/0.1 ml solution.

Unless otherwise indicated, the treatment and tumor induction dosage schedules consisted of daily subcutaneous injections of 0.2 mgm 3-methylcholanthrene in 0.1 ml of sesame oil over a period of 4 weeks. Control animals received 0.1 ml of sesame oil subcutaneously. All injections were made into the ventral abdominal wall unless otherwise indicated.

The C3H mice used were from the Roscoe B. Jackson Memorial Laboratories, Bar Harbor, Maine (C3H/Jax) or from the Cumberland Farms, Clinton, Tennessee (C3H/Cumb). The strain, sex, and age are indicated for each experiment. The animals were given the Rockland Mouse Diet complete and water ad libitum. When the date of birth was not identical, the mice were randomized among experimental and control groups.

In experiments with transplantable tumors, the myeloma X5563 was routinely passed subcutaneously to the dorsal surface of C3H/Jax mice by the trocar technique. Male and female mice 10 to 14 weeks of age were inoculated. In the first experiment, injections of sesame oil or 3-methylcholanthrene were given starting 3 days after transplantation. The animals were inspected and weighed at weekly intervals, and after four weeks were weighed and sacrificed. Tumors de-

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*Based on a paper appearing in Cancer Research, 20:1214, 1960.

†Department of Pathology, University of Chicago.

[a]Eastman Organic Distillation Products Industries.

[b]Mann Research Laboratories.

tectable at autopsy were carefully dissected out and weighed. Samples of tumor, spleen and liver were fixed in Zenker's formol solution, imbedded in paraffin, and stained with haematoxylin-eosin-azure, for histological examination.

In the second experiment, the mice were divided into 4 groups three days after transplantation of the myeloma. Daily treatment with sesame oil, 3-methylcholanthrene, dihydrotestosterone, or dihydrotestosterone and 3-methylcholanthrene was continued for 4 weeks. The animals were then weighed and sacrificed. The tumor mass was removed and weighed, but no histological preparations were made.

In the third experiment, the tumor was allowed to grow until detectable, and the animals were then randomized on the basis of tumor size. Therapy with methylcholanthrene or sesame oil was instituted 30 days after tumor transplantation, and the tumors were measured over a period of 4 weeks at weekly intervals. A continuous record of time of death was maintained. After 4 weeks, treatment was stopped, but the survivors were followed to trace the course of any possible "cures."

Induced-tumor experiments. For induced-tumor studies, only C3H/Cumb females 12 to 14 weeks of age were used. In the first experiment, 30 mice received sesame oil and 30 received 0.2 mgm of 3-methylcholanthrene injected subcutaneously into the ventral abdominal area daily for 4 weeks.

Fourteen weeks after tumor induction, all the surviving animals were weighed. Total white blood count and hematocrit determinations were made, the mice were autopsied, and tumors removed. The liver, spleen, kidney, and tumor were weighed and sections taken for histological examination.

In the second experiment tumors were induced in a group of 80 mice by treatment with 3-methylcholanthrene as in the first experiment, but injection of the carcinogen was begun two weeks later. Approximately 14 weeks after institution of induction treatment, the survivors were divided into two groups alike as far as general condition, body weight, and tumor size were concerned. Tumor size was estimated as the product of two dimensions measured with calipers. One of these groups received four weeks of therapy with 3-methylcholanthrene begun 10 weeks after the end of the induction period. The other group received therapy with sesame oil for a like period and served as a control. Injections were made subcutaneously into the dorsal surface away from the tumors. The mice were inspected regularly and mortality recorded accurately until the last animal expired. Tumor size was measured on the 7th, 15th, and 28th days of the therapeutic regime.

RESULTS

The influence of 3-methylcholanthrene on the transplantable myeloma X5563 is shown in Table 1 which summarizes the data pooled from 3 separate experiments, all performed in identical fashion. It can be seen that growth of the myeloma X5563 in C3H mice was consistently depressed by 3-methylcholanthrene if treatment with this carcinogen was instituted within 3 days of tumor transplantation. In those mice with a palpable tumor mass the average weight of the resected tumors treated with sesame oil was twice as great as in those treated with 3-methylcholanthrene.

The histological picture revealed a distinct contrast between tumors from mice treated with 3-methylcholanthrene and those from controls. In the latter, necrosis was usually not a

Table 1

THE EFFECTS OF 3-METHYLCHOLANTHRENE ADMINISTERED 3 DAYS AFTER TRANSPLANTATION ON THE GROWTH OF THE MYELOMA X5563 AND ON THE HOST C3H MICE

Treatment group	Sex	No. Mice	Effect on tumor growth			Effect on body weight of host	
			No. + total*	Without tumor (per cent)	Ave. weight of tumor† (g)	Ave. gross weight change‡ (g)	Ave. net weight change** (g)
Sesame oil	M	36	$\frac{35}{36}$	3	3.5	+ 4.6	+ 1.0
	F	19	$\frac{19}{19}$	0	3.3	+ 5.1	+ 1.8
3-Methylcholanthrene	M	36	$\frac{23}{36}$	36	1.3	+ 0.7	- 0.1
	F	28	$\frac{16}{28}$	43	1.1	+ 3.3	+ 2.7

*Number of mice with tumors/total number injected.

†Average weight based only on those mice with tumors.

‡Gross weight change = the weight of the mice with or without tumor after 28 days of therapy less their weight at the onset of therapy.

**Net weight change = the weight of these mice minus the weight of the tumor when present less their weight at the onset of therapy.

prominent feature. It sometimes occurred in very large tumors, but always in a central location sparing the peripheral borders. Mitotic figures were quite prominent throughout and almost no fibrous tissue was present. The carcinogen-treated tumors revealed a great reduction in number of mitotic figures, many of which were bizarre. Peripheral necrosis and fibrosis were prominent features. In transition from these areas of frank necrosis, bizarre cells with disrupted nuclei and vacuolated cytoplasm were seen. A distinct infiltration with leucocytes, or normal plasma cells, however, was not characteristic. Figures 1 and 2 demonstrate some of these features.

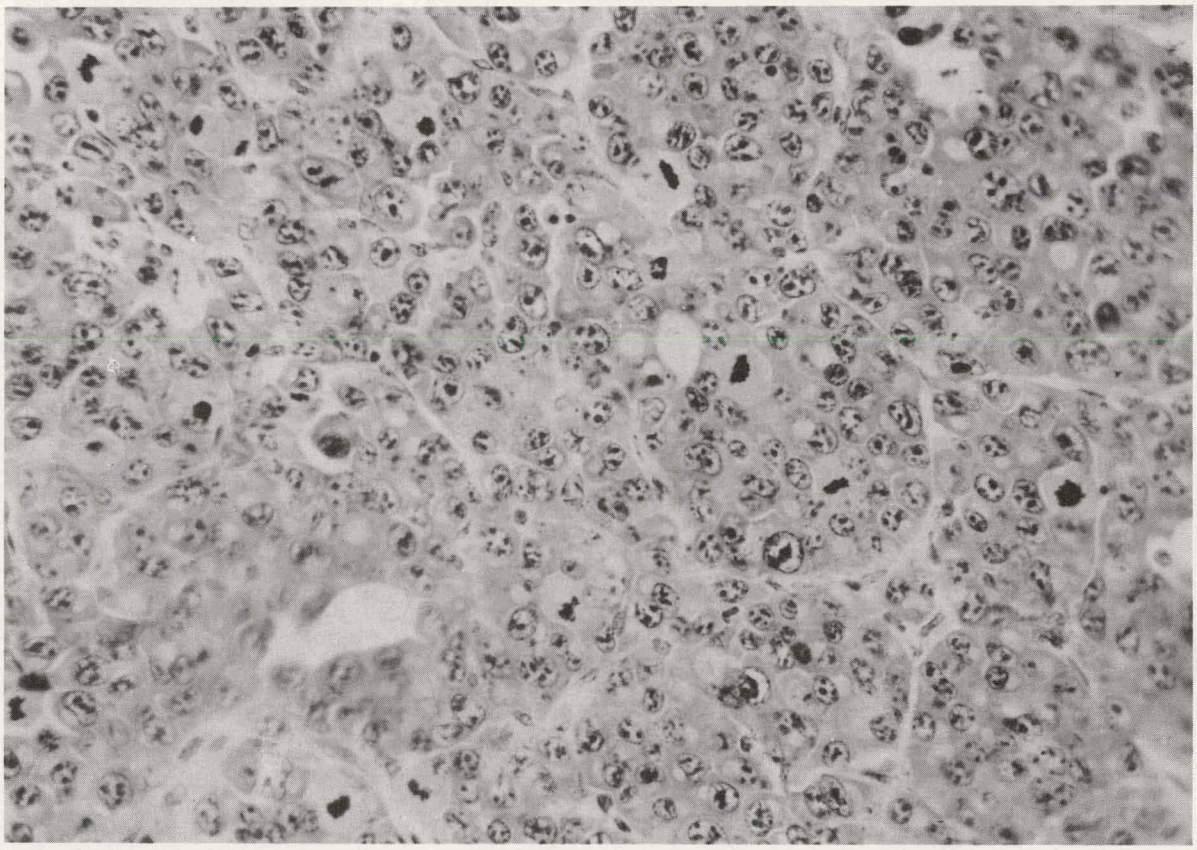

Figure 1. Photomicrograph of the myeloma X5563 excised after 28 days of therapy with control sesame oil. Tumor weighed 4.4 g. Note at least 14 mitotic figures are present. (Hematoxylin-eosin-azure, X 470).

Table 1 further demonstrates that the tumor-inhibitory effect of the carcinogen was not associated with general retardation of growth in the host. Excluding the weight of the excised tumors, the net difference in body weight between the control and 3-methylcholanthrene-treated animals is negligible.

Further evidence that tumor inhibition is not dependent on a general growth suppressive effect exerted by 3-methylcholanthrene is demonstrated in Table 2. Administration of dihydrotestosterone led to a slight increase over the expected net weight change in mice treated with sesame oil or 3-methylcholanthrene. A striking increase in weight resulted when this androgenic hormone was combined with 3-methylcholanthrene for 4 weeks of therapy, although it had no demonstrable influence on the transplanted myeloma.

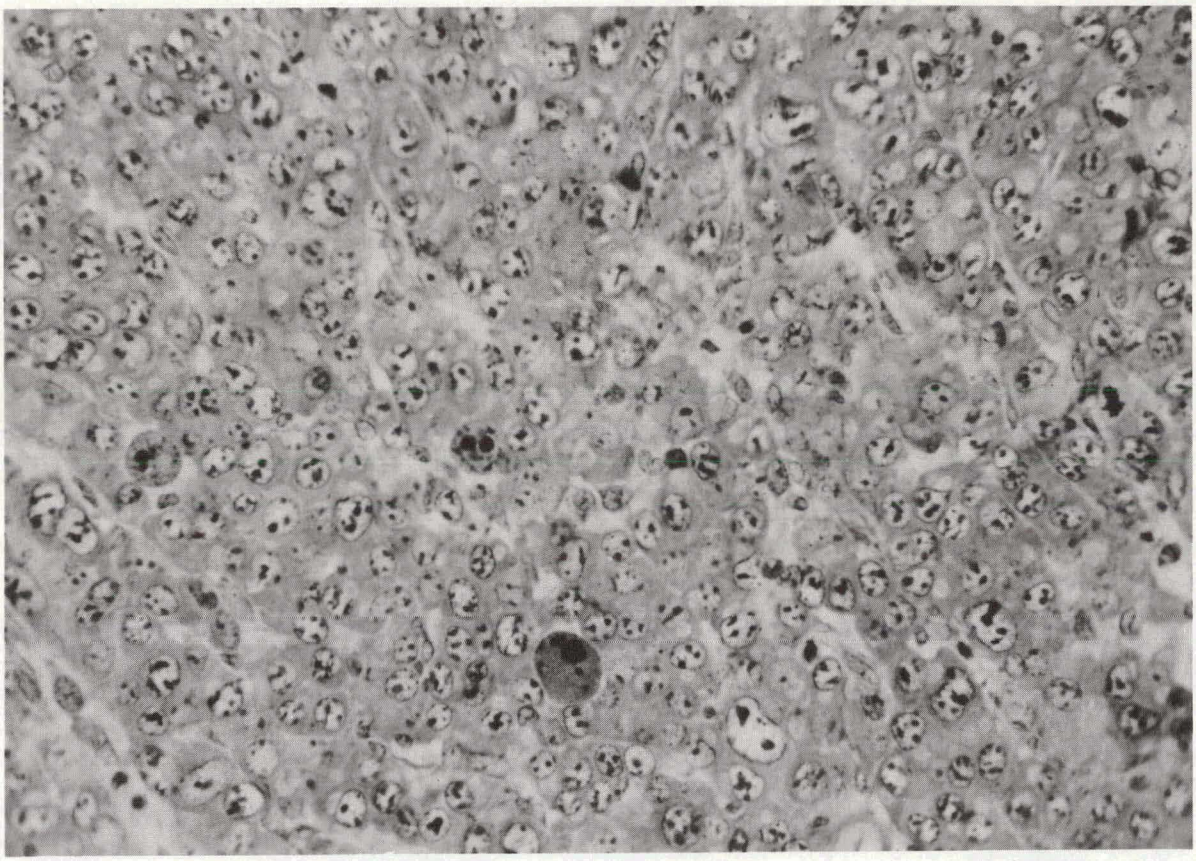

Figure 2. Photomicrograph of the myeloma X5563 excised after 28 days of therapy with 3-methylcholanthrene. Tumor weighed 2.1 g. Note the reduced mitotic figures and the large bizarre cells. (Hematoxylin-eosin-azure, X 470).

The phenomena of tumor retardation and/or regression induced by the carcinogenic agent are demonstrated in the third experiment. Those animals with palpable masses 30 days after inoculation were divided into two groups on the basis of tumor size. Figure 3 shows that the last control animal died 29 days after the beginning of treatment, when 42 per cent of the carcinogen-treated animals were still alive. Furthermore, the 4 animals which still survived at 65 days gave no evidence of the presence of a transplantable tumor. If the tumor was considered "small" at the onset of therapy, complete regression occurred.

Of the group of 30 C3H ♀ mice receiving 3-methylcholanthrene subcutaneously for a period of 28 consecutive days, 18 were alive 14 weeks after institution of therapy. These animals appeared chronically ill with hunched posture, diarrhea, and ruffled hair. The ventral abdominal surface was nearly replaced by one or more poorly-defined tumor masses. Bleeding necrotic ulcers were frequently present in the tumor.

All 30 sesame oil controls were alive at this time and appeared to be grossly normal. The average body weight of mice with chemically-induced tumors was 20.5 g, the hematocrit 36.4 per cent, and the white blood count 30,200. Compared with these values were an average body weight of 23.1 g, hematocrit of 51.7 per cent, and a white blood count of 12,700 in the controls.

Autopsy of the surviving mice revealed that no gross or histological lesions were present

Table 2

THE INFLUENCE OF DIHYDROTESTOSTERONE AND/OR 3-METHYLCHOLANTHRENE ON THE GROWTH OF THE MYELOMA X5563 AND THE HOST C3H MICE ADMINISTERED 3 DAYS AFTER TRANSPLANTATION

Treatment group	Sex	Growth of tumor			Growth of C3H mice	
		No. + / total*	Without tumor (per cent)	Weight of tumor† (g)	Gross weight change** (g)	Net weight change‡ (g)
Sesame oil	M	7/7	0.0	1.9	+ 2.5	+ 0.6
	F	8/8	0.0	1.8	+ 3.6	+ 1.8
3-Methylcholanthrene	M	5/6	17	0.6	+ 1.3	+ 0.7
	F	6/10	40	0.6	+ 3.9	+ 2.4
Dihydrotestosterone and sesame oil	M	9/9	0.0	1.6	+ 4.2	+ 2.6
	F	9/9	0.0	1.8	+ 5.6	+ 3.8
Dihydrotestosterone and 3-methylcholanthrene	M	6/9	33	0.4	+ 7.4	+ 7.2
	F	5/8	38	0.5	+ 7.3	+ 6.8

*Number of mice with tumors/total number injected.

†Average weight based only on those mice with tumors.

**Gross weight change = the weight of the mice with or without tumors after 28 days of therapy less their weight at the onset of therapy.

‡Net weight change = the weight of these mice minus the weight of the tumor when present less their weight at the onset of therapy.

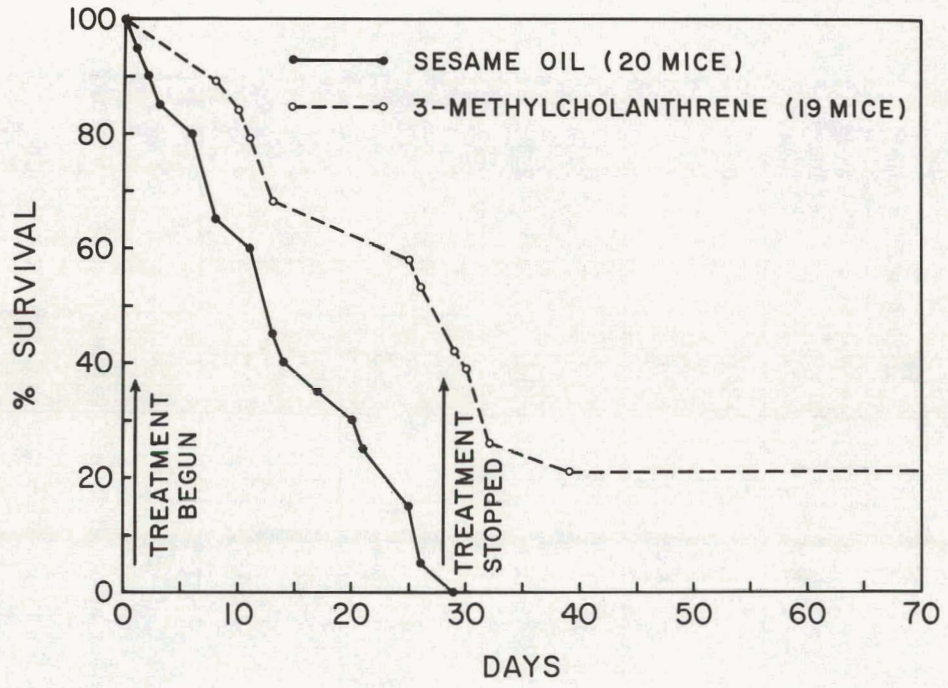

Figure 3. The effect of 3-methylcholanthrene on the survival of C3H mice transplanted 30 days previously with myeloma X5563.

in the control animals. The tumors in the carcinogen-treated group were locally quite invasive, involving the skin, subcutaneous tissue and muscle, and even extending into and through the peritoneum, although no gross distant metastases were detected. The spleen was usually enlarged, averaging more than 3 times the weight of that in the control animals. No other gross abnormalities were detected. Histological examination revealed that the induced neoplasms included 13 fibrosarcomas, 1 squamous cell carcinoma, 2 lesions that were either epitheliomas or epidermal carcinomas. Also, mixed carcinosarcomatous lesions were present in 2 animals injected subcutaneously with 0.2 mgm 3-methylcholanthrene for 28 days. Figure 4 illustrates one of those tumors classified as a carcinosarcoma. Metastases were not demonstrated microscopically in the organs examined. Extramedullary myelopoiesis was greatly increased in the spleens of all the tumor-bearing mice. The liver was involved to a lesser degree and in some cases myelopoiesis was present in the kidneys.

The influence of 3-methylcholanthrene on the induced tumors is evident in the explosive onset of induced tumors which was again remarkable in the group of 80 animals treated with the 3-methylcholanthrene. Fourteen weeks after the beginning of the induction phase, 67 of these mice were found to possess tumors grossly identical with those of the previously described groups.

Table 3 demonstrates the suppressive influence of subsequent 3-methylcholanthrene therapy on growth of these induced tumors as compared with sesame oil treatment. When one considers that only the surviving animals were measured on the 7th, 15th and 28th days, the effect may be more dramatic than these figures indicate. It should be stressed that accurate measurement of

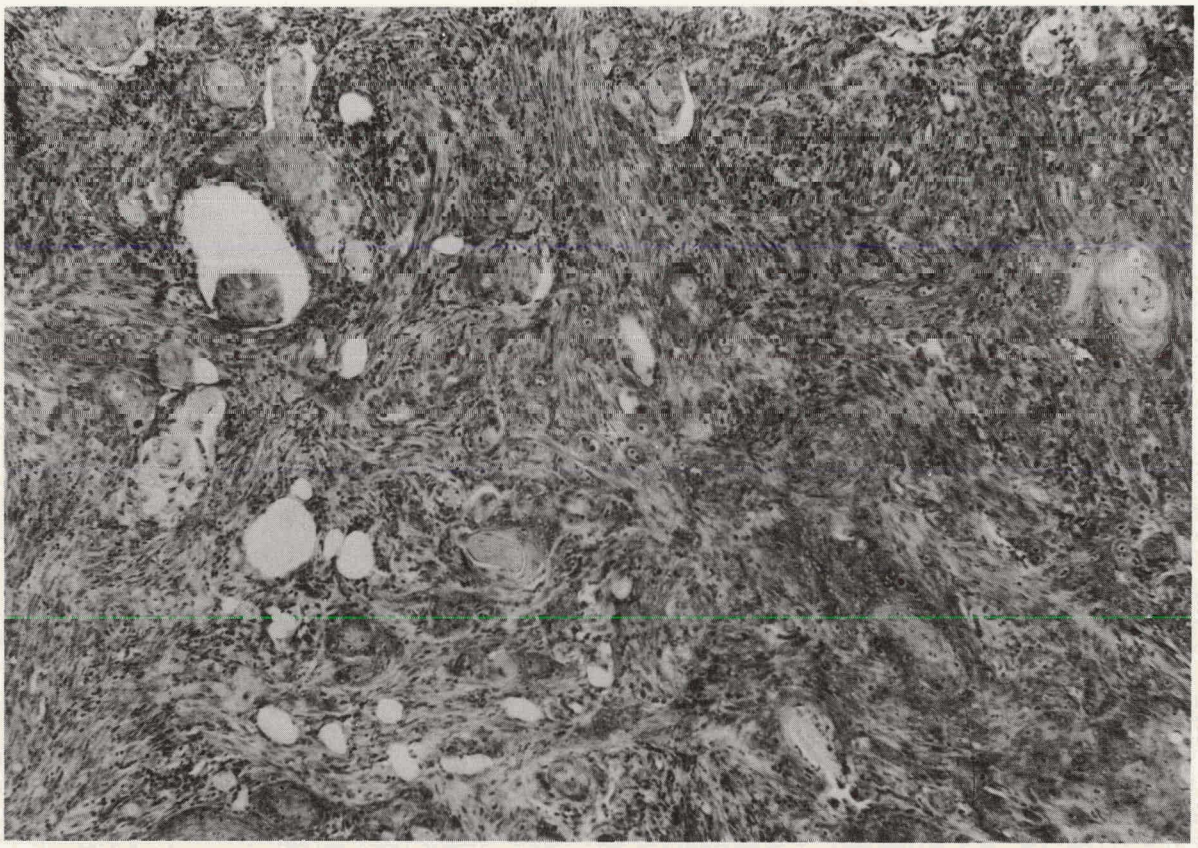

Figure 4. Photomicrograph of a tumor induced by 3-methylcholanthrene. Note the mixed elements of fibrosarcoma and epidermoid carcinoma. (Hematoxylin-eosin-azure, X 125).

Table 3

THE EFFECT OF 3-METHYLCHOLANTHRENE ON THE GROWTH OF 3-METHYLCHOLANTHRENE-INDUCED NEOPLASMS IN C3H MICE

	0 days†		7 days†		15 days†		28 days†	
	N	T	N	T	N	T	N	T
Sesame oil	34	6	27	7	15	7	0	0
3-methylcholanthrene	33	6	33	5	26	5	21	5

† = Days after institution of therapy.
N = Total number of surviving mice.
T = The average of the product of two dimensions measured with calipers.

these induced tumors was not possible due to their highly invasive character. In general, those animals with larger tumors or with tumors that ulcerated early, died more rapidly. A definite prolongation of life was achieved by the carcinogen therapy. After a 28-day course of this treatment, 21 of 33 animals survived, whereas the last of the 34 controls expired on this day. No further treatment was given and all the experimental animals died within another 27 days.

DISCUSSION

Green[3,10] has suggested that the tumor-inhibitory properties of the polycyclic hydrocarbons are dependent on their ability to enhance the natural immunity of the host. He has pointed out that the highest incidence of transplantable tumor regression following therapy with one of these agents occurs when the incidence of natural regression is itself the highest.[3] Although this information comes almost entirely from studies on transplantable tumors in relatively non-inbred strains of rats,[1-4,6,7] Rubin[11] has recently reported data suggesting that this is also true in mice.

Green[3] has expressed the view that if a tumor grows without natural regression in one highly inbred strain of mice only, its inhibition by the distal application of polycyclic hydrocarbons is much less likely.

The data here presented are not in agreement with this theory. The myeloma X5563 arose spontaneously in the C3H mouse, has since been serially passaged in this inbred strain by Potter,[12] and growth in any other strain has not been reported. When treatment with the carcinogen was begun within the first 3 days of transplantation, growth was inhibited in 39 per cent of the animals. In the controls, growth occurred in all but one instance. Furthermore, suppression and complete regression still took place when therapy was delayed until 30 days after transplantation. In contrast we have encountered no instance of spontaneous regression of this tumor once established in several hundred C3H/Jax mice.

It is Green's belief[3,10] that inhibition of either spontaneous or induced tumors by a polycyclic hydrocarbon should not occur, because there is no host-tumor resistance under these circumstances. Carcinogenic agents have now been described, however, which occasionally alter growth of chemically-induced sarcomas in rats,[6] suppress growth of spontaneous mammary carcinomas in mice,[5,7] and sometimes initiate regression of mammary carcinomas[8] and skin cancer[9] in the human. We have further demonstrated a suppressive effect exerted by 3-methylcholanthrene on epidermoid carcinomas and fibrosarcomas induced in C3H mice.

Although the tumor-inhibitory action of carcinogenic agents may sometimes be due to a general depression of somatic growth,[7,13] or to the dietary protein content,[14] this is not always so. Our studies, designed with reference to the findings of Huggins and Pollice[4] and Glenn and coworkers,[15] reveal a potentiation by 3-methylcholanthrene and dihydrotestosterone as shown by change in body weight of mice inoculated with the myeloma X5563. Here it is interesting to note that potentiation of weight increase did not extend to the myeloma, thus suggesting that the tumor-inhibitory property of 3-methylcholanthrene is distinct from its ability to influence somatic growth.

To conclude that some degree of immunological incompatibility cannot occur between a host and its spontaneous or externally-induced tumor, would be extremely hazardous. If such exists, tumor inhibition may in part be due to an enhancement of this immunity, although Rubin's results[11,16] suggest that the effect of 3-methylcholanthrene is to depress rather than augment the immunological response in mice.

Finally, it is possible that there is a more direct attack on the cancer cell. Shubik and Porta[17] have presented strong evidence that the effects of 3-methylcholanthrene, 3,4-benzpyrene, and 9,10-dimethyl-1,2-benzanthracene administered as large single doses to normal adult mice, are strikingly akin pathologically to those induced by X-rays[18] or nitrogen mustard.[19] Our tentative opinion is that the characteristics of peripheral necrosis, reduction of mitotic figures,

without a prominent infiltration of host leucocytes and plasma cells are similar to those seen when tumors are exposed to other cytotoxic agents.

ACKNOWLEDGMENT

We are indebted to Dr. Charles B. Huggins whose encouragement and advice stimulated the completion of these experiments.

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CHARACTERISTICS AND MANUFACTURE OF RADIOISOTOPES FOR MEDICAL PURPOSES AT THE ARGONNE CANCER RESEARCH HOSPITAL*

By

P. V. Harper

With a view to adding to the number of radioisotopes at present available from such sources as Oak Ridge, Brookhaven and various commercial firms, we have been interested at the Argonne Cancer Research Hospital in exploring the problems involved in the production and medical application of new and untried radioisotopes. Many facilities are available to us for production purposes, and we are fortunate in having at our disposal the resources of the Atomic Energy Commission's installations throughout the country.

Activation experiments involving reasonably short-lived isotopes are carried out at the nearby CP-5 reactor at Argonne National Laboratory, having at present a maximum thermal-neutron flux of 3×10^{13}, which is to be doubled in the near future. For experiments requiring a higher thermal-neutron flux, the facilities of the Materials Testing Reactor at Idaho Falls, Idaho, have been used extensively. On occasion we have also made use of the Brookhaven reactor, the Oak Ridge reactor, and the small reactor at the Armour Research Foundation in Chicago. For experiments requiring proton bombardment, we use the Argonne National Laboratory cyclotron as well as the 450 MEV instrument at the University of Chicago. For production purposes, however, the 86-inch cyclotron at Oak Ridge, which has a beam current of up to 2 milliamperes, promises to be of the greatest help. We have received only the most enthusiastic cooperation from the scientists and engineers at all these installations.

Our equipment at Argonne Cancer Research Hospital consists of a small cave with an 18-inch thick zinc bromide window equipped with remote control manipulators, and is fully adequate for most purposes. For experiments that involve the handling of extremely high activities, the large caves at Argonne National Laboratory are available. In connection with our own cave are a well-equipped general chemical laboratory, as well as unlimited facilities for animal work. In addition to the usual instrumentation, the Argonne type 256 channel pulse height analyzer has been invaluable for detecting trace impurities and following the course of separations. The Argonne Cancer Research Hospital itself is equipped with an excellent machine shop as well as with an electronic shop of very high caliber. Two additional potential facilities are the shielded low-level counting room which has five 5 x 5 inch sodium iodide crystals integrally mounted in photomultiplier tubes, and the 50 MEV electron linear accelerator. The latter is capable of producing useful quantities of short-lived radioisotopes by the gamma, n reaction after converting the electron beam to bromstrahlen in a high z converter plate. The beam current presently available is not yet sufficient for the production of useful quantities of short-lived materials, such as O^{15}, but plans for an improved cathode design are in progress, by which it is hoped to increase the available beam current by a factor of 10.

*This paper was delivered at the 7th International Electronics and Nuclear Exposition in Rome, Italy, June 20 - 26, 1960.

In producing a radioisotope for clinical and particularly for therapeutic purposes, one of the prerequisites is that it should be readily available at any time at a reasonable cost, and much of our work has been involved in engineering problems directed toward these goals. Our own particular field of interest has been those isotopes decaying by electron capture and those emitting low energy gamma rays. These have peculiar advantages in ease of handling because shielding problems become negligible, and also because when used as agents for implant therapy they produce a relatively much more localized radiation field. In diagnosis they permit the use of much lighter and more efficient collimators and at the same time are capable of producing a much higher degree of contrast in cold nodules such as are seen in the thyroid or liver, due to the fact that the absorption of soft x-rays and low energy gamma rays is significant in a cm, or two of tissue.

Patients for trial of experimental procedures come to us through my own contacts as a practicing surgeon and especially through the courtesy of Department of Radiology, University of Chicago. During treatment with radioactive materials or in subsequent follow-up studies, these patients are housed on the hospital floors of the Argonne Cancer Research Hospital, which functions as a wing of the University of Chicago Hospital.

RADIOISOTOPES AS THERAPEUTIC AGENTS

As a surgeon frequently concerned with the care of patients suffering from advanced malignancy, I have been interested in finding therapeutic agents suitable for use in conjunction with surgical procedures which are relatively safe, since such use almost necessarily involves exposure hazards for the operator. Palladium-103 at least partially fulfills these requirements. It decays by electron capture to rhodium-103 which then emits characteristic fluorescent radiation of rhodium the useful portions of which are the K alpha line (20.2 KEV, 80 per cent) and the K beta line (23 KEV, 20 per cent). The accompanying gamma rays are either extremely weak (10^{-4} to 10^{-6} photons/disintegration) or are almost completely converted in the L (.040 KEV) or the K (.053 KEV) shells. For practical purposes, there are then only the very weak gamma, the intense K lines, the conversion electrons and L x-rays which are completely attenuated by a few microns of tissue, and thus cause little or no tissue damage when the isotope is deposited in the tissue as a number of discrete sources. In considering the dosimetry of this material two factors lead to great simplification. First, most of the attenuation of the radiation of the tissue is by photoelectric absorption so that the effects of scattering are minimal. Thus, for practical purposes, the dose gradient around a point source of tissue may be considered as an exponential absorption superimposed upon an inverse square attenuation. Second, the amount of radiation scattered (25 per cent), is not degraded significantly in energy. Radiation doses in phantoms calculated on this basis agree well with measurements using ferrous sulfate which in this range is not significantly energy dependent. The half-value depth in tissue of this radiation is approximately 1 cm, so that the implant should be as fine grained as possible. Distribution rules vary with the size of the implant. In a uniform implant which is large when compared with the half-value layer, the radiation dosage in the central region considering only the K x-rays, is 22,000 rads/millicurie destroyed/cm^3 of tissue. In small implants where the size is negligible compared to the half-value layer using Paterson-Parker distribution, the value is approximately one quarter of this with intermediate values for intermediate size implants. Another feature to be considered is that the palladium itself strongly absorbs its own K x-rays (μ/ρ about 10) so that our final solution to this problem has consisted in depositing small quantities of finely divided palladium

black in suspension in an appropriate distribution, rather than as metallic wires or seeds throughout the tissue.

Production of palladium-103 by neutron activation of palladium-102 has presented some problems since the precursor palladium-102 has a natural abundance of only 1 per cent. The long irradiation time and high thermal-neutron flux cause extensive decomposition of the organic material, and therefore it has not been easy to use the Szilard-Chalmers reaction with such substances as palladium phthalocyanine. This might be feasible however, if highly enriched palladium-102 were used as the precursor. To maintain a steady supply of substantial quantities of the material on hand for routine clinical purposes we have made use of the high flux available at MTR to activate a 1 to 2 g sample each cycle (3 weeks). In this way, 500 to 1,000 millicuries are produced, these quantities being reduced to approximately one-half by the time the short-lived activities have decayed sufficiently to permit handling. The silver isotopes 110 and 111 are produced in substantial quantities and are separated from the palladium by dissolving it in aqua regia and scavenging it with silver chloride four times. The solution is then neutralized and reduced with formate to palladium black which is washed and then suspended in 6 per cent gelatin and sterilized. The material is assayed by counting the K photons using a beryllium window crystal in a defined geometry, looking only at the 20 KEV line with the 256 channel pulse height analyser. Photons originating from the electron capture process and photons originating from internal conversion in the K shell give a total of 0.80 photons/disintegration.

In preparing this material for clinical use we do not attempt to make the suspension of palladium black colloidal. As long as the material is fine enough to go through a medium gauge needle without clogging, we feel that this coarse suspension will remain at the implantation site in the tumor much better than a very fine suspension and will have less tendency to migrate through the lymphatics or appear in the liver as has been observed with colloidal gold and yttrium. Essentially we are attempting to tattoo the tumor. These assumptions have been fully confirmed by experiments in animals and experience with patients. As anticipated, the handling problems in the operating room have been reduced to negligible levels. Protection of the material with thin lead foil is more than adequate to eliminate any significant radiation to the operator's hands. As far as clinical results are concerned, it can be said on the basis of a very limited experience that radioactive palladium appears to be quite innocuous and that some patients appear to have received very significant palliative benefit from it. Care is necessary when working with this material since its relatively long half-life of 17 days makes particular precautions against contamination imperative. One great advantage is that it is handled in particulate form rather than in solution so that spills may be cleaned up with relative ease.

One of the difficulties with palladium-103 as produced by neutron bombardment has been the delay resulting from the short-lived activities induced during the process. Not infrequently samples are considerably lower in specific activity than is desirable, thus necessitating the injection of considerable quantites of metallic palladium. In an effort to overcome this we have investigated the possibilities of making carrier-free palladium in large quantities by the proton bombardment of rhodium. This would keep the amount of carrier injected into the implant at a minimum and control it independently of the amount of radioactivity. In the production of palladium-103 from rhodium-103 the thick target yield is stated to be 520 millicuries/milliampere/hour for 22 MEV protons. The limiting factor in such bombardments is the large amount of power (20 or 30 kilowatts) which must be dissipated in the target. This requires that the target foil

be very evenly and substantially bonded to the copper target backing which is cooled with water. In preliminary experiments in which target foils were tested to destruction, it was found that they could withstand the currents of 1-1/2 milliamperes. The activation function of rhodium-103 to palladium-103 was studied by bombarding a stack of foils in the external beam. The maximum cross section was found to be 400 millibarns for 12 MEV protons and the thick target yield was approximately half the value previously stated. It was felt, nevertheless, that it would be worthwhile to pursue this project and plans for this are going forward. The high cost of rhodium suggested that a more economical operation might be to bombard a relatively thick rhodium target and then machine off the top two- or three-thousandths of an inch containing the activity before rebombarding. It would thus be feasible to achieve a yield of 1-1/2 or 2 curies of carrier-free palladium-103 from a 4- or 5-hour bombardment with an expenditure of approximately fifty dollars for target materials. Separation of the carrier-free palladium may be carried out in a number of ways. The rhodium must first be dissolved, which is the most difficult step and the palladium may then be precipitated as the glyoxime and extracted with a small amount of carrier, or precipitated on selenium, carrier-free. If this project is successful, it will facilitate greatly the use of palladium-103 as a therapeutic agent.

We have also explored some of the possibilities of using Cs^{131} as a source of therapeutic radiation. This material decays with a half-life of 10 days by pure electron capture and emits the characteristic 30 KEV fluorescent x-rays of xenon. These photons penetrate tissue rather readily with a half-value layer of about 5 cm and we were at first hopeful that the material would prove useful as a therapeutic agent for the treatment of malignancies. No compound of cesium, however, has been found sufficiently insoluble for use as an implant, so that treatment of tumors is necessarily limited to sealed sources, in which the isotope is in solution. Opportunities of using such applicators are not sufficiently frequent to justify keeping the isotope on hand. Production presents few problems; one to two hundred-gram slugs of barium metal are irradiated in the thermal-neutron flux; the barium-130 captures a neutron to become barium-131 which decays with a 12-day half-life to Cs^{131}. This may then be distilled from the molten barium metal in an inert atmosphere onto a cold finger with little difficulty, so that curie quantities can readily be made available.

One other isotope under study at the Argonne Cancer Research Hospital as a source of therapy is cobalt-60. In designing a teletherapy source it is of course desirable to have the highest possible specific activity so that the source itself may be small and the field of treatment may have sharp edges. A substantial improvement in the specific activity of such a cobalt source was found to be possible several years ago by Dr. L. S. Skaggs and Dr. L. H. Lanzl. A pilot experiment was performed in which a model cobalt source was irradiated in fragments which were subsequently reassembled, thus avoiding flux depression during the time of irradiation, and consequently achieving a higher activity. A full scale irradiation of this sort has just been completed at MTR and the assembled source has been installed at Argonne Cancer Research Hospital. Measurements on this source indicate that 31 per cent of the cobalt was transmuted to Co^{60} which corresponds to a specific activity of 330 curies/g.

RADIOISOTOPES IN DIAGNOSIS

In the field of diagnosis, it has also been our hope that isotopes emitting soft x-rays will be useful. In liver scanning, for instance, when gold-198 or iodine-131 are used, cold nodules (me-

tastatic tumor) are not readily visible against the background of high energy gamma radiation penetrating through them from the underlying liver tissue. It seemed likely that a much higher degree of contrast could be achieved by using an isotope emitting soft x-rays, although this would of course preclude the detection of nodules deep within the substance of the liver. (These, however, are not visible in any case under the presently available circumstances.) Our first idea was to use palladium-103 in a soluble complex such as the phthalocyaninesulfonate. This does indeed localize to a very large extent in the liver but unfortunately it remains there so that a very substantial radiation dosage is delivered to the liver when sufficient isotope to produce a good scan is used. In this connection, an isotope overlooked for many years came to our attention, I^{125}. This isotope decays by electron capture and emits a soft (35 KEV) gamma ray which is largely converted in the K shell. The 60-day half life which at first appeared alarming has some definite advantages, the shelf life of the material being 7-1/2 times that of I^{131}, an item of great importance where cost and convenience are concerned. The difference in half life is of little importance in liver scanning using tagged rose bengal since this material is eliminated from the liver within a few hours and from the body within a day. In addition, since I^{125} decays by electron capture there is no beta radiation and the energy absorbed in the tissue/disintegration is less than that of I^{131} by a factor of 10 or 20 to 1 depending on the size and shape of the organ involved, which more than compensates for the longer half life.

The radiations from I^{125} consist of the gamma ray which is 35.5 KEV (.07 photons/dis.), the tellurium K alpha which is 27.3 KEV (1.12 photons/dis.) and the K beta line which is 31.4 KEV (.24 photons/dis.). The total number of photons/disintegration is 1.43 as compared to I^{131} which has only .8 photons/disintegration of the useful 360 KEV energy. One matter that concerned us greatly in using this material for scanning purposes was the fact that the attenuation of radiation in this particular energy range is largely by scatter, rather than absorption and we feared that there would be a large background of scattered radiation. Experiments were carried out with more or less realistic phantoms and this fear was not borne out. The contrast obtained from a cold nodule in the liver is not as great as that obtained with palladium-103, but is considerably better than that obtained with I^{131}.

The radiation from I^{125} is, as one would expect, attenuated to a greater extent than that of I^{131} in passing through the intervening tissues of the abdominal wall, so that in this respect, I^{131} has a definite advantage. Of greater weight, however, is the fact that it is possible to design very efficient collimators for the soft radiations of I^{125} and palladium-103. In fact, the limiting factor in this connection appears to be not the penetration of the collimator material by the radiation, but the structural strength of the walls between the collimator holes. We were rather at a loss for a method of evaluating all these factors in order to arrive at a figure of merit in comparing I^{125} and I^{131} for scanning purposes. In fact, even in our final conclusions it is apparent that the figure of comparison depends largely on what you are looking at. When the figure of merit is expressed as bits/second/sq. cm scanned/rad of tissue dose, the I^{125} is of an order of magnitude or more, better than I^{131} and this does not include the factors involved in the shelf life and the fact that 1/2 mm rather than 2 cm of lead shielding is required, as well as a much thinner crystal. In this formulation, a "bit" represents a sufficient number of counts to permit a decision of whether the count rate is normal or abnormal. I^{125} thus appears to represent a real advance as a tool for the diagnosis of thyroid and liver disease.

Production of I^{125} is by the neutron bombardment of xenon-124 which captures a neutron to

become xenon-125 which subsequently decays with an 18-hour half life to iodine-125. The recently reported cross section of 75 barns for this reaction first suggested that it might be feasible to produce useful quantities of the isotope. Recent measurements in our laboratory indicate that the cross section is 175 ± 15 barns which is considerably more favorable, so that if it should prove technically possible to irradiate xenon gas under pressure of 100 to 200 atmospheres, then curie quantities of I^{125} will be readily available. Investigations are in progress to test the feasibility of using as an irradiation vessel a thick-walled cylinder of Zircaloy 2. One additional problem with I^{125} is that it has a cross section of 1400 ± 100 barns so that substantial amounts of I^{126} are produced. This material was identified by comparing its spectrum to that of I^{126} produced from I^{127} using the linear accelerator. Shortening of radiation time should minimize this problem and since the I^{126} has a half-life of 2 weeks, the material can be stored until it decays sufficiently so that its presence no longer causes interference.

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

By

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The exact location and extent of brain tumors are often difficult to define. With the advent of radioactive tracer techniques, non-traumatic methods have been developed which use radioactive compounds having an affinity for tumor tissue. The use of radioactive diiodofluorescein-I^{131} in the diagnosis and localization of brain tumors was described in 1948,[1] and an extensive study on its use was published in 1950.[2] Radioactive phosphorus has been studied as a means of localizing brain tumors.[3] It was reported in 1951 that radioactive iodinated human serum albumin was useful as a brain tumor localizing agent.[4]

The present study was designed to test the efficacy of I^{131}-labeled octoiodofluorescein (OIF) in localizing brain tumors in mice. This compound was selected because diiodofluorescein, which is an analogue of OIF, is known to localize in tumors. Since OIF is 76 per cent iodine by weight and opaque to x-rays, its radio-opaque properties were also studied with a view to its use in cerebral radiography. As no previous biological data on this compound have been published, its toxicity, distribution, excretion and staining properties were investigated.

MATERIALS AND METHODS

Octoiodofluorescein was synthesized according to the method of Pratt and Perkins.[5] After repeated failures to purify OIF by forming the acetonate as described in the literature, chemical purity was established by using chromatographic columns prepared with Whatman cellulose powder and developed with increasing concentrations of acetone in carbon tetrachloride. By this method, there was no separation or movement of the dye, thus indicating the absence of contamination by acetone-soluble lower iodinated fluorescein derivatives which are known to migrate from the origin. Using ascending paper chromatography, only one peak was obtained with an Rf of 0.85 in a 50 per cent ethanol-water solvent system. With a 70 per cent methanol-water solvent system one peak with an Rf of 0.75 was obtained. A melting point could not be determined since the dye decomposes above 250° C.

As a qualitative determination for the presence of lower iodinated fluorescein compounds, the fluorescent properties of these dyes were investigated. By comparison with fluorescein, diiodofluorescein, tetraiodofluorescein and hexaiodofluorescein, all of which fluoresce under ultraviolet light, OIF was found to be almost completely free from the lower iodinated compounds. OIF in a weak base fluoresces very slightly with a pink color.

An absorption spectrum analysis was performed using Beckman DK automatic spectrometer

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and compared with the values given by Holmes.[6] Results of this analysis are shown in Table 1.

Table 1

AQUEOUS ABSORPTION MAXIMUM OF IODINATED FLUORESCEINS

Dye	μ	After Holmes μ
Fluorescein	490.2	490
Diiodofluorescein	512	
Hexaiodofluorescein	511.6	
Tetraiodoerythrosin (OIF)	548	550

The method of labeling OIF with I^{131} was that of ion exchange chromatography. The procedure was as follows,

Thirty ml of glacial acetic acid and 200 mg of powdered OIF were placed in a 250-ml centrifuge tube and heated in an oil bath for 6 hours to aid solution. To 1 ml of carrier-free I^{131} were added 2 drops of 0.1 N ascorbic acid, the pH was adjusted to 4 with acetic acid and within a few minutes trace amounts of iodate were reduced to iodide. The I^{131} was added to the OIF solution and the container was rinsed twice with 5 ml of acetic acid which was also added. The solution was stirred under a hood at 100° C overnight. It was then diluted to 240 ml with distilled water whereupon a precipitate of OIF was formed. This was centrifuged for 15 minutes, the supernatant removed and the precipitate washed 4 times with water containing 5 drops of acetic acid. The washed precipitate was dissolved in 5 ml of 1.0 N NaOH and buffered at pH 7.6 with 10 ml of 0.1 M dipotassium acid phosphate. The OIF-I^{131} was transferred to an injection vial and autoclaved.

The final solution was assayed by comparing a diluted aliquot with standard I^{131} solution. By this method of labeling, from 40 to 50 per cent of the initial quantity of I^{131} was transferred to the fluorescein molecule.

Two experimental mouse tumors were used in the studies. The first was a C3HBA mammary gland adenocarcinoma obtained from the National Cancer Institute, and carried in C3H female mice from Jackson Memorial Laboratories, Bar Harbor, Maine. The second was a brain tumor carried in a substrain of white Swiss mice and was obtained from the Brookhaven National Laboratories.* The original brain tumor was characterized as a mixed cell type which converted into an ependymoblastoma after repeated transplantation.

The mammary gland adenocarcinoma was transplanted by subcutaneous injections of homogenized tumor cells suspended in sterile saline. Transplants grew rapidly with 100 per cent "take."

Transplantation of the brain tumor was by direct injection of 0.02 to 0.05 ml of a homogenized tumor cell suspension in saline solution into the brain of unanesthetized young Swiss mice. After a latent period of 3 weeks, approximately 50 per cent of the population developed symp-

*We are indebted to Dr. George C. Cotzias of the Brookhaven National Laboratories for making this tumor available to our group.

toms of brain tumor characterized by lethargy, hemiplasia, protrusion of the skull, spinning when held in the air by the tail and weight loss. After this stage (Figure 1), the mice rarely lived more than 48 hours.

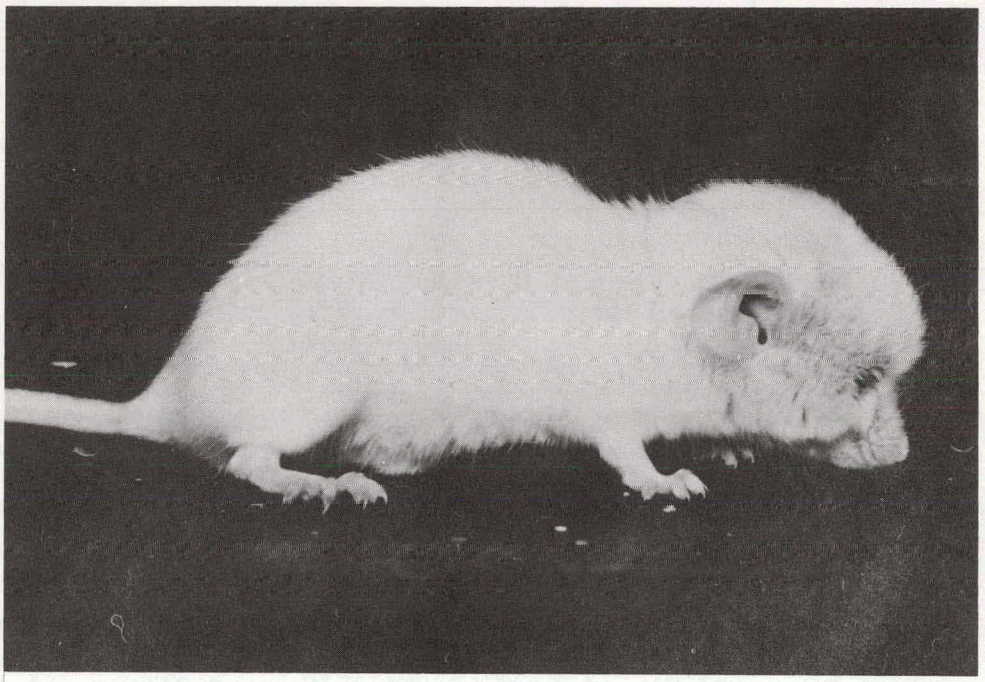

Figure 1. Swiss mouse with ependymoblastoma approximately three weeks after implantation. Note protrusion of skull due to the brain tumor.

EXPERIMENTAL RESULTS

In toxicity studies, CF No. 1 adult female mice were divided into four groups, and each group given a measured amount of dye by intravenous injection into the tail. The doses administered ranged from 435 to 730 mg/Kg as single injections. Time of death was recorded for all groups. The results of the LD_{50} determination are shown in Table 2. The LD_{50} obtained by probit analysis was 510 mg/Kg. Gross symptoms produced by toxic doses were lethargy, anorexia and weight loss. Since OIF has a deep red hue, a slight pink coloration was noted around

Table 2

MORTALITY OF MICE AFTER INTRAVENOUS ADMINISTRATION OF OIF-I^{131}

Dose (mg/Kg)	No. Mice	Number of deaths per day											Total deaths	Deaths (per cent)
		1	2	3	4	5	6	7	8	9	10	11		
435	15						1		1	1		1	4	26.6
568	16	1	2	2	1		4		1				11	68.6
666	15	1	4	4	2	2	1						14	93.3
730	6		6										6	100.0

the eyes, nose, ears, feet, skin and tail of white mice. A dose of 225 mg/Kg, representing approximately one-half of the LD_{50} dose resulted in a pink coloration which disappeared 39 days after injection, and it was then no longer possible to distinguish treated from control mice. All mice lived and showed no toxic effects of this dosage level.

Distribution and excretion studies were carried out with normal C3H and normal Swiss mice using tracer doses of OIF-I^{131}. Each mouse received 0.05 ml of OIF-I^{131} via the tail vein. Groups of four mice were sacrificed at stated time intervals, the organs removed, weighed and counted in a well-type crystal scintillation counter. Counts were compared with a standard preparation of OIF-I^{131}. The data were calculated as per cent of injected dose per total organ and per cent of injected dose per gram of wet weight of organ. These results are shown in Tables 3 and 4.

Table 3

DISTRIBUTION OF OIF-I^{131} PER TOTAL ORGAN

Time	Per cent of OIF-I^{131} injected					
	Liver	Spleen	Int.	Kidneys	Thyroid	Brain
Normal C3H						
2 min	21.2	0.30	1.93	2.15	0.03	0.32
5 min	30.0	0.42	5.33	1.60	0.0	0.17
10 min	31.9	0.41	5.16	0.93	0.0	0.11
20 min	39.7	0.39	14.1	1.28	0.0	0.13
30 min	33.8	0.36	22.9	1.09	0.0	0.0
1 hr	23.1	0.34	34.5	1.05	0.0	0.0
2 hr	21.6	0.47	32.4	0.87	0.0	0.0
4 hr	10.9	0.36	55.5	0.96	0.0	0.0
Normal Swiss						
1 hr	26.0	0.30	33.2	1.07	0.0	0.04

Distribution studies showed a rapid decrease of the dye in the blood followed by a rapid increase in the liver where it reached a maximum value of about 40 per cent of the injected dose in 20 minutes. The concentration in the liver then decreased as the concentration in the intestinal contents increased. The concentration of dye in the spleen remained relatively constant within 4 hours after injection. Radioactivity was not detected in subcutaneous fat at any time. The kidneys appear to contain a constant concentration of dye after an initial rapid decrease. The thyroid was found to contain no radioactivity indicating little or no liberation of free iodine-131 within 4 hours after injection. No OIF-I^{131} was found in the brain 20 minutes after injection suggesting that the dye does not cross the normal blood brain barrier. The small amount noted in the brain within the first 20 minutes is believed to represent vascular contamination.

To determine the major pathways of excretion, eight C3H mice were injected intravenously with 0.1 ml of OIF-I^{131} and placed in pairs in metabolism cages. After 24 hours the urine and feces were assayed. Each urine sample was brought to a volume of 5 ml of which 1 ml was used for counting. Samples were compared to a standard preparation. The results of the excretion

study are shown in Table 5. After 24 hours about 60 per cent of the dye was excreted, 10 per cent in the urine and 50 per cent in the feces.

Table 4

DISTRIBUTION OF OIF-I^{131} PER GRAM OF ORGAN

Time	Per cent of OIF-I^{131} injected						
	Liver	Spleen	Int.	Kidneys	Thyroid	Brain	1 ml blood
Normal C3H							
2 min	17.2	2.5	0.54	6.65	0.0	0.75	21.4
5 min	24.4	3.4	1.5	5.0	0.0	0.4	11.9
10 min	25.9	3.3	1.45	2.9	0.0	0.25	8.4
20 min	32.2	3.1	2.9	3.9	0.0	0.3	6.1
30 min	27.5	2.9	6.5	3.4	0.0	0.0	6.6
1 hr	18.8	2.7	9.7	3.2	0.0	0.0	5.1
2 hr	17.5	3.8	9.1	2.7	0.0	0.0	4.5
4 hr	8.9	2.9	15.6	3.0	0.0	0.0	2.0
Normal Swiss							
1 hr	21.1	2.4	9.1	3.3	0.0	0.0	3.4

Table 5

OIF-I^{131} RECOVERED FROM URINE AND FECES AFTER TWENTY-FOUR HOURS

	Per cent recovered				
	Cage 1	Cage 2	Cage 3	Cage 4	Average ± S.D.
Urine	8.8	9.0	8.5	12.0	9.8 ± 1.05
Feces	51.0	48.0	58.5	50.5	52.0 ± 4.8

To study localization of OIF-I^{131} in tumor tissues, fourteen C3H mice having C3HBA mammary tumors were injected intravenously with 0.1 ml of OIF-I^{131}. They were sacrificed at specific time intervals, the tumors removed and cleared of necrotic tissue. The tumors were weighed, assayed for radioactivity, and the data calculated as per cent of dose per gram of tumor and per cent of dose injected per total tumor. Results for the mammary gland adenocarcinoma are presented in Table 6.

The average concentration of OIF-I^{131} in the mammary tumors was 1.1 ± 0.51 (SD) per cent of the dose per gram of tumor. This concentration was found at all times studied and for various tumor sizes indicating that the dye builds up rapidly in that tissue and is slowly released from it.

For the localization of OIF-I^{131} in ependymoblastoma, Swiss mice were injected with 0.1 ml of OIF-I^{131} as soon as neurological symptoms were observed. After one hour the animal was sacrificed by anesthesia with ether, the brain removed and washed free of surface blood (Figure 2).

Table 6

LOCALIZATION OF OIF-I^{131} IN C3HBA MAMMARY GLAND ADENOCARCINOMA

Time	Weight of tumor in g	Per cent of dose per total tumor	Per cent of dose per g tumor
20 min	1.72	1.72	1.00
20 min	3.03	1.2	0.40
30 min	5.21	5.1	0.97
2 hrs	1.19	1.47	1.23
3 hrs	6.62	11.1	1.70
4 hrs	0.13	0.29	2.20
6 hrs	4.59	2.1	0.46
6 hrs	8.99	5.7	0.64
17 hrs	5.08	4.9	0.98
17 hrs	3.47	2.95	0.85
17 hrs	1.64	2.53	1.55
24 hrs	2.54	1.54	0.65
24 hrs	1.04	1.79	1.72
24 hrs	2.46	2.38	0.97

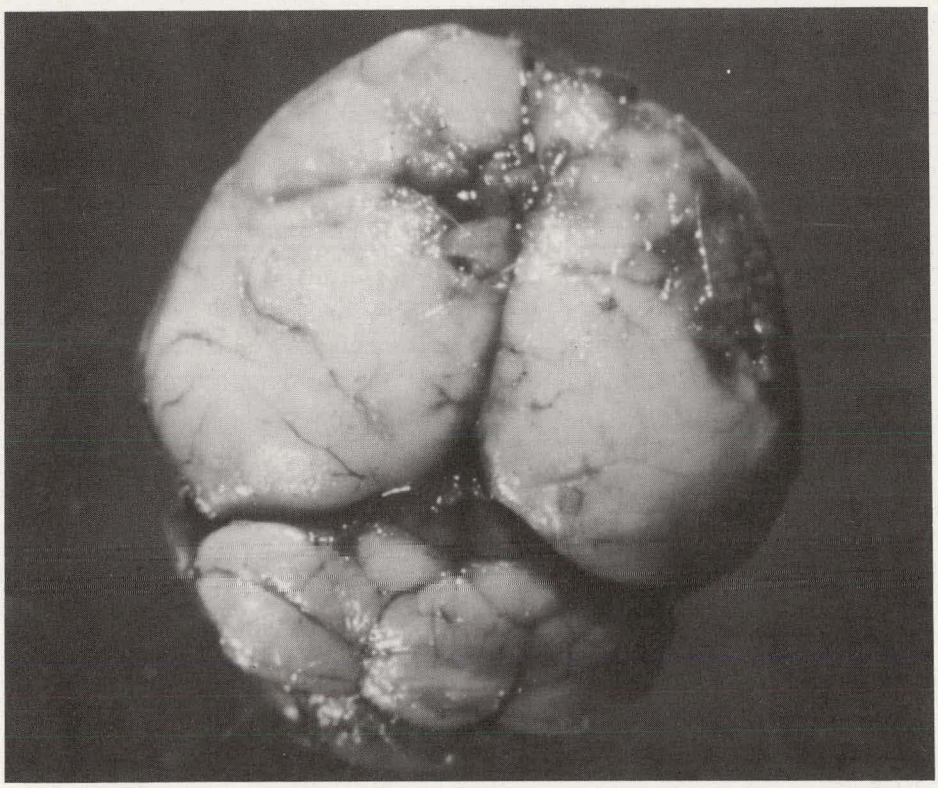

Figure 2. Ependymoblastoma lesion in right cerebral hemisphere of Swiss mouse. Note staining of lesion with octoiodofluorescein-I^{131} dye.

The tumor was dissected as free as possible from normal brain tissue, fixed in formalin and weighed. Both normal and tumor tissues were assayed for radioactivity. The results were calculated as per cent of dose per gram of tumor and normal brain tissue and the ratio of tumor radioactivity to normal brain tissue radioactivity. The results from a total of 12 mice are shown in Table 7.

Table 7

DIFFERENTIAL UPTAKE OF OIF-I^{131} IN EPENDYMOBLASTOMA TUMORS OF SWISS MICE

Mouse	Tumor per cent dose/g	Normal per cent dose/g	Ratio
1	0.54	0.063	8.5
2	0.79	0.075	10.55
3	0.92	0.096	9.61
4	1.18	0.065	17.6
5	1.50	0.086	17.5
6	2.54	0.144	17.0
7	0.707	0.15	4.69
8	0.997	0.124	8.05
9	0.665	0.141	4.72
10	0.50	0.158	3.15
11	5.68	0.173	32.8
12	9.54	0.39	24.4

The results of OIF-I^{131} localization in the brain tumor were more variable than in the case of the mammary tumor. The range of concentrations found was from 0.50 to 9.54 per cent of the dose per gram of tumor. The average value was 2.13 ± 2.75 (SD) per cent per gram. Animals No. 11 and No. 12 (Table 7) represent tumor tissue completely free from normal tissue and give a more valid figure for concentration in the tumor. The range of concentrations of OIF-I^{131} in normal tissue taken from mice one hour after injection was from 0.063 to 0.39 per cent of the dose per gram with an average value of 0.139 ± 0.168 (SD) per cent per gram. The ratios of tumor to normal ranged from 3.15 to 32.8.

In an attempt to determine radio-opaque properties of OIF with a view to its possible use in cerebral radiography, 10 special plastic vials were designed to contain 1 ml of OIF solution. OIF solutions at concentrations of from 0 to 180 mg/ml were placed in the vials and irradiated at 150 KV, 5 milliamps for 10 seconds with 0.25 mm copper filter. The dose of radiation to the film was 36.5 mr from a Maxitron X-ray unit 85 cm from the film. The film was developed under standard conditions and read on a densitometer.

DISCUSSION

The acute LD_{50} dose of OIF in mice was found to be 510 mg/Kg, a value which compares favorably with other iodinated fluorescein derivatives.[7] The faint pink coloration caused by the

dye was transitory, and disappeared completely after 39 days.

The localization of I^{131}-labeled OIF in brain tumors as demonstrated by a concentration ratio of between 3.15 to 32.8 indicates that OIF-I^{131} may prove to be a suitable agent for localizing brain tumors in humans. The wide variation in the results may be due to several factors. First, the diffuse nature of the tumors makes it extremely difficult to remove one completely free of normal tissue. Second, several of the brain tumors were too small to be individually visible, and the whole area of tissue stained with the dye at the site of inoculation was removed. Third, in several mice the normal tissue was found to contain an amount of radioactivity greater than that found in brain tissue of similar mice one hour after injection. This may be due to diffuse growth of the tumor or to the elaboration of some substance which permits a higher concentration of dye to localize in the surrounding tissues. This latter condition has been reported by Scott.[8] All three conditions tend to decrease the ratio of tumor activity to normal activity and result in a large amount of variation.

Following the completion of this preliminary study in mice, the efficacy of OIF-I^{131} as a brain tumor localizing agent in humans was investigated. The results of this clinical evaluation will be reported elsewhere.

OIF was found to be opaque to x-rays at concentrations above 60 mg/ml. However, at this concentration, it is felt that this compound will not be a useful radio-opaque agent for cerebral radiography of brain tumors since the dosage required will be too large. It may be toxic to the patient and may also produce an objectionable pink coloration in the skin.

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THE EFFECT OF IRRADIATION ON NORMAL HEMAGGLUTININS AND BACTERICIDINS OF MICE*†

By

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Increased susceptibility to bacterial infection is one important result of total-body exposure to a mid-lethal dose of x-radiation. In the mouse, microorganisms from the intestinal tract commonly invade the blood stream during the second week postirradiation and give rise to fatal bacteremias.[1-3] The effect of irradiation on the cellular elements of the mouse's defense mechanism has been well studied,[4-6] but it is also possible that its humoral elements, i.e., natural antibodies, may be affected.

In rabbits, Donaldson and Marcus[7] found that irradiation caused loss or reduction of normal bactericidins for Bacillus subtilis but not of agglutinins for this microorganism or for sheep cells. Talmage et al.[8] reported that irradiation of rabbits reduced their natural hemolysins for sheep cells.

The present investigations were undertaken to find out whether the loss of some antibacterial substance from the blood of the irradiated mouse might contribute to its increased susceptibility to bacterial infection. Part I deals with the effect of irradiation on the natural hemagglutinins in mice, Part II with its effect on their natural bactericidins.

Part I. The Effect of Irradiation on the Natural Hemagglutinins in Mice

After a preliminary unsuccessful attempt to demonstrate agglutinins in the sera of normal CF No. 1 mice for several microorganisms responsible for postirradiation bacteremias, a search was made for other natural antibodies on which the effects of irradiation might be studied. Finally, agglutinins for red cells of the chicken were chosen, because these cells were agglutinated regularly and in high titer by the sera of CF No. 1 mice.

MATERIALS AND METHODS

CF No. 1 female mice 8 to 16 weeks of age were used in all experiments. They were housed in metal cages on wood shavings. Rockland mouse pellets and tap water were available at all times.

Total-body irradiation in a single exposure of 550 to 700 r was delivered at 250 kv, 30 ma, at a distance of 79.25 cm using a 1/4-mm copper and a 1-mm aluminum filter at a rate of approximately 60 r/minute. The LD_{50} (30 days) for these mice is about 550 r.

Mice selected at random were bled under light anesthesia from a severed brachial artery. Serum pools from irradiated mice were prepared from 9 or 10 individuals, normal pools from

*This report summarizes the contents of the following publications: Kornfeld, L. and C. P. Miller. J. Immunol., 84:73, 1960; Kornfeld, L., C. W. Hammond and C. P. Miller. J. Immunol., 84:77, 1960.

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4 to 30 individuals.

Heparinized blood from White Rock chickens mixed with an equal volume of modified Alsever's solution and stored no longer than 2 weeks in the refrigerator served as the source of red cells. Immediately before use, the cells were washed three times in physiologic saline.

For the hemagglutination test, 0.1-ml quantities of a 0.25 per cent red cell suspension were added to equal volumes of serial 2-fold dilutions of serum in saline and incubated for 1 hr in a water bath at 37° C. The tubes were shaken, centrifuged at 1000 rpm for 5 min and read after shaking. The titers recorded were the reciprocals of the highest final dilutions of serum showing agglutination distinctly visible to the naked eye. Each experiment included a titration of a reference serum.

RESULTS

It can be seen from Table 1 that agglutinin titers for chicken red blood cells of 40 pools of normal mouse serum were quite consistent and that they were practically identical with those of 39 pools of serum obtained from mice at various intervals after exposure to 550 r. Exposure to 700 r also produced sera with titers essentially like those of normal pools.

Table 1

HEMAGGLUTININ TITERS OF POOLED SERA FROM NORMAL AND IRRADIATED (550 r) MICE

	No. of pools tested	No. of pools with titer of			
		32	64	128	256
Normal	40	2	19	18	1
Days postirradiation					
1-3	6		2	3	1
4-6	8		5	2	1
7-9	9	1	4	4	
10-12	6		4	2	
13-15	3		1	2	
16-18	3		2	1	
19-21	4		3	1	
Total irradiated	39	1	21	15	2

Table 2 reveals however, that a radiation effect became evident when the sera of individual mice were examined. Here are shown the distribution of titers of 77 normal and 382 irradiated (550 r) mice, including the geometric mean titer (antilogarithm of the mean of the logarithms of the titers) and the geometric standard deviation (antilogarithm of the standard deviation of the logarithms of the titers) for each group. A total of 4 negative sera (0.86 per cent), one among normal, three among irradiated mice were omitted from the tables and calculations. The calculated mean titers reveal a gradual depression of hemagglutinins during the first 10 to 12 days followed by a rise to normal. Statistical analysis by means of a simple χ^2 test indicates that

Table 2

HEMAGGLUTININ TITERS OF INDIVIDUAL SERA FROM NORMAL AND IRRADIATED (550 r) MICE

	No. of sera tested	No. of sera with titer of						Geometric mean titer	G.S.D.*
		8	16	32	64	128	256		
Normal	77			4	33	37	3	90.5	1.59
Days postirradiation									
1-3	60		5	14	17	12	12	73.5	2.38
4-6	79	3	7	24	15	18	12	60.9	2.57
7-9	86	2	12	28	26	12	6	48.5	2.25
10-12	58	1	12	16	20	7	2	43.7	2.16
13-18	60	5	5	10	21	11	8	58.0	2.65
19-21	39			5	24	8	2	72.5	1.65
Total irradiated	382								

*Geometric Standard Deviation (antilogarithm of the standard deviation of the logarithms of the titers), by which geometric mean titer is to be multiplied and also divided, in order to include two thirds of the population.

the apparent changes in mean titer with time must be regarded as substantially real.

The table also shows a difference in the distribution of hemagglutinin titers of normal and irradiated (550 r) mice. Whereas 90 per cent of the titers of normals were 64 or 128, the titers of irradiated varied widely, particularly between the 3rd and 17th days postirradiation as indicated by the significantly larger geometric standard deviations.

Because of the high mortality of mice exposed to 700 r, fewer observations could be made and no data are presented; the changes in hemagglutinin titers were essentially like those produced by 550 r.

DISCUSSION

Ideally, in such a study as this, the serum samples should be collected from the same animals before and after irradiation. Since repeated blood specimens of sufficient quantity cannot be obtained from the same mouse, different groups of animals were bled each time, in the hope that the pooling of sera would equalize differences among individuals. Pools of sera from normal and from irradiated mice had consistent titers, but when sufficient numbers of individual sera were tested to permit statistical analysis of the data two effects of irradiation were brought out: a) the gradual decline of mean hemagglutinin titers during the first 10 to 12 days postirradiation followed by the return to normal in the next 10 days, and b) the much greater variation of titers of sera from irradiated as compared with those from normal mice.

The decline of hemagglutinin titers in the postirradiation period was small and this may have accounted for the failure to detect it with pooled sera. The difference between the lowest mean titer and the normal value was approximately 2-fold, a difference which would not have been considered significant between two separate titrations. However, conclusions drawn from the data presented here are based on average values, all but one calculated from 60 or more

titrations representing several experiments. Furthermore, both decrease and increase of titers follow a smooth and similar trend for both x-ray doses. Statistical analysis indicates a significance level of 0.0005, clearly supporting this reasoning. In this connection it is noteworthy that Talmage et al.,[8] using a far more accurate method for titrating normal antisheep hemolysins and following individual rabbits, observed a decline of similar magnitude by the 10th day postirradiation. The possible significance of these findings will be discussed in Part II.

Part II. The Effect of Irradiation on Natural Bactericidins of Mice

A strain of Escherichia coli, susceptible to killing by normal serum and complement was used as the test microorganism.

A recent review of the literature[9] reveals that most investigators agree that the killing of gram-negative bacteria by normal serum results from the combined action of three factors—a relatively stable heat component, complement and Mg^{++}. In this respect, bactericidal activity is comparable to the hemolytic activity of normal serum.

The failure of fresh mouse serum to lyse sensitized erythrocytes under most experimental conditions is due to a deficiency in the second component of complement, the addition of which results in hemolytic activity.[10-13] Similarly, bactericidal activity requires the addition of fresh guinea pig serum or end piece.[14,15] This may explain why mouse serum was reported to be devoid of bactericidins.[16]

MATERIALS AND METHODS

Mice were irradiated and sera obtained as described in Part I. Each serum sample was prepared from the blood of 4 to 5 mice. In contrast to our findings with hemagglutinins, pooled sera proved to be satisfactory for demonstrating the effect of irradiation on normal bactericidal activity.

A strain of Escherichia coli was isolated from the intestinal tract of a normal mouse and carried on nutrient agar. An 18-hr subculture was washed with saline and diluted to contain approximately 1000 organisms/ml.

Complement was obtained from guinea pigs (300 to 400 g) exposed to 200 or 300 r total-body irradiation and exsanguinated 48 hrs later. The serum was stored at -20° C and diluted 1:10 before use. This serum was devoid of demonstrable bactericidal activity, yet had the ability to lyse sensitized sheep cells and to "activate" mouse serum in bactericidal tests.[17]

Sterile physiologic saline (0.85 per cent) was used for all dilutions. Since the addition of Mg^{++} in recommended amounts[18] did not increase the sensitivity of the test, it was assumed that an adequate supply of this ion was present in the relatively large quantity of guinea pig serum in each tube.

For the bactericidal test, 2-fold serial dilutions (0.2 ml) of each mouse serum were prepared in Wassermann tubes. Complement (diluted 1:10) and bacterial suspensions (approximately 1000 organisms/ml) were mixed in proportions of 2:1, and 0.2 ml of the mixture immediately added to each serum dilution. After incubation in a water bath at 37° C for 6 hrs with occasional shaking, 0.1 ml samples were transferred to eosin-methylene blue agar plates. These were incubated at 37° C for 18 hrs, and colony counts made. Titers were expressed as the highest dilutions of serum from which no colonies, or only occasional colonies developed.

EXPERIMENTAL RESULTS

Bactericidin titers of a large number of normal mouse sera obtained over several months ranged from 16 to 64. Repeated tests indicated that the results were reproducible within one 2-fold dilution. Titers remained unaltered after heating at 56° C for 30 min, or after storage in the refrigerator for 2 weeks, or in the deep freeze for 6 months.

The method employed to demonstrate bactericidal activity differs from those used by most investigators chiefly in the length of incubation. Figure 1 illustrates colony counts of aliquots taken at frequent intervals from two of a series of serum dilutions. During the first 3 hours, the population increased in both tubes. In the next 2 or 3 hours multiplication continued exponentially

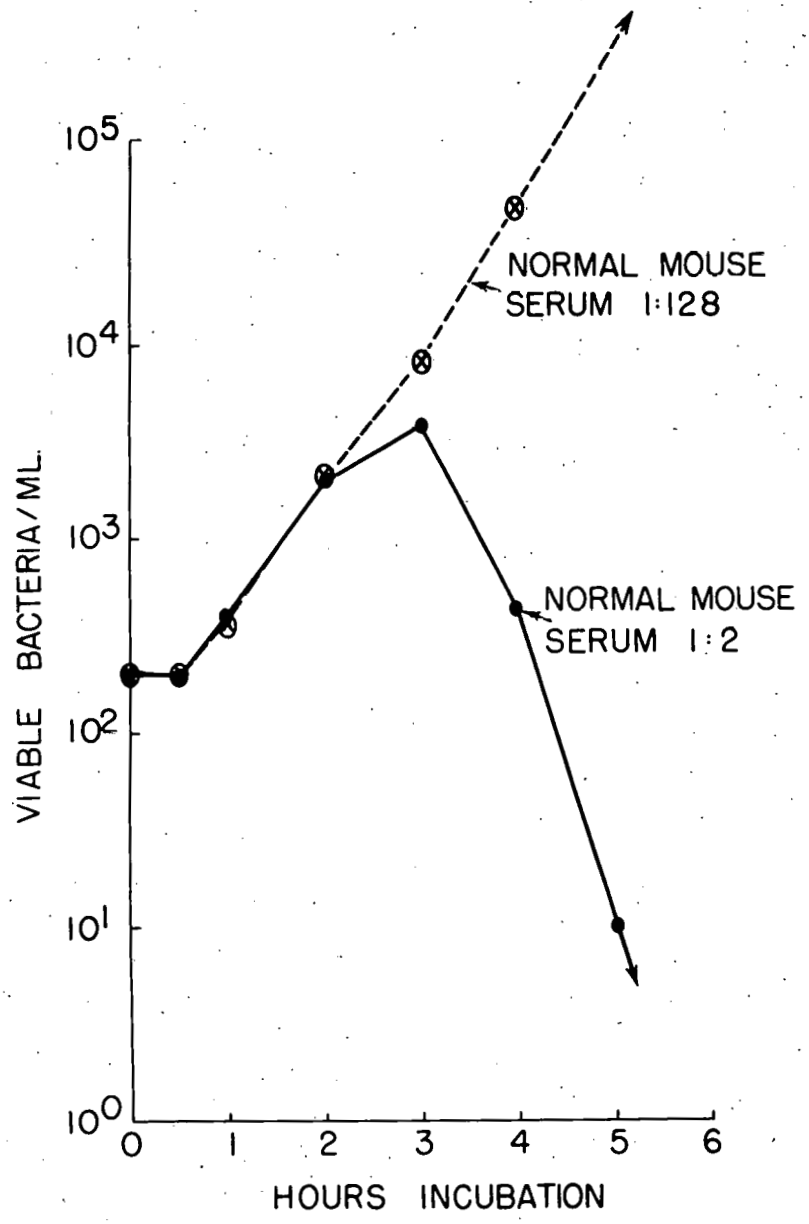

Figure 1. Colony counts of Escherichia coli in two dilutions of normal mouse serum (plus complement).

in the higher dilution, while the number of viable microorganisms fell rapidly in the lower dilution. In dilutions showing incomplete bactericidal activity, the lowest viable count was always observed after 6 hrs. Thereafter the survivors multiplied freely. Since no bactericidal activity occurred after 6 hrs, plating at this time was adopted as routine.

Results similar to those shown in Figure 1 were obtained when sera from irradiated rabbits were used as source of complement, or when fresh sera from normal rabbits and guinea pigs were used to study bactericidal action. Chick[19] obtained identical growth-death curves when investigating the bactericidal power of normal rabbit serum against E. coli.

Table 3 shows the effect on the bactericidin titers of sera from groups of mice bled before and after irradiation (600 r). Bactericidal activity was increased slightly a few hours after irradiation, then declined rapidly until it was undetectable after 12 hrs, returning to normal by the

Table 3

EFFECT OF IRRADIATION (600 r) ON BACTERICIDINS FOR ESCHERICHIA COLI (CF NO. 1 MICE, 1956)

Bleeding time	Bactericidin titer					
	Experiment no.					
	1	2	3	4	5	6
Preirradiation	64	16	16	32	16	16
Postirradiation						
Hours						
1	64		16	64	32	64
3	256	32	64	16	32	64
6	16	8	2	8	16	64
9	<2	2	<2	32	<2	32
12	<2	<2		4		
24	<2	<2		<2	<2	<2
Days						
7	<2				<2	<2
11	<2				8	2
14	64				64	32
17	64					
21	64					

end of the second week postirradiation. The rise in titer was usually only 2-fold, rarely 4-fold, but it always occurred. Exposure to 300 r results in similar though more moderate changes. Since no interference with the bactericidal activity could be demonstrated in vitro after mixing the sera of normal and irradiated animals, it was considered unlikely that the lack of activity after irradiation was due to the presence of an inhibitory substance in sera from irradiated mice.

To test whether the rapid decline of bactericidal activity postirradiation might be due to the

absorption of bactericidins in vivo by enteric bacteria through the damaged intestinal mucosa, gram-negative bacilli were eliminated from the intestinal tract by the administration of 40,000 units of streptomycin by stomach tube 17 hrs before exposure to 600 r. This treatment was repeated on the following day in those mice held for the 24-hr postirradiation bleeding. Sera collected before and after irradiation were tested in the presence and absence of complement in order to rule out the inhibition of bacterial growth by any traces of antibiotic that might have entered the blood stream. It was found that a) no bactericidal effect was observed in the absence of complement, and b) streptomycin did not alter the normal serum bactericidin levels, or affect postirradiation changes in bactericidal activity.

Additional evidence that absorption by coliform bacteria was not responsible for the loss of bactericidins following irradiation was obtained by comparing mice possessing specific antibodies with nonimmune controls. Mice given a series of intraperitoneal injections of heat-killed E. coli were exposed to 600 r 14 days after the last immunization. Sera were assayed for agglutinins and bactericidins with E. coli. The results showed that a) specific agglutinins in immunized mice persisted undiminished after irradiation and b) serum bactericidin titers were unaltered by immunization but followed the same postirradiation patterns in the immune and nonimmune groups.

The experiments so far described were carried out between January and July 1956. When work was resumed 3 months later, the normal CF No. 1 mice although healthy and apparently normal in other respects, were lacking in serum bactericidins. All efforts to explain this loss, in terms of environmental and experimental conditions proved fruitless. Our suspicion that some change had taken place in the CF No. 1 mice, which are not highly inbred, was strengthened when it was found that after July 1957 normal bactericidins were again present, but only in approximately half the individuals tested.

Table 4 illustrates the response to irradiation at a time when bactericidal activity was lacking in normal CF No. 1 mice. Exposure to 400 or 600 r resulted in the transitory appearance of bactericidins during the first few hours postirradiation, and again after the second week; 200 r had only a slight immediate effect.

A number of highly inbred strains were examined, and C57BL/6 mice (males at least 4 months old) were selected for further study because of their uniformly high bactericidin titers. Over a period of 18 months, bactericidins were found regularly. As shown in Table 5, these mice respond to exposure to 600 r very much as did the original CF No. 1 except that the bactericidal activity is lost more slowly and returns to normal several days earlier.

DISCUSSION

In the first part of this report, it was shown that the exposure of CF No. 1 mice to mid-lethal doses of x-ray caused a moderate, but definite decline of agglutinin titers for chicken cells. While it is highly unlikely that agglutinins for chicken cells influence resistance to infection, it is possible that their behavior parallels that of some other serum constituent which does influence resistance. This is suggested by the observation that the period of reduced hemagglutinin titers following irradiation coincides with that of enhanced susceptibility to infection.[1]

In considering the effects of x-rays on the bactericidins, one might expect, a priori, a more direct relationship between loss of serum bactericidal activity and increased susceptibility to infection, particularly since E. coli is frequently responsible for postirradiation bacteremias in

Table 4

EFFECT OF IRRADIATION ON BACTERICIDINS FOR ESCHERICHIA COLI (CF NO. 1 MICE, 1957)

Bleeding time	Bactericidin titer		
	200 r	400 r	600 r
Preirradiation	<2	<2	<2
Postirradiation			
Hours			
1	16	64	32
3	16	8	128
6	<2	<2	2
9	4	16	<2
12		<2	8
24	<2	<2	<2
Days			
7	2	<2	<2
14	<2	16	16
21	2	8	8

mice.[1] However, the bactericidins disappear within a few hours of irradiation, several days before the usual appearance of bacteremias,[1] and return about the end of the second week after irradiation, at a time when bacteremias are still occurring.[1] Moreover, the absence or presence of bactericidins depended much more on length of time after irradiation, than on fitness of the individual at the time the serum was obtained. Mice killed early in the experiment, when they were still quite lively, had no detectable serum bactericidins, whereas after the second week even obviously moribund animals had normal titers. It is very doubtful therefore, that loss of serum bactericidal activity is an important factor in the development of postirradiation bacteremias in mice.

The speed with which the bactericidins disappear after irradiation is of considerable interest. The experiments using antibiotics appear to rule out the possibility that it is due to absorption by gram-negative bacilli through the intestinal mucosa.

Table 3 shows that the half disappearance time of bactericidins in mice is a few hours, a much greater rate than previously reported for antibody degradation. Bactericidins in rabbits measured by the same method fell much more slowly after irradiation.[17] Talmage[21] pointed out that disappearance rates vary for a given antibody in different animal species and also for different antibodies in the same species.

As is also shown in Table 3, bactericidins were absent from mouse sera for more than 10 days after irradiation, indicating that production of this substance had been interrupted by radiation injury. The slight but consistent rise in titer 1 to 3 hrs after irradiation may be due to release of preformed bactericidin from its site of origin, possibly cells of the highly radiosensitive lymphoid system. Morphologic evidence of injury to lymph nodes has been observed as early as 2 hrs after moderate doses of whole-body irradiation.[22,23]

Table 5

EFFECT OF IRRADIATION (600 r) ON BACTERICIDINS FOR ESCHERICHIA COLI (C57BL/6 MICE)

Bleeding time	Bactericidin titer				
	Experiment no.				
	1	2	3	4	5
Preirradiation	128	128	64	64	64
Postirradiation					
Hours					
1	128		128		
3	256	128	64		
6	256	128	128		
9	256	256			
12	128	128			
24	8	< 4	< 4	< 4	< 4
Days					
2	< 4	< 4		< 4	< 4
3	< 4	8		< 4	< 4
4				8	< 4
7	16	16		64	32
10	32	32			
14		256			

No satisfactory explanation can be offered for the variation in bactericidal activity in the sera of CF No. 1 mice after October 1956.

Despite similarities between the bactericidal substance studied and properdin, a component of normal sera described by Pillemer et al.[24] several differences were found which indicate that they are not identical.[24,25]

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STUDIES ON THE Cx-REACTIVE PROTEIN. III. THE EFFECT OF IRRADIATION OF RABBITS ON THE ACUTE PHASE PROTEIN SYSTEM*†

By

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The occurrence in the rabbit of an abnormal protein analogous in many of its properties to the human C-reactive protein has been demonstrated.[1] This rabbit acute phase protein reacts only with a special form of the pneumococcal somatic polysaccharide, C_x, whereas human C-reactive protein reacts both with Cx and the classical C polysaccharide. Both human and rabbit proteins as they occur in the blood are bound to lipid and both lipid-protein complexes are precipitable by calcium ion if the salt concentration is low.

Both acute phase proteins are antigenic in several laboratory animals.[1-3] Lipid free preparations of them act as antigens which stimulate in sheep the production of specific antisera which do not react with normal serum. Reciprocal immunological cross reactions employing sheep antisera to C- and Cx-reactive proteins have recently been demonstrated, which although partial, are appreciable.[3]

Virtually nothing is known concerning any natural biological function of these acute phase proteins which explains their appearance and disappearance from the blood. There is, however, some evidence that the Cx-reactive protein is elaborated in response to appropriate stimuli by some cellular component of the reticulo-endothelial system.[4] Blockade of the reticulo-endothelial system with thorotrast reduces the capacity of rabbits to elaborate the abnormal protein.

Attempts have been made to elicit the Cx-reactive protein response in rabbits by the use of agents which do not produce an inflammatory reaction, but which have a direct cytotoxic effect on the bone marrow and the lymphoid tissues.[5,6] It was found that x-radiation and one of the radiomimetic compounds caused the appearance of Cx-reactive protein 24 hours after exposure.

The present investigation was undertaken to determine the pattern of Cx-protein response caused by varying doses of radiation to rabbits, and its possible relation to their susceptibility to subsequent endogenous bacteremia. The effect of total-body exposure to x or gamma radiation on the susceptibility of rabbits and mice to bacterial infection has been the subject of inquiry by the junior authors for a number of years.[7-9] Postirradiation infection of enteric origin was found to occur in rabbits following irradiation with 900 r, but serial blood cultures during life and culture of organs at autopsy were often required to demonstrate such infection.

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EXPERIMENTAL PROCEDURE

Three general types of experiments were carried out. In the first, rabbits were exposed to a single dose of x-radiation which ranged from 50 to 1200 r. Daily serial bleedings were tested for the presence of Cx-reactive protein. In the second set of experiments, autopsy cultures were made on rabbits which died or were sacrificed at different times postirradiation. In the third set of experiments, irradiated rabbits in which an initial Cx-protein response had subsided, were inoculated intravenously with live or killed suspensions of Escherichia coli.

MATERIALS AND METHODS

Precipitating antiserum of high titer was obtained from a sheep 4 weeks following the intracutaneous injection into 6 separate skin sites of purified Cx-reactive protein incorporated into complete adjuvant. The immune serum which gave a slight cross reaction with normal rabbit serum was absorbed with dried normal rabbit serum.

The precipitin tests with antiserum to Cx-reactive protein were carried out in capillary tubes according to the procedure described by Anderson and McCarty.[10] This method is based on the capillary precipitin method for serological typing of Group A streptococci described by Swift, Wilson and Lancefield.[11] The quantity of precipitate formed was measured in millimeters.

The rabbits were healthy young adults, weighing 2 to 3 kg, obtained from a snuffle-free colony. After arrival at the laboratory they were housed in separate cages in a room from which other animals were excluded and were cared for by a man who had no contact with other rabbits. Food and water were available at all times.

Most of the rabbits were bled every day (except Sundays) beginning the day before irradiation. Five found to have positive reactions before irradiation were discarded. Blood was drawn aseptically from a marginal ear vein into a small syringe, allowed to clot overnight in a centrifuge tube, and centrifuged the following day. The serum was removed and stored in the refrigerator.

The animals were irradiated by exposure to x rays from a General Electric Maxitron 250, after they had been placed in perforated aluminum boxes of proper size to prevent undue movement. The radiation dose was delivered half to one side of the body and half to the other. The x-ray factors were: 250 kv, 30 ma, 0.5 mm Cu and 1 mm Al filter; approximately 45 r/minute; distance from tube to target (midpoint of rabbit) 79-80 cm.

For autopsy cultures the body cavities were opened aseptically. One or two ml of blood was aspirated from the heart and a drop was cultured on plates of blood agar and eosin-methylene blue agar, the remainder in brain heart infusion broth. Six pieces of the liver (totaling about 1/15 of the whole) were excised from different parts, including areas showing any gross pathological change. The pieces of liver were cultured in 50 ml of brain heart infusion broth. The spleen was removed, cut in several places, and dropped into a flask of broth. The lungs of most of the sacrificed rabbits were cultured by the same procedure as the liver. After 24 and 72 hours incubation, subcultures were made onto appropriate diagnostic media.

The bacterial suspensions used for intravenous inoculation were derived from a streptomycin-resistant strain of E. coli, of rabbit origin, grown for 24 hours on nutrient agar, suspended in saline and diluted to contain approximately 10^8 bacilli/ml. The numbers of viable bacilli were confirmed by plating, in quadruplicate, 0.1 ml of 10^{-6} and 10^{-7} dilutions.

Suspensions of killed bacteria were prepared from the same strain of E. coli in the same

manner, killed by heating in boiling water for 30 minutes, washed thrice by centrifugation, and using a Coleman spectrophotometer, diluted to contain approximately 10^9 bacilli/ml.

RESULTS

The appearance of Cx-reactive protein in the blood of irradiated rabbits was usually a diphasic phenomenon. It first appeared 24 to 48 hours after irradiation, persisted two to four days and then disappeared. This initial appearance, designated the primary phase, was observed in all but 5 of 97 rabbits exposed to 500 r or more, but in only 4 of 16 rabbits exposed to 200 r or less (see Table 1). Ten of 14 rabbits irradiated with 1200 r died during the primary phase as did 9 of 43 irradiated with 900 r.

Table 1

APPEARANCE OF Cx-REACTIVE PROTEIN FOLLOWING VARIOUS DOSES OF IRRADIATION

Irradiation dose (r)	No. rabbits tested	No. rabbits Cx positive in:		Deaths
		Primary phase	Secondary phase	
1200	14	14	4	14 (all by 9th day)
900	43	42	28	35
700	36	32	2 of 13*	0
500	4	4	1	0
200	4	2	0	0
100	4	0	1	0
50	8	2	0	0

*All but 13 of the 700 r rabbits were used in the intravenous inoculation experiments.

The primary phase which lasted two to four days was followed by a negative phase, i.e., a period in which Cx-reactive protein was absent from the blood. It reappeared during the second week postirradiation in many, but not all the rabbits exposed to the two highest doses of x ray (900 and 1200 r). This secondary phase occurred in only 2 of 13 rabbits in the 700 r series and in 2 of 20 exposed to 500 r or less.

Figure 1 illustrates various patterns of response following exposure to 900 r. The amount of Cx-reactive protein in each serum is recorded as mm of precipitate formed.

Rabbits A and B showed only the primary phase, which varied in duration from one to four days. Rabbit A was sacrificed on the 9th day for autopsy cultures, all of which showed no growth. Rabbits C and D showed both primary and secondary phases with a negative phase intervening. Rabbit C which was sacrificed during the secondary phase was found to have a generalized infection with E. coli. Rabbit D was sacrificed for autopsy cultures on the 22nd day after the secondary phase. Growth occurred in none of its cultures.

The effect of a second irradiation with 900 r was demonstrated when 3 rabbits which had been exposed to 900 r and allowed to recover were irradiated a second time with 900 r on the 49th day. Cx-reactive protein promptly reappeared in their blood, as it had following the initial exposure.

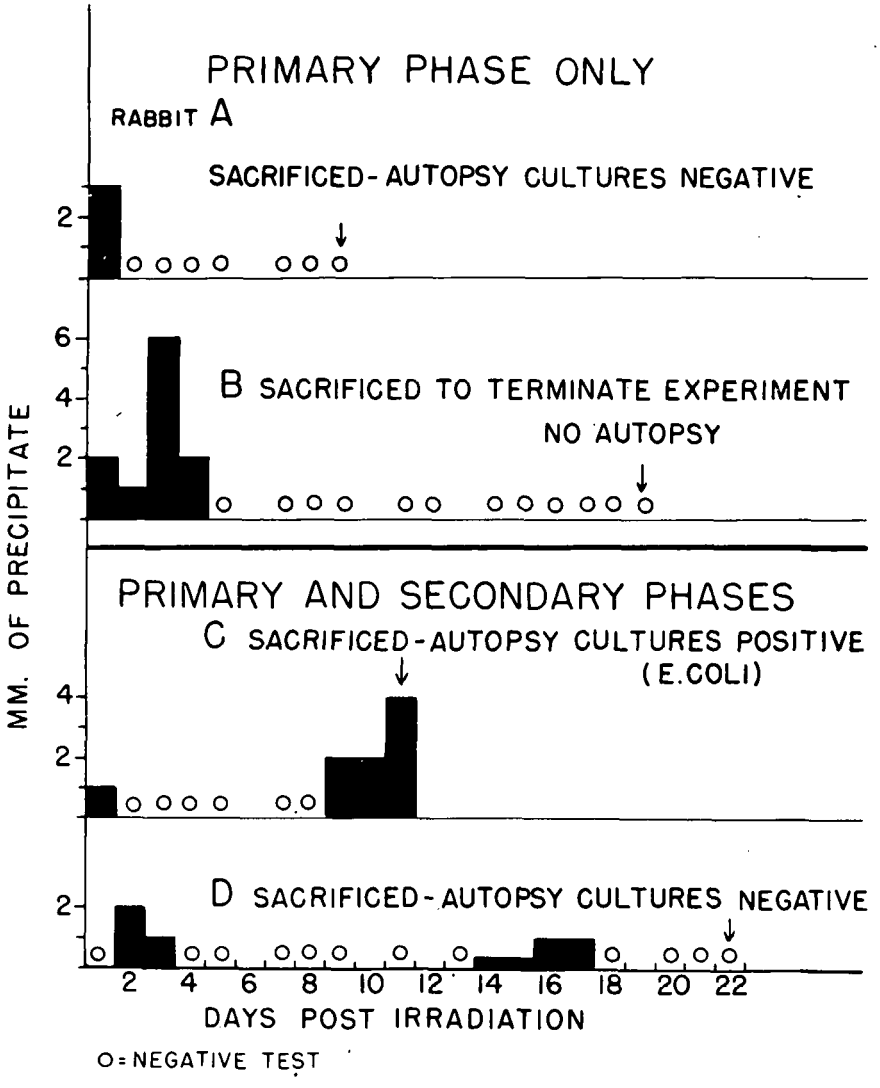

Figure 1. Determinations of Cx-reactive protein in the blood of 4 rabbits illustrating different patterns of response to irradiation with 900 r.

Autopsy cultures in the 900 r series showed no growth in cultures of heart's blood, liver, spleen or lung of 6 rabbits sacrificed during the primary phase and one which died during the intermediate or negative phase. Nor were any positive cultures obtained from 6 rabbits sacrificed and one which died during the post-secondary period, i.e., after the second disappearance of Cx-reactive protein (Table 2).

Bacteria were recovered, however, from all 15 animals which died and 6 of 9 sacrificed during the secondary phase, i.e., bacterial infection was demonstrated in all but 3 of 24 rabbits autopsied at this time. More exhaustive cultures of organs might possibly have revealed a focus of infection in these 3 rabbits. Earlier studies showed that the bacteria present in organs of irradiated (900 r) rabbits might be few in number.[7]

The microorganism most frequently recovered in autopsy cultures was E. coli which was

Table 2

RESULTS OF AUTOPSY CULTURES AND TESTS FOR Cx-REACTIVE PROTEIN AT DIFFERENT TIMES POSTIRRADIATION (900 r)

Phase	Time postirradiation (days)	Rabbits autopsied	Cx reaction at death or sacrifice	Culture results
Primary	1st - 5th	6 sacrificed	6 positive	6 negative
Negative	3rd - 11th	1 died	negative	negative
Secondary	4th - 12th	15 died 9 sacrificed	15 positive 9 positive	15 positive 6 positive, 3 negative
Postsecondary	13th - 21st	1 died 6 sacrificed	negative 6 negative	negative 6 negative

found in 14 rabbits. An unidentified gram-negative bacillus was recovered from 3. Less frequently found, sometimes associated with E. coli, were Staphylococcus aureus, a spore-forming rod, and an alpha Streptococcus. When bacterial infection was present, it was always found in the liver, and usually in the spleen and blood. With very few exceptions, the same microorganism or pair of microorganisms was recovered from all cultures of an infected rabbit. Thus the secondary, but not the initial appearance of Cx-reactive protein in the blood of irradiated rabbits was usually associated with bacterial infection.

In an attempt to suppress the secondary phase by treatment with antibiotics, 50 mg of penicillin* and 100 mg of dihydroxystreptomycin* were administered intramuscularly twice daily but failed to prevent the reappearance of Cx-reactive protein, presumably because the infections themselves produced sufficient trauma to stimulate its appearance. This explanation is based on the observation that similar injections into unirradiated rabbits caused the appearance of Cx-reactive protein.

The response to intravenous inoculation of viable bacteria during the negative phase was investigated by the following experiments which were designed to answer two questions: (a) did Cx-reactive protein disappear after the primary phase because the animals were no longer able to produce it, i.e., was the negative phase due to inability to elaborate Cx-reactive protein?, and (b) was the reappearance (secondary phase) caused by endogenous bacterial infection? (Table 3).

Small groups of rabbits were irradiated with 900 or 700 r and tested daily for the presence of Cx-reactive protein. The primary phase was demonstrated in all of them. After a rabbit had become negative, i.e., lost Cx-reactive protein from its blood, it was inoculated intravenously with a suspension containing 10^8 viable bacteria (E. coli). Cx-reactive protein reappeared in the blood of every rabbit within 24 hours—in many as early as 6 hours after infection. Two rabbits which were inoculated on the 47th day postirradiation, after a long period of negative tests, responded in a similar fashion. Cx-reactive protein persisted in the blood of every rabbit until it

*The antibiotics used in these experiments were generously supplied by the following: Lederle Laboratories, Charles Pfizer and Company, Inc., and E. R. Squibb and Sons.

Table 3

APPEARANCE OF Cx-REACTIVE PROTEIN FOLLOWING INTRAVENOUS INJECTION OF VIABLE OR DEAD BACTERIA (E. COLI) DURING NEGATIVE PHASE

Radiation dose (r)	Bacteria injected	Time of injection day postirradiation	No. rabbits	Duration of positive reactions*	Day of death postirradiation	Days observed postirradiation	No. surviving to end of experiment
900	10^8 viable	5th	8		6, 7, 8, 8, 9, 9, 9, 9	9	0
	10^8 viable	9th	4	Until death or	10, 11, 24	25	1
	10^8 viable	47th†	2	termination	-	78	2
700	10^8 viable	3rd	10	of	6, 8, 11, 15	16	6
	10^8 viable	4th	4	experiment	10, 17	19	2
none	10^8 viable	-	8		9	16	7
700	10^9 dead	3rd	4	1 - 2 days	-	9	4
	10^9 dead	4th	6	1 - 2 days	13	19	5
none	10^9 dead	-	3	1 - 4 days	-	18	3

*All became positive 6 - 24 hours after injection.

†These 2 rabbits were in the period of recovery from radiation injury.

died or was sacrificed to terminate the experiment.

All the rabbits irradiated with 900 r and inoculated with 10^8 E. coli died, except one which was sacrificed and the two which were inoculated on the 47th day postirradiation, i.e., after they had recovered from radiation injury. Deaths were less numerous among the 700 r rabbits inoculated with viable bacteria.

The response to injection of killed bacteria was tested when 10 rabbits irradiated with 700 r were injected intravenously with 10^9 killed E. coli on the 3rd or 4th day postirradiation. Cx-reactive protein appeared in the blood of each 24 hours after inoculation (none was tested at 6 hours) but disappeared again after 24 to 48 hours. In three cases, it reappeared 7 days after inoculation and disappeared 2 days later.

Three normal rabbits were injected intravenously with the same suspension of killed E. coli. The sera of all contained Cx-reactive protein 24 hours after inoculation. In two cases it disappeared within 48 hours but not until the 5th day in the third. The secondary phase did not occur in any of the normal rabbits.

DISCUSSION

These findings demonstrate that the appearance of Cx-reactive protein in the blood of rabbits can be elicited by exposure to ionizing radiation as well as by bacterial infection. In rabbits irradiated with 900 r, the pattern of response was usually biphasic. The initial appearance of the protein within 48 hours of exposure was followed by a secondary appearance after an intermediate period of several days during which it was absent from the blood. This secondary response was associated with bacterial infection, either spontaneous or induced.

While these observations give no indication of a possible biological function of the acute-phase protein, they emphasize the importance of identifying the agent or process responsible for its appearance, since many types of injuries have been shown to elicit it. Even in carefully controlled laboratory experiments, caution must be exercised in ascribing the appearance of Cx-reactive protein to a single known cause.

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STUDIES ON SUSCEPTIBILITY TO INFECTION FOLLOWING IONIZING RADIATION V. COMPARISON OF INTRAPERITONEAL AND INTRAVENOUS CHALLENGE AT INTERVALS FOLLOWING DIFFERENT DOSES OF X-RADIATION*†

By

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

Enhanced susceptibility to bacterial infection is one of the important changes which follow whole body exposure of mammals to moderate doses of ionizing radiation. Its occurrence during the postirradiation period has been well documented by laboratory and clinical observations, but the time of its onset and duration has not been studied as systematically as it deserves.

Schechmeister, Bond and Swift[1] infected mice by inhalation of an aerosol of Streptococcus zooepidemicus at intervals after total-body exposure to 350 r x-radiation. Their results showed that susceptibility to this air-borne infection increased steadily until the 15th day postirradiation, then declined at the same rate to the 30th day and slowly returned to normal by the 41st day.

Kaplan, Speck and Jawetz[2] related susceptibility to time postirradiation (450 r) by plotting average day of death after intramuscular injection of a standard inoculum (100 LD_{50}) of a beta hemolytic streptococcus (strain C203). By these criteria, susceptibility of the mice was maximal between the 3rd and 7th days after x-radiation. However, Schechmeister, Paulissen and Fishman[3] reported that they found susceptibility of mice to infection with Salmonella enteritidis inoculated intraperitoneally to be maximal 8 hours after irradiation with 350 r; but survival times were not reported, and it is well known that Salmonella enteritidis infection may not kill for a number of days after inoculation.

Marston et al.[4] found that susceptibility did not continue to increase beyond the 3rd day postirradiation (475 r) among mice injected subcutaneously with a standard inoculum of a virulent strain of Proteus vulgaris (Kf7).

Hammond, Colling, Cooper and Miller[5] inoculated RAP female mice, 9 to 10 weeks old, with approximately 10^7 Pseudomonas aeruginosa by stomach tube at 2 hours, 5 days and 11 days after whole body exposure to 550 r. Mortality from Pseudomonas bacteremia was greatest among the mice inoculated on the 11th day postirradiation and least among those inoculated 2 hours after irradiation.

In the experiments herein described, young adult mice were exposed to 300, 400, 475,** 500, or 600 r—in order to determine the effect of each dose on susceptibility to an experimental bacterial infection during the first three weeks postirradiation. This is a period when many patho-

*Based on a paper appearing in J. Exptl. Med., 111:773, 1960.

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‡Department of Medicine, University of Chicago.

**A single experiment at 475 r.

logical changes occur in rapid succession. It was necessary, therefore, to make frequent determinations and to assay susceptibility by inducing an acute, rapidly fatal infection so that the lapse of time between inoculation and death should be as short as possible. Pseudomonas aeruginosa was used because it kills more quickly than any microorganism we have tried for this purpose.

MATERIALS AND METHODS

The mice used were CF No. 1 females, ten weeks old, weighing 20 to 25 g. They were 8 weeks old on arrival, and were held in quarantine and examined daily for 2 weeks. If any evidence of Salmonella or other epizootic infection appeared, the whole shipment was discarded. All the mice challenged at one time came from a single shipment. They were housed on bedding of wood shavings, in groups of 10 to 20, in stainless steel cages measuring 15 x 9 x 7 inches. Cages were changed and autoclaved twice a week. Rockland mouse pellets were available at all times as was tap water in sterilized water bottles which were changed daily.

The mice were irradiated with a 250 KV, 30 ma Maxitron 250 (General Electric) machine using 0.25 mm copper and 1 mm aluminum filters at a target distance of 70 cm and a dose rate of approximately 60 r per minute. For details of the method of exposure see Hammond, Ruml, Cooper, Miller.[6]

The largest series of experiments was made with mice exposed to 500 r for two reasons: (a) this dose was usually sublethal, e.g., it killed only 3 among 100 uninoculated controls in 8 runs; and (b) from early experiments it was known to produce a marked effect on susceptibility to infection. Lower and higher doses were used in smaller series of experiments—300, 400, 475, and 600 r. Results in this last series were not wholly comparable with the others because 600 r was an LD_{50-70} for these mice.

The challenge microorganism was a streptomycin-resistant strain of Pseudomonas aeruginosa which has been used for challenge in this laboratory for a number of years. Its virulence for unirradiated CF No. 1 mice, maintained by weekly passage, has been remarkably constant. Its streptomycin resistance served merely to differentiate it in autopsy cultures from any other strains of Pseudomonas aeruginosa which the mice might have been carrying in their intestinal tracts.

An 18-hour culture on an agar plate was suspended in 5 ml saline, transferred to a 25-ml Erlenmeyer flask containing a few glass beads and agitated on an electric rotator for 15 minutes to disperse any clumps of bacteria. The suspension was then diluted to a standard density (approximately 10^9 microorganisms per ml) by means of a Coleman spectrophotometer. Ten-fold dilutions were made and the bacterial content checked by plating, in quadruplicate, 0.1 ml of the 10^{-6} and 10^{-7} dilutions.

The same suspensions were used for intravenous and intraperitoneal inoculations. Plate counts showed that practically no change occurred in the numbers of viable microorganisms in the suspensions during the time required to inject all the mice in the larger experiments.

The challenge inoculations were planned so that the largest inocula would kill all of the mice and the smallest none.

The mice to be inoculated were randomly distributed into groups of 10 to 20 and were then injected intraperitoneally or intravenously (into the dorsal tail vein) with 0.5 ml quantities using a 27-gauge needle. This volume was used because it could be measured more accurately than a smaller one and was not too large for intravenous inoculation. Great care was taken with all in-

travenous injections and any mouse was discarded if the needle failed to enter the vein on the first attempt or if any leakage of inoculum into the perivenous tissue was detected. Survivors were observed for 30 days after inoculation.

Both intraperitoneal and intravenous routes of inoculation were used in the 400 r and 500 r series, but only the intraperitoneal in the 300 r and 600 r series.

Non-irradiated controls were not included in every challenge inoculation because their LD_{50}'s were in such close agreement. Those included came from the same shipment as the irradiated mice.

Both least squares and probit analysis were used to calculate the LD_{50}'s. The values obtained by the two methods were in very close agreement, varying not more than 0.3 of a log. The LD_{50}'s listed in Tables 1 and 2 and the points plotted in Figures 1, 2, and 4 were those obtained by the method of least squares.

Every mouse which died was autopsied for culture of heart's blood unless it had been eaten by its cage mates. A few mice which died with negative blood cultures or with blood cultures positive for some other microorganism were excluded from the mortality data.

On the days indicated in Figure 3 and Table 3, white blood counts were made on blood drawn from a dorsal tail vein of uninoculated mice in each series.* No mouse was used more than once for this purpose. Differential counts were also made on representative samplings of mice.

RESULTS

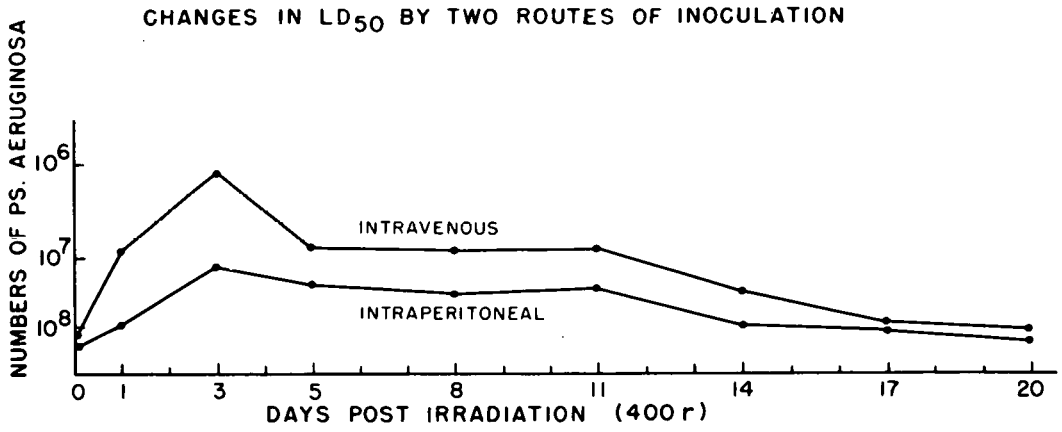

Figure 1. Changes in LD_{50} following 400 r.

400 and 500 r series. Both intravenous and intraperitoneal routes of inoculation were used in these series. Tables 1 and 2 list: (a) the LD_{50} of the inocula in each challenge, (b) the average of these for each day of challenge (postirradiation interval), and (c) the LD_{50} computed from the pooled mortality data for each day of challenge. It is these last computations (LD_{50} of the pooled mortalities) which are plotted in Figures 1 and 2. Table 2, which presents the data on the 500 r series, also includes the results, in parentheses, of a single experiment on mice irradiated with 475 r.

*The leucocyte counts were made by Frances S. Vandervoort, Betty Wolfe, and Gilbert Claudio.

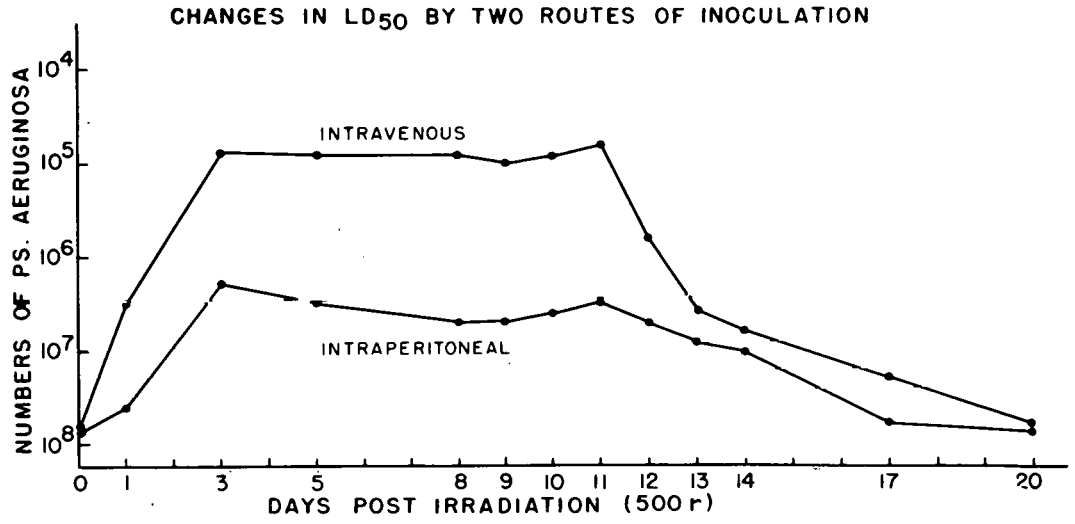

Figure 2. Changes in LD_{50} following 500 r.

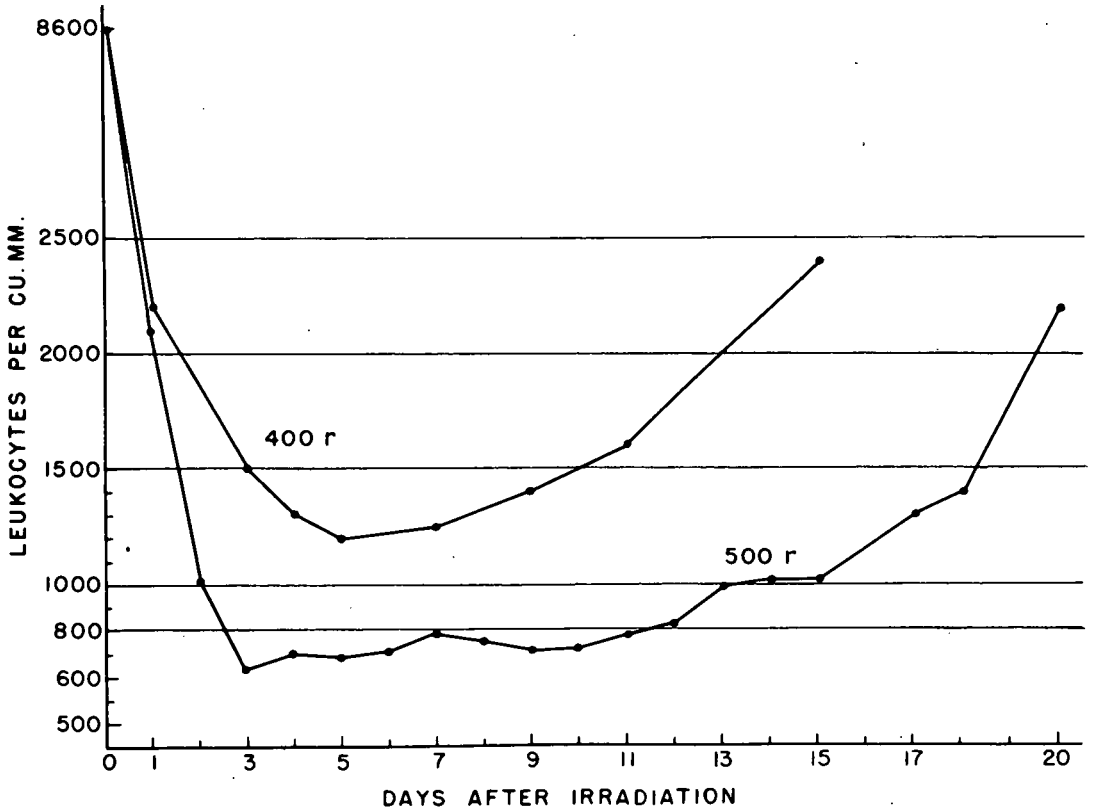

Figure 3. Geometric means of leucocyte counts of mice irradiated with 400 and 500 r.

Table 1

RESULTS OF CHALLENGE INOCULATIONS - 400 r SERIES

Day post-irradiation	Intravenous				Intraperitoneal			
	LD_{50}	No. mice	Averaged LD_{50}'s	LD_{50} of pooled mortality data	LD_{50}	No. mice	Averaged LD_{50}'s	LD_{50} of pooled mortality data
1	4.8×10^6	40	9.4×10^6	9.4×10^6	4.7×10^7	40	7.3×10^7	7.0×10^7
	1.4×10^7	60			1.0×10^8	67		
3	1.0×10^6	80	2.1×10^6	1.5×10^6	8.7×10^6	96	1.2×10^7	1.4×10^7
	3.2×10^6	96			2.6×10^7	72		
5	3.0×10^6	40	8.2×10^6	8.1×10^6	1.4×10^7	40	2.4×10^7	2.0×10^7
	6.6×10^6	40			2.0×10^7	40		
	1.5×10^7	80			4.0×10^7	80		
8	3.2×10^6	80	1.0×10^7	9.5×10^6	2.8×10^7	80	2.9×10^7	3.0×10^7
	1.8×10^7	80			3.0×10^7	80		
11	6.8×10^6	80	8.4×10^6	8.2×10^6	9.5×10^6	80	2.5×10^7	2.4×10^7
	1.0×10^7	95			4.0×10^7	76		
14	3.1×10^7	80	3.1×10^7	3.1×10^7	4.8×10^7	80	5.0×10^7	5.1×10^7
	3.2×10^7	80			5.2×10^7	80		
17	4.0×10^7	80	5.8×10^7	5.4×10^7	6.8×10^7	60	7.6×10^7	7.3×10^7
	7.6×10^7	76			8.4×10^7	57		
20	6.2×10^7	80	7.2×10^7	7.3×10^7	6.4×10^7	60	7.7×10^7	8.0×10^7
	8.3×10^7	54			9.1×10^7	54		
Normal controls	7.1×10^7	30	7.5×10^7	7.5×10^7	7.4×10^7	30	7.9×10^7	8.1×10^7
	7.3×10^7	30			7.6×10^7	30		
	7.5×10^7	24			8.0×10^7	24		
	7.9×10^7	30			8.2×10^7	30		
	8.0×10^7	30			8.3×10^7	30		

In both series, susceptibility to the experimental infection increased rapidly to the 3rd day postirradiation. In the 400 r series, it declined thereafter; but in the 500 r series, it was maintained until the 11th day. In both series susceptibility had returned to normal by the 20th day postirradiation.

300 and 600 r series. These mice were challenged at only 3 intervals after irradiation and only by intraperitoneal inoculation. Exposure to 300 r caused but slight increase in susceptibility to the experimental infection. Exposure to 600 r, however, was followed by a very marked increase, a not unexpected result since this dose of radiation is an LD_{50-70} for these mice.

As was pointed out in the Introduction, Pseudomonas aeruginosa was chosen as the test microorganism because it produces a rapidly lethal infection. Deaths among unirradiated mice usually occurred within 18 to 30 hours, occasionally as late as 48 hours, only rarely thereafter. Although most deaths among irradiated mice followed this time schedule, some were delayed beyond it. With few exceptions these late deaths resulted from intravenous inoculation of the lower doses of Pseudomonas aeruginosa. As they were shown by positive heart's blood culture to have been due to Pseudomonas infection, and as they constituted only a small fraction of the total, they were included in the mortality data.

The geometric means of the leucocyte counts on 120 uninoculated mice exposed to 400 r, and

Table 2

RESULTS OF CHALLENGE INOCULATIONS - 500 r SERIES
(and one experiment with 475 r*)

Day post-irradiation	Intravenous				Intraperitoneal			
	LD_{50}	No. mice	Averaged LD_{50}'s	LD_{50} of pooled mortality data	LD_{50}	No. mice	Averaged LD_{50}'s	LD_{50} of pooled mortality data
1	1.0×10^6	30	3.1×10^6	3.2×10^6	2.6×10^7	30	3.8×10^7	4.3×10^7
	5.2×10^6	56			5.0×10^7	50		
3	8.7×10^4	50	1.4×10^5	9.2×10^4	1.0×10^6	40	2.6×10^6	2.3×10^6
	2.0×10^5	50			4.2×10^6	40		
5	3.0×10^4	30	1.6×10^5	9.4×10^4	1.0×10^6	50	3.9×10^6	3.4×10^6
	2.3×10^5	50			3.4×10^6	50		
	2.7×10^5	78			7.4×10^6	65		
8	6.2×10^4	55	9.4×10^4	9.5×10^4	4.3×10^6	44	5.8×10^6	5.8×10^6
	7.1×10^4	120			6.2×10^6	80		
	1.5×10^5	78			7.1×10^6	78		
9	1.6×10^4	78	9.1×10^4	1.3×10^5	9.8×10^5	80	5.6×10^6	5.9×10^6
	5.8×10^4	114			5.8×10^6	78		
	2.0×10^5	80			1.0×10^7	120		
	(4.1×10^5)*	80			(6.5×10^7)*	114		
10	6.1×10^4	65	7.4×10^4	7.6×10^4	9.7×10^5	77	3.2×10^6	4.5×10^6
	6.3×10^4	133			1.0×10^6	110		
	7.2×10^4	40			2.3×10^6	40		
	1.0×10^5	40			8.8×10^6	40		
11	6.6×10^3	60	7.0×10^4	5.2×10^4	8.3×10^5	30	2.9×10^6	3.2×10^6
	3.5×10^4	50			1.0×10^6	50		
	5.2×10^4	50			2.5×10^6	50		
	7.7×10^4	36			3.3×10^6	60		
	1.8×10^5	60			7.1×10^6	80		
	(2.3×10^5)*	80			(3.4×10^6)*	60		
12	5.9×10^5	72	1.3×10^6	7.0×10^5	6.7×10^6	72	6.9×10^6	6.2×10^6
	2.1×10^6	84			7.1×10^6	60		
13	1.0×10^6	94	7.0×10^6	4.4×10^6	8.7×10^6	65	8.8×10^6	8.9×10^6
	1.3×10^7	65			9.0×10^6	61		
	(4.2×10^5)*	80			(1.1×10^7)*	80		
14	2.0×10^6	40	6.5×10^6	7.1×10^6	6.6×10^6	40	1.4×10^7	1.0×10^7
	1.1×10^7	72			2.3×10^7	72		
17	1.6×10^7	65	1.6×10^7	2.4×10^7	5.0×10^7	60	6.1×10^7	6.8×10^7
	1.6×10^7	75			7.2×10^7	40		
20	7.2×10^7	54	7.2×10^7	7.2×10^7	7.4×10^7	42	7.4×10^7	7.4×10^7
Normal controls	7.3×10^7	30	7.6×10^7	7.7×10^7	7.4×10^7	30	8.0×10^7	8.1×10^7
	7.4×10^7	30			7.5×10^7	30		
	7.5×10^7	24			7.7×10^7	30		
	7.7×10^7	30			8.0×10^7	30		
	8.0×10^7	24			8.4×10^7	30		
	8.1×10^7	30			8.5×10^7	30		

*Results of one experiment with 475 r are shown in parentheses, but not included in averaged or pooled LD_{50}'s.

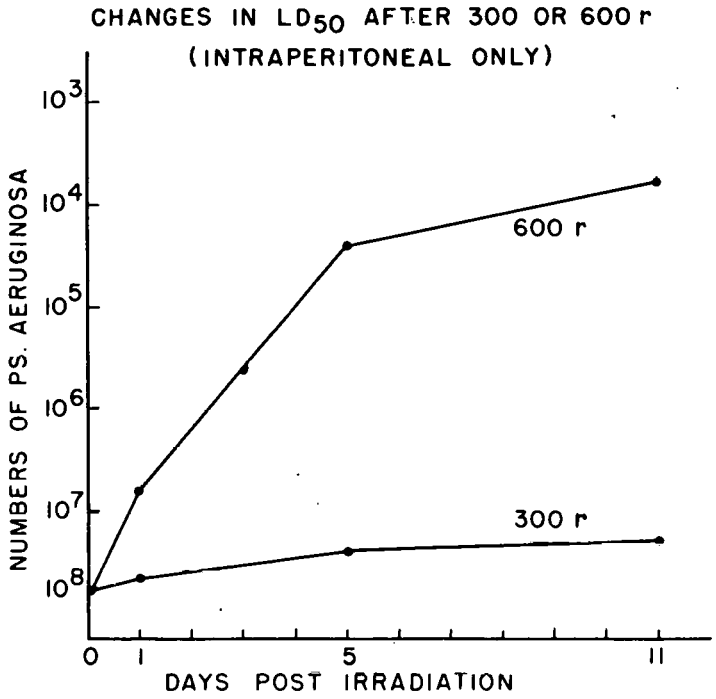

Figure 4. Changes in LD_{50} following 300 r and 600 r.

on 596 exposed to 500 r, are plotted in Figure 3. In the 400 r series, the lowest level (1200) occurred on the 5th day postirradiation, after which the counts rose steadily. In the 500 r series, they fell much more steeply to a minimum of 640 on the 3rd day and remained below 800 until the 12th day.

The geometric means of the leucocyte counts on 90 uninoculated mice exposed to 300 r, and 111 exposed to 600 r are shown in Table 3.

Table 3

TOTAL LEUCOCYTE COUNTS

Days postirradiation	300 r	600 r
1	3000	2100
5	2400	500
11	4200	750

Differential leucocyte counts are presented in Table 4 as percentages of lymphocytes and polymorphonuclear heterophiles. The other types of leucocytes have been omitted from the table, as they were never numerous and became very scarce during the periods of leucopenia. The most interesting finding was the almost total disappearance of polymorphonuclears on the 11th day in the 500 and 600 r series.

According to the curves in Figures 1 and 2 and the data in Tables 1 and 2, irradiated mice

were more susceptible to the experimental infection when it was initiated by intravenous inoculation. This difference was due to the fact that equivalent numbers of Pseudomonas aeruginosa caused more deaths when they were injected intravenously than when they were injected intraperitoneally. Among unirradiated mice, however, there was practically no difference in the mortalities resulting from the two routes of inoculation.

Table 4

DIFFERENTIAL LEUCOCYTE COUNTS LYMPHOCYTES (L) AND POLYMORPHONUCLEAR HETEROPHILES (P) PER CENT
AVERAGES OF 10-15 MICE

Day postirradiation	400 r		500 r		600 r	
	L	P	L	P	L	P
1	66	34	58	42	66	34
5	70	30	79	21	92	8
11	80	20	99	1	99	1

Unirradiated Controls: lymphocytes 70 per cent; polymorphonuclear heterophiles 25 per cent.

In search of an explanation for this disparity, additional experiments were carried out. The results, summarized below, show the greater lethality of intravenously induced infection in irradiated mice to have been due to the establishment of a focus of infection resulting from leakage of a minute fraction of inoculum into the perivenous tissues at the site of injection.

(a) Leakage into the perivascular tissues during intravenous inoculation was demonstrated by injecting India ink into the tail vein with the same care as was used in the inoculations. After amputation, dehydration, and clearing, a small spot of India ink was distinctly visible by transmitted light at the site of the injection.

(b) Microscopic studies were made of sections of the tails of mice inoculated intravenously with staphylococci, which could be identified in stained preparations more accurately than Pseudomonas. As late as forty-eight hours after injection, staphylococci were to be seen in and around the inoculated vein. In unirradiated mice the perivenous tissue was infiltrated with leucocytes, while in irradiated mice (5 days after 500 r) almost no leucocytes were present and the staphylococci were much more numerous, i.e., had multiplied in the irradiated mice.

(c) Normal and irradiated mice (on the 5th day after 500 r) were inoculated intravenously with Pseudomonas aeruginosa, the normals with 10^7 and the irradiated mice with a tenth that number (10^6), since the larger dose would have been lethal for them. One minute later and on the 3 succeeding days, mice were killed and the tissues about the site of injection were excised, homogenized in streptomycin broth, and cultured by plating 0.1 ml of 10-fold dilutions.

The results, plotted in Figure 5 show that shortly after an intravenous injection, approximately one ten-thousandth of the bacteria in each inoculum was recovered from the subcutaneous tissues at the site of inoculation. They also show that during the next 3 days, the average numbers of these bacteria increased in the irradiated mice but decreased in the unirradiated mice.

The irradiated (leucopenic) mice were unable to dispose of these extravascular bacteria

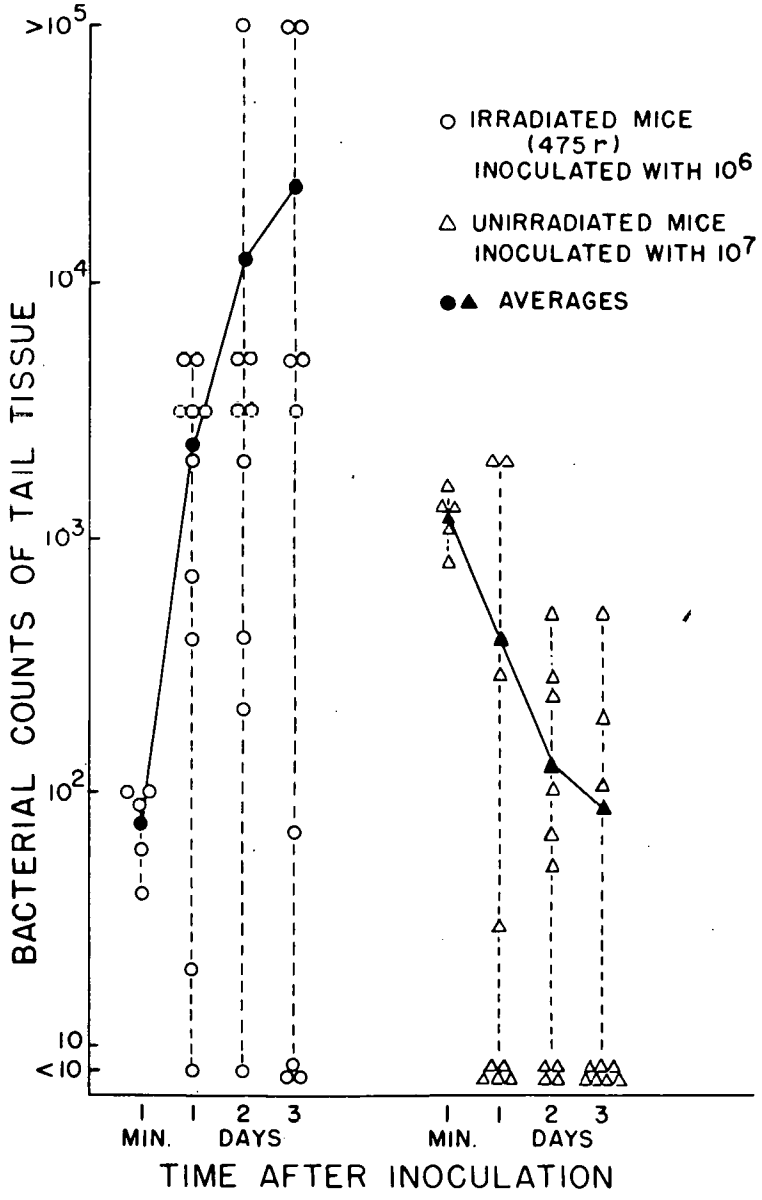

Figure 5. Numbers of Ps. aeruginosa recovered from homogenates of tissue at site of intravenous inoculation.

which were able to multiply and create a focus of infection, a process which may have been facilitated by the toxin produced by Pseudomonas aeruginosa.

Such foci of infection were not found at the site of intraperitoneal inoculation even in irradiated mice.

DISCUSSION

In this investigation the effect of four levels of x-radiation on susceptibility to bacterial infection was studied by comparing a series of LD_{50}'s, determined at intervals postirradiation, with those obtained on unirradiated mice. The LD_{50}'s were estimated by customary methods

from mortality data on mice challenged with graded inocula of the test microorganism, Pseudomonas aeruginosa. Such a method of biological assay inevitably involves uncontrollable factors which are increased in an animal host suffering from the radiation syndrome.

As is well known, exposure to doses above 300 r initiates a sequence of pathological changes which do not develop at the same rate or to the same degree in all members of a mouse population. In other words, innate or at least inherent differences in radiosensitivity exist among individuals of the same age and origin. The mice used of necessity in these experiments were not a highly inbred strain. To compensate for their lack of uniformity, large numbers were used and the results checked by repetition.

Although our quantitative estimations of susceptibility to the bacterial infection (LD_{50}'s) must be regarded as approximations, the results do show certain definite effects: Intraperitoneal inoculations show, as expected, that the larger the dose of radiation, the greater the effect on susceptibility to the experimental infection. Exposure to 300 r caused practically no change; the effect of 400 r was moderate but transitory, and 500 r produced a marked and prolonged effect. 600 r produced the most marked change, but caused so many radiation deaths that the results are not comparable with the others.

In the 400 and 500 r series, in which two routes of inoculation were used, susceptibility to the experimental infection appeared to have been increased much more in the mice challenged by intravenous inoculation than in those challenged intraperitoneally.

Among irradiated mice higher mortalities resulted from equivalent inocula injected into the tail vein because a local infection was established at the site of injection. Their leucopenia prevented the irradiated mice from combating this infection which added substantial numbers of bacteria to those already given by inoculation. The establishment of the local infection was probably facilitated by the toxin produced by the test microorganism. Such foci of infection were not found at the site of intraperitoneal injection, by which route there is a minimum of mechanical injury. Injection into a tail vein is a slower and more painstaking procedure and inevitably results in some degree of local trauma and leakage of a minute fraction of the inoculum into the surrounding tissues which have a poorer vascular supply than those of the abdominal wall.

Comparison of the curves in Figures 1, 2, and 3 brings out the correlation of severity of leucopenia with increased susceptibility to infection. It is particularly striking in intravenously inoculated mice in the 500 r series, presumably for the reasons described. Smith et al. have emphasized the importance of leucopenia, especially granulocytopenia, as a factor in postirradiation susceptibility to bacterial infection.[7,8]

ACKNOWLEDGMENTS

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OPTIMUM TREATMENT TIME IN COMBINATION THERAPY WITH ACTINOMYCIN AND IRRADIATION*

By

K. Ranniger† and M. L. Griem

Among the drugs that have been used in conjunction with x-rays in the treatment of malignancies are several Actinomycin compounds derived from cultures of Streptomyces.[1] These antibiotics are reported to promote cytostatic activity in several experimental animal and human tumors,[2-4] and to potentiate the skin reaction of radiation.[5] Whether they are capable of enhancing the effect of x-rays upon tumors is still under discussion.

The following experiments were designed to determine the optimum time of administration of Actinomycin D‡ in combination with irradiation in laboratory animals.

METHODS

Normal, Sprague-Dawley strain female rats weighing 220 to 250 g and bearing a Walker 256 carcinoma about 1 cm in diameter in the hind leg were selected for treatment. Before irradiation these rats were given single intraperitoneal injections of 150 μg/kg of Actinomycin D, freshly dissolved in physiological saline. They were then immobilized in 5 mm thick protective lead boxes leaving only the tumor-bearing hind leg exposed. The animals were not anesthetized.

Radiation was administered to the tumor-bearing hind legs, one hour, 4 hours, and 24 hours after injection of the drug. The x-ray dose was 2500 r measured in air. Technical factors were: 250 kv, HVL 1.5 mm Cu, FSD 62 cm.

Each group of experimental animals consisted of about 50 rats, divided into six treatment groups as follows:

A. Control animals receiving no treatment,

B. Animals receiving drug only,

C. Animals receiving x-ray only,

D. Animals treated with x-ray one hour, 4 hours, and 24 hours after injection of the drug.

The experiment was repeated until the data were considered statistically significant.

RESULTS

Figure 1 represents the average tumor size produced by each treatment in comparison with the progressive growth of tumors in untreated animals. It can be seen that treatment with Actinomycin alone does not produce a marked decrease in tumor size. Irradiation alone retards tumor growth in comparison to the controls and to Actinomycin-treated animals. When radiation is given one and four hours after injection of the drug, the results are similar to those obtained by irradiation alone. If irradiation is delayed until 24 hours after Actinomycin injection, the ra-

*Summary of a paper presented at May 1960 meeting of the Radiation Research Society.

†Department of Radiology, The University of Chicago.

‡Actinomycin D was courteously supplied by Dr. Elmer Alpert, Merck, Sharp, and Dohme Research Laboratories, West Point, Pennsylvania.

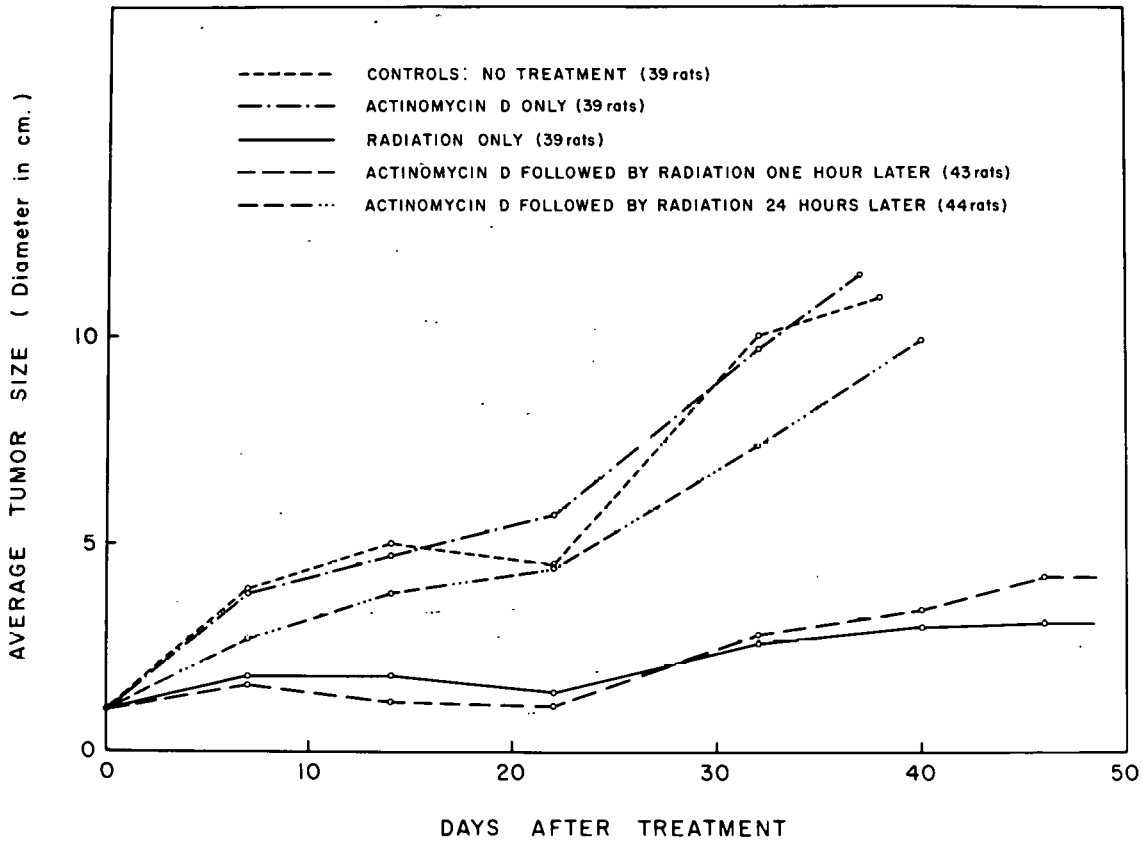

Figure 1. Average tumor size at various times following combination therapy with Actinomycin D and radiation as compared to controls.

diation effect is diminished and the tumor grows as fast as in an untreated animal.

Figure 2 shows that the length of survival agrees with the rate of tumor growth. Non-treated animals and animals injected with Actinomycin D die at approximately equal rates. Irradiation alone prolongs survival as do Actinomycin injections followed by irradiation after one hour.

Radiation administered 24 hours after drug injection appreciably shortens the length of survival compared to irradiation alone, and the survival time is the same as in non-treated animals.

DISCUSSION

These experiments confirm the results of those investigators who found that a single dose of 150 μg/kg of Actinomycin D had no cytostatic effect upon Walker 256 carcino-sarcoma. They show further that irradiation one hour and four hours following the administration of the drug may only slightly enhance the radiation effect. It is of interest to note that Actinomycin followed by irradiation after 24 hours depresses the cytostatic effect of radiation on this tumor. Hackman[3] found that following a single injection of Actinomycin C there was an increased number of mitoses in mouse tumors during the first five hours followed by a marked depression of the mitotic count to below normal. This depression reaches its maximum about 22 hours after drug injection. Radiation sensitivity is known to be greatest during the early phases of mitosis and lowest during the resting phase of the cell.[6] This may explain why radiation administered 24 hours

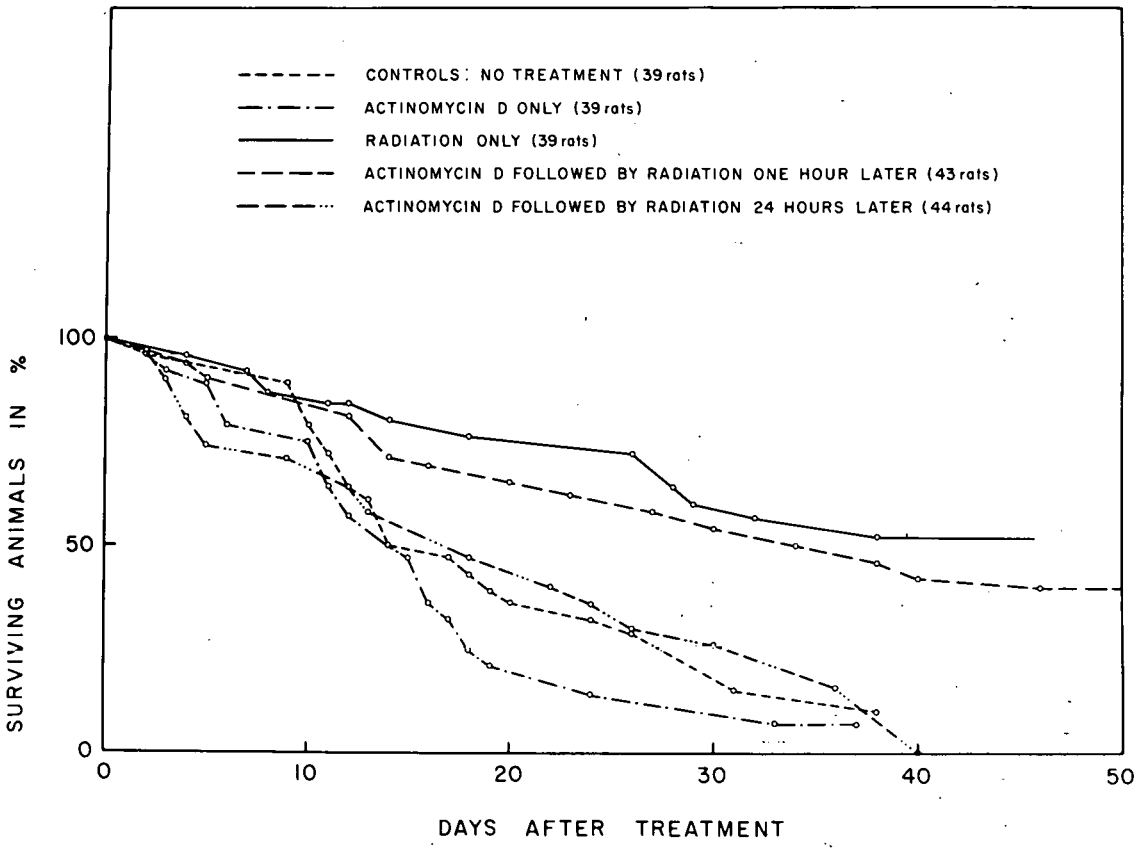

Figure 2. Survival time following combination treatment with Actinomycin D and radiation as compared to controls.

after injection of Actinomycin D is relatively ineffective, since at this time the number of cells in mitosis is low.

Similar experiments on other animal tumors indicate that the findings described here for the Walker tumor will be confirmed. A time relationship has not been established in human cancers, but these results suggest that in combined treatment of malignancies with x-ray and Actinomycin D, radiation should be given not later than five hours following administration of the drug. The experimental data presented cover only the first 24 hours following administration of Actinomycin D, and the relationship between the drug and irradiation past this time has not yet been investigated.

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STEROID FEVER STUDIES: PHYSIOLOGICAL DIFFERENCES BETWEEN BACTERIAL PYROGENS AND ENDOGENOUS STEROID PYROGENS OF MAN*

By

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

Previous studies from these laboratories have shown that a number of steroid metabolites of the 5β type, derived from gonadal and adrenocortical hormones, provoke intense fever when injected into humans, and several aspects of the relation between chemical structure and thermogenic activity of these endogenous hormonal transformation products have been reported earlier.[1-4] These pyrogenic steroids include 19- and 21-carbon compounds of both the 11-desoxy and 11-oxygenated series such as etiocholanolone, 11-hydroxyetiocholanolone, pregnanediol, pregnanolone and 11-ketopregnanolone. Typical examples of the fever produced in man by these compounds are shown in Figure 1. The chart in the lower right hand corner of this figure demonstrates the pyrogenic reaction provoked in man by injection of the endogenous biliary substance lithocholic acid,[5] and indicates that the fever-producing action of steroids extends to 24 carbon compounds of appropriate chemical structure.

Studies on the mechanism by which these steroids produce fever in man have led to a comparison of their biological properties with those of bacterial pyrogens and a number of differences have been demonstrated

Steroid pyrogen fever is associated with a significant leukocytosis which generally parallels the course of the temperature elevation as shown in Figure 2. It has not been possible, however, to demonstrate at any time during the initial hours following steroid injection, the transient, early and profound leukopenia which is one of the most characteristic hematologic features of bacterial pyrogen fever.[6,7]

Bacterial endotoxin-induced fever can be provoked in man and a number of experimental animals as well.[6,7] In contrast, steroid pyrogen-induced fever appears to have a high degree of species specificity.[8] The upper left hand chart in Figure 3 shows the typical fever produced in man by intramuscular injection of 11-ketopregnanolone in a dose of 0.2 mg/kg body weight. The remaining charts demonstrate the temperature response in 11 species of laboratory and domestic animals injected with a dose of this steroid 5 times as large. The temperature variations shown are within the normal daily limits for these animals except for the non-specific hypothermic reaction of the mouse. It has not been possible to provoke significant or consistent fevers in these animals by intramuscular, intravenous or intracerebral injection of a variety of steroid pyrogens in doses ranging up to 25 times those which provoke intense fever in man.

Bacterial pyrogen fever is characterized by a latency period of approximately 20 to 90 minutes and a peak temperature elevation which occurs 2 to 3 hours after injection.[6,7] An example

*This paper was presented at a meeting of the Association of American Physicians in Atlantic City, May 1960, and appears in Trans. Assoc. Am. Physicians, 73:176, 1960.

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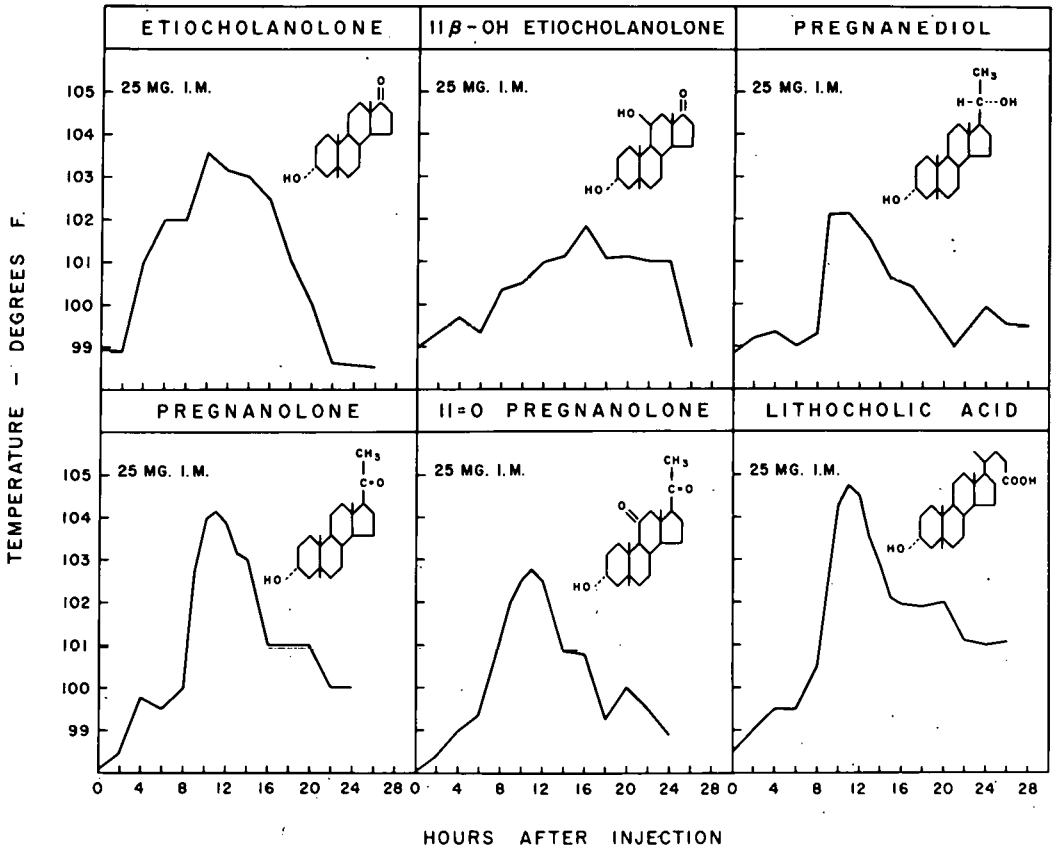

Figure 1. Examples of the fever produced in man by I.M. injection of endogenous steroid pyrogens.

of this type of fever in man is shown on the left side of Figure 4. In contrast, steroid pyrogen fever is characterized by a latency period of approximately 4 hours or more and a peak temperature elevation which occurs 9-12 hours after injection as shown in the fever curves on the right side of this figure. A somewhat more abrupt fever can be provoked by larger doses of the more potent compounds of this type. However, it is of interest that the latency period of fever produced by intravenous injection of steroid pyrogens is frequently as prolonged as that seen in fever produced by intramuscular injection of these compounds.

Steroid pyrogen fever is most consistently and effectively produced in man by intramuscular injection of these metabolites. Intravenous administration of these compounds does not, for unexplained reasons, always produce fever, but it has been observed in some subjects that fever production following intravenous injection of these steroids is dependent in part on the length of the period of infusion. These responses are demonstrated in Figure 5 in a subject receiving the same dose of steroid pyrogen by intramuscular injection and by rapid and slow intravenous injection.

In contrast bacterial pyrogens consistently produce fever in susceptible animals by immediate intravenous injection; the extent of this fever is generally not influenced by prolonged intravenous infusion; and these agents provoke fever irregularly by intramuscular injection.[6,7]

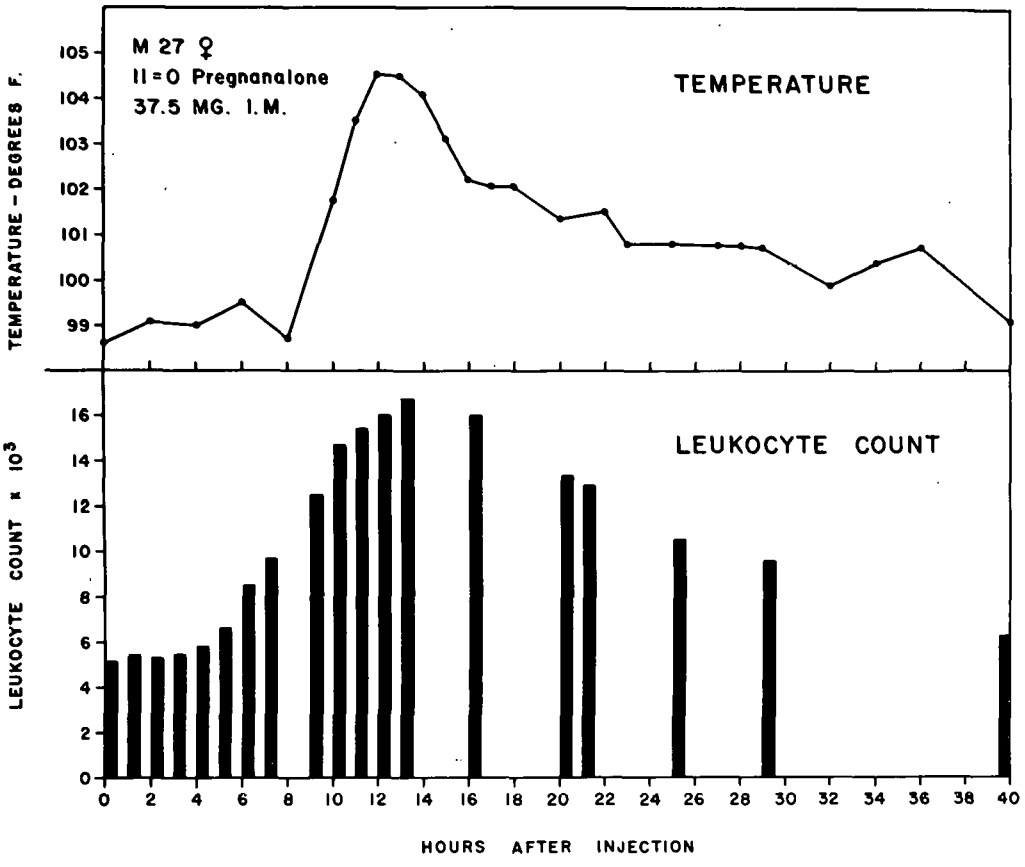

Figure 2. Example of the granulocytosis produced by steroid pyrogen injection in man.

Fever production diminishes markedly following repeated injection of bacterial pyrogens in man and experimental animals.[6,7] This phenomenon, described as the development of "tolerance," has been shown to be due in part to the functional status of the reticuloendothelial system.[9-11]

Repeated injection of steroid pyrogens, however, does not result in the development of tolerance to the fever-producing activity of this group of substances. Figure 6 shows a representative study of this type in two subjects receiving frequent small injections of 11-ketopregnanolone. The fever produced by this steroid pyrogen remains undiminished and this compound has been shown to provoke recurrent fevers of the same magnitude for periods as long as several weeks. Comparable results have been observed with other steroid pyrogens, such as etiocholanolone.[2]

Bacterial endotoxins stimulate release of a secondary, biologically distinct thermogenic substance into the blood stream of animals during the course of experimental fever. This substance called "endogenous pyrogen," is derived from leukocytes, provokes immediate temperature elevation following passive serum transfer, does not produce tolerance and undoubtedly plays a prominent role in the pathogenesis of bacterial endotoxin-induced fever.[12-19]

It has not been possible to demonstrate the presence of significant amounts of a substance

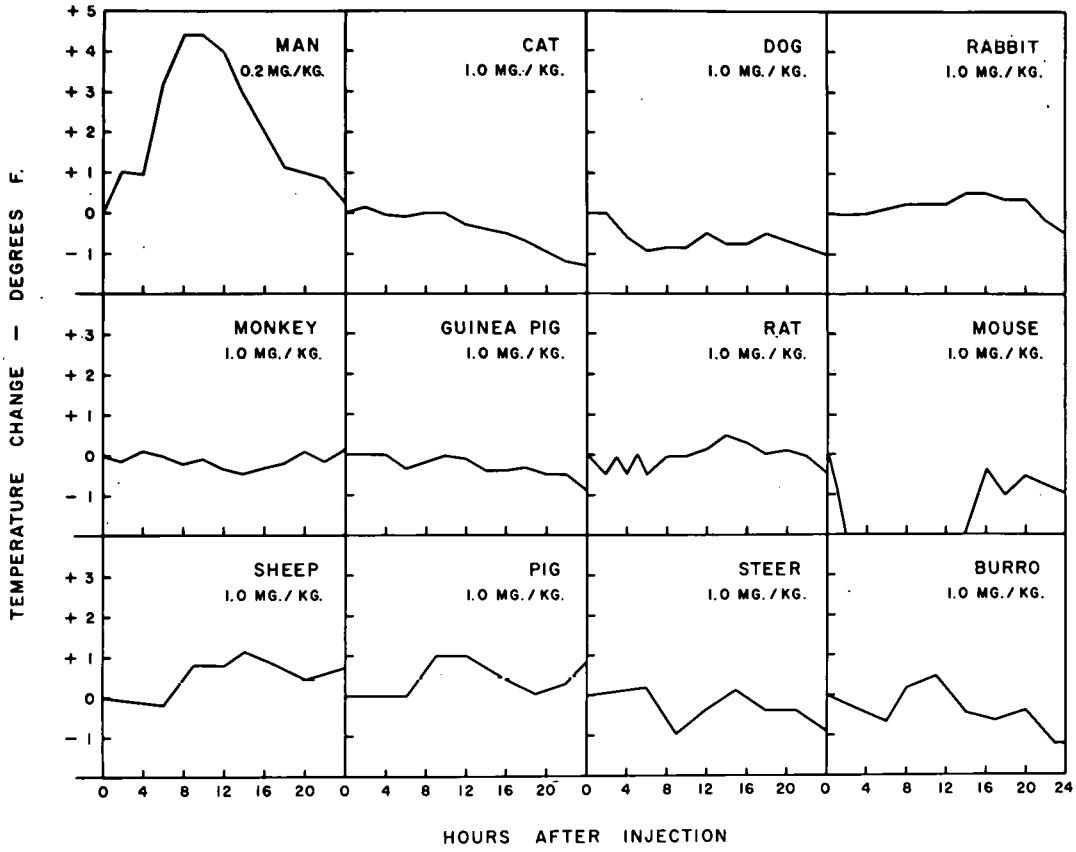

Figure 3. Comparative effect on temperature produced by I.M. injection of 11-ketopregnanolone in man and various experimental and domestic animals.

comparable to this leukocyte pyrogen in the plasma of humans during the course of steroid-induced fever.

Figure 7 shows the results of plasma transfer experiments in two subjects in whom studies were conducted as follows: subjects A and B each received 3 to 4 repeated injections of steroid pyrogen over several days. At the peak of each fever, as indicated by the arrows in the model fever curve on the left in Figure 7, a phlebotomy was done. The red blood cells were separated and returned to the donors and the plasma was pooled. When the donors were afebrile, 1000 ml or more of the plasma was rapidly injected into each and temperature was carefully observed. As shown, neither subject developed significant fever following these plasma transfer experiments.

Figure 8 shows the results of comparable experiments in two subjects, from whom plasma was obtained at the end of the latency period of steroid fever, as indicated by arrows G and H in the model fever curve on the left. Plasma transfer in these experiments also failed to provoke significant temperature elevation in the recipients. A total of 10 plasma transfer experiments of the types described here, were done. All involved plasma transfer in the amounts of 1000 ml or more, and in no instance was the presence of significant amounts of a fever-producing substance in the plasma demonstrated.

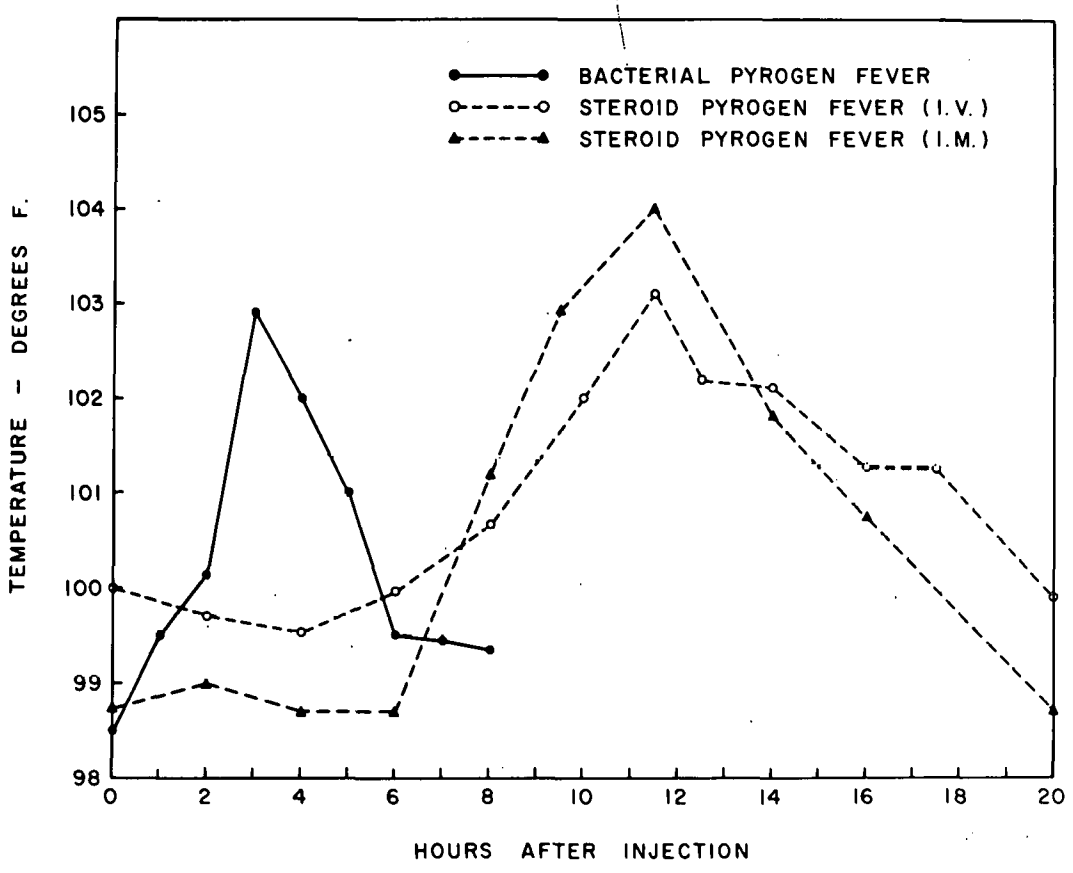

Figure 4. Comparison of latency period of fever produced by bacterial and steroid pyrogens in man.

In summary, the physiological properties of bacterial pyrogens and endogenous steroid pyrogens of man are shown to differ in several important respects, and these differences confirm the identity of pyrogenic steroids as a new, potent and biologically distinct class of fever-producing compounds. Thermogenic substances which have previously been isolated from animal tissues, share the physico-chemical properties characteristic of either polysaccharides[20] or proteins.[21] The pyrogenic steroids which have been described in this and previous reports, however, are pure compounds of lipid nature, and thus the chemical basis of potential fever-producing substances of endogenous origin is considerably broadened.

The physiologic significance of the prolonged latency period of steroid fever is not clear at the present time. This delay in onset of fever is consistent with several possible mechanisms of steroid pyrogen action including the development of a secondary thermogenic substance, comparable to the leukocyte pyrogen, which then becomes the proximate cause of fever. Consideration was given this possibility in the present studies, but no evidence for the release of such a substance into the blood stream of man could be obtained. Moreover, it is difficult to believe that significant amounts of a circulating endogenous pyrogen of the leukocyte type could escape detection in passive transfer experiments involving 1000 ml or more of plasma.

It seems reasonable to conclude, therefore, that the physiologic differences between bacterial and steroid pyrogens noted in these studies imply that these substances provoke fever by

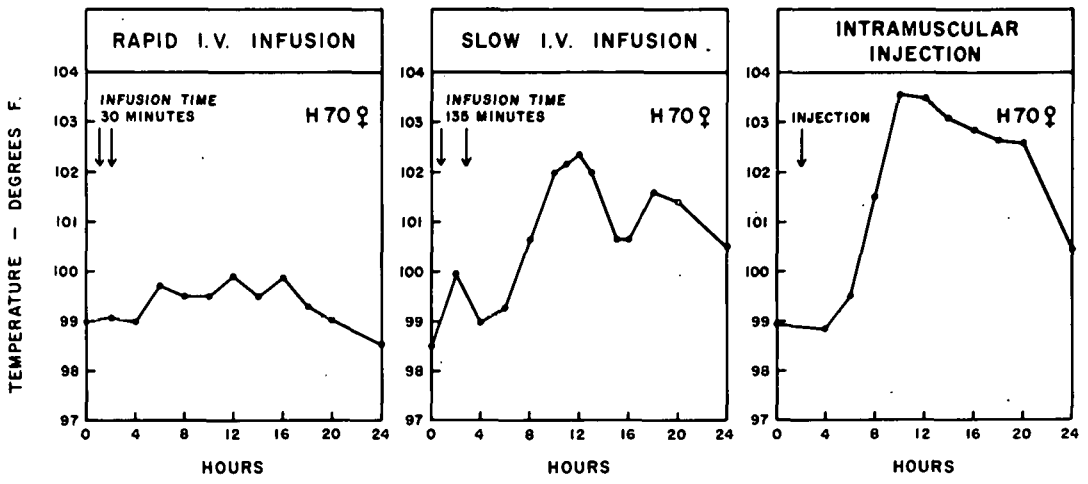

Figure 5. Effect of mode of administration of steroid pyrogen on fever production in an adult human.

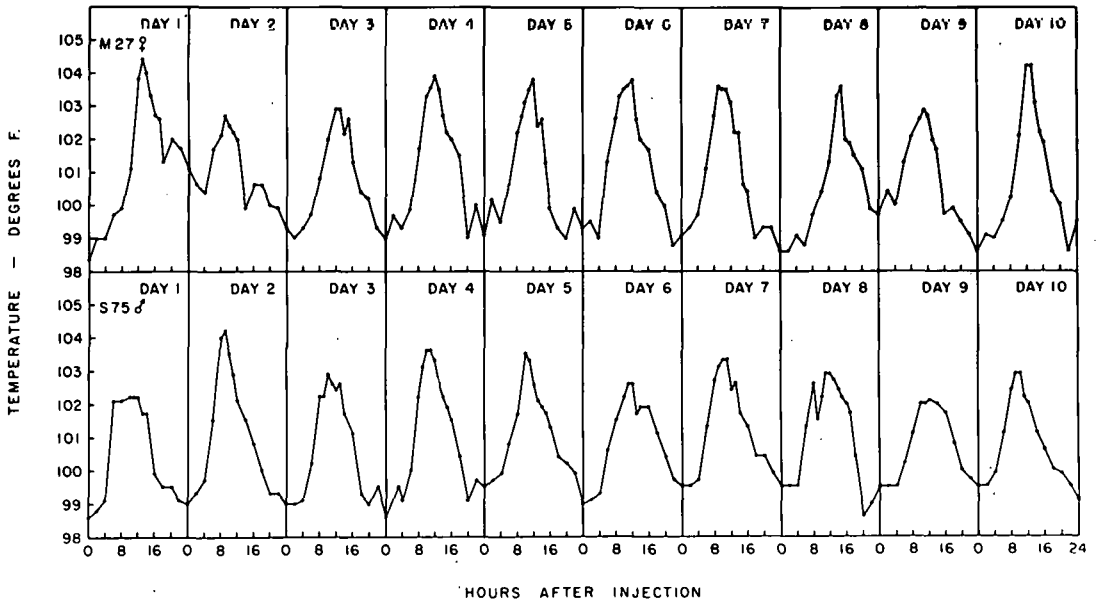

Figure 6. Temperature response to I.M. injection of 10 mg of 11-keto-pregnanolone daily for 10 days.

dissimilar mechanisms of action. It follows then, as previously suggested,[22] that bacterial endotoxin-induced fever in animals may not be a completely accurate experimental prototype of fever produced by non-microbial agents in man. Moreover, the demonstrated absence of circulating endogenous pyrogen in fevers produced by epinephrine,[23] Koolin[24] or dinitrophenol[24] in animals, together with our own observations in steroid fever in man, do not support the view that any presently known circulating endogenous pyrogens is consistently involved in the mediation of all

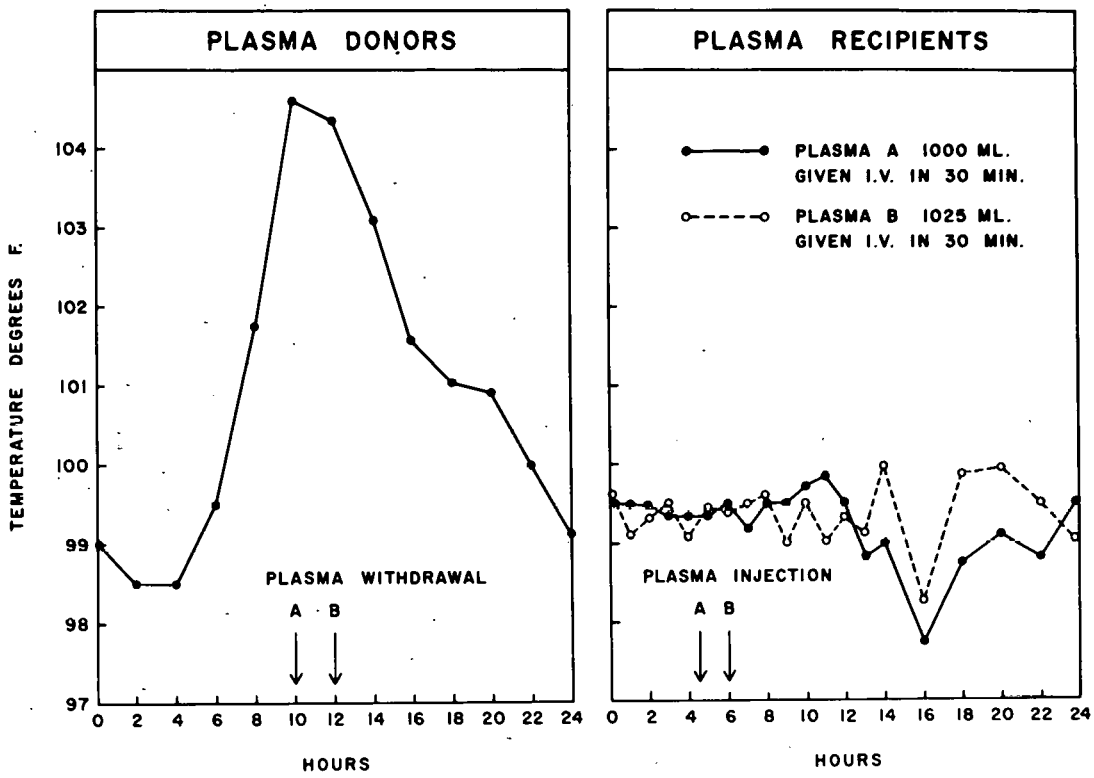

Figure 7. Plasma transfer experiments in which donor plasma was withdrawn at the peak of fever produced by 11-ketopregnanolone injection.

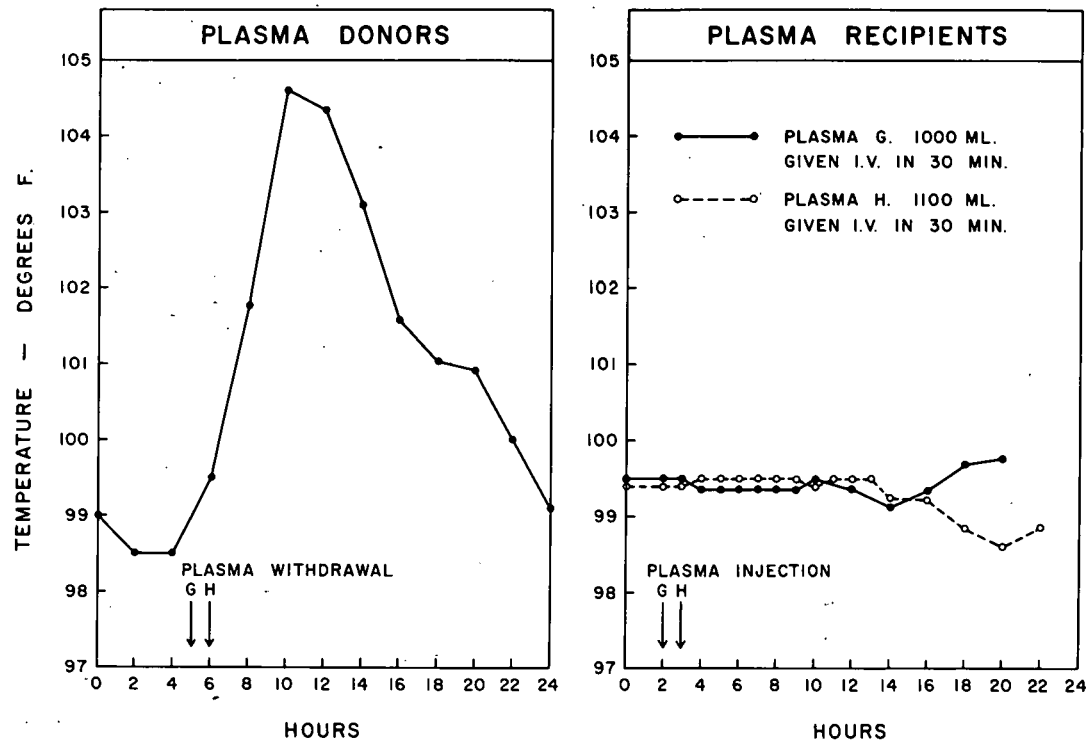

Figure 8. Plasma transfer experiments in which donor plasma was withdrawn at the end of the latency period of fever produced by 11-ketopregnanolone injection.

forms of experimental or naturally-occurring fever.

Finally, the high degree of species specificity of steroid fever makes it evident that the type of experimental animal, as well as the chemical nature of pyrogen used in studies on the mechanism of fever, must be taken into particular account when evaluating such studies.

The physiologic basis for the species specificity of steroid-induced fever is not clear since the mode of action of these substances in man is not known. In addition to being of intrinsic biologic interest, however, it may imply the special significance of these or structurally related endogenous compounds to thermogenic processes under certain circumstances in humans. The probable participation of one steroid pyrogen, etiocholanolone, in the febrile mechanism of disease has indeed already been indicated by the studies of Bondy and associates[25] and it is not unreasonable to suggest that related steroids of the type described in these studies may be significantly involved in the pathogenesis of other forms of naturally occurring fevers in man.

ACKNOWLEDGMENTS

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ENZYMATIC INCORPORATION OF RIBONUCLEOSIDE TRIPHOSPHATES INTO THE INTERPOLYNUCLEOTIDE LINKAGES OF RIBONUCLEIC ACID*

By

S. B. Weiss

Two separate reactions have been described for the enzymatic polymerization of nucleotides. In 1955, Grunberg-Manago and Ochoa[1] reported that an enzyme isolated from the microorganism Azotobacter vinelandii catalyzes the synthesis of highly polymerized ribonucleotides from 5'-ribonucleoside diphosphates with the release of inorganic phosphate. Because of its similarity to the action of phosphorylase on polysaccharides, i.e., its reversible phosphorolysis, the enzyme was called polynucleotide phosphorylase. Since polynucleotide phosphorylase acts on single as well as mixtures of ribonucleoside diphosphates, to form single or mixed polymers, its role as a general mechanism for the biosynthesis of specific ribonucleic acids has been debated.[2] This enzyme is widely distributed in microorganisms and its presence in higher forms has been briefly reported by Hilmoe and Heppel.[3]

DNA† polymerase was first described in 1956 by Kornberg, Lehman and Simms[4] in extracts from E. coli. Further investigations by this group[5] clearly showed that this enzyme catalyzes a net synthesis of deoxyribonucleic acid which is dependent on the presence of all four deoxynucleoside triphosphates, as well as magnesium ions and "primer" DNA, and occurs with the elimination of inorganic pyrophosphate. A mammalian system which catalyzes a reaction similar to DNA polymerase has been reported.[6]

Terminal addition of one or more ribonucleotides to RNA molecules is another reaction observed by a number of investigators.[7-12] This reaction is specific for the ribonucleoside triphosphate and results in the release of inorganic pyrophosphate. However, no extensive polymerization occurs.

In view of the importance of specific ribonucleic acid molecules as carriers for individual amino acids in protein synthesis,[13,14] and the widely held view that RNA may provide templates for the synthesis of specific proteins, the present work was undertaken. In the course of this investigation, a new type of reaction for the incorporation of ribonucleotides has been found. This paper will describe the enzymatic incorporation of ribonucleoside triphosphates into the interpolynucleotide linkages of RNA, by an enzyme system of mammalian origin, which requires that all four triphosphates be present. In this respect, the reaction resembles the action of DNA polymerase rather than polynucleotide phosphorylase.

A preliminary account of some aspects of this work has been published.[15]

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*This paper appears in Proc. Nat. Acad. Sci., 46:1020, 1960.

†The abbreviations used in this report are: DNA, deoxyribonucleic acid; RNA, ribonucleic acid; CTP or cyt-P-P-P, ATP, UTP and GTP for the tri- and CDP, ADP, UDP and GDP for the di- and CMP, AMP, UMP and GMP for the monophosphates of cytidine, adenosine, uridine and guanosine; Tris, tris-(hydroxymethyl)-aminomethane; TCA, trichloroacetic acid; c.p.m., counts per minute; Pi, inorganic orthophosphate; P-O-P, inorganic pyrophosphate.

MATERIALS AND METHODS

Preparation of labeled cyt-P^{32}-P-P: 4 mc of P^{32} and 100 μmoles of phosphoric acid were dried overnight in an oven at 100°. To the tube, 20 mg of PCl_5 was added and thoroughly mixed. The tube was tightly stoppered and incubated for 30 minutes in a 45° bath. 0.20 ml of dimethylformamide was added and the tube incubated for another 30 minutes to insure complete formation of $POCl_3$. 22 mg of dried 2',3'-bensylidene-O-cytidine, prepared by the method of Baddiley,[16] was added, mixed, and the vessel incubated at 45° for four hours. The reaction was stopped by the addition of 2 ml of 0.20 N HCl and the tube heated in a boiling water bath for 12 minutes. After cooling, the mixture was diluted to 200 ml, the pH adjusted to 9, passed over a column of Dowex-1 formate resin and eluted with a gradient elution system containing 0.04 N formic acid in the upper reservoir as previously described.[17] The P^{32}-cytidine 5'-phosphate emerged from the column after an earlier material believed to be dicytidine monophosphate. The 5' isomer peak was identified with the aid of the specific 5'-nucleotidase of Crotalus adamanteus venom. No 2' or 3' isomer was detected. In this manner, 14 μmoles of labeled CMP were prepared.

Labeled cyt-P^{32}-P-P was prepared from labeled CMP by a crude brewers yeast extract.[18] The yeast extract was purified 2.5 fold, with respect to its cytidylate kinase activity, by ammonium sulfate fractionation. The reaction mixture consisted of 250 μmoles of $MgCl_2$, 1.5 mmoles of Tris buffer of pH 7.4, 50 μmoles of ADP, 200 μmoles of phosphopyruvic acid, 500 μgrams of pyruvic kinase, 30 μmoles of labeled CMP and 28 mg of the yeast extract in a final volume of 50 ml. After incubation at 37° for two hours, the reaction was stopped by heating in a boiling water bath for two minutes and then placed immediately in ice. The denatured protein was removed by filtration and the labeled CTP isolated by chromatography on a column of Dowex-1 chloride resin as described by Lehman et al.[19] The yield of CTP was 80% based on the starting CMP. The final product had a specific activity of 3.5×10^7 c.p.m. per micromole. Analysis of the labeled nucleotides synthesized in this report are given in Table 1.

UTP^{32}, labeled in the ester phosphate only, was prepared from cyt-P^{32}-P-P by the procedure of Lohman.[20] The labeled UTP was purified by chromatography on a column of Dowex-1 chloride resin as described by Bessman and co-workers.[21] UTP was obtained in 67% yield with

Table 1

ANALYSIS OF THE RIBONUCLEOSIDE TRIPHOSPHATES

Labeled nucleotide	Absorbance ratios*		Per cent radioactivity†		
	λ250/λ260	λ280/λ260	Monophosphate	Diphosphate	Triphosphate
CTP	0.45	2.09	0.20	1.8	98
UTP	0.75	0.35	0.15	1.3	98
ATP	0.84	0.23	0.30	5.5	94

*All values were determined at pH 2.

†Labeled compound was added to a mixture of appropriate ribonucleoside mono-, di-, and triphosphates. The mixture was subjected to paper electrophoresis in 0.025M citrate buffer of pH 4.6, at 250 volts for 16 hours. The individual nucleotides were located under ultraviolet light and their radioactive content determined. The per cent radioactivity is based on the total number of counts placed on paper.

a specific activity identical to CTP.

ATP^{32}, labeled in the ester phosphate only, was prepared from P^{32}-labeled adenosine 5'-phosphate. The labeled mononucleotide was synthesized by a method suggested by Goldwasser,[22] using the isopropylidene derivative of adenosine as starting material. 50 μmoles of inorganic phosphate, containing 6 mc of P^{32}, was dried in a small vial at 110° overnight. 1 ml of dimethylformamide, 40 mg of dried 2',3'-O-isopropylidene adenosine and 0.50 ml of dicyclohexylcarbodiimide were added and the stoppered vial stirred for 12 hours by magnetic mixing. The reaction was stopped by the addition of 6 ml of 0.05 N HCl and allowed to stir for another 12 hours. The solution was neutralized with 2 N NaOH and the insoluble dicyclohexylurea product removed by filtration. The filtrate was extracted with ether and after removal of dissolved ether by a gentle stream of air, passed over a column of Dowex-1 formate resin. AMP^{32} was eluted with a gradient elution system with 1.0 N formic acid in the upper reservoir and 250 ml of water in the mixing chamber. Three radioactive peaks were located containing adenine. In the order eluted, these peaks were diadenosine monophosphate, adenosine monophosphate and adenosine diphosphate as determined by the ratio of radioactivity to adenine nucleotide. The fractions containing AMP^{32} were concentrated and samples subjected to paper electrophoresis as described in Table 1. Although partially contaminated with the other two adenosine reaction products, the labeled AMP isolated after paper electrophoresis was shown to be the 5' isomer by the action of the specific 5'-nucleotidase of snake venom. The yield of labeled AMP was 3 μmoles. It was diluted 3 fold with unlabeled AMP and converted to ATP^{32} using the crude yeast extract described above which also contained adenylate kinase. The conditions for the reaction were essentially as described for CTP formation except that no ADP was present and 1 μmole of ATP was added to initiate the reaction. Labeled ATP was isolated in a similar manner described for CTP. 6.3 μ-moles of ATP were obtained with a specific activity of 3.4×10^7 c.p.m. per micromole.

Preparation of rat liver enzyme: A 20% rat liver homogenate, in 0.25 molar sucrose containing 0.001 M $MgCl_2$, was prepared with the aid of a loose fitting stainless steel homogenizer. The homogenate was filtered through gauze and centrifuged at approximately 600 x g for six minutes at 4°. The supernatant was decanted and the loosely packed pellet washed twice more with twenty volumes of homogenizing medium. Microscopic examination of the pellet at this stage showed a high concentration of nuclei. The residue was washed once more in isotonic sucrose to remove excess $MgCl_2$, suspended in twenty volumes of 0.05 M Tris buffer of pH 7.4, and allowed to stand at 0° for 10 minutes. The lysed nuclei were centrifuged at 10,000 x g for 10 minutes. The highly colored supernatant was discarded leaving a somewhat white pellet behind. The residue obtained from 40 grams of rat liver in this manner was suspended to a final volume of 16 ml with 0.05 M Tris buffer of pH 7.4. 2 M KCl was added to the suspension dropwise, with adequate stirring, to a final concentration of 0.40 M KCl. Within a few minutes a white aggregate formed that could be separated from solution by lifting on glass rods. The isolated aggregate was washed twice in the Tris-KCl medium and finally suspended in 0.05 M Tris of pH 8.1 by vigorous homogenization with a glass homogenizer to give a protein concentration of 10-12 mg per ml of suspension. This preparation was used as the source of enzyme for most of the experiments reported here and shall be referred to as the aggregate-enzyme.

Enzyme assay: After incubation, the reaction was stopped by the addition of 5 ml of cold 5% TCA. The acid-insoluble precipitate was washed twice with cold 5% TCA, twice with ethanol-ether (3:1) and extracted twice with 4 ml of 10% NaCl, at pH 8, in a boiling water bath for 30

minutes. 2 mg of carrier RNA was added with each extraction. The combined extracts were precipitated with 2 volumes of cold ethanol, the precipitate redissolved in 5 ml of water and 2 ml samples dried and assayed in a windowless gas flow counter.

Isolation of ribonucleic acid: RNA, from various preparations, was isolated by the Kirby modification[23] of the procedure of Gierer and Schram.[24] P^{32}-labeled RNA, isolated in this way, was exhaustively dialysed against 0.05 M KCl until no further counts could be detected in the dialysing medium.

Hydrolysis of ribonucleic acid: The conditions for alkaline hydrolysis are described in Table 4. After hydrolysis, the solution was neutralized with 18% perchloric acid. After standing for 2 hours at 0°, the precipitated salt was removed by centrifugation. Separation of nucleotides was accomplished by paper electrophoresis in 0.025 M citrate buffer of pH 3.5, for 20 hours at 400 volts, and by chromatography on columns of Dowex-1 formate resin. The isolated nucleoside 2'- and 3'-monophosphates were assayed for radioactivity.

Enzymatic hydrolysis of labeled RNA was accomplished with the aid of Crotalus adamanteus venom. The reaction conditions and procedure are given in Figures 6 and 7.

Phosphorus was determined by the method of Gomori.[25] Inorganic-P^{32} was separated from organic-P^{32} by extraction into organic solvents as described by Borkenhagen and Kennedy.[26] Protein was determined by the method of Lowry.[27] Optical density measurements were made in a Zeiss spectrophotometer. The nucleotides used in this work were products of the Pabst Laboratories. Other materials used in this report were of commercial origin. Crotalus adamanteus venom was bought from Ross Allen's Reptile Institute, Silver Springs, Florida.

EXPERIMENTAL

Enzymatic incorporation of nucleoside triphosphates into RNA: When rat liver homogenates were incubated with cyt-P^{32}-P-P, a significant incorporation of label into the acid-insoluble residue was observed. Further experiments indicated that the nuclear fraction was particularly active in this respect and that optimal incorporation occurred when ATP, UTP and GTP were present. It was found that after lysis of the nuclei, the DNA in these preparations could be aggregated by the addition of the proper amount of salt. At higher salt concentrations (1 molar), the aggregate becomes soluble and a highly viscous suspension develops. Removal of the aggregate from solution and subsequent assay for nucleotide incorporation indicated that this preparation contained the entire activity of the original nuclear fraction and still maintained the requirement for all four ribonucleotides (Table 2). This requirement is also shown for labeled ATP with whole nuclei. The incorporation of labeled substrate is increased from 8 to 10 fold as the concentrations of each of the unlabeled nucleotides are simultaneously increased (Figure 1).

If ribonucleoside diphosphates are substituted for the unlabeled ribonucleoside triphosphates a considerable reduction in the incorporation is observed (Table 3). This would suggest that the incorporation of ribonucleotides into RNA occurs at the triphosphate level and that the reaction proceeds by the elimination of inorganic pyrophosphate similar to DNA polymerase[5] and the terminal addition type reactions.[12] Such a reaction mechanism would be in agreement with the observation that inorganic pyrophosphate, but not inorganic orthophosphate, inhibits the incorporation (Table 3).

Identification of the radioactive product as RNA: The labeled product, isolated from the reaction mixture, is acid-insoluble and nondialysable. Treatment with crystalline ribonuclease, but

Table 2

NUCLEOTIDE REQUIREMENT FOR THE INCORPORATION OF LABELED NUCLEOTIDES INTO RNA

Experiment number	Labeled substrate	Nucleotides added	Labeled substrate incorporated into RNA
			$\mu\mu$moles
1*	CTP^{32}	Complete	50.0
		Omit ATP	10.0
		Omit GTP	6.0
		Omit UTP	6.8
		Omit ATP, UTP, GTP	4.7
		Complete except 1 μmole each of ATP, UTP, GTP	570.0
2*	UTP^{32}	Complete	32.0
		Omit ATP	5.5
		Omit GTP	4.3
		Omit CTP	3.5
		Omit ATP, UTP, GTP	2.8
		Complete except 1 μmole each of ATP, UTP, GTP	280.0
3†	ATP^{32}	Complete	62.0
		Omit GTP	12.1
		Omit UTP	16.5
		Omit CTP	13.9
		Omit GTP, UTP, CTP	12.4

*The complete system contained 5 μmoles of $MgCl_2$, 100 μmoles Tris, buffer of pH 8.05, 10 μmoles of cysteine, 0.06 μmoles of CTP^{32} or UTP^{32} (2.1×10^7 c.p.m. per micromole), 0.06 μmoles each of ATP, UTP, GTP, and CTP, except as indicated, and aggregate-enzyme containing 6 mg of protein. The final volume of the system was 1.0 ml. The vessels were incubated for 15 minutes at 37°.

†The reaction mixture was the same as above except that in addition, each vessel contained 60 μmoles of KCl, 20 μmoles of NaF and 0.06 μmoles of ATP^{32} (1.4×10^7 c.p.m. per micromole) as the labeled substrate. 12 mg of twice washed nuclei served as the enzyme source.

not desoxyribonuclease, alters these properties. The product may be isolated further under conditions where DNA is not extracted.[23] Treatment with alkali, under conditions known to hydrolyze RNA, or with snake venom, results in the formation of acid-soluble labeled material. The products formed by alkaline hydrolysis can be separated by paper electrophoresis or by ion exchange chromatography and have been identified as the mononucleotides of cytidine, adenosine, uridine and guanosine.

Optimal pH for incorporation of CTP into RNA: When the hydrogen ion concentration of the reaction mixture was varied over a considerable range (Figure 2), the highest incorporation took place in the range of pH 8 to 9. The activity falls off rapidly at values below pH 7.5, and

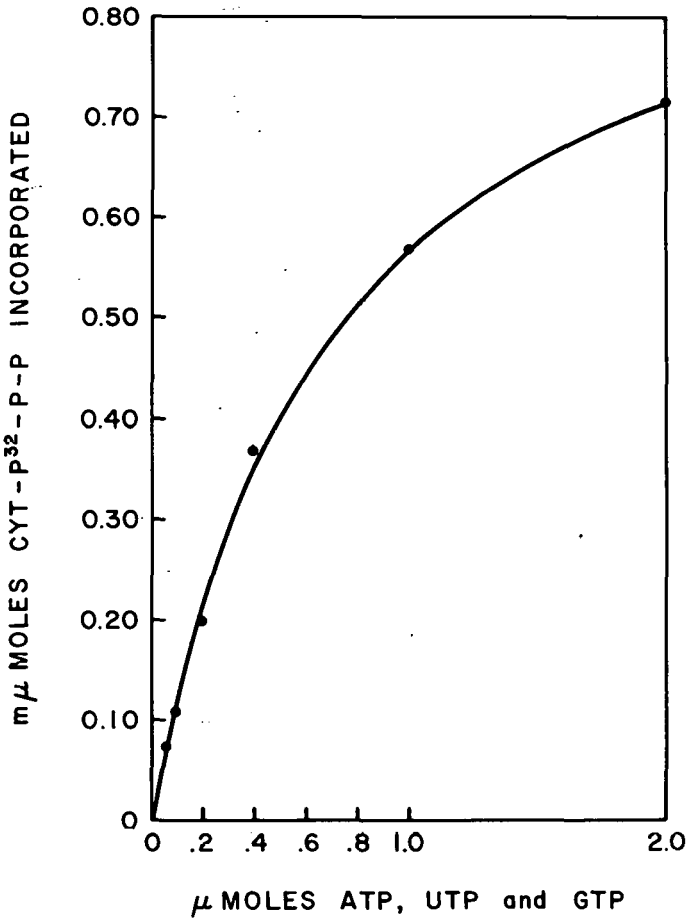

Figure 1. Incorporation of CTP^{32} at various concentrations of ATP, UTP and GTP. Each tube contained 5 μmoles of $MgCl_2$, 100 μmoles Tris buffer of pH 8.05, 10 μmoles of cysteine, 0.06 μmoles of CTP^{32} (9×10^6 c.p.m. per micromole), rat liver aggregate-enzyme (see methods) containing 6 mg of protein and ATP, UTP and GTP each, in the quantities shown. The final volume of the system was 1.1 ml. The tubes were incubated for 15 minutes at 37°.

above pH 9.

Requirement for divalent cations: The enzymatic incorporation of ribonucleotides into RNA requires the presence of divalent cations. At low concentrations, both magnesium and manganese can activate the system; magnesium was more effective. However, at higher concentrations both cations become inhibitory; the inhibition with manganese was more pronounced (Figure 3). Calcium was unable to activate the enzyme system. Low concentrations of calcium (0.001 M), in the presence of optimal amounts of magnesium ion, were inhibitory.

The incorporation of CTP into RNA as a function of time: Under conditions of these experiments, optimum reaction time for the incorporation of CTP into RNA occurred in ten minutes (Figure 4). The diminished incorporation observed for longer periods of incubation most probably represents product breakdown. The reaction was linear for the first two minutes with half

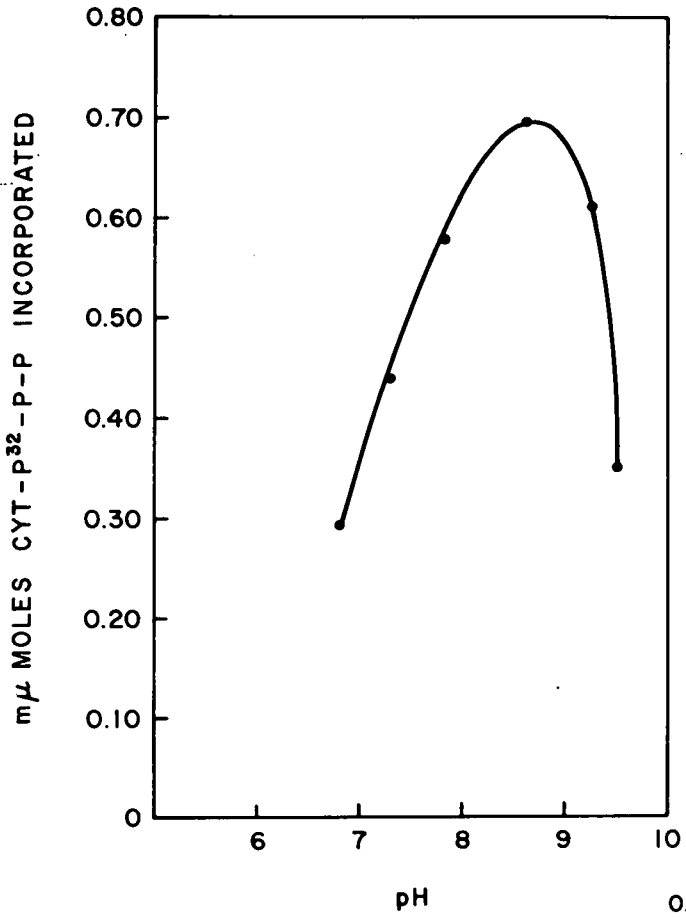

Figure 2. The incorporation of CTP^{32} as a function of pH. The conditions of the enzyme assay were identical with those shown in Figure 1, except that each tube contained 100 μ-moles of an equimolar mixture of phosphate and Tris. One μmole each of ATP, UTP, and GTP per reaction vessel was used. The hydrogen ion concentration of the Tris-phosphate buffer was varied to give the pH values shown, which were measured with a glass electrode after the addition of all reaction components. The tubes were incubated for 15 minutes at 37°.

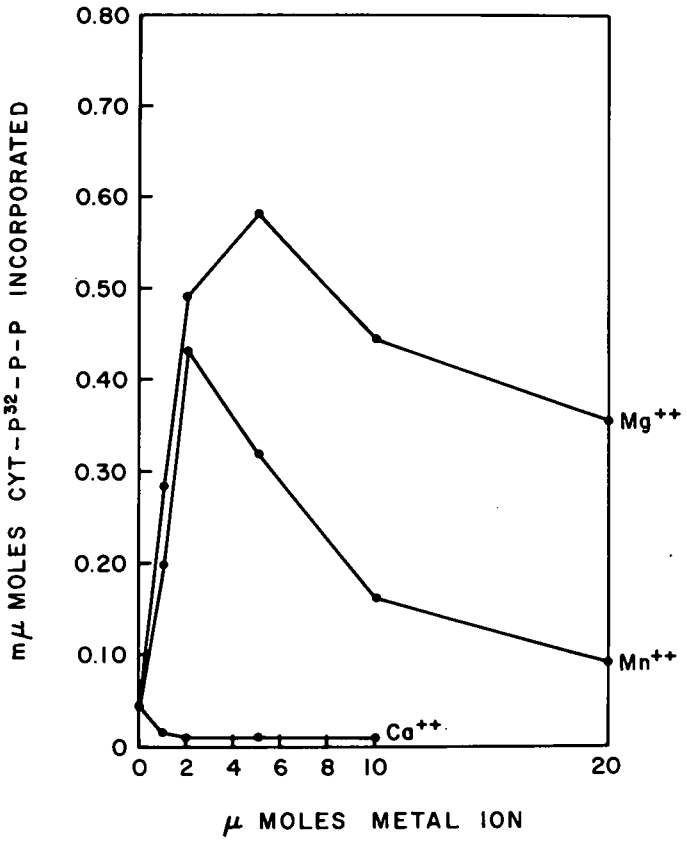

Figure 3. The requirement of divalent cations for the incorporation of CTP^{32} The conditions of the enzyme assay were identical with those shown for Figure 1, except that the time of incubation was 15 minutes and the added divalent cation concentration varied as indicated. One μmole each of ATP, UTP and GTP per reaction vessel was used.

Table 3

COFACTOR REQUIREMENTS FOR THE INCORPORATION OF LABELED NUCLEOTIDES INTO RNA

Experiment number	Labeled substrate	Additions	Labeled substrate incorporated into RNA
			$\mu\mu$ moles
1	CTP^{32}	Complete	61.0
		Complete: ADP, UDP, GDP in place of ATP, UTP, GTP	18.0
		Complete: +5 μ moles of P-O-P	22.0
		Complete: +10 μ moles of P-O-P	3.7
		Complete: in 100 μ moles of Pi buffer pH 7.5 (no Tris)	60.0
2	UTP^{32}	Complete	32.0
		Complete: ADP, CDP, GDP in place of ATP, CTP, GTP	20.0
		Complete: +5 μ moles of P-O-P	11.0
		Complete: +10 μ moles of P-O-P	1.2
		Complete: in 100 μ moles of Pi buffer pH 7.5 (no Tris)	32.0

The contents of the complete system and the conditions of the reaction were identical to that described in Table 2, except that 10 μmoles of $MgCl_2$ were used. The ribonucleoside di- and triphosphates used were 0.06 μ moles each.

maximum incorporation occurring at the end of one minute.

Position of the nucleotide incorporated into RNA: When P^{32}-labeled RNA, prepared enzymatically with whole nuclei or aggregate-enzyme, was subjected to alkaline hydrolysis, each of the nucleoside 2'- and 3'-monophosphates separated by paper electrophoresis or by ion-exchange chromatography were labeled (Table 4). An electropherogram of the products separated after hydrolysis is shown in Fig. 5. The slight amount of ultraviolet absorbing radioactive material migrating more slowly than 2'(3')-CMP in Figure 5, is believed to be some unhydrolyzed dinucleoside monophosphate. Although these results suggest that the incorporation of substrate is into the inner linkages of the RNA chain, the possibility of a terminal addition to different end groups of different ribonucleic acids, present in the enzyme preparation, cannot be excluded by this evidence alone.

The phosphodiesterase of snake venom has been shown to attack nucleic acid, in a stepwise fashion from the 3'-hydroxyl end of the chain, to liberate nucleoside 5'-monophosphates.[28] The free nucleoside 5'-monophosphates may be attacked next by the phosphomonoesterase of snake venom to form nucleosides and inorganic phosphate. If terminally labeled P^{32}-RNA is digested with crude snake venom, one might expect a disproportionate release of inorganic-P^{32} and inorganic phosphate if the kinetics of the overall reaction were essentially those of the diesterase activity. That such is the case, may be demonstrated by the reaction of snake venom

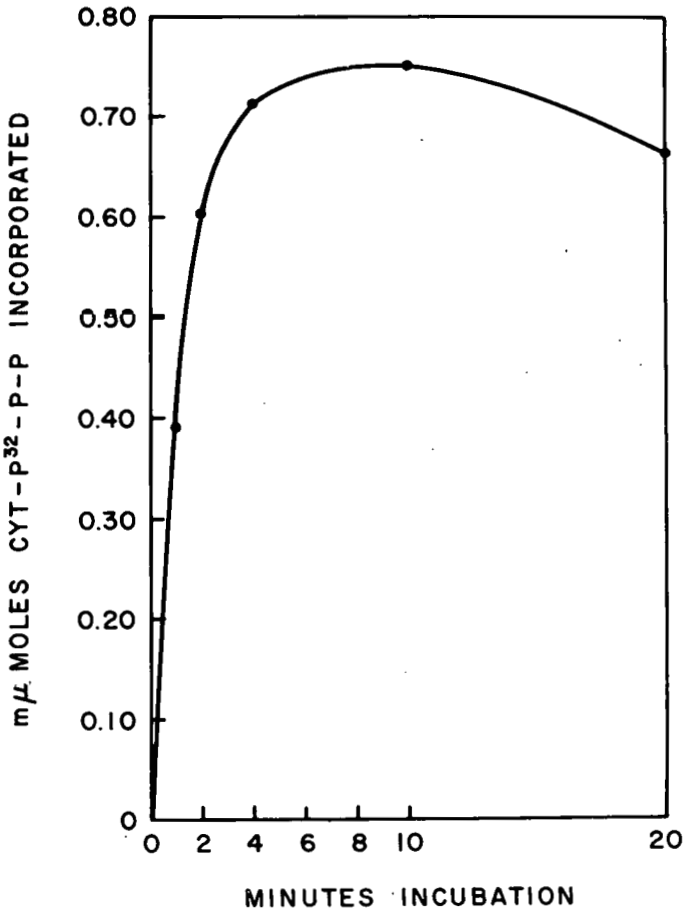

Figure 4. Incorporation of CTP^{32} as a function of time. The contents of each vessel were the same as indicated for Figure 1, except that 1 μmole each of ATP, UTP and GTP were used. The final volume of the system was 1.1 ml. The reaction was incubated at 37° for the time intervals shown.

Table 4

ALKALINE HYDROLYSIS OF P^{32}-RNA

Labeled substrate used	Total RNA counts	Counts in nucleotides isolated after alkaline hydrolysis				Per cent recovery of total counts
		2'(3')CMP	2'(3')AMP	2'(3')GMP	2'(3')UMP	
CTP^{32}*	83,200	20,200	8,350	18,850	22,900	84.5
UTP^{32}†	40,000	15,400	4,200	5,800	13,000	96.0
ATP^{32}‡	31,900	6,090	6,500	8,000	8,100	90.0

Labeled RNA was prepared by the incubation of CTP^{32}, UTP^{32} and ATP^{32} with the nuclei preparations as shown in Figure 2. The P^{32}-RNA was isolated by the phenol procedure.

*Hydrolysis carried out in 0.20N KOH for 3 hours at 80°. The mononucleotides were separated by paper electrophoresis in 0.025 M citrate buffer of pH 3.5, at 400 volts for 20 hours.

†Hydrolysis carried out in 0.30N KOH for 18 hours at 37°. The separation of mononucleotides was by paper electrophoresis.

‡Hydrolysis same as for CTP^{32}-labeled RNA. The mononucleotides were separated by gradient elution with formic acid from a column of Dowex-1 formate resin (10 per cent cross-linked).

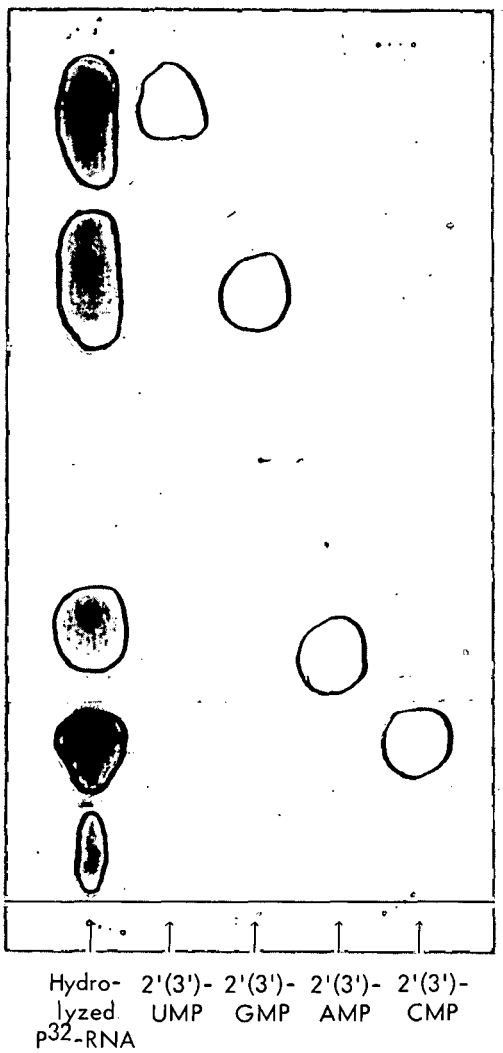

Figure 5. Paper electrophoresis of P^{32}-RNA, labeled with CTP^{32}, after alkaline hydrolysis. The conditions for alkaline digestion and paper electrophoresis are indicated in Table 4. The ultraviolet absorbing areas are encircled. The exposed dark areas indicate the presence of radioactivity.

with terminally labeled P^{32}-RNA,[13] prepared by the "pH 5 enzyme" and RNA isolated from the soluble fraction of rat liver homogenates (SRNA). Under these conditions, more than 90% of the radioactivity was released while less than 10% of the RNA chain had been degraded (Figure 6). However, venom digestion of RNA labeled with CTP^{32} or UTP^{32} by the aggregate-enzyme (NRNA), results in the release of inorganic-P^{32} and inorganic phosphate at nearly identical rates (Figure 7A and 7B). The slight discrepancy in rates observed in Figure 7B indicates that the distribution of labeled uridylate in the RNA chain is not statistically uniform. Higher concentrations of venom were used in the NRNA experiments so that digestion of 80 to 90% of the material could be achieved in a reasonable length of time.

DISCUSSION

A number of bacterial and mammalian cell-free systems have been described which incorporate labeled ribonucleotides into RNA.[1,7-12,29] Most of these, with the exception of polynucleotide phosphorylase, have been shown to catalyze the terminal addition of nucleotides to RNA chains. Goldwasser[30] and Edmonds and Abrams[10] have presented evidence which suggests that mammalian preparations can also catalyze incorporation of ribonucleotides into the framework

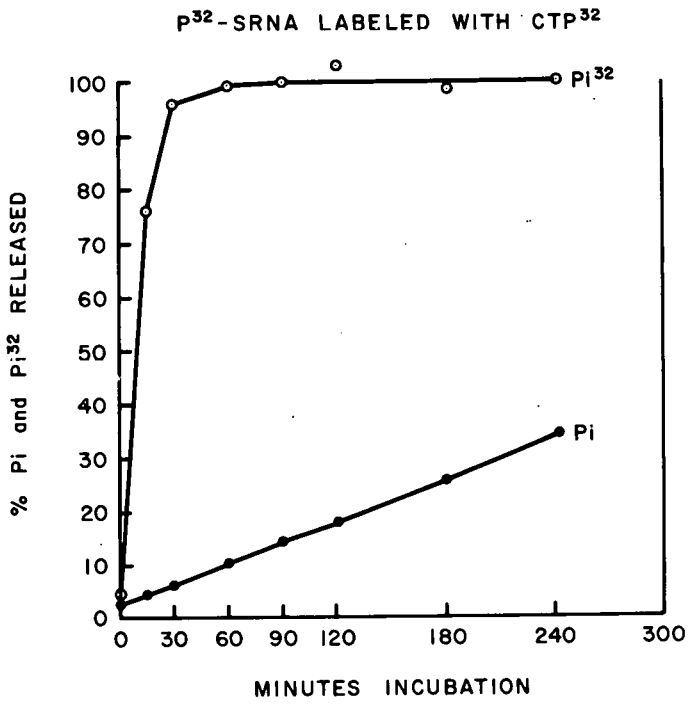

Figure 6. The enzymatic hydrolysis of P^{32}-labeled SRNA with snake venom. Terminal-labeled P^{32}-SRNA was prepared as follows: 4 tubes were used and each contained 10 μmoles of $MgCl_2$, 100 μmoles of Tris buffer of pH 8.05, 20 μmoles of NaF, 0.06 μ-moles of CTP^{32} (3.4×10^7 c.p.m. micromole), 1.2 mg of SRNA and "pH 5 enzyme" containing 6-7 mg of protein, in a final volume of 1 ml. After 20 minutes at 37°, the SRNA was isolated by the phenol procedure (see methods) with 3 mg more of carrier SRNA being added.

The snake venom incubation reaction contained 20 μmoles of $MgCl_2$, 200 μmoles of glycine buffer of pH 8.4, P^{32}-labeled SRNA containing 144,000 total c.p.m. and 13 μmoles of total phosphate, and 8 mg of crude Crotalus adamanteus venom, in a final volume of 4 ml. The reaction mixture was incubated at 37°. At the time intervals shown, 0.50 samples were removed and the reaction was stopped by the addition of 5% TCA. 2 mg of carrier albumin was added to insure complete precipitation. The precipitate was removed by centrifugation and appropriate aliquots were taken for inorganic phosphate and inorganic-P^{32} determinations as described under methods. The per cent Pi and Pi^{32} released is based on the total phosphate and radioactivity content of the RNA used in the reaction.

of RNA. However, none of the above systems demonstrate a specific requirement for all four ribonucleotides.

Recently, Edmonds and Abrams[31] found that extracts from calf thymus nuclei catalyze the formation of single adenylate polymers from ATP. It is apparent that incorporation studies with C^{14}-labeled nucleotides alone cannot in itself determine whether natural or atypical polynucleotide polymers have been formed. The synthesis of homopolymers by the enzyme reported here may be excluded since alkaline hydrolysis of the P^{32}-RNA results in the formation of all four nucleoside 2'- and 3'-monophosphates, each containing significant radioactivity.

The present experiments show that the omission of any one of the four ribonucleoside tri-

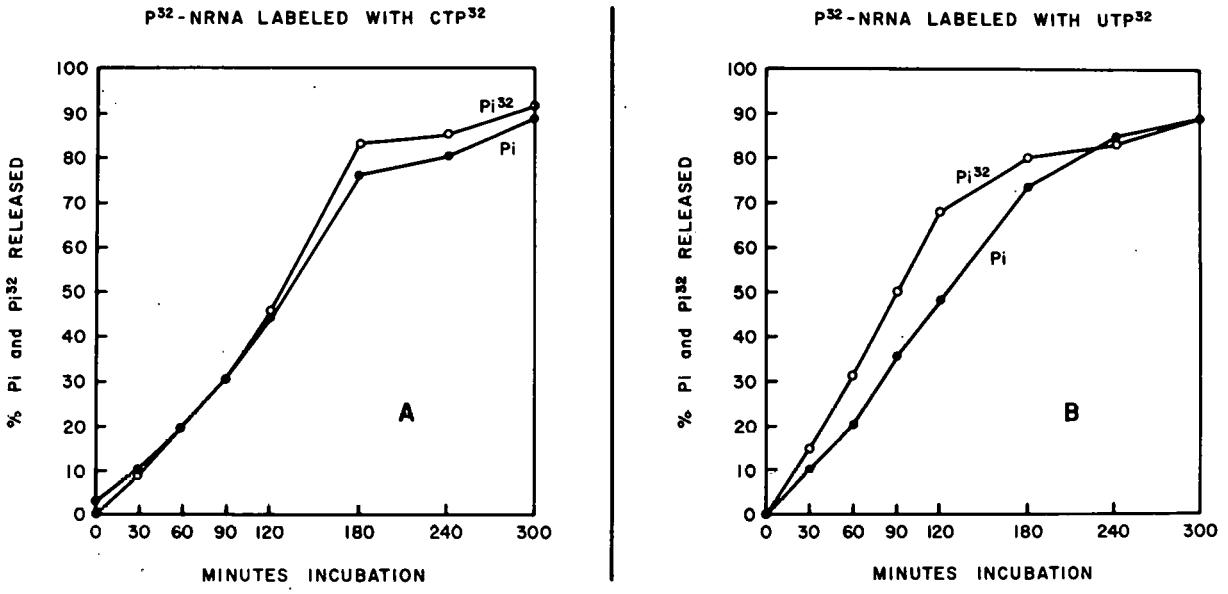

Figure 7. The enzymatic hydrolysis of P^{32}-labeled NRNA with snake venom. P^{32}-labeled NRNA was prepared by the incubation of either CTP^{32} or UTP^{32} with the aggregate-enzyme as described previously. The reaction system was identical to that shown in Figure 2, and 4 tubes were incubated with each label. After 15 minutes at 37°, the NRNA was isolated as described in Figure 6, except that unlabeled NRNA was added as carrier.

The conditions for the snake venom reaction and procedure were identical with that shown in Figure 6, except that 16 mg of crude Crotalus adamanteus venom was used. The P^{32}-NRNA used in the reaction system for Figure 7A contained a total of 16,000 c.p.m. and 14.66 μmoles of total phosphate. The P^{32}-NRNA used in the reaction system for Figure 7B contained a total of 19,400 c.p.m. and 14.2 μmoles of total phosphate. Per cent Pi and Pi^{32} released was calculated as described for Figure 6.

nucleoside triphosphates resulted in a marked reduction of label incorporated when CTP, UTP and ATP were the labeled substrates. In this respect, and also because the ribonucleoside triphosphate rather than the diphosphate appears to be required, the system is similar to the action of DNA polymerase and different from polynucleotide phosphorylase. The hydrolysis experiments with snake venom clearly demonstrate that the labeled RNA formed by the aggregate-enzyme is quite different from terminally labeled RNA. Indeed, the similar rates of release of inorganic phosphate and inorganic-P^{32} can only be interpreted to mean that the labeled substrate had been incorporated throughout the entire polynucleotide chain. This information, coupled with the four ribonucleotide requirement, suggests that polynucleotide synthesis had taken place. The same type of activity shown here for extracts from rat liver nuclei can be shown to occur with nuclei from calf thymus and ascites cells. The reaction is not stimulated by the addition of nuclear RNA, however, addition of soluble RNA causes terminal addition products besides those described above. It is also interesting that preincubation of the aggregate-enzyme with extremely small quantities of desoxyribonuclease completely inactivates the system. Similar treatment with ribonuclease is not as effective. Elucidation of the exact requirements for this enzyme must await further purification.

SUMMARY

An enzyme system isolated from rat liver nuclei was found to catalyze the incorporation of labeled ribonucleoside triphosphates into ribonucleic acid. The enzymatic activity was dependent on the presence of all four ribonucleoside triphosphates.

Evidence is presented which shows that the incorporation of labeled ribonucleoside triphosphate occurs throughout the entire polynucleotide chain.

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