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

Semiannual Report to

THE ATOMIC ENERGY COMMISSION

SEPTEMBER 1959

LEON O. JACOBSON, M.D.
Editor

MARGOT DOYLE
Associate Editor

OPERATED BY THE UNIVERSITY OF CHICAGO
UNDER
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INTERMEDIATES OF CARBOHYDRATE METABOLISM IN HUMAN ERYTHROCYTES*

By

T. Necheles and E. Beutler

An understanding of the metabolism of the human erythrocyte is of basic importance in study of the normal function and survival of these cells, both in vivo and in vitro. It is also quite probable that the basis of many hemolytic anemias, especially those in which an intracorpuscular defect is present, lies in abnormalities in the enzymatic pathways of intermediary metabolism. Several such abnormalities have already been demonstrated. Gibson has presented evidence that congenital methemoglobinemia is due to a defect in the enzymatic pathways for DPN-linked hemoglobin reduction. More recently, it has been shown that a decrease in glucose-6-phosphate dehydrogenase activity is present in the red cells of subjects who are sensitive to the hemolytic effect of a variety of drugs and to fava beans. Dacie and co-workers found defective glucose utilization in at least some of the congenital spherocytic hemolytic anemias. Studies of red cells from some patients with hereditary spherocytosis have demonstrated a decreased rate of incorporation of P^{32} orthophosphate into some of the intracellular phosphorylated intermediates including ATP and DPG. The absence of glucose-6-phosphate dehydrogenase activity has also been reported by Newton and Bass in the red cells of several patients with nonspherolytic congenital hemolytic anemia.

With the recent publication by Bartlett of an elegant method for the separation by column chromatography, of the phosphorylated metabolic intermediates of human erythrocytes, it has become possible to study from this point of view the erythrocytes of patients suffering from several hemolytic states. Such an investigation is reported here.

METHODS

Washed, packed human erythrocytes were extracted twice with trichloroacetic acid (TCA). The neutralized extracts were chromatographed on Dowex-1 resin columns using the method described by Bartlett.

The following abbreviations were used:

AMP	= adenosine monophosphate	GDP	= glucose 1,6 diphosphate
ADP	= adenosine diphosphate	MPG	= monophosphoglycerate
ATP	= adenosine triphosphate	DPG	= diphosphoglycerate
G-6-P	= glucose-6-phosphate	RP	= ribose phosphate
F-6-P	= fructose-6-phosphate	PEP	= phosphoenolpyruvate
HMP	= hexose monophosphate	DPN	= diphosphopyridine nucleotide
FDP	= fructose 1,6 diphosphate	DPNH	= reduced diphosphopyridine nucleotide
HDP	= hexose diphosphate		

*This paper has been submitted for publication in Blood.

The following chemical and physical determinations were carried out on the collected fractions. The volume of each fraction was measured. Total phosphate was measured by a modification of the method of Fiske and Sabbrow. Total hexose was measured by the anthrone method. Fructose was determined by the cysteine-carbazole method. Glucose was usually determined by the differences between total hexose and fructose, and was in addition determined occasionally by the aminobiphenyl method. Trioses were demonstrated by appropriate spectra with the cysteine-carbazole reagent. Mono- and diphosphoglycerate were demonstrated by appropriate spectra with chromatropic acid, but were usually determined by the total phosphate concentration in these peaks. Nucleotides were estimated by

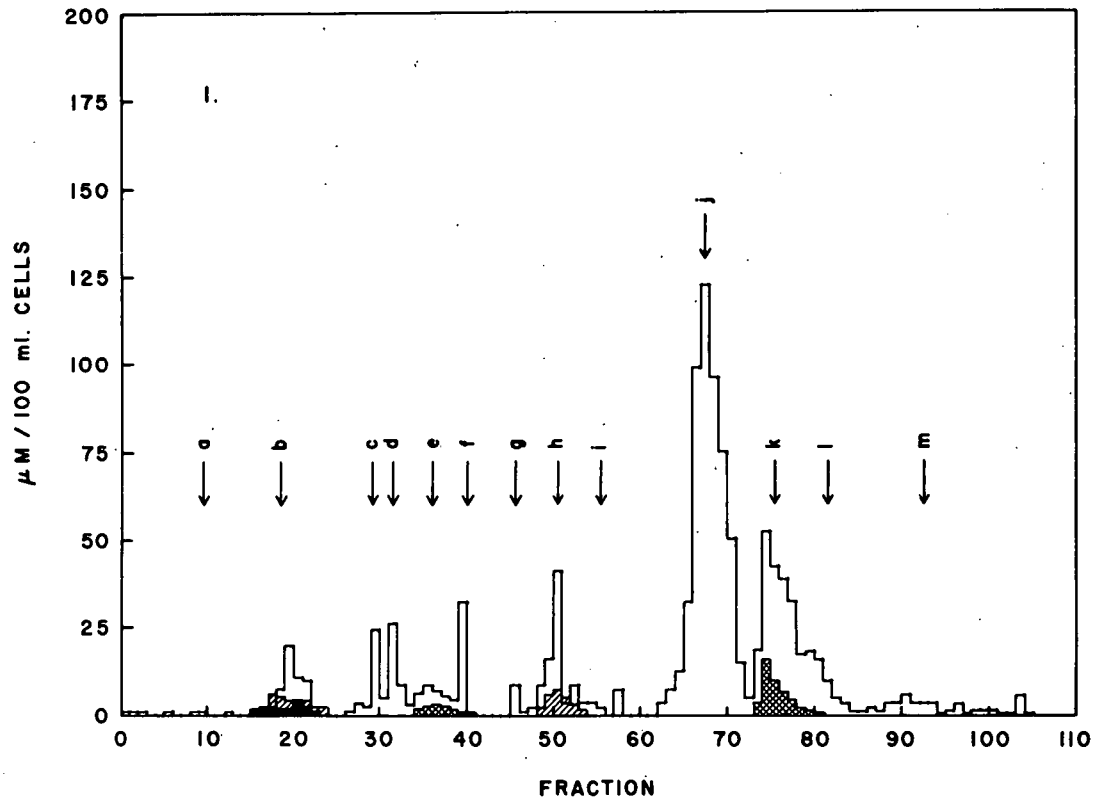

Figure 1. Chromatographic pattern obtained from TCA extract of the washed erythrocytes from a normal subject (#1). Line indicates phosphate concentration in micromoles per 100 ml packed erythrocytes, cross-hatched areas represent 260 absorption calculated as adenosine, diagonally-hatched areas are glucose-phosphate concentration, and solid areas represent fructose phosphate concentrations. The tentative identification of the peaks is discussed in the text.

absorption at 260 mμ and calculated as adenosine derivatives using an average molar extinction coefficient of 15×10^3.

All concentrations are expressed as micromoles per 100 ml packed erythrocytes. The following peaks were identified on the column using known compounds obtained from Sigma Chemical Corp.: G-6-P, F-6-P, R-5-P, 6-PG, and ATP.

A summary of the clinical material studied is included in Table 1. The chromatographic patterns obtained for the patients studied are illustrated by Figures 1-15.

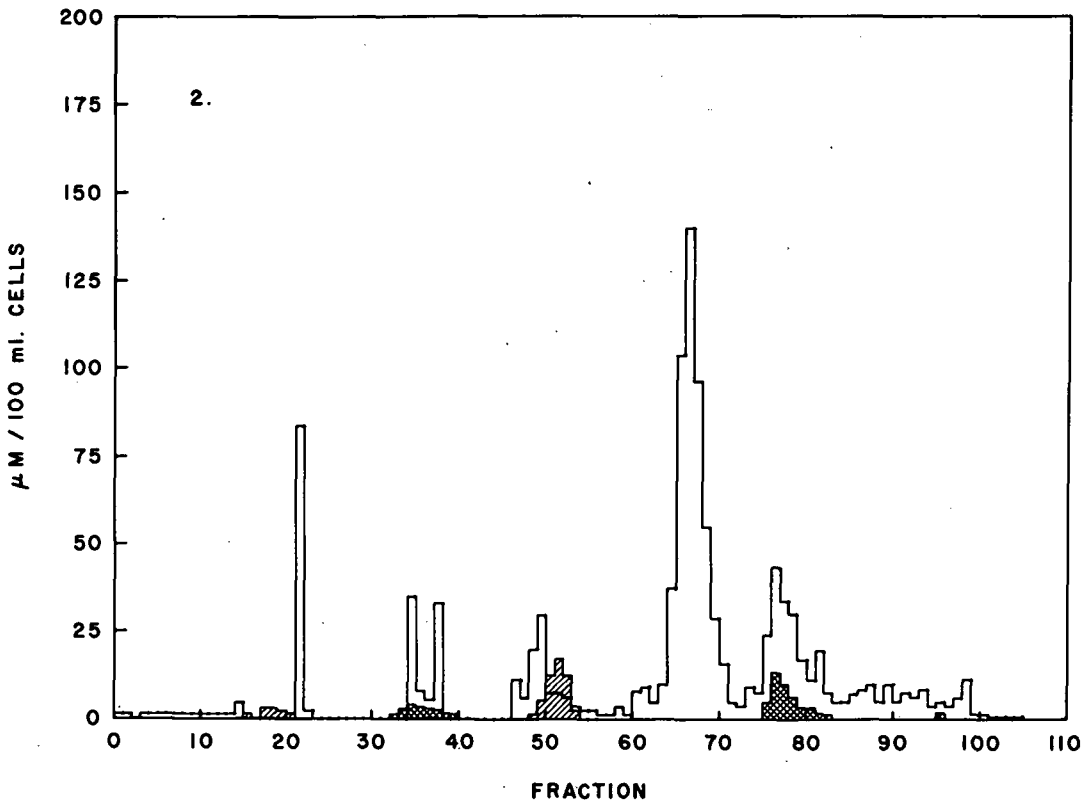

Figure 2. Normal cells. Chromatographic pattern obtained from TCA extract of the washed erythrocytes from a normal subject (#1).

RESULTS

Representative normal elution patterns are shown in Figure 1. The following peaks were observed in normal erythrocytes:

a - a small phosphate peak was observed in the first fractions. This was accompanied by variable amounts of nucleotides. According to Bartlett, this probably represents a combination of uric acid and AMP.

b - this large peak which, using appropriate analyses, appeared to consist of inorganic phosphate, AMP (and other mononucleotides), glucose monophosphate, and fructose monophosphate.

c - a phosphate peak which eluted in the same position as ribose monophosphate.

d - a phosphate peak, which upon reaction with chromotropic acid, gave an optical spectrum characteristic for monophosphoglycerate.

e - a large phosphate peak with absorption at 260 mμ probably consisting largely of ADP, although optical spectra in the ultraviolet region indicate that this is a mix-

Table 1

SUMMARY OF CLINICAL STUDIES

Patient	Diagnosis		Initial peripheral counts					After preparation of washed cells				
			RBC (10^6/cmm)	WBC (per cmm)	Hb (g %)	Hct. (%)	Retic. (%)	WBC (per cmm)	Retic. (%)	RBC (10^6/cmm)	Hct. (%)	Hb (g %)
(1) R.B.	Normal - 33 yr Male		5.85	5,500	17.8	54			0.8	9.27	85	28.4
(2) T.N.	Normal - 25 yr Male		5.00	10,000	16.7	49		12,000	0.8	8.62	84	27.5
(3) E.B.	Normal - 51 yr Female		4.3	8,000	13.8	42		8,000	0.6	9.39	91	31
(4) Y.L.	Normal - 24 yr Female				13.2	40			0.9		76	
(5a) H.M.	Normal	23 June 1959	5.96	8,500		50	0.5	3,100	0.8	13.10	94	
(5b)		27 Feb. 1958	4.48	7,000		45		9,200	1.1	9.28	84	
(6) F.S.	Idiopathic hemochromatosis undergoing phlebotomy (500 ml/wk) - 60 yr Male		4.28	10,500	13.2	39	1.0	4,600	0.8	9.49	86	29.4
(7) V.J.	Treated iron-deficiency anemia - 51 yr Female		4.81	6,950	13.0	41	2.1		2.1		82	
(8) M.J.	Treated iron-deficiency anemia with reticulocytosis - 46 yr Female							13,400	11.4	9.94	89	24.4
(9) B.S.	Primaquine-sensitive - 28 yr Male								0.5		83	
(10a) B.H.	Paroxysmal nocturnal hemoglobinuria - 33 yr Female	20 Feb. 1958	3.30	8,800	11.3	33		10,500	1.5	8.49	92	31
(10b)		4 Feb. 1958	2.18	2,300	8.9	36		5,000	1.8	8.85	90	
(10c)		7 July 1959	3.95	5,100	10.0	34	0.6	6,850	0.8	8.85	25.7	
(11) S.R.	Paroxysmal nocturnal hemoglobinuria - 44 yr Female		2.24	5,350	6.8	19	5.3	5,400	11.0		91	
(12a) B.N.	Ovalocytosis plus hemolytic anemia - 56 yr Female	3 April 1958	3.98	3,800	8.6	27	3.0	7,000	2.4	14.0	92	
(12b)	Ovalocytosis	14 July 1958	3.14	3,300	9.5	33	2.1		3.0		90	
(13) H.G.	Ovalocytosis - 66 yr Male		5.31	5,500	13.5	40			1.8		90	
(14) H.R.	Compensated chronic acquired idiopathic hemolytic anemia - 26 yr Male		5.07	8,000	14.7	42	1.5	5,400	1.0	10.25	84	27.6
(15) A.C.	Acanthocytosis - 15 yr Male		5.00	5,100		47	1.9	5,550	2.2	7.74	85	

ture of nucleotides.

f - a small phosphate peak, unidentified chemically, which gave an optical spectrum with the cysteine-carbazole reagent that was not characteristic of fructose, glucose, or ribose.

g - a small phosphate peak, unidentified chemically, which did not react with anthrone, cysteine-carbazole, or exhibit any absorption in the ultraviolet range.

h - a large phosphate peak which reacted with both anthrone and cysteine-carbazole reagents and appeared to consist of a mixture of glucose diphosphate and fructose diphosphate.

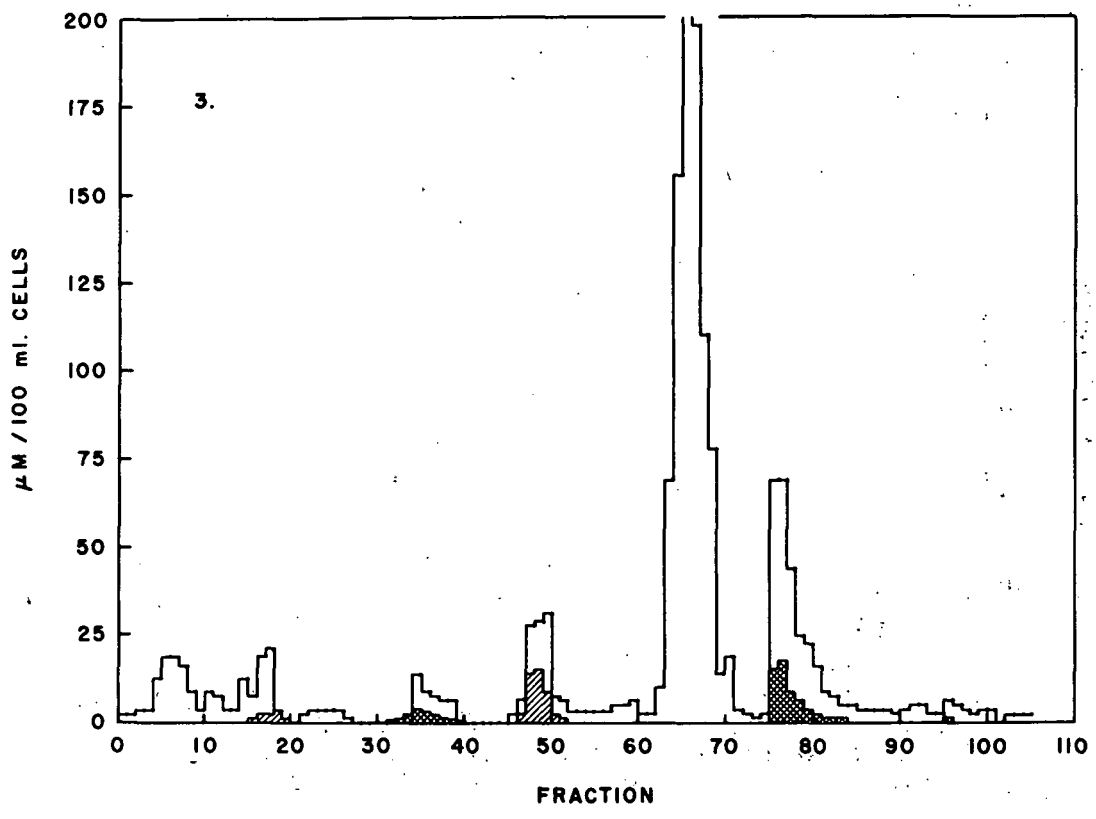

Figure 3. Normal cells. Chromatographic pattern obtained from TCA extract of the washed erythrocytes from a normal subject (#1).

i - a small phosphate peak characterized by high absorption at 220 mμ and tentatively identified as phosphoenolpyruvate.

j - a major phosphate peak identified by characteristic optical spectra with chromotropic acid as diphosphoglycerate.

k - a peak exhibiting optical absorption at 260 mμ and probably largely ATP, although optical spectra in the ultraviolet region indicate the presence of additional nucleotides.

l - various minor peaks, unidentified chemically.

m - a small peak absorbing in the ultraviolet range with an optical spectrum characteristic of adenosine and probably ATP.

The concentration of phosphorylated intermediates as determined in 5 normal subjects are given in Table 2. These values agree well with those found by Bartlett using essentially identical techniques. The results obtained with reticulocyte-rich red cells and from patients suffering from various hemolytic states are also given in Table 2 and the differences summarized in Table 3. The only noteworthy change in patients with reticulocytosis is a two-fold increase of the hexose monophosphate fraction. In patients with hemolytic states, various abnormal patterns were found (Tables 2 and 3).

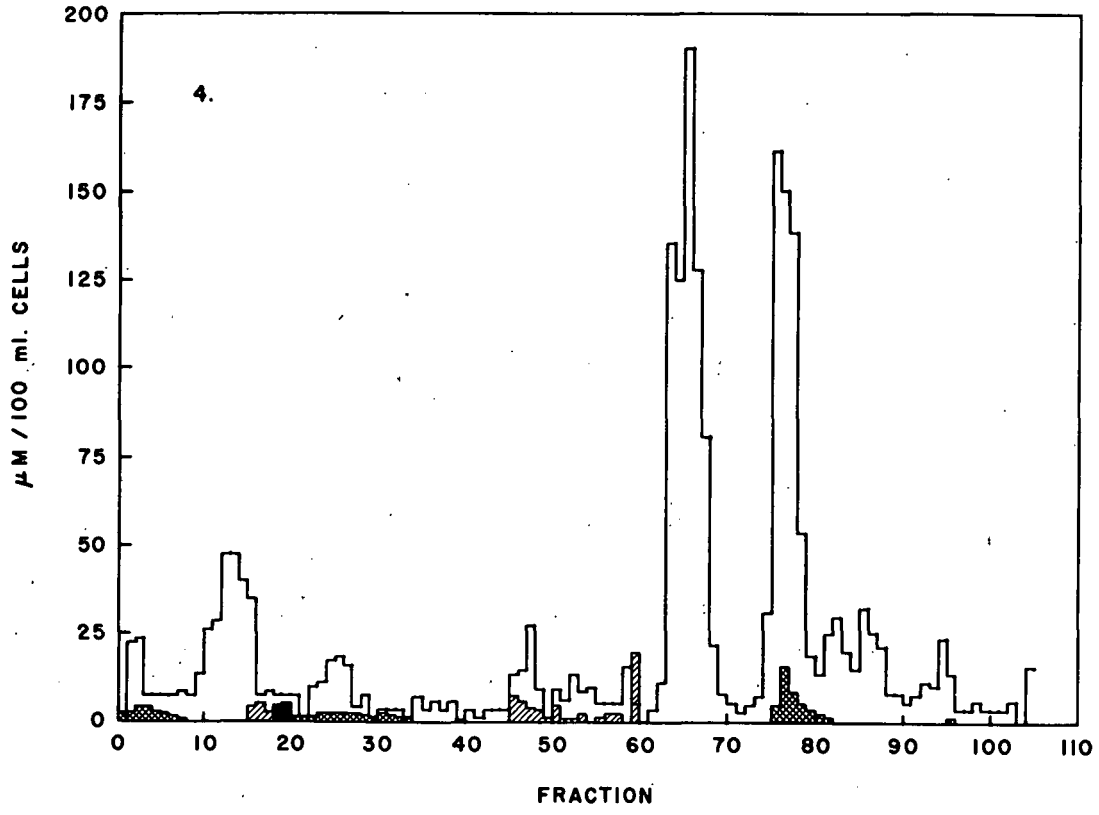

Figure 4. Normal cells. Chromatographic pattern obtained from TCA extract of the washed erythrocytes from a normal subject (#1).

DISCUSSION

The results described originally by Bartlett in normal subjects have been confirmed and have been extended to include several hemolytic states. Many of the peaks described by him as minor in the normal erythrocyte have been shown to be larger under abnormal circumstances. This is to be expected when activities of red cell enzymes are altered. The concentration of the various intermediates in a steady state system is determined by

Table 2

PHOSPHORYLATED INTERMEDIATES IN HUMAN ERYTHROCYTE CONCENTRATIONS EXPRESSED IN μM/100 ml CELLS

	Peak identification note	a ← b → c						d	e	f	g ← h → i			j	k	l	m	Total nucleotides
		(1)	AMP (2)	GMP (3)	FMP (4)	Triose (5)	RP (5)	MPG (1)	ADP (2)	(1)	(1)	GDP (3)	FDP (4)	(1)	DPG (1)	ATP (3)	(1)	(1)	(3)
1	Normal	10.2	6.3	22.9	14.0	-	31.8	38.9	19.0	25.8	45.4	22.6		16.6	262	46.1	149.2	15.8	72.3
2	Normal	21.3	-	11.6	4.9	-	0.0	15.2	22.5	20.9	21.0	54.9		7.9	270	54.3	118.4	29.2	76.8
3	Normal	125.6	12.0	13.0	4.5	-	17.6	0.0	18.0	0.0	39.1	39.5		22.0	416	58.0	121.4	31.2	88.0
4	Normal	304.7	51.8	20.8	13.6	-	74.7	27.1	8.9	16.4	20.2	61.5		31.6	354	52.1	6930	59.2	115.2
5a	Normal	34.2	25.6	4.7	6.6	-	54.5	3.9	55.3	3.2	17.5	39.9		23.2	468	39.6	310.5	31.7	135.7
5b	Normal	4.8	11.1	3.3	6.1	Trace	8.3	7.4	23.9	5.4	0.0	18.5	19.3	15.4	185	98.4	0.0	68.6	133.4
Ave.		83.5	20.2	12.7	8.3	-	31.2	15.3	24.6	12.0	23.9	42.7		19.1	326	58.1	232.1	39.3	103.6
SD		80.7	18.5	8.1	4.3		28.7	14.7	15.9	10.5	16.2	13.3		8.6	87	20.8	246.8	20.1	28.3
YOUNG RED CELL POPULATION																			
6		36.9	22.5	13.0	4.4	-	0.0	71.1	31.0	0.0	36.4	72.2		940.7	433	90.0	91.4	36.9	143.5
7		23.0	46.9	19.5	5.2	-	0.0	0.0	17.9	10.5	65.2	171.4		1381.0	690	63.1	1276.6	282.1	127.9
8		6.3	9.4	2.4	7.7	55.4	73.9	12.0	14.8	39.9	0.0	13.6	18.7	514.0	257	56.6	0.0	9.8	80.8
Ave.		22.1	26.3	11.6	5.7		24.6	27.7	21.2	18.1	33.9	92.3		978.6	450	69.9	458.0	109.6	117.4
SD				8.6	2.2							71.6			218				32.6
PRIMAQUINE-SENSITIVE																			
9		58.9	25.9	44.0	10.0	-	1196.7	73.6	40.4	0.5	0.0	38.6	54.9	438	372	55.7	32.6	777.9	144.7
PAROXYSMAL NOCTURNAL HEMOGLOBINURIA																			
10a		32.9	20.8	31.2	6.0	-	10.3	0.0	45.0	0.0	39.5	91.5		151.3	609	71.7	227.4	29.2	127.5
10b		31.8	12.5	23.4	2.0	-	10.9	5.6	26.2	0.0	45.4	34.6		161.9	744	850	309.8	26.0	123.7
10c		27.5	17.3	13.9	7.9	-	3.0	4.1	31.9	1.0	0.0	24.3	24.3	1.9	759	116.52	0.0	0.0	165.7
11		12.3	21.3	11.9	7.7	46.2	19.4	9.3	43.7	5.6	71.3	90.8		21.7	683	65.6	14.4	23.6	171.6
Ave.		26.1	17.8	20.1	6.2		10.9	4.8	36.7	1.7	44.1	66.4		84.2	699	84.7	137.9	19.7	147.1
SD				8.9								29.2			68				25.0
OVALOCYTOSIS																			
12a		54.4	31.2	59.3	23.3	-	19.8	18.1	41.6	310.1	0.0	120.6		220.0	1745	54.1	720.6	62.1	132.0
12b		325.9	60.3	87.7	59.8	-	163.0	233.0	11.8	260.7	0.0	98.3		163.2	416	48.1	259.0	5.7	131.1
13		60.5	29.1	31.1	14.4	-	44.9	32.8	14.2	37.4	76.9	84.9		12.8	421	28.3	172.2	8.3	82.3
Ave.		146.9	40.2	59.4	32.5	-	75.9	44.6	22.5	202.7	25.6	101.3		132.0	861	43.5	383.9	25.4	115.1
SD				28.3	24.0							18.0			766				28.4
IDIOPATHIC HEMOLYTIC ANEMIA																			
14			25.1	12.5	12.6				45.9			53.5			359	31.2			111.2
ACANTHOCYTOSIS																			
15			6.4	5.5	16.7	-			17.3			29.5			184	74.2		10.3	184.6

NOTES: (1) determined as phosphorus
(2) determined as 260 mμ absorption
(3) determined as glucose
(4) determined as fructose
(5) determined as triose
(Blank spaces indicate - not determined)

the relative activities of the several enzyme systems involved. Any change in the relative activities of one or more such enzyme systems will change the steady state concentrations of some of the intermediate compounds. If an individual enzyme has a relatively high activity, the concentration of its substrate, under steady state conditions, will usually be low. If the activity of this enzyme becomes decreased, the concentration of the substrate will increase until a new steady state condition is achieved. To the extent that they reflect the in vivo intracellular concentrations of carbohydrate metabolism, the chromatograms described here indicate the steady state concentrations of metabolic intermediates in the erythrocytes in the various conditions studied. The great variability sometimes observed in repeated samples, even from the same subject, may represent true physiological variations, but more likely is due to the technical difficulties involved in preparing a TCA extract from the red cells without changing concentrations of unstable substances from those present in the living state.

Table 3

SUMMARY SHOWING THE DEVIATIONS FROM NORMAL IN THE PHOSPHORYLATED INTERMEDIATES OF ERYTHROCYTES IN HEMOLYTIC STATES

	AMP	ADP	ATP	Total nucleotides	GMP	FMP	MPG	HDP	DPG
Reticulocytosis	N	N	N	N	N	N	N	2+	1+
Paroxysmal nocturnal hemoglobinuria	N	N	1+	1+	N	N	N	1+	2+
Ovalocytosis	N	N	N	N	2+	2+	1+	2+	2+
Primaquine-sensitive	N	N	N	1+	2+	N	2+	2+	N
Idiopathic hemolytic anemia	N	1+	1-	N	N	N		N	N

N - concentration not significantly different from that found in normal erythrocytes.
(-) - concentration slightly less (1-3 S.D.) than that found in normal erythrocytes.
1+ - concentration slightly increased (1-3 S.D.) over that found in normal erythrocytes.
2+ - concentration markedly increased (> 3 S.D.) over that found in normal erythrocytes.

The consistent, significant elevations from normal which were obtained could, however, be related to specific enzymatic lesions within these cells.

For example, primaquine-sensitive erythrocytes are deficient in glucose-6-phosphate dehydrogenase. Thus, it is possible to correlate the changes in the chromatogram with the known defect in enzyme activity. An increase is noted in the glucose monophosphate fraction. This might be predicted, since glucose-6-phosphate dehydrogenase deficiency results in decreased metabolism of glucose monophosphate via the hexose monophosphate shunt. Similarly, the increase noted in the fructose diphosphate fraction may be explained by increased metabolism via the glycolytic pathway without sufficient increase in the aldolase

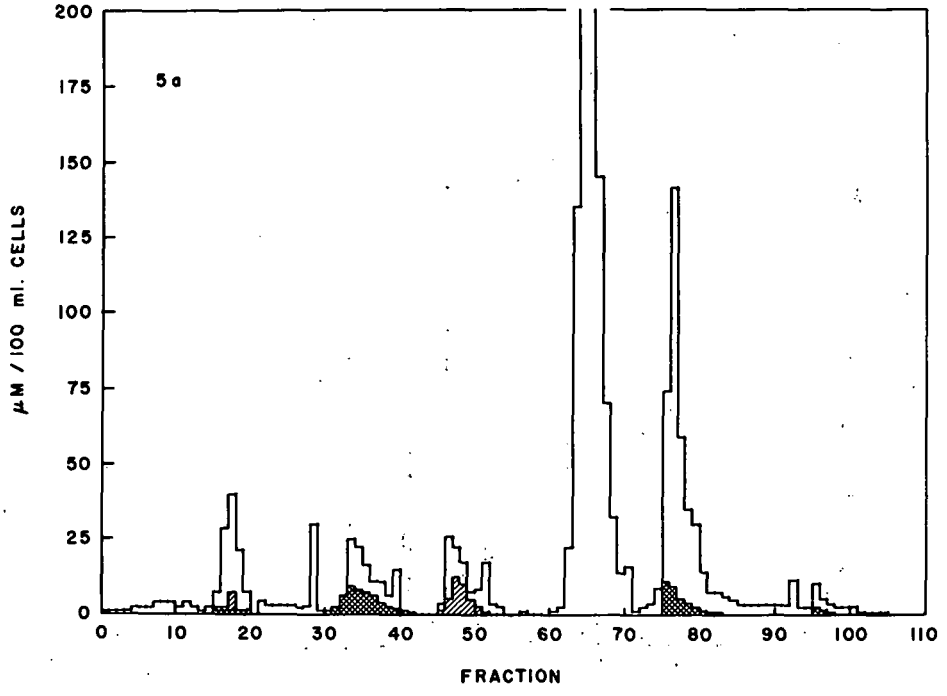

Figure 5a. Normal cells. Chromatographic pattern obtained from TCA extract of the washed erythrocytes from a normal subject (#1).

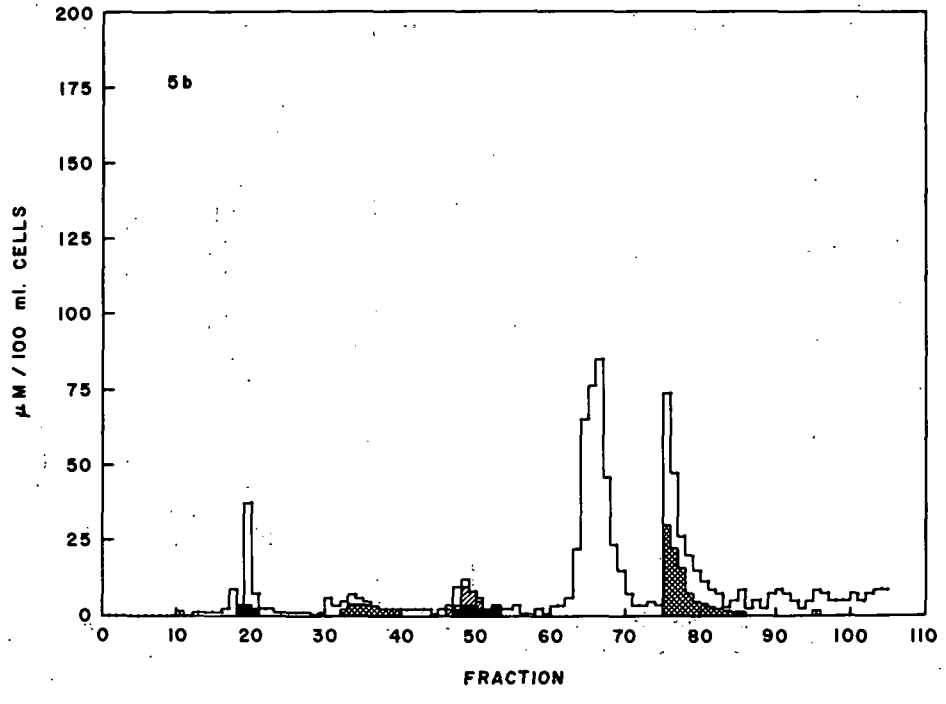

Figure 5b. Normal cells. Same donor as #5a. Chromatographic pattern obtained from TCA extract of the washed erythrocytes from a normal subject (#1).

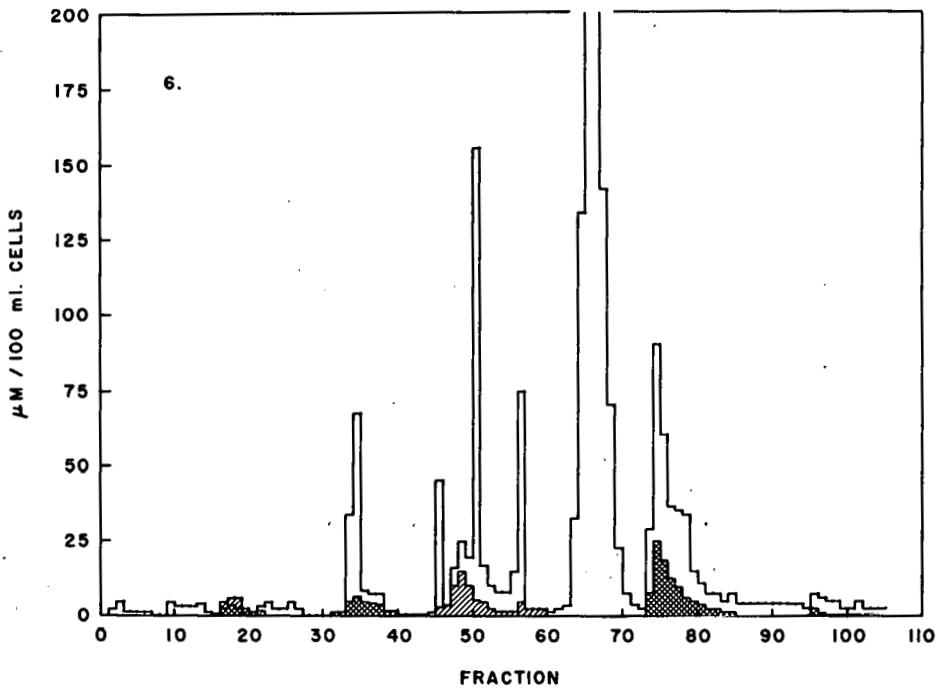

Figure 6. Idiopathic hemochromatosis—young cell population. Chromatographic pattern obtained from TCA extract of the washed erythrocytes from a normal subject (#1).

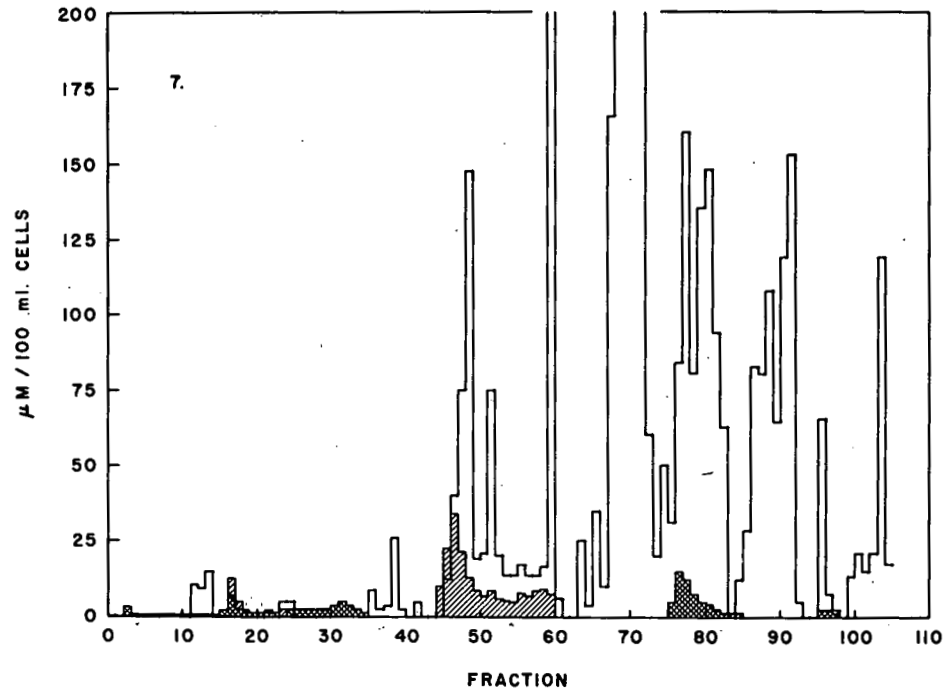

Figure 7. Treated iron deficiency—young cell population. Chromatographic pattern obtained from TCA extract of the washed erythrocytes from a normal subject (#1).

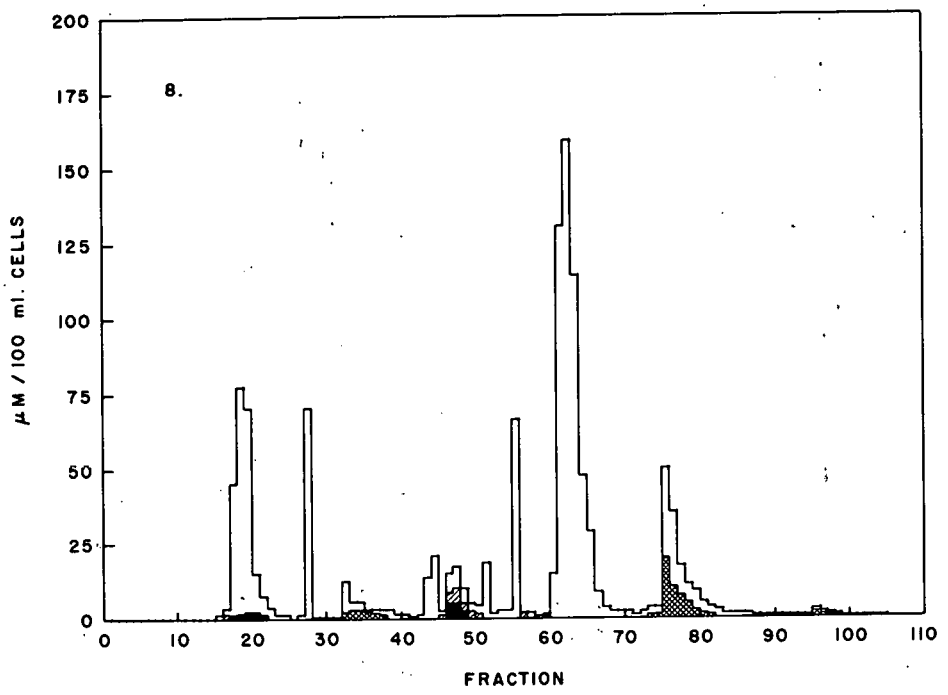

Figure 8. Treated iron deficiency—young cell population. Chromatographic pattern obtained from TCA extract of the washed erythrocytes from a normal subject (#1).

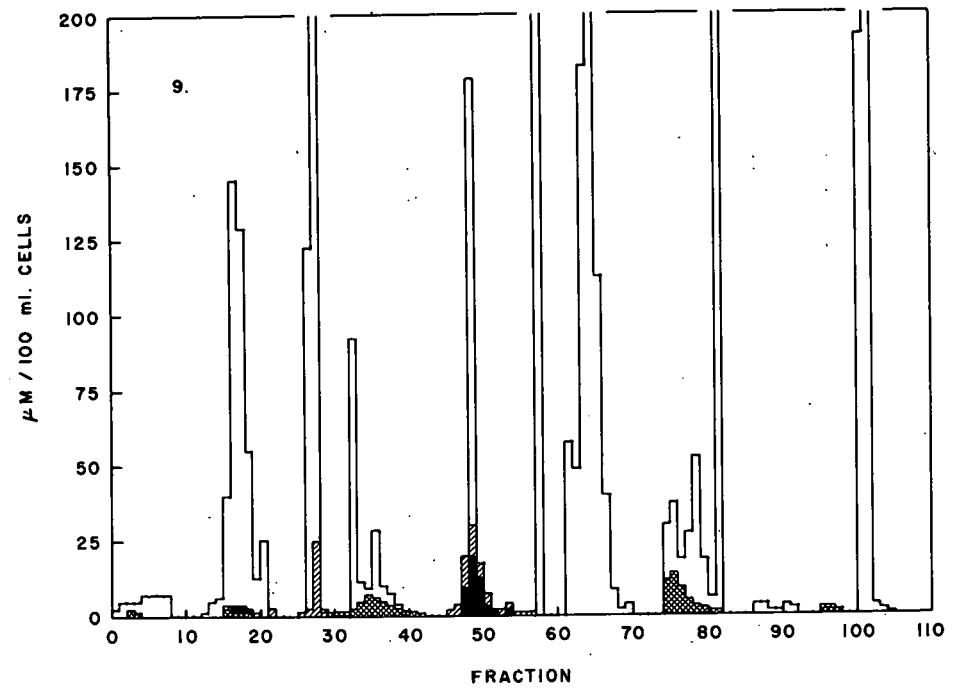

Figure 9. Erythrocytes from a primaquine-sensitive donor. Chromatographic pattern obtained from TCA extract of the washed erythrocytes from a normal subject (#1).

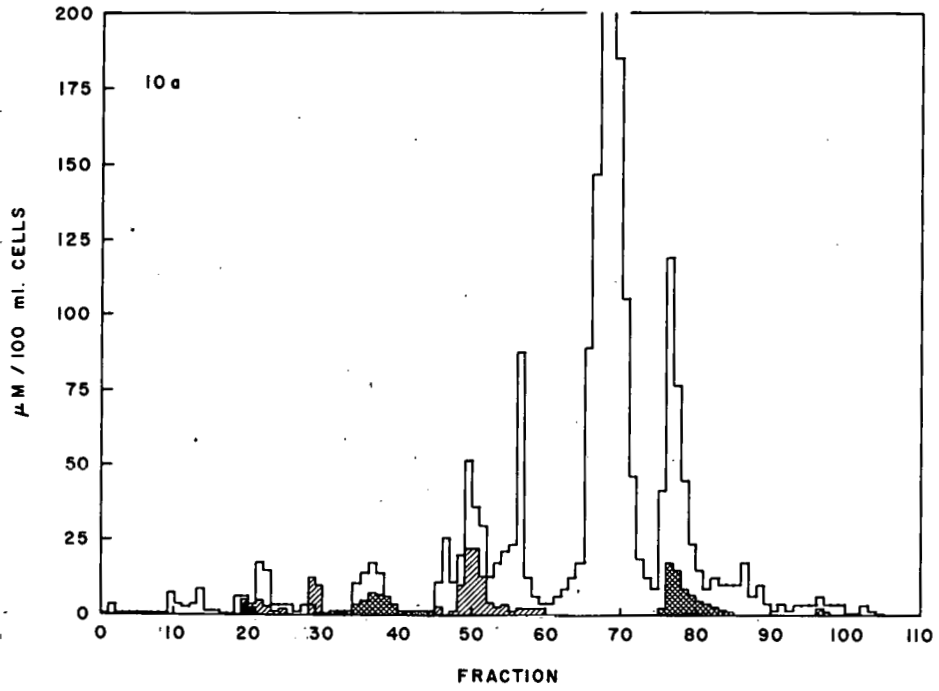

Figure 10a. Paroxysmal nocturnal hemoglobinuria. Chromatographic pattern obtained from TCA extract of the washed erythrocytes from a normal subject (#1).

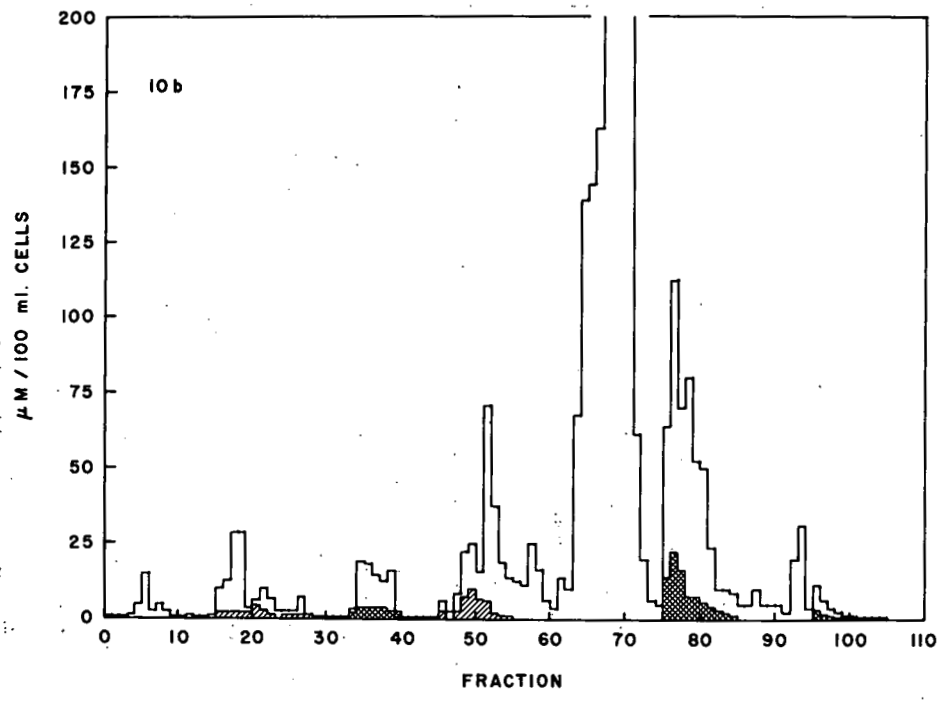

Figure 10b. Paroxysmal nocturnal hemoglobinuria during crisis. Same donor as 10a. Chromatographic pattern obtained from TCA extract of the washed erythrocytes from a normal subject (#1).

activity to counterbalance the increased substrate (fructose diphosphate). Increased activity of this enzyme has been demonstrated, but apparently this increase is not functionally sufficient to handle the load in the glycolytic pathway. The diphosphoglycerate fraction is essentially normal, although the monophosphoglycerate and phosphoenolpyruvate fractions are increased. The latter may be due to the relative decrease in the DPNH available for the production of lactic acid from pyruvate.

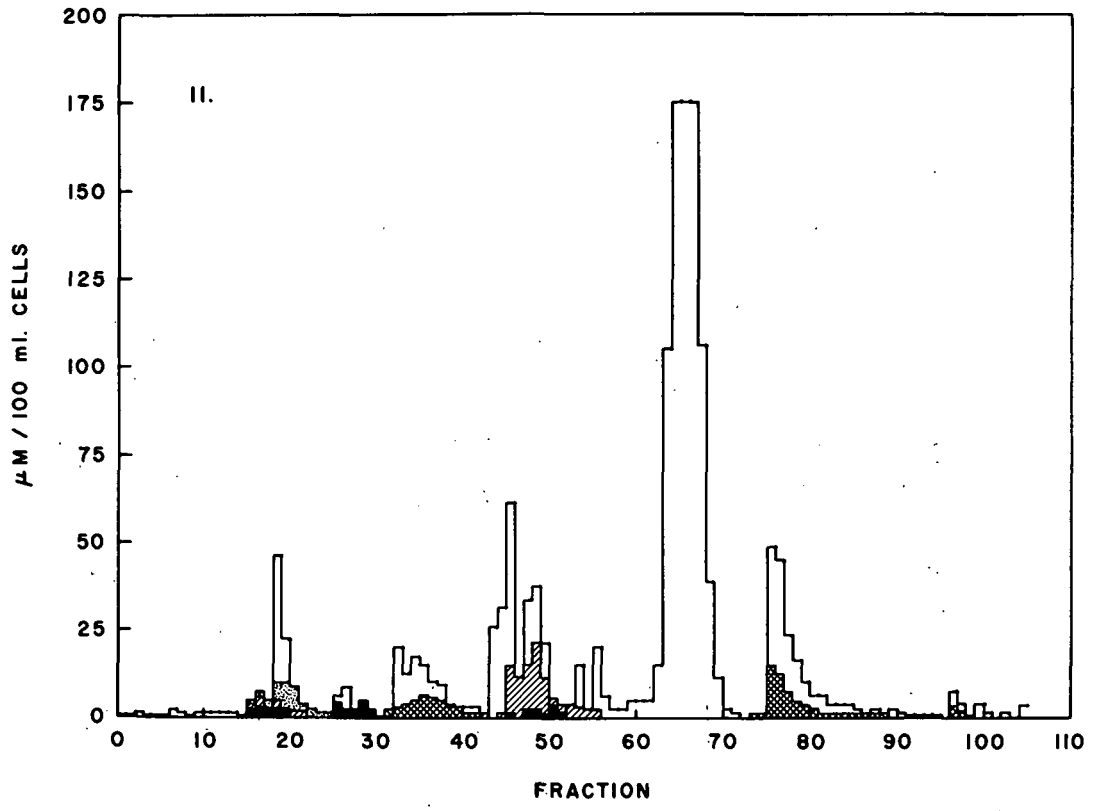

Figure 11. Paroxysmal nocturnal hemoglobinuria. Chromatographic pattern obtained from TCA extract of the washed erythrocytes from a normal subject (#1).

In contrast to the above, little is known of the basic lesion of PNH erythrocytes, although changes in lipids of the stroma, and in cholinesterase activity have been demonstrated. In these cells, in agreement with the data found by Altman, et al., the diphosphoglycerate fraction was found to be slightly increased. This increase in diphosphoglycerate suggests a decrease in enzymatic activity in one of the steps leading from phosphoglycerate to lactate. Such changes could be due to either a decrease in enzyme concentration, or, to a decrease in available reduced co-enzyme, or, to many other factors, such as electrolyte concentration, pH in the cell, etc., which might affect enzyme activity. Lactic dehydrogenase activity was found to be normal in the red cells of patient #10 at the time sample 10c was examined. The reason for the consistent increase in total nucleotides is obscure.

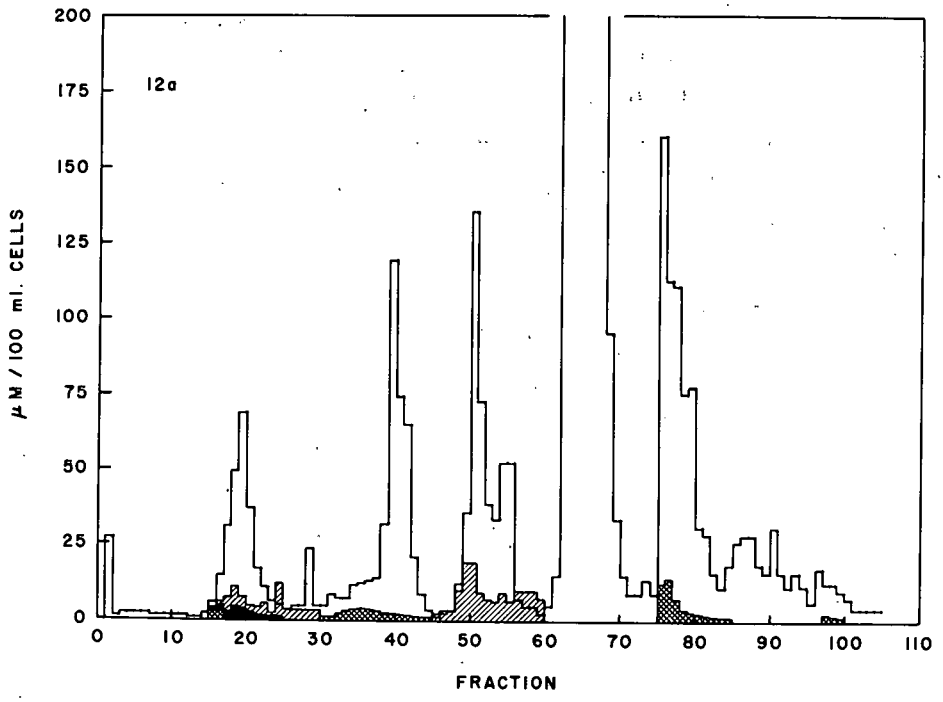

Figure 12a. Ovalocytosis with hemolytic anemia of unknown origin. Chromatographic pattern obtained from TCA extract of the washed erythrocytes from a normal subject (#1).

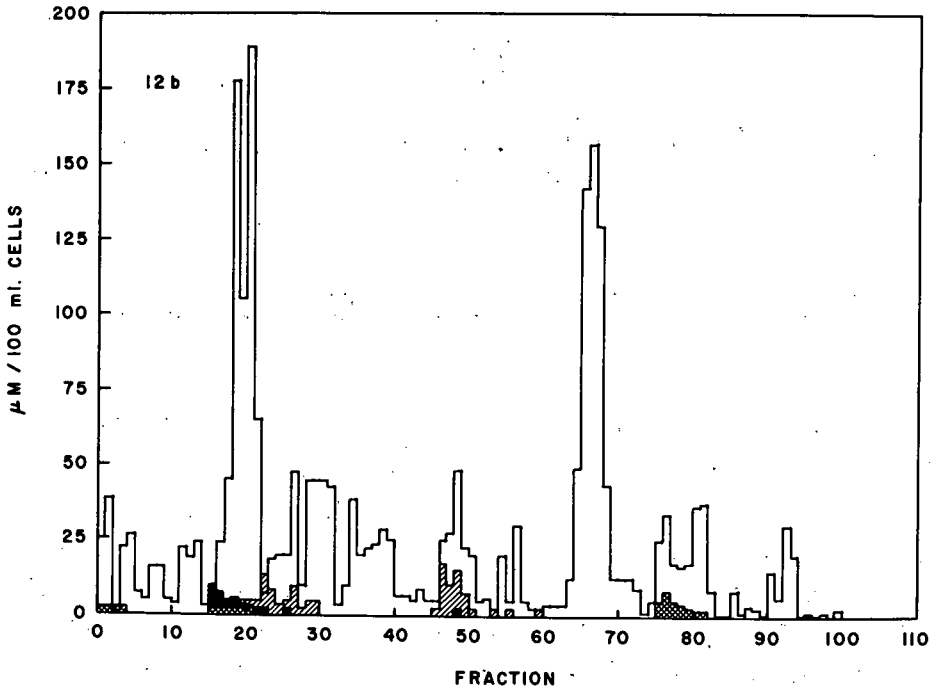

Figure 12b. Ovalocytosis with hemolytic anemia of unknown origin 4 months later. Chromatographic pattern obtained from TCA extract of the washed erythrocytes from a normal subject (#1).

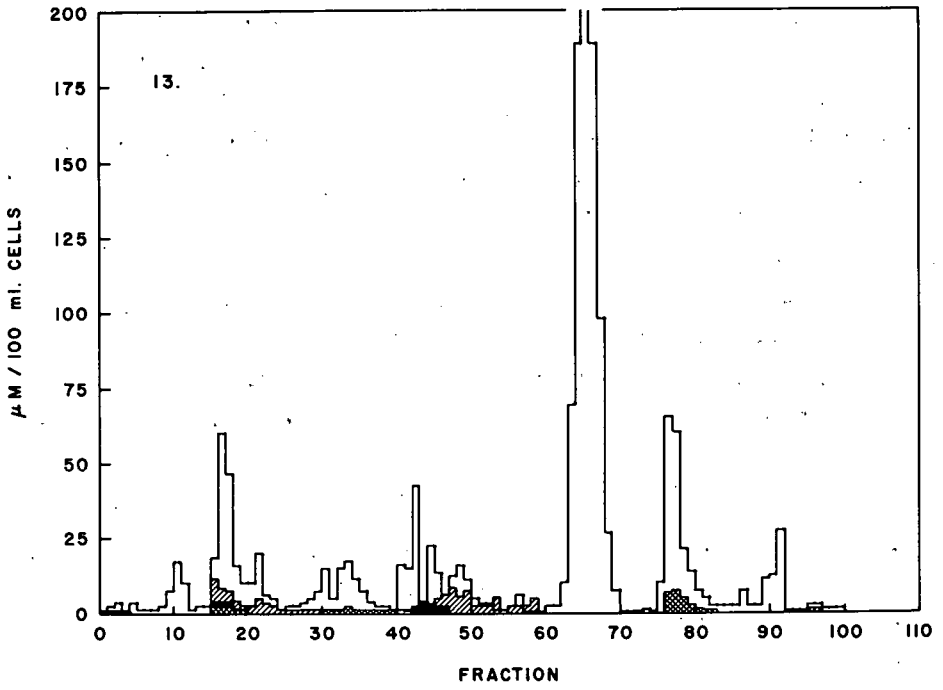

Figure 13. Ovalocytosis. Chromatographic pattern obtained from TCA extract of the washed erythrocytes from a normal subject (#1).

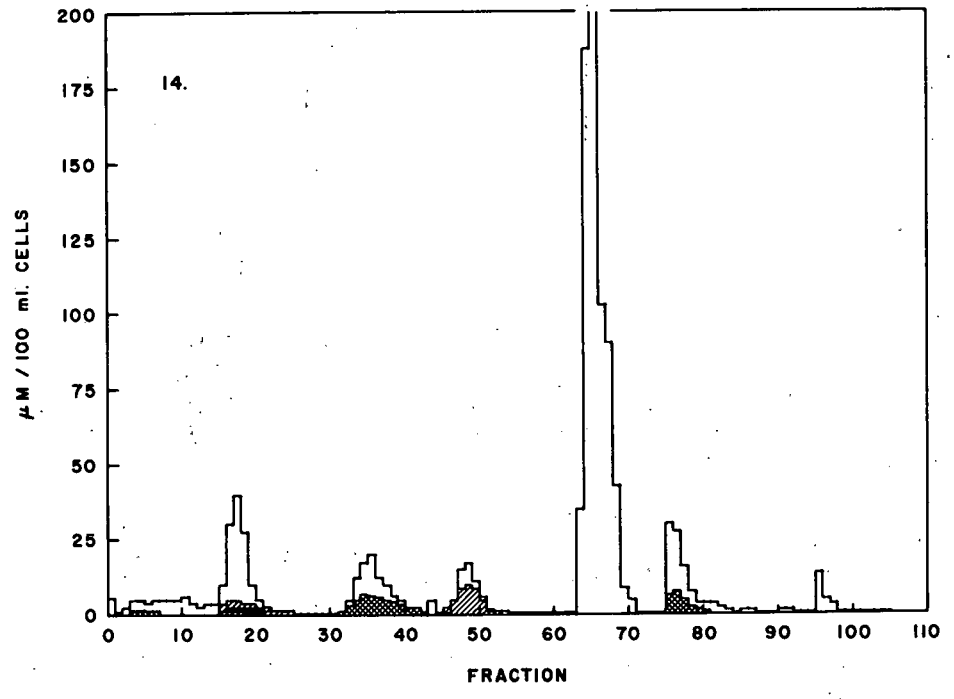

Figure 14. Compensated chronic-acquired, idiopathic hemolytic anemia. Chromatographic pattern obtained from TCA extract of the washed erythrocytes from a normal subject (#1).

Although the extracts from the two patients are not identical, the pattern of changes in concentrations of the intermediates is sufficiently similar to suggest that the same lesion is found in both.

Unfortunately, such good agreement was not found in extracts from patients with ovalocytosis. The abnormality which was found consistently, both in replicate extracts from the same patients and between patients, was a marked increase in glucose diphosphate. In addition, there was a more or less marked increase in glucose monophosphate and a lesser increase in fructose monophosphate. The diphosphoglycerate fraction in the various determinations varied widely from a five-fold increase over normal in one patient at one time, to an essentially normal concentration in the same patient on the second determination. Unidentified peaks were prominent, the significance of which is not presently understood.

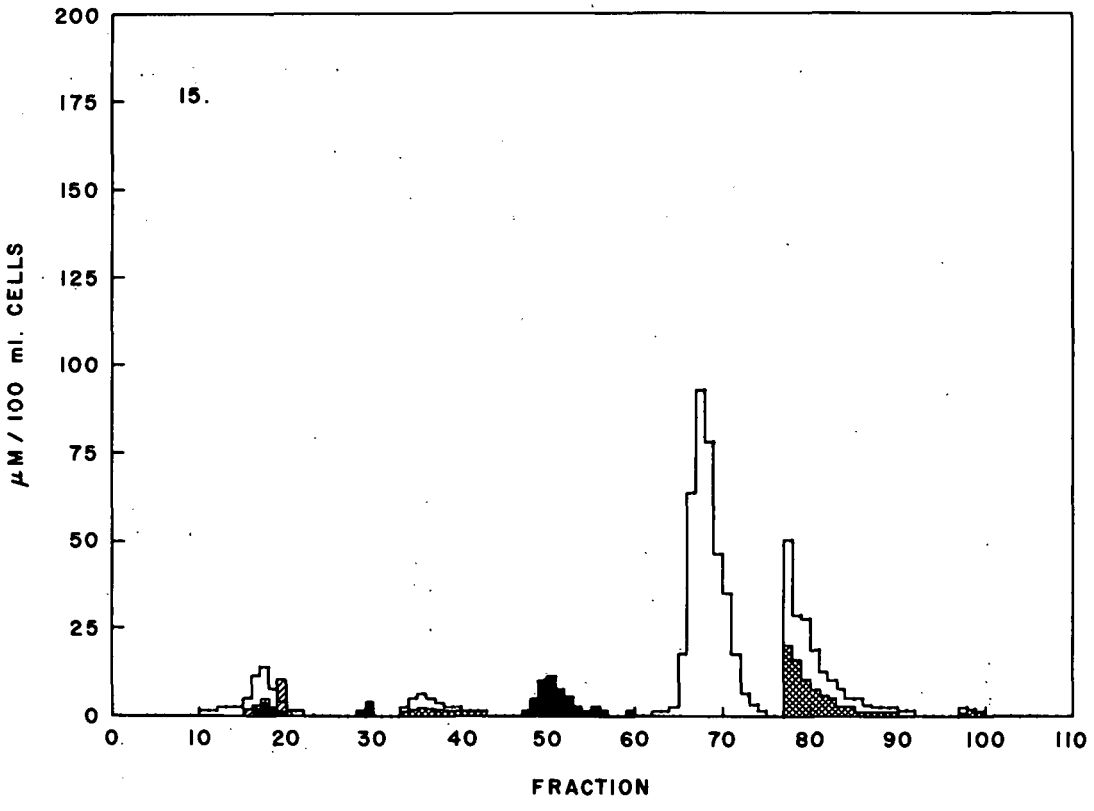

Figure 15. Acanthocytosis. Chromatographic pattern obtained from TCA extract of the washed erythrocytes from a normal subject (#1).

Finally, no abnormalities could be found in the red cells of a patient with a chronic compensated hemolytic state of unknown etiology.

The investigations reported here have demonstrated abnormalities in the concentrations of the phosphorylated intermediates in erythrocytes from patients with paroxysmal

nocturnal hemoglobinuria, hereditary ovalocytosis, and primaquine sensitivity. These changes do not define the enzymatic defect or defects which may underlie the hemolytic process, but they may provide leads for further investigations on the enzymatic level.

THE HISTOPATHOLOGY OF DELAYED DEATH IN IRRADIATED MICE TREATED WITH HOMOLOGOUS CELLS*

By

Joanne D. Denko, Eric L. Simmons, and Robert W. Wissler

INTRODUCTION

The results of Jacobson and co-workers[1,2] established the fact that lethally irradiated mice show less acute radiation injury at 30 days if their spleens are shielded or if isologous spleen cells are injected. Rapid proliferation of splenic tissue and repopulation of bone marrow in these animals were observed histologically. Subsequently, Lorenz, Congdon, and their colleagues[3,4] demonstrated that injection of homologous or heterologous bone marrow enhanced survival at 28 days and was accompanied by histologic regeneration of bone marrow and spleen.

In 1954, however, Barnes and Loutit[5] observed that lethally irradiated mice, whose lives had been spared in the 30-day period by homologous cell injections, subsequently developed diarrhea, underwent progressive weight loss, and succumbed at a more rapid rate during the ensuing 30 to 200 days than did irradiated mice treated with isologous cells. This reaction is now well-recognized by radiobiologists[6-8] and has come to be known as "delayed death," "secondary disease," "bone marrow disease," "homologous disease," etc. It is this disease that has proved a discouraging deterrent to more active therapeutic usage of bone marrow in treating the human being.

Congdon and Urso[8] have reported their autopsy findings on "bone marrow disease" when (101 x C3H)F_1 mouse marrow was injected into irradiated LAF_1 mice. In view of the interest that is being shown in the postirradiation effects after the injection of bone marrow, and the many differences in reactions seen with the array of mouse strains being used, it seemed of value to present the histopathological changes that we have observed for yet another pair of mice, the DBA/2 and CF No. 1.

MATERIALS AND METHODS

Test animals were 12- to 14-week-old CF No. 1 female mice (homozygous for aabbcc; obtained from Carworth Farms, Inc., New City, New York) and DBA/2 female mice (obtained from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine). Previous experience in our laboratory had shown that these mice died of "delayed irradiation death" after total-body irradiation and injections of bone marrow cells of the other strain.

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All mice were given 750 r in air while confined in perforated Lusteroid tubes which were rotated on a Lucite wheel having a capacity for 16 tubes at a time. The irradiation source was a G.E. Maxitron 250, 250 kvp, 30 ma, target distance 79 cm, filtration 0.25 ma Cu and 1.0 mm Al, half-value layer 1.04 mm Cu. After irradiation, the CF No. 1 mice were given intravenously by tail vein 10 or 20 million DBA/2 young adult (6- to 8-week-old) bone marrow cells. The DBA/2 mice received 5, 10, or 20 million young adult CF No. 1 bone-marrow cells. The animals were examined, starting at 24 days, for signs of illness —i.e., emaciation, weight loss, ruffled fur, and hunched back. If an animal showed these signs and was considered moribund, it was put to death by cervical dislocation. If it died spontaneously and was found soon enough before decomposition, an autopsy was performed. The longest survivor in this portion of the study died at 77 days.

Moribund animals that had been given 750 r and various treatments (isologous, homologous, and heterologous) 200 to 400 days previously were also included in this study. Such animals are of interest for comparison, even though it is unlikely at these late stages that their death was due to "bone marrow disease."

RESULTS

Repeated peripheral blood counts at varying intervals after infusion of homologous cells and terminal blood counts on moribund animals showed no consistent hematologic pattern. The presence of anemia, granulocytopenia, or lymphopenia was rare, and in fact, the tendency was for elevation of cell counts. This may have been due in part at least to hemoconcentration, if we assume that the emaciated appearance of moribund mice was indicative of a dehydrated state. Blood volume studies were not done.

The pathologic study of the tissues indicated death from a variety of causes, but in some cases no cause was discernible, although the animals were very emaciated. The general categories to be discussed are infection, focal necroses (heart and liver), other liver disease, kidney (glomerular) disease, abnormal erythropoiesis, abnormal myelopoiesis, and reticuloendothelial change. In addition, 2 animals had carcinoma of the intestine. Many contained the intestinal parasite Hymenolepsis, which frequently infests normal mice as well. The findings in the late survivors will be described separately.

In assembling the data under these various headings, the findings on all animals showing a particular disorder were surveyed. The diagnoses were checked to make sure there was approximately the same percentage incidence in the animals which died and those which were killed so as to rule out two possibilities: (1) that in the very last hours of life a different condition might not have arisen that we could have missed in the animals put to death, or (2) that the animals considered moribund might have recovered because their condition was not really a fatal one. There was no appreciable gross or microscopic difference between the animals found dead and the animals killed. The one possible exception was the almost complete absence of immature reticuloendothelial cells and the markedly decreased incidence of anaplastic reticuloendothelial cells, of the kind to be described, in animals which were found dead, suggesting early postmortem decomposition of this cell

type. In the tables which follow, no attempt is made to separate animals dying spontaneously from those that were put to death.

The times of death were broken down by 10-day intervals to see whether death might have been caused by different conditions at various time intervals after irradiation. For the mice dying 24 days or more after X-irradiation, no definite pattern of pathological changes could be distinguished that would be correlated with elapsed time after irradiation. Therefore, the data in the tables are not organized in relation to interval after X-irradiation. Ten CF No. 1 and 41 DBA/2 mice, succumbing between 24 and 77 days after X-irradiation, were studied in the autopsy series.

Infection. Infection was found in 5 of the 10 CF No. 1 mice and in 15 of the 41 DBA/2 animals. The infections found most commonly were pneumonia and bronchitis, septic liver infarcts, pyelonephritis, and lower small intestine and cecal abscesses. Usually there was only one area of infection in a given animal. Pneumonia was found most frequently and occasionally occurred together with kidney infection. The distribution of infections is shown in Table 1.

Table 1

DISTRIBUTION OF INFECTION

Strain	Lung	Liver	Kidney	Intestine	Heart	Skeletal muscle	Skin (para-urethral)
CF No. 1 given DBA/2 cells	3/10	-	-	1/10	1/10	1/10	-
DBA/2 given CF No. 1 cells	8/41	4/41	8/41	2/41	2/41	-	1/41

Because of the more rapid decomposition of tissues in animals dying with infection and/or fever, this sample is a smaller proportion of the total deaths than it would have been had it been possible to examine all animals histologically. The sites of infection are probably represented in approximate proportion to their true incidence, however.

Focal necroses (heart and liver). In hearts, focal necroses were never observed grossly but were seen often microscopically as very small subepicardial areas of brilliant purple deposition of calcium in necrotic muscle fibers surrounded by fibrosis. As shown in Figure 1, these were sometimes adjacent to a blood vessel, but this was not necessarily a characteristic location. With just one exception, they appeared too small to have had much effect on the circulatory dynamics. The cardiac necroses were found in 2 of 10 CF No. 1 mice and in 9 of the 41 DBA/2 strain.

Multiple lesions in livers were sometimes observed grossly as discrete yellowish spots. Microscopically, they consisted in necrosis of liver parenchymal cells with relative sparing of stromal cells. No vascular thrombi were observed to account for these

findings. In some instances of widespread necrosis, there was secondary infection with little cellular reaction, probably because the infection was a terminal event. Liver necrosis was seen in 8 of the 41 DBA/2 mice and in 2 of the 10 CF No. 1 mice. Heart and liver were involved simultaneously in 2 of the 9 DBA/2 animals showing the lesion, and these conditions coincided in 1 of the 2 CF No. 1 mice. An example of this type of pathological change is shown in Figure 2.

Liver cytologic damage. In addition to the liver necroses just mentioned, many of the livers showed minimal to marked variation in liver parenchymal cell size, with or without pyknosis of nuclei. This lesion was present in 5 of the 10 CF No. 1 mice and in 26 of the 41 DBA/2 animals. In none was it severe enough to be a significant factor in mortality.

Kidney disease. Many of the kidneys showed changes of a mild to moderate nature, particularly in the glomeruli. In some, the glomerular size was quite variable. In others, there was a mild to moderate thickening of the basement membranes as seen with hematoxylin-eosin-azure stain (HEA) and also with periodic acid-Schiff stain (PAS). One kidney had basophilic protein deposition in the tubules. There was no far-advanced glomerular disease or nephrosclerosis or tubular change of cystic type as observed in rats by Lamson et al.[9] Figure 3 shows an example of relatively severe glomerular basement membrane thickening with apparent ischemia of the glomerular tuft. In most cases it was felt to be too mild to be of significance in producing the animal's death. The various findings are tabulated in Table 2.

Table 2

RENAL LESIONS

Strain	Glomeruli		Tubule protein deposition
	Thickened basement membrane	Variation in glomerular size	
CF No. 1 given DBA/2 cells	1/10	-	-
DBA/2 given CF No. 1 cells	14/41	8/41	1/41

To investigate the possibility that these lesions were contributing to death in the animals, other animals were watched for signs of approaching death, with the intention of studying terminal blood nonprotein nitrogen. The results are given in Table 3. Their heart blood and spleen were cultured and found negative for pathogenic bacteria. It is probable that the moderate rise in 2 of the mice is not enough to account for death. In fact, the elevation observed could have resulted from dehydration, but these mice had several glomerular lesions at autopsy.

Erythropoiesis. In studying the hematopoietic organs, we found that many of the animals showed a diminution in erythropoietic elements in spleen, sometimes to the virtual

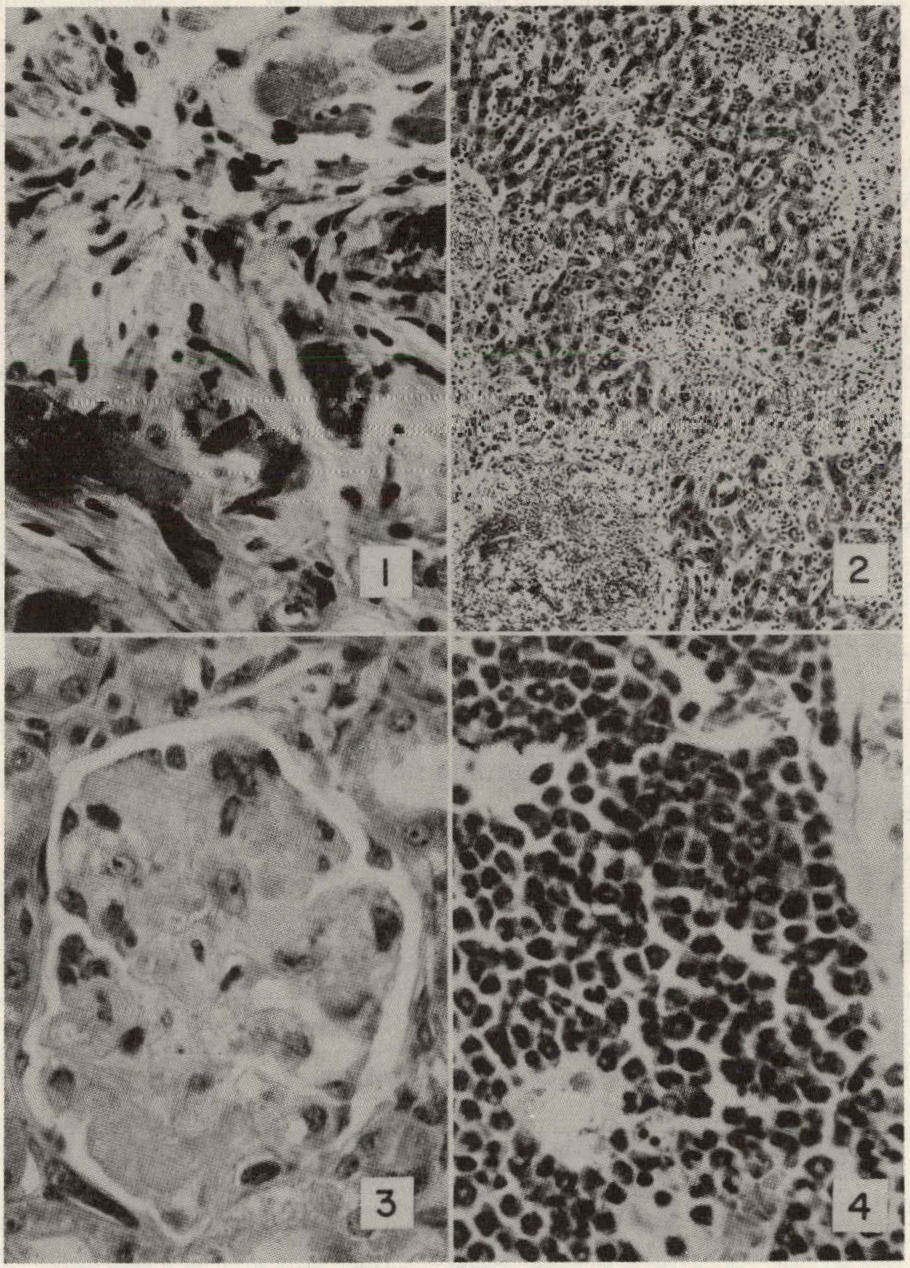

Figure 1. Cardiac infarction in DBA/2 given 750 r and 10 million CF No. 1 bone marrow cells, dead on day 34. HEA stain. (510x).

Figure 2. Liver necrosis with hemorrhage in DBA/2 given 750 r and 5 million CF No. 1 bone marrow cells, killed on day 28. HEA stain. (125x).

Figure 3. Kidney disease in DBA/2 given 750 r and 10 million CF No. 1 bone marrow cells, dead on day 55. PAS stain. (700x).

Figure 4. Mature myeloid cell predominance in bone marrow of CF No. 1 given 750 r and 10 million CF No. 1 baby liver cells, dead on day 322. HEA stain. (700x).

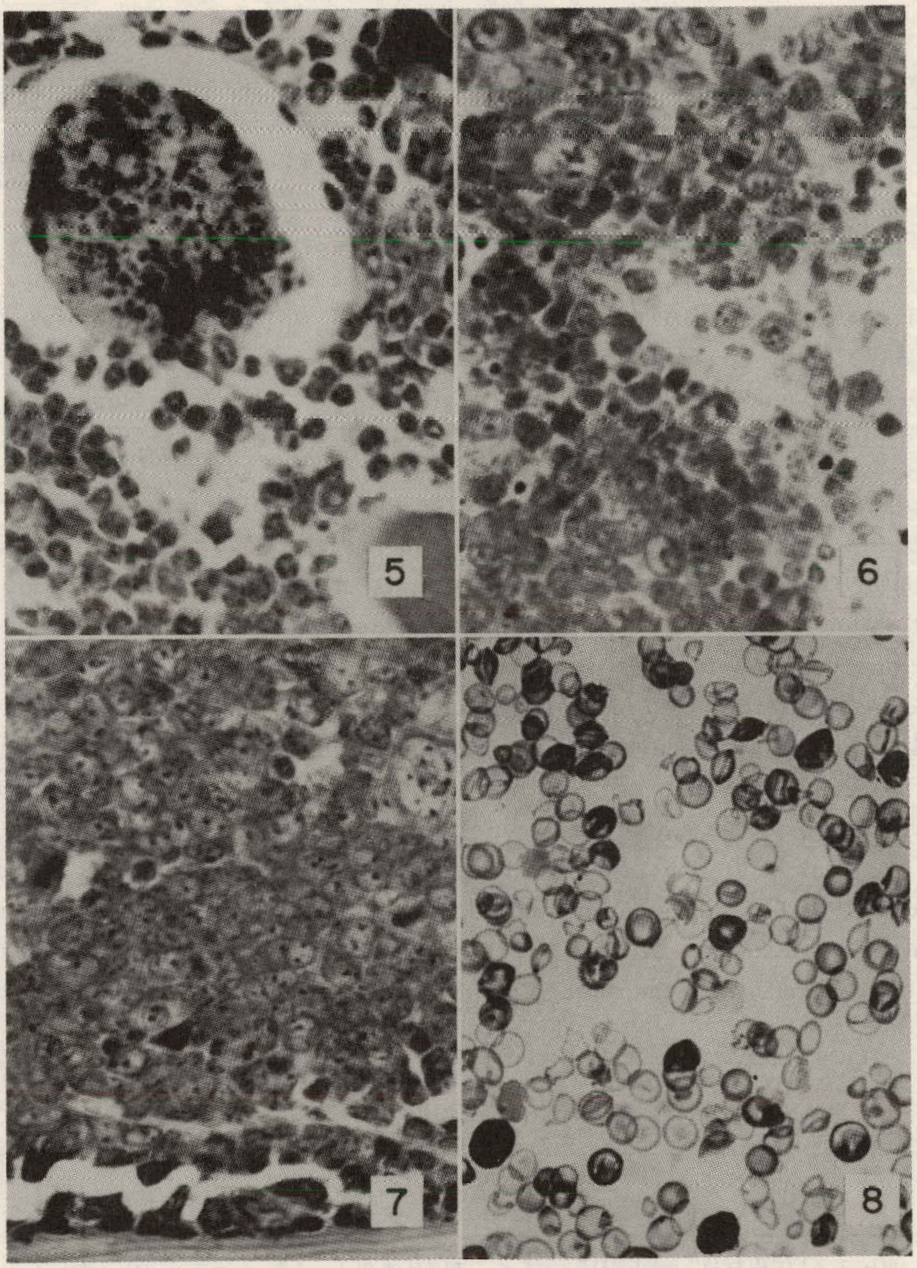

Figure 5. Clump of disintegrating myelocytes in bone marrow of DBA/2 given 750 r and 5 million CF No. 1 bone marrow cells, killed on day 53. HEA stain. (700x).

Figure 6. Anaplastic reticuloendothelial cells in bone marrow of DBA/2 given 750 r and 20 million CF No. 1 bone marrow cells, killed on day 26. HEA stain. (700x).

Figure 7. Immature reticuloendothelial cells in bone marrow of DBA/2 given 750 r and 10 million CF No. 1 bone marrow cells, killed on day 40. HEA stain. (700x).

Figure 8. Anemic blood smear of DBA/2 given 750 r and 4 million BALB/c baby spleen cells, dead on day 242. HEA stain. (700x).

Table 3

NONPROTEIN NITROGEN DETERMINATIONS IN MORIBUND MICE

Strain	NPN (mg %)	Culture of spleen and heart blood
Normal CF No. 1	27-33	Negative
CF No. 1 given DALB/c cells	29	Negative
CF No. 1 given DBA/2 cells	62.8	Negative
DBA/2 given CF No. 1 cells	57.2	Negative
DBA/2 given Locke's solution	36	Negative

exclusion of these elements. Table 4 shows the incidence of depressed erythropoiesis. In the first column, "active" erythropoiesis is interpreted broadly, so that animals with any amount greater than "little" appear here. Animals in which autolysis or poor fixation made interpretation uncertain have been placed in the last column. Notably, there was frequently less erythropoiesis evident in the bone marrow of femur and sternum than in the spleens. Table 5 shows the distribution of erythropoiesis in bone marrow. Erythropoiesis was found to be practically absent in the spleen in 2 DBA/2 mice with evidence of adequate blood formation in their bone marrow. In many mice, however, it was impossible to find definite histological evidence of erythropoiesis in either bone marrow or spleen. The possibility arose that this lack of erythropoiesis might be a major factor in the decreased life span of the irradiated and homologously injected mice. Therefore, attempts were made to do complete blood counts when animals appeared moribund. Table 6 correlates the results of this phase of the work, with the subsequent histological findings. Normal erythrocytic

Table 4

ERYTHROPOIESIS IN SPLEEN

Strain	Active	Little	Absent	Cannot interpret
CF No. 1 given DBA/2 cells	4/10	-	5/10	1/10
DBA/2 given CF No. 1 cells	15/41	3/41	15/41	8/41

Table 5

ERYTHROPOIESIS IN BONE MARROW

Strain	Adequate	Little	Virtually absent	Cannot interpret
CF No. 1 given DBA/2 cells	-	-	10/10	-
DBA/2 given CF No. 1 cells	2/41	-	38/41	1/41

Table 6

CORRELATION BETWEEN BLOOD COUNTS AND AUTOPSY FINDINGS

Strain	Erythrocytes (cells x 10^6)	Total leukocytes (cells x 10^3)	Erythropoiesis	
			Spleen	Bone marrow
CF No. 1 given DBA/2 cells	12.64	12 (infection)	Moderate	Cannot interpret
DBA/2 given CF No. 1 cells	9.88	7.5	Good	Moderate
DBA/2 given CF No. 1 cells	7.1	13 (infection)	Present	None
DBA/2 given CF No. 1 cells	8.1	9.5	Present	None
DBA/2 given CF No. 1 cells	12.08	5	None	None

counts for DBA/2 females in this laboratory ranged from 9.2 to 10.2 million, and for the CF No. 1 female mice, from 9.9 to 11.2 million. It is difficult to explain the concurrence of a red cell count of 12.08 million and the finding of no apparent erythropoiesis in spleen or bone marrow, unless one postulates marrow exhaustion from rapid discharge into the blood. The reverse of this (low erythrocytic count and adequate erythropoiesis) was seen in 2 of the late survivors (vide infra). It is of interest that Congdon et al.[10] have reported secondary destruction of hematopoietic elements in spleen and bone marrow in heterologously treated mice within the acute 30-day period. Their animals showed hemorrhage, anemia, and infection.

In addition to a marked diminution in erythropoiesis, some animals showed an abnormal predominance of myelopoiesis in bone marrow and, to a lesser extent, in spleen (Table 7). In fact, this was sometimes so pronounced that the spleen showed nothing but masses of mature myelocytes, giving the appearance of a chronic noninvasive myeloid leukemia. In spleens showing this condition, lymphopoiesis was virtually absent. Figure 4 shows an example of almost exclusive granulopoiesis from the bone marrow of an animal in the group of late survivors but is quite typical of all ages.

Discrete clumps of disintegrating myelocytes were found in the bone marrow and spleen of 2 mice (Figure 5).

Table 7

CHARACTERISTIC CELL TYPES IN RETICULOENDOTHELIAL SYSTEM

Strain	Mature myeloid		Anaplastic reticuloendothelial		Immature reticuloendothelial	
	Spleen	Femur	Spleen	Femur	Spleen	Femur
CF No. 1 given DBA/2 cells	8/10	8/10	7/10	2/10	1/10	3/10
DBA/2 given CF No. 1 cells	20/41	26/41	23/41	7/41	0/41	8/41

Other reticuloendothelial changes. In spleen, and to a lesser extent in bone marrow and occasionally even in the lymph node, animals showed one or the other of two very unusual changes. The first (Figure 6) consisted of masses of large, irregular, pale anaplastic cells with large, prominent, darkly staining nucleoli. The second (Figure 7) was similar except for a more uniform cell type, with smaller, more regular, pale, immature cells of the reticuloendothelial system. Table 7 shows the frequency of occurrence of these changes, as well as the masses of mature myelocytes described above. Often the two patterns were observed in the same animal, and even in the same organ. In general, however, the immature reticuloendothelial cells appeared to occur mainly in the bone marrow, and the anaplastic cells more frequently in the spleen. In no case did these changes suggest fully developed leukemia or lymphoma.

Summary of pathological findings. Of the 10 CF No. 1 mice studied, 3 died of severe lung infections (overlapping with bowel infection in 1 case); 1 had multiple larger areas of liver necrosis and decreased to absent erythropoiesis; and 1 had intestinal carcinoma. Four mice had only immature reticuloendothelial cells and/or clumps of mature myelocytes in the blood-forming organs, but this condition also overlapped with many of the other findings described above.

In the 41 DBA/2 animals reported, often more than one condition that could result in death was found to be present. These are as follows: 6 died with pneumonia alone or together with other infections, 3 with severe liver infection, 1 with infections of liver and lung, and 7 with infections in other areas (including 5 in kidney and 2 in bowel). Five mice died with severe multiple liver necrosis, in some cases coinciding with liver cell anisocytosis. Two additional animals showed liver cell anisocytosis without necrosis, 13 showed diminished to absent erythropoiesis, and 1 had intestinal carcinoma. In 14 mice, there was no apparent cause of death. In attempting to determine the cause of death, the mere presence of immature reticuloendothelial cells has not been considered an adequate factor unless erythropoiesis was absent.

Late deaths. In the course of this study, it became apparent (1) that animals were showing a diversity of lesions, and (2) that certain animals from earlier experiments were surviving much longer than the animals selected for this investigation. Hence, we decided to study these additional animals that had also been given 750 r and supportive injections of isologous, homologous, or heterologous cells. Twelve mice that died between 200 and 400 days after irradiation were studied, and the findings are listed in Table 8. There were only two entirely new findings: (1) hemorrhage into the gastrointestinal tract in 1 CF No. 1 mou e protected with newborn guinea pig spleen cells, and (2) a very bizarre type of blood smear appearing in 2 DBA/2 mice, 1 of which had received spleen cells from newborn DALB/c mice and 1 of which had received young adult CF No. 1 bone marrow. This change consisted of a severe anisocytosis, pallor of the erythrocytes, and poikilocytosis, similar to pernicious anemia in the human being. The cause of this anemia is unknown. It is difficult to understand a red cell count of 1.1 million when erythropoiesis in the spleen is apparently adequate except as a failure of the red cells to be discharged into the circu-

Table 8

HISTOLOGIC FINDINGS IN MICE DYING BETWEEN 200 AND 400 DAYS AFTER 750 r PLUS INJECTIONS OF SUPPORTIVE CELLS

Strain	Type of cells given	Number of cells given (10^6)	Day of death	Findings
CF No. 1	CF No. 1 BM[a]	10	252	Glomerular basement membrane thickening and variation in size; liver cell anisocytosis. No adequate cause of death observed.
CF No. 1	Newborn guinea pig S[a]	4	252	Old hemorrhage into gastrointestinal tract; pyelonephritis; unilateral deposition of protein in tubules of one kidney; liver cell anisocytosis.
CF No. 1	Guinea pig S	14	258	Small areas of cardiac necrosis. No satisfactory cause of death observed.
CF No. 1	CF No. 1 baby L[a]	10	322	Pyelonephritis; patchy bronchopneumonia; mild liver cell anisocytosis; mild glomerular basement membrane thickening.
CF No. 1	DALB/c BM	5	361	Urinary cystitis. No adequate cause of death observed.
CF No. 1	CF No. 1 baby L	3	378	Mammary carcinoma; immature and anaplastic reticuloendothelial cells in bone marrow and spleen.
DBA/2	DALB/c baby S	4	242	Cataracts; anemia with smear resembling pernicious anemia (5.65 million) with moderate erythropoiesis in spleen; anaplastic reticuloendothelial cells in bone marrow.
DBA/2	CF No. 1 BM	5	300	Severe anemia (1.1 million) with smear resembling pernicious anemia; adequate erythropoiesis in spleen; anaplastic and immature reticuloendothelial cells; small areas of cardiac necrosis; glomerular basement membrane thickening; liver cell anisocytosis.
DBA/2	DBA/2 embryo L	20	320	Bronchitis and bronchopneumonia.
DBA/2	DBA/2 BM	1	322	Cardiac hypertrophy; liver cell anisocytosis. No adequate cause of death observed.
DBA/2	DBA/2 baby L	10	324	Bronchopneumonia; renal abscesses; small cardiac infarct; glomerular basement membrane thickening; dilatation of Bowman's spaces.
DBA/2	CF No. 1 baby L	2.5	333	Mature myeloid cells predominate in bone marrow, with 3 normal red cell counts; marked liver cell anisocytosis; marked glomerular basement membrane thickening.
DALB/c	DBA/2 baby L	5	237	Liver cell anisocytosis; anaplastic reticuloendothelial cells in bone marrow.
DALB/c	DBA/2 baby L	2.5	301	Slight thickening of glomerular basement membranes. No adequate cause of death observed.

[a]BM stands for bone marrow, S for spleen, and L for liver.

lation. Figure 8 shows an example of this type of blood smear.

DISCUSSION

It is now well-established from the work of many investigators[11-17] that transferred foreign hematopoietic cells colonize in lethally irradiated mice. Depending on the genetic relationship of the two strains of mice involved, the type and number of cells injected, and the irradiation dosage administered, a variety of immunological interactions have been postulated to account for the secondary disease that results.

1. With the recovery of the irradiated animal's immune mechanism there may be destruction of the foreign colonized hematopoietic tissue. Our findings for DBA/2 to CF No. 1 and vice versa, in agreement with those of Congdon and Urso[8] with (101 x C3H)F_1 bone marrow to LAF_1 mice, furnish evidence of postirradiation destruction of hematopoietic tissue in an occasional animal, but it is not clear whether this is recovered tissue from the irradiated animal, or hematopoietic tissue from the donor. In both studies there was some indication in a few animals of acute necrosis of blood-forming cells.

2. Congdon and Urso[8] also discuss the possibility that the host is so overwhelmed by the foreign antigens in its body that its spleen and lymph nodes become fibrotic. We did not find this reaction in the DBA/2 and CF No. 1 combinations used.

3. Barnes and colleagues[18] have suggested that the grafted tissues, which are known[19] to be capable of antibody formation themselves, are stimulated by the antigens in the host's body and produce degenerative changes in it by continual production of anti-host antibodies.

4. Van Bekkum[20] has pointed out that the possibility also exists that the immunologic defenses of the hosts are weakened by lethal irradiation, so that chronic infections start and provoke the symptoms of secondary disease. Curtis and Healey[21] have shown that uninjected mice that survive irradiation suffer irreparable damage in the form of diminished tolerance to stress as well as shortened life, and it is known[22] that irradiated mice treated with isologous cells have a shorter life span than do nonirradiated untreated mice, although, in general, they do survive longer than those receiving homologous or heterologous cells. Although some of our mice apparently did die of massive infections, in some cases, at least, there was good mobilization of granulocytes.

In studying the tissues of our animals given foreign bone marrow, we have attempted to evaluate changes in the host body that might help us to elucidate the possible interaction between host and donor cells. Some mice showed varying degrees of kidney or liver abnormality, but in most instances this did not appear severe enough to produce death. Some had generalized anaplastic or immature reticuloendothelial cell proliferation, but this was not invasive and did not necessarily limit function. Similarly, the presence of increased numbers of mature myelocytes in the bone marrow was not considered to be harmful, except for possible interference with erythropoiesis. The finding of clumps of disintegrating myelocytes in reticuloendothelial organs may be interpreted as some indication of a reaction between host and recipient cells. This was observed in only 2 animals, however,

and then only focally. We could not ascertain in this study whether the necrotic cells were host or donor cells. Nor could we be certain that an immune mechanism really was involved.

Our observations with DBA/2 and CF No. 1 mice are essentially similar to the earlier findings of Congdon and Urso[8] with LAF_1 mice given (101 x C3H)F_1 bone marrow cells. They reported infections in lungs, kidneys, and gastrointestinal tract, infarcts and abscesses in liver, increase of granulocyte formation in bone marrow, and a few areas of focal necrosis of blood-forming cells in the spleen. Two of their reported findings, not seen in our study, were the presence of hemorrhagic lesions in the pylorus and large intestine and the fibrosis of lymphocytic tissues such as lymph nodes and spleen.

In our studies there was no apparent single pattern of histopathologic change that could be held responsible for producing delayed death in either CF No. 1 or DBA/2 mice given 750 r and injections of bone marrow of the opposite strain. One finding that may be of more significance than we realize was the frequent appearance of an almost uniform cell type in the hematopoietic tissues of these irradiated, injected mice. Under these conditions the normally diverse hematopoietic tissues appear to be developing in only one direction, particularly toward a mature granulopoietic cell type or into an immature reticular cell type.

At present it is difficult for us to correlate the histopathologic changes seen in these mice with any of the existing hypotheses regarding host and donor interaction. The general appearance at the time of death suggested premature aging, senility, and emaciation—a delayed X-irradiation effect found by others in studies of mice and of other species.

SUMMARY

To investigate the nature of late deaths that occurred after successful recovery beyond the acute 30-day period, CF No. 1 and DBA/2 female mice were irradiated with 750 r and were given bone marrow cells of the other strain. Surviving mice were sacrificed for histologic study when they appeared moribund. Death could not be ascribed consistently to impaired or arrested hematopoietic function, and no single common histopathologic pattern was found to exist. Rather, the mice seemed "weakened" and died with a variety of conditions, including pneumonia, necrotizing and infected liver lesions, and rarely with carcinoma or ischemic renal glomerular lesions. The cause of death was not always apparent.

ACKNOWLEDGMENT

The authors gratefully acknowledge the technical assistance of Janet Kolar and Dorothy Carter.

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ELECTRON THERAPY WITH A SCANNING-BEAM SYSTEM USING A 50-MEV LINEAR ACCELERATOR*

By

L. S. Skaggs and L. H. Lanzl

The system of electron therapy developed at the Argonne Cancer Research Hospital is now being used on patients in the Clinics. It consists of two principal units; a linear electron accelerator and an electromagnetic beam deflector. Their application to cancer therapy and the dose distributions obtained differ significantly from those of conventional X-rays.

The electron source consists of a 50-million-electron-volt traveling-wave linear accelerator, a schematic drawing of the microwave system of which is shown in Figure 1.

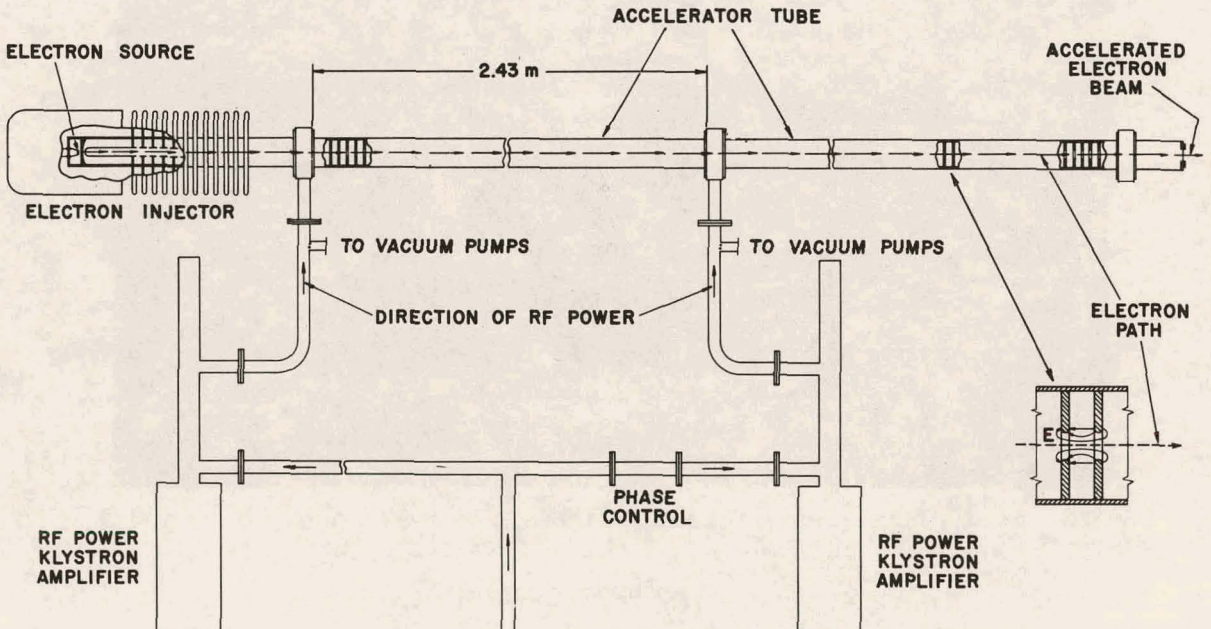


Figure 1.

*Paper presented at the 9th International Congress of Radiology, Munich, Germany, July, 1959.

The magnetron is the source of a 2,857-megacycle radio wave, which is directed to a tee junction with part of the wave going to one klystron amplifier and the remaining part going to a second klystron. The resulting amplified wave is then fed in cascade to the linear accelerator proper.

The electron accelerating agent is the electric field component of the electromagnetic wave which lies in the direction of the axis of the accelerating tube. At the time when the wave fills the tube, electrons are injected. They emerge from the tube with a predetermined energy of between 5 and 50 Mev. One method of energy selection is by the use of a phase shifter (see Figure 1).

Figure 2 is a photograph of the accelerator taken without the beam-scanning device in place. The two klystron stations are shown together with a spare one. The accelerator tube is contained in the protective housing on the left of the picture.

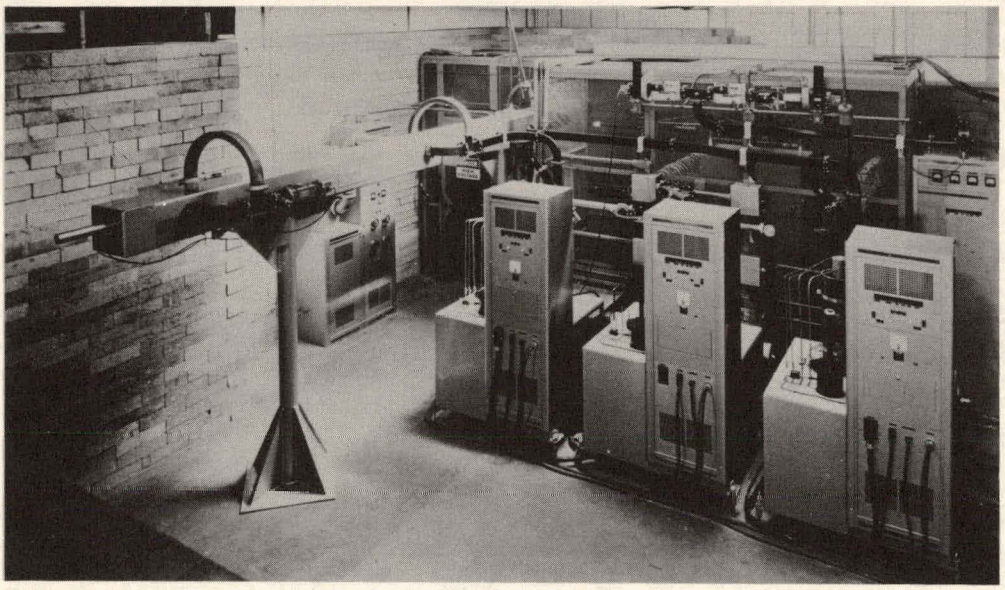


Figure 2.

Figure 3 illustrates one type of electron spectrum from the unit. The ordinate gives the energy in Mev and the abscissa, the relative beam current. Although this spectrum is not as sharp as the spectrum from a betatron, it is more than adequate for therapy.

The linear accelerator was chosen in preference to the betatron for this work because it is capable of providing free electron beams over a wide energy range much more easily.

The spectrum obtained under different conditions of tuning is shown in Figure 4. These data represent six separate runs taken over a 40-minute period. The machine can be tuned with the peaks arbitrarily separated or with a single peak. It has been found pos-

sible to predict the double peak theoretically. The high degree of reproducibility of the data indicates the stability of the machine.

After leaving the accelerator, the electrons are directed to the beam-deflecting and scanning system, which consists essentially of a series of three magnets (Figure 5). Here they are first deflected through 45 degrees and analyzed by means of an electromagnet. Actual energy selection occurs at a collimator, and the electrons selected there are again deflected through 45 degrees by a second magnet, giving a beam in the same direction as the initial beam but offset by 71 cm. The beam then enters a third magnet where it is bent

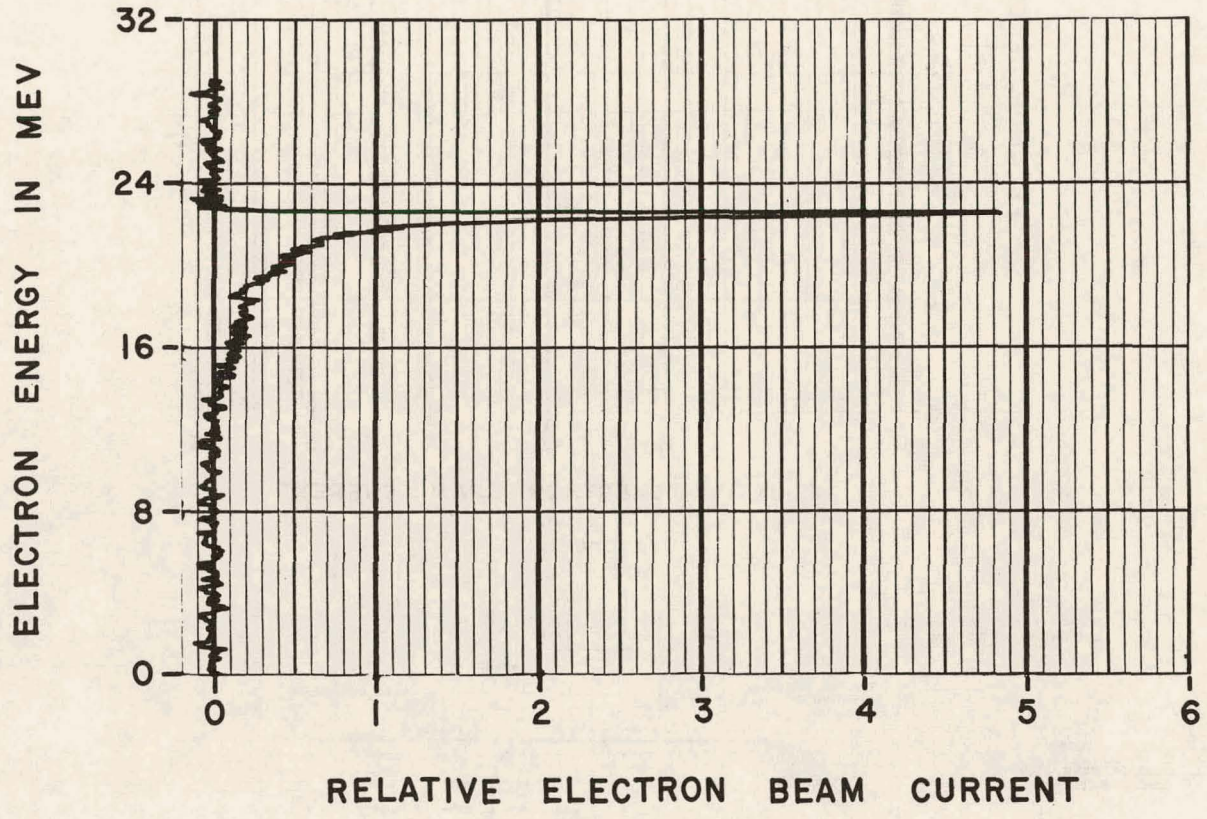


Figure 3.

through 90 degrees. The first two magnets provide horizontal beam focusing; the third, both horizontal and vertical focusing. During this process, the beam path is still enclosed in a vacuum chamber to avoid scattering by air. On emerging from the exit window, the beam is directed onto the patient who is protected from stray radiations by an X-ray and neutron shield between him and the energy collimator. The cross-sectional area of the beam is about 1/2 cm diameter.

Figure 6 is a photograph of the deflection system taken during installation. Vacuum system, magnets #1, #2, and #3, and the supporting structure are visible.

To irradiate a given tumor area with the 1/2-cm beam, the magnet system is made

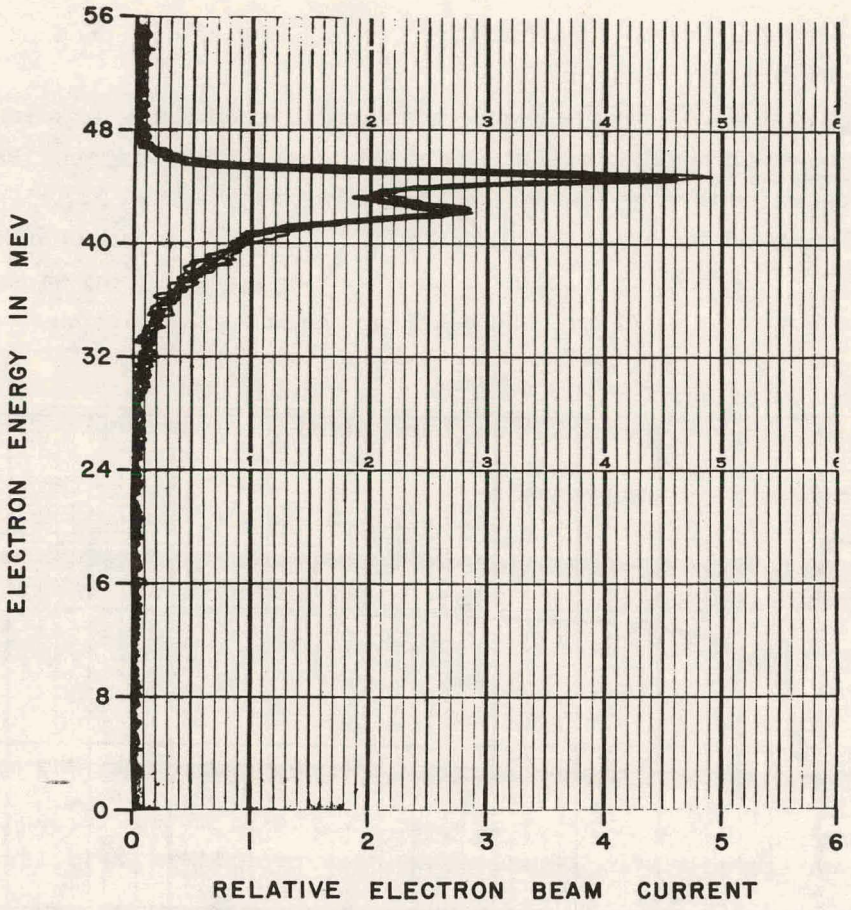


Figure 4.

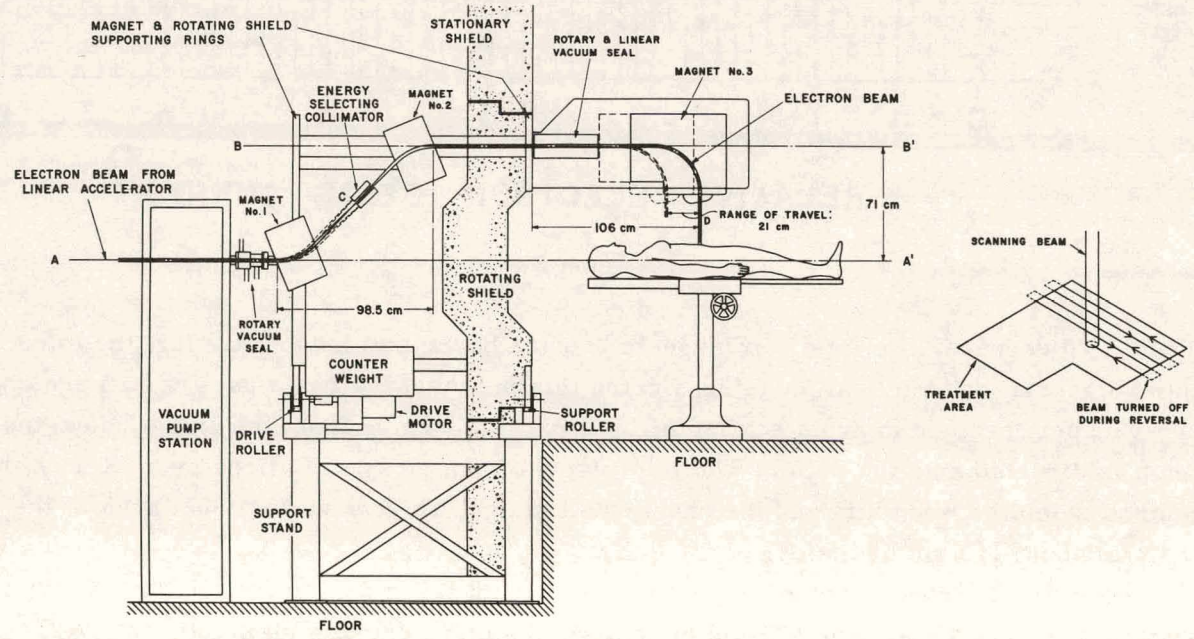


Figure 5.

to move so as to scan automatically over that area. This can be done in one of two basic ways; either by "arc therapy" or by "field therapy."

In "arc therapy" all three magnets rotate about the initial beam direction and in addition, magnet #3 moves linearly along the offset beam direction. Arbitrary arcs up to 360° and linear dimensions up to 20 cm are provided for. The motions of magnet #3 are shown schematically in Figure 7.

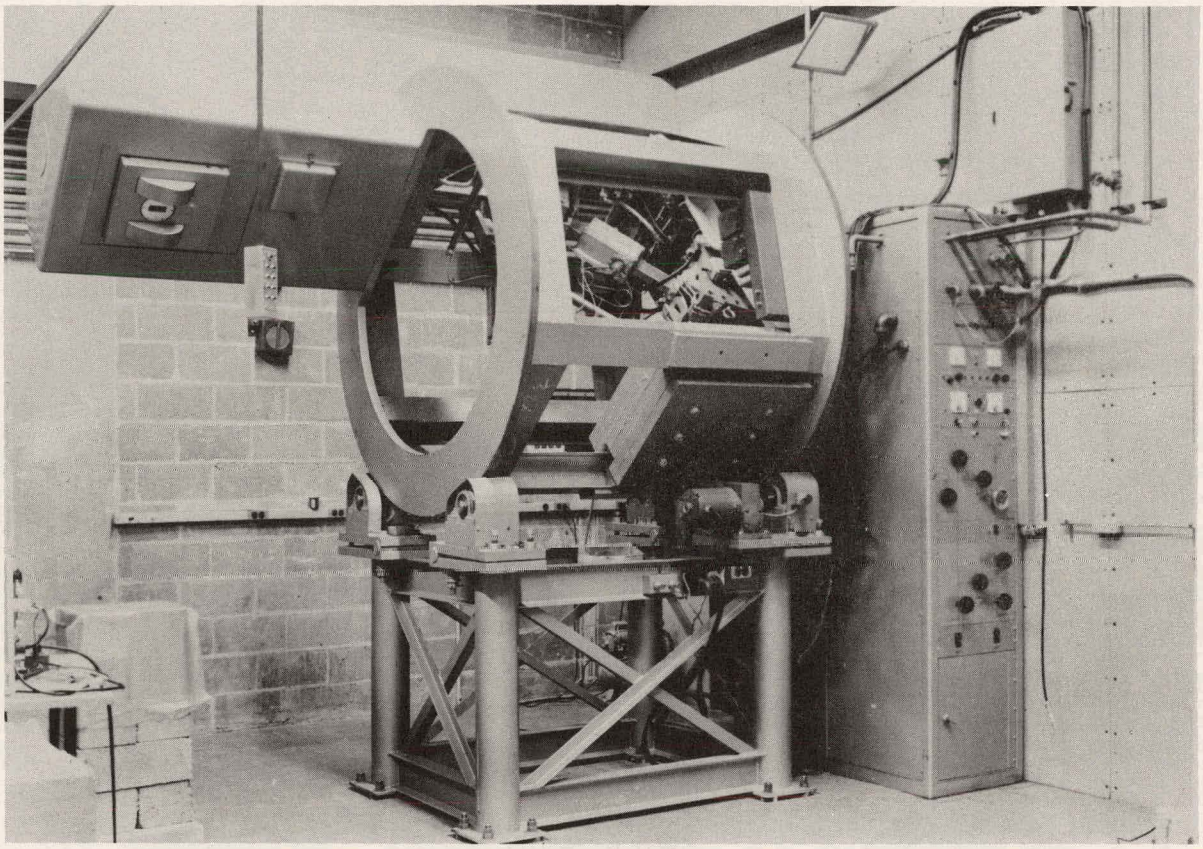


Figure 6.

The film exposures reproduced in Figure 8 were made with this set-up and show blackening from 5-, 10-, and 15-Mev electrons. If the surface of the phantom is placed near the center of rotation of the magnet system, depth doses of two and three times the surface dose are obtainable. On the other hand, if a uniform dose is required under these conditions, double or multiple passes can be made using different energies and intensities.

In "field therapy" a linear motion of magnet #3 is combined with a rotational motion of magnet #3 alone. The axis of rotation is not along the initial beam direction but along that of the offset beam. Figure 9 illustrates the setup for the film exposures shown in Figure 10.

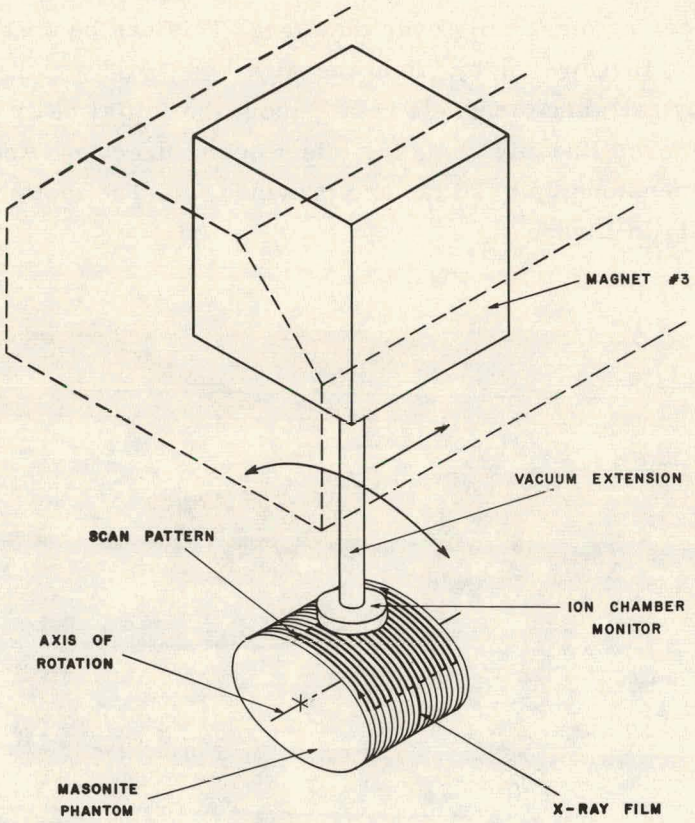


Figure 7.

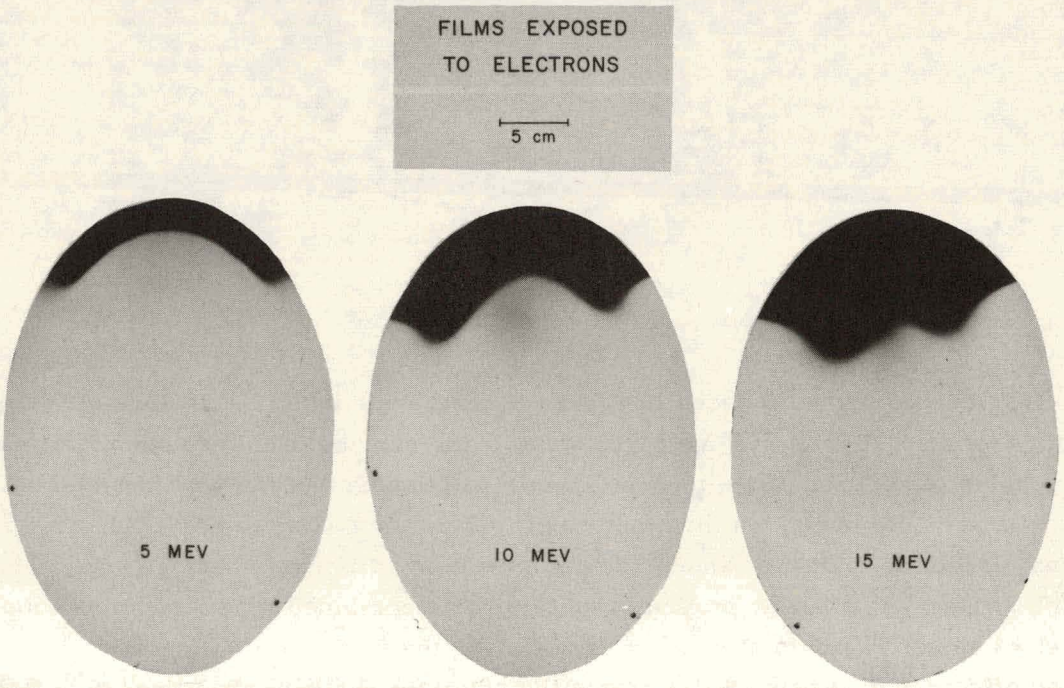


Figure 8.

These patterns were obtained by exposing films at 30 Mev with square field patterns of from 4 x 4 cm to 16 x 16 cm. Sizes up to 20 x 20 cm are available. The form of the pattern is not restricted to the conventional squares, rectangles, and circles, but almost any arbitrary shape can be covered uniformly. Field sizes are determined by a template made of either masonite or lucite and provided with an opening equal to that of the desired field.

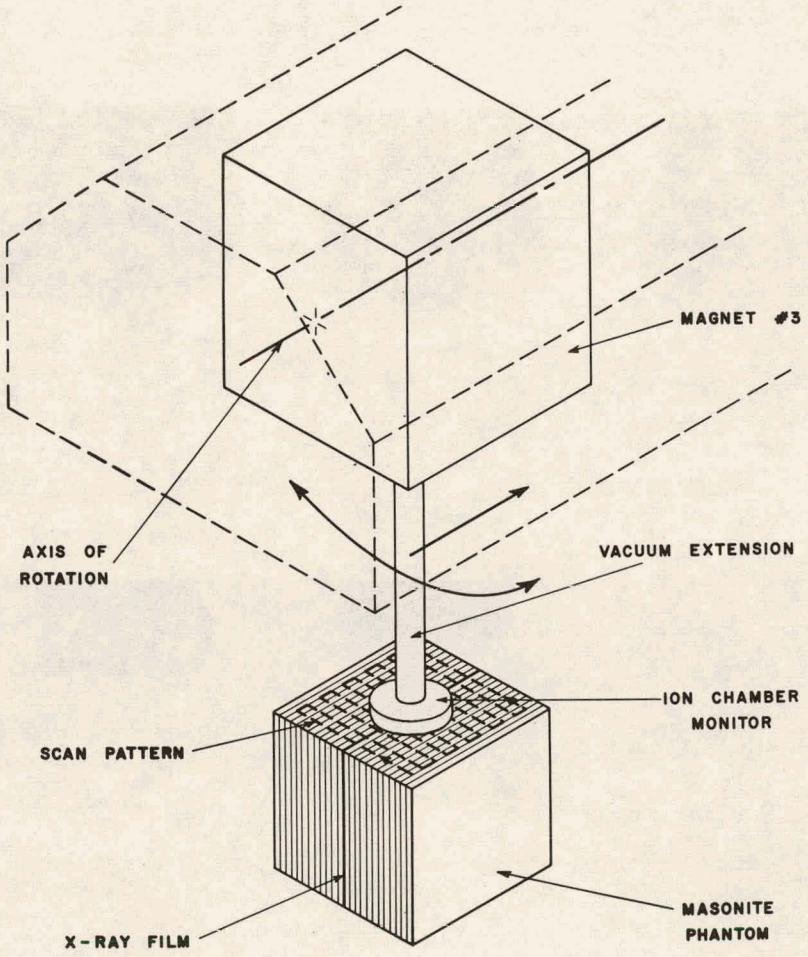


Figure 9.

After a template is installed and the unit turned on, the magnet system automatically scans the whole area within the template aperture. A probe moves within this aperture, exactly as the beam moves within the radiation field. Upon touching an edge of the aperture, the probe sends a guiding signal to the magnet motors for the proper change in motion. It also controls the turning on and off of the beam during the scanning cycle. When the area has been completely scanned once, the unit automatically turns off.

Figure 11 demonstrates the patterns obtained by exposing films to 10-, 15-, 20-, 25-,

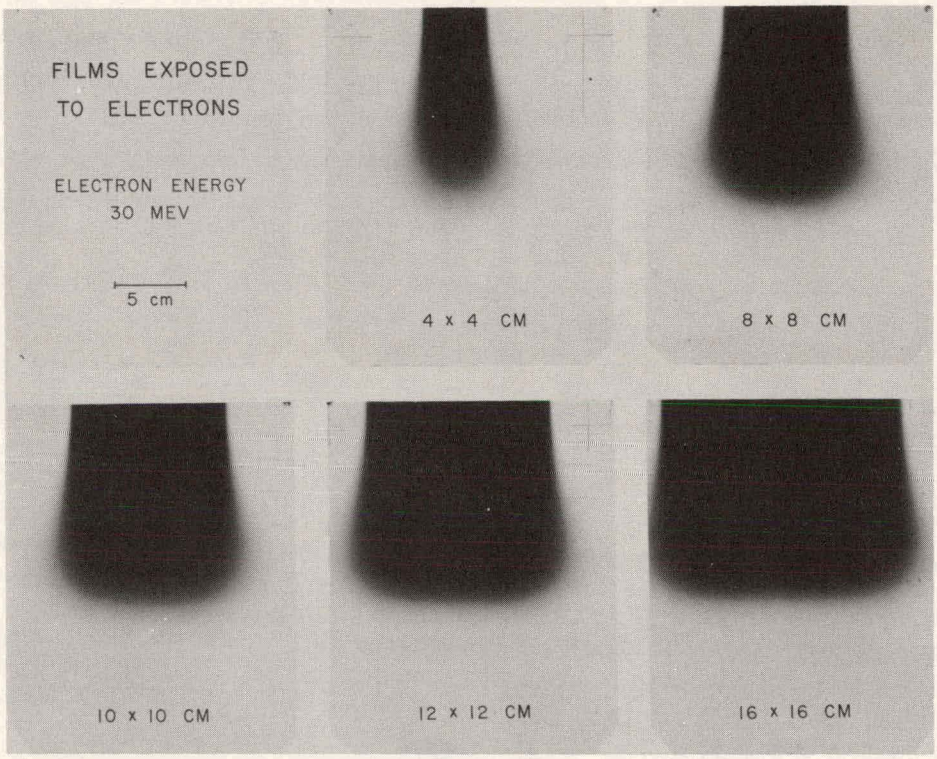


Figure 10.

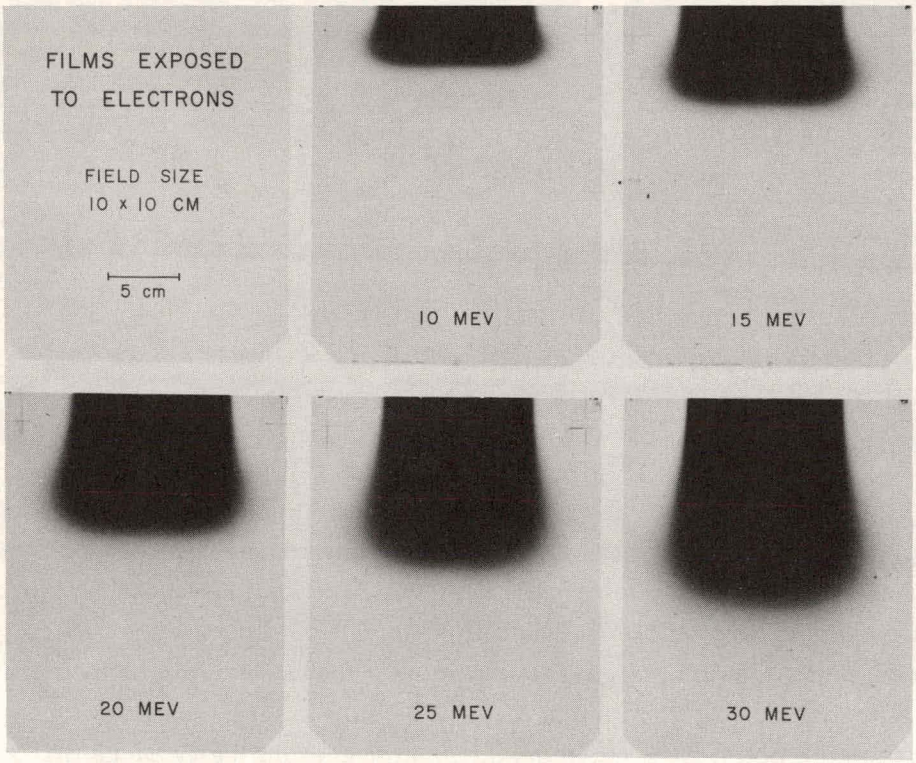
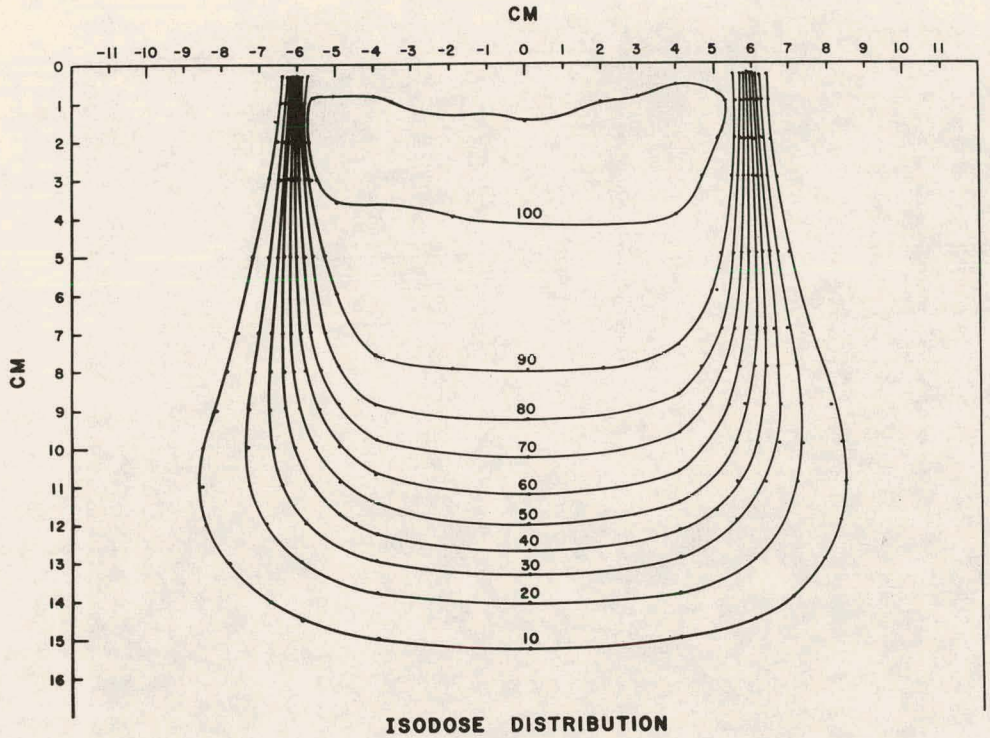
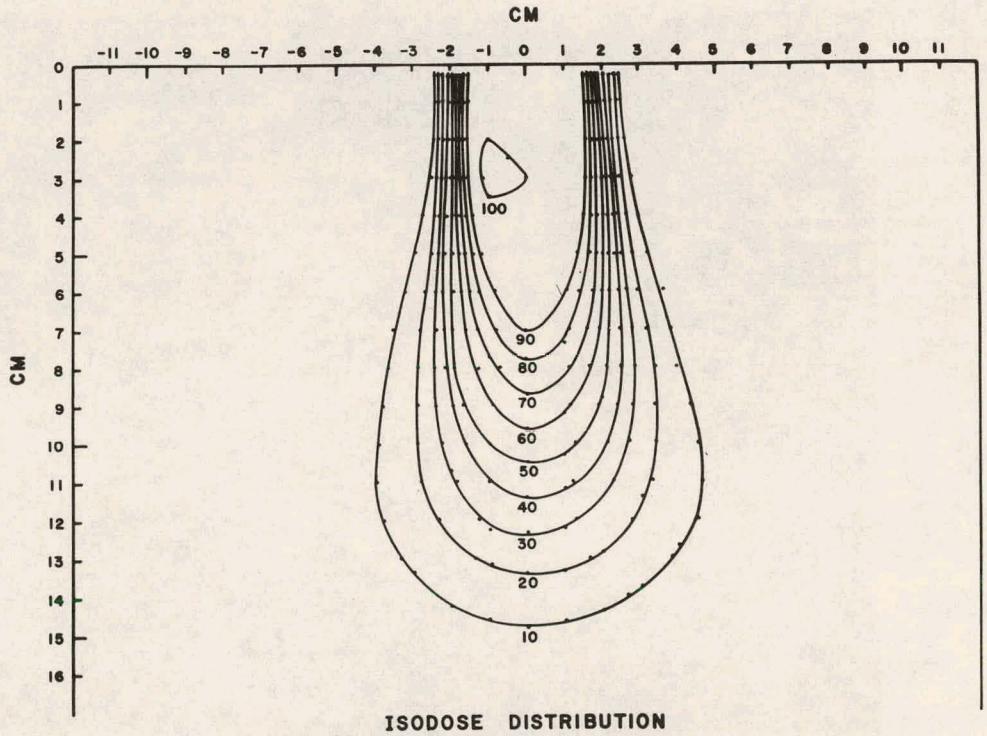


Figure 11.



ISODOSE DISTRIBUTION
30 MEV ELECTRONS
FIELD SIZE 12 x 4 CM

Figure 12



ISODOSE DISTRIBUTION
30 MEV ELECTRONS
FIELD SIZE 4 x 4 CM

Figure 13

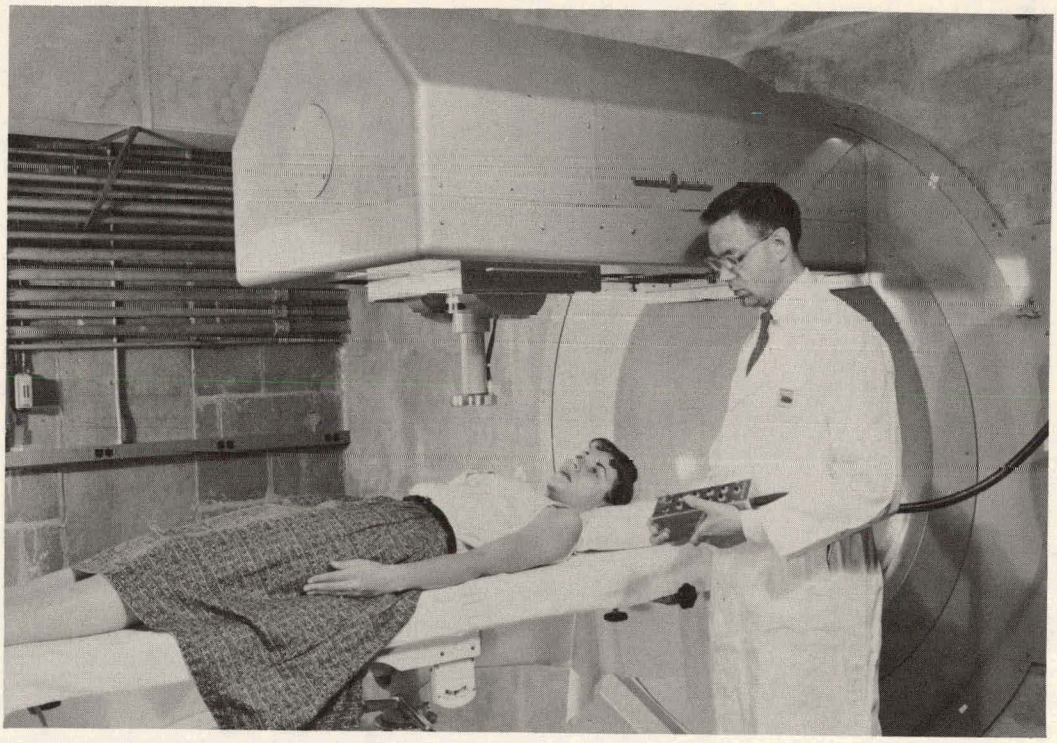


Figure 14.

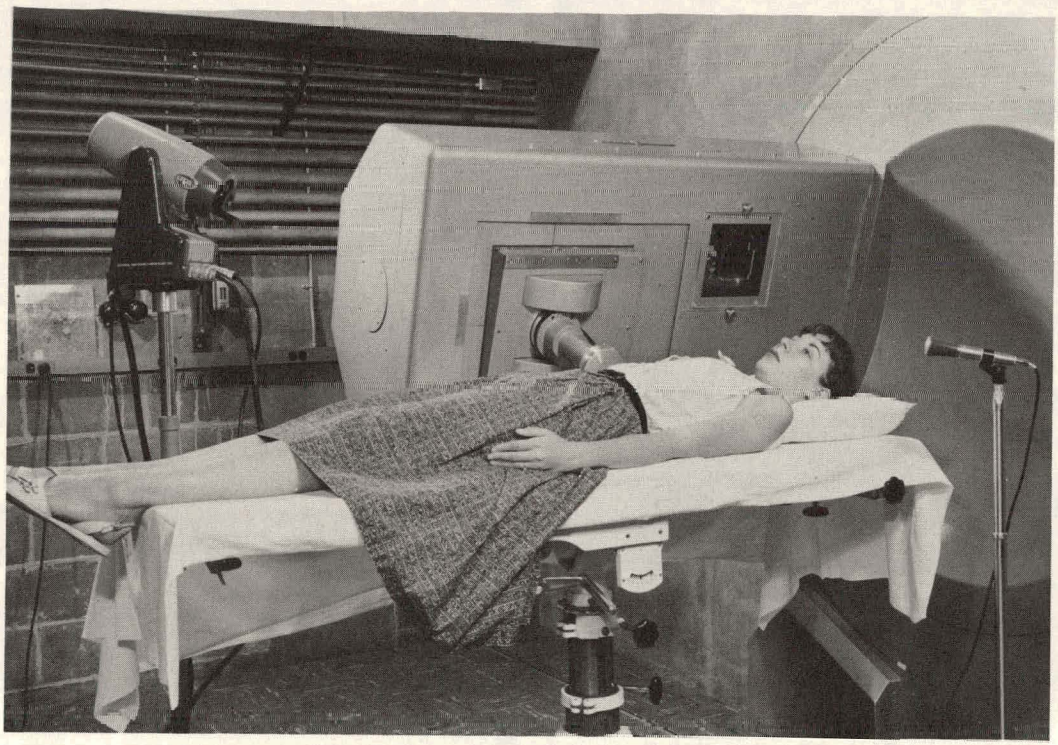


Figure 15.

and 30-Mev electrons, with a square field of 10 x 10 cm. The sharp cutoff of the beam in the depth is to be noted. The scale is shown in the upper left-hand corner of the film.

The isodose distributions as measured on film for rectangular fields of 12 x 4 cm and 4 x 4 cm are shown in Figures 12 and 13. The small penumbra near the surface is to be noted.

The final magnet enclosed in trim is illustrated in Figure 14. A pendant containing the magnet movement controls makes patient line-up quite simple.

Figure 15 is another photograph of the enclosed third magnet. The template in use here has an irregular shape. This means of field size control avoids the use of heavy collimators and facilitates rapid patient line-up. Also shown in the photograph is the beam monitor, which is a transmission ion chamber.

In conclusion, the depth dose patterns indicate that a uniform dose over a large portion of the field, with little radiation outside, is obtainable by the use of this apparatus. Thus, lesions which start near the body surface and extend to any depth below it can be treated with a minimum of radiation to underlying and adjacent tissues. Depth dose distributions with a peaking of the dose below the skin are obtainable with arc therapy techniques and here again, underlying tissues are spared. Also, cross-fire techniques used through the trunk of the body result in an excellent dose distribution.

The machine is currently in use on patients. Setup time is no longer than with standard x-ray equipment.

EFFECT OF CONTINUOUS GAMMA IRRADIATION OF MICE ON THEIR LEUKOCYTE COUNTS AND SUSCEPTIBILITY TO BACTERIAL INFECTION*†

By

Carolyn W. Hammond, Sonia K. Anderle, and C. Phillip Miller‡

INTRODUCTION

A single acute, whole-body exposure to X- or γ-radiation of midlethal intensity increases susceptibility to bacterial infection, whether the infection be "spontaneous"—i.e., endogenous in origin—or the result of experimental inoculation. This effect of acute irradiation has been adequately demonstrated in mice and other small laboratory animals by a number of investigators.[1] Smith and her co-workers[2,3] have emphasized the correlation between increased susceptibility and the leukopenia which follows total-body X-irradiation.

By way of comparison with the effect of such single acute exposures, the present study was undertaken in 1955 to determine the effect of prolonged, continuous γ-irradiation on the susceptibility of mice to bacterial infection. Mice were exposed to three levels of γ-radiation from a cobalt-60 source for various periods and challenged by intraperitoneal injection with graded inocula of Pseudomonas aeruginosa. Susceptibility to infection was determined by comparing the resulting mortalities with those of unirradiated controls similarly inoculated.

Leukocyte counts (total and differential) and hematocrits were followed during the exposure period and in two of the three series for 9 to 10 weeks thereafter, i.e., during recovery, on unchallenged mice set aside for this purpose.

MATERIALS AND METHODS

Mice. In all experiments the mice, CF-1 females, were 10 weeks old when irradiation was begun. They were housed in groups of 10 in Lucite cages measuring 7-1/2 x 12 x 7 inches, covered with perforated aluminum tops with aluminum feed mangers in which Rockland mouse pellets were available at all times. The floors were covered with wood shavings or sawdust. Tap water was supplied in sterilized bottles which were changed every day. The cages were changed and disinfected once a week.

*This research was supported jointly by the U. S. Atomic Energy Commission Contract AT(11-1)-46, research grant E-1259 from the National Institute of Allergy and Infectious Diseases, Public Health Service, the USAF under Contract AF18(600)-945 monitored by the USAF School of Aviation Medicine, Randolph Air Force Base, Texas, and the A. B. Kuppenheimer Fund, University of Chicago.

†Reprinted from Radiation Research, 11:242 (1959).

‡Department of Medicine, University of Chicago.

Irradiation. The mice were exposed continuously, except as noted, to γ-radiation from a nominal 10-curie cobalt-60 source, at the three dosage levels* shown in Table 1, which lists the range of each and its average. The variation was due to decay of the cobalt source. The average is used to designate dosage in the tables and graphs which follow. All these doses are admittedly approximate; they are based on the assumption that distribution of the mice was random within each cage. Furthermore, they disregard the shielding effect of the mice on each other.

Table 1

RADIATION DOSAGE

Daily exposure (hr/day)	Range (r/day)	Average (r/day)
22	34-34	34
24	72-67	69
22	130-127	128

The dose measurements were made with a Victoreen ionization chamber (calibrated by the National Bureau of Standards) placed in the center of one of the Lucite mouse cages with its aluminum top in place. The ionization chamber was covered with a close-fitting 4-mm Lucite cap of sufficient thickness to provide electronic equilibrium for cobalt-60 γ-rays. The accuracy of these measurements is estimated to be ±3%.†

The mice were exposed in an air-conditioned room located in the subbasement of the Argonne Cancer Research Hospital, USAEC, at the University of Chicago. The cages were placed on a curved wooden rack so that their centers were equidistant from the source. For the 34- and 128-r series, the source was shielded for 2 hours each day, reducing the exposure time to 22 hours per day. This 2-hour interval afforded time enough for the routine care of the mice and for drawing blood for leukocyte counts. In the 69-r series, exposed 24 hours a day, all interruptions in exposure were recorded and deducted from the total elapsed time in computing the accumulated radiation dose.

In order that all the mice in a given experiment could be challenged on the same day with the same culture of the test microorganism, they were placed in the cobalt room at different times and were all removed on the day of challenge. All the mice in any experiment, including the unirradiated controls, came from the same shipment. Before transfer to the cobalt room, they were kept in a room reserved for normal mice. The temperature

*These doses differ from those given in our preliminary report because the initial measurements were made under conditions of less scatter than these.

†For these measurements we are indebted to Drs. Lester S. Skaggs, Professor of Medical Physics, and Lawrence H. Lanzl, Assistant Professor of Medical Physics, University of Chicago.

of this room also was automatically controlled throughout the year.

Challenge inoculations. The test microorganism was a streptomycin-resistant strain of Pseudomonas aeruginosa which has been used in this laboratory for several years to challenge mice subjected to a single acute exposure to X-radiation. Virulence has been maintained by weekly passage through mice. Its streptomycin resistance served to differentiate it from any other strain which might have been encountered.

An 18-hour agar culture was washed off an agar plate and suspended in 5 ml of saline and shaken for 15 minutes on a rotator in a flask containing glass beads to disperse any clumps of bacteria. The suspension was diluted to a standard density (10^9 per milliliter) by means of a Coleman colorimeter (filter 430 mm μ). A series of tenfold dilutions was made, and the bacterial content checked by plating in quadruplicate 0.1-ml samples from the sixth and seventh tubes.

The challenge inoculations were made by injecting 8 to 20 mice intraperitoneally with 0.5 ml of each of enough dilutions to be sure that the lowest would kill all the mice and the highest none.

Before challenge the mice were removed from their cages and redistributed systematically in order to separate cage mates.

Autopsy cultures. A heart's blood culture was made on every mouse which died, unless it had been too badly eaten by its cage mates. With very rare exceptions, these blood cultures were positive for the test microorganism.

Leukocyte counts and hematocrit determinations. In every experiment, white blood counts were made on representative samples of mice.* In the 69-r and 128-r series groups of mice were set aside for total and differential counts and hematocrit determinations during their irradiation and for 9 to 10 weeks after they were removed from the cobalt room. These mice were identified by markings in order to space the bleedings at long intervals. No individual was bled more than three times during the course of an experiment. Blood was obtained by nicking a tail vein with a razor blade. A total of 3984 blood counts was made.

Hematocrit determinations to measure the volume of packed red cells were made by drawing blood into a heparinized capillary tube, which was then sealed at one end and centrifuged in a microhematocrit centrifuge.

Deaths among unchallenged mice. The cumulative mortalities among the mice set aside for blood counts are shown in Table 2. In both series very few mice died until the last week of their irradiation, but they continued to die after removal from the cobalt room. In fact, among the 128-r mice there was a rather marked increase in mortality during the first week postirradiation. This was not surprising, as their leukocyte counts, which had already fallen to a very low level, continued to decline during this period. (See Figure 2.)

Among the mice which had white counts made during this critical time, comparison

*These were made by Gilbert Claudio and Frances S. Vandervoort.

was made of an individual's leukopenia and its death or survival. All that can be said is that mice usually died if their counts had fallen below about 600. Some mice with still lower counts survived, however, and others died although their counts had never dropped below 1000 or even 1200.

It should be borne in mind that these low counts are not very accurate. The scarcer the leukocytes, the more difficult was their precise enumeration by the standard methods employed.

Table 2

CUMULATIVE MORTALITIES AMONG UNCHALLENGED MICE DURING AND AFTER EXPOSURE[a]

	Per cent mortality after exposure to:	
	69 r/day	128 r/day
Weeks of exposure		
1	0	0
2	0.7	0.7
3	1.0	6.8
4	1.7	
5-8	5.1	
Weeks after exposure		
1-2	6.0	20.9
3-4	8.4	21.6
5-6	9.3	21.9
7-8	11.7	22.6
9-10	13.1	24.5

[a]No deaths occurred among mice exposed to 34 r/day.

RESULTS OF CHALLENGE INOCULATIONS

The results of all the challenge inoculations in all the runs are summarized in Table 3, which also includes the geometric means of the leukocyte counts of each group at the time of challenge. The numbers of Pseudomonas in the challenge inocula have been expressed in logarithms (base 10) and have been rounded to the nearest unit. The maximum variation was 0.2 log unit.

Controls. Among the unirradiated controls the results from Pseudomonas inoculation were reproduced consistently—i.e., the mortalities from inoculation with 10^9, 10^8, and 10^7, were, respectively, 100%, 61 to 70%, and none. This strain of Pseudomonas has given similar results in unirradiated CF-1 mice for a number of years.

The 34-r series. Comparison of the mortalities of the controls with those of the mice receiving 34 r/day shows that 9 weeks' exposure at this dose rate (total accumulation 2142 r) caused no significant increase in susceptibility to the experimental infection.

The 69-r series. The mice irradiated with 72 to 67 r/day showed no increase in mor-

Table 3

SUMMARY OF CHALLENGE INOCULATIONS WITH PSEUDOMONAS AERUGINOSA

Radiation			Per cent mortality from inoculation with approximately:[a]									Total mice	Number of runs	Leukocyte counts[b]
Dose rate (r/day)	Duration (weeks)	Total accumulated (r)	10^9	10^8	10^7	10^6	10^5	10^4	10^3	10^2	10			
34	Controls	0	100	61	0							36	2	10,000
	3	715	96	57	0	0						107	2	4,400
	6	1430	100	44	0	0						108	2	3,100
	9	2140	100	64	3	0						124	2	2,600
69	Controls	0	100	70	0							132	5	10,000
	2	980	100	80	0	0						40	1	2,200
	3	1470	100	90	20	0						40	1	1,600
	5	2470		100	80	0	0					90	2	1,300
	6	2925		100	65	12	3	0				179	4	1,100
	7	3410		100	57	30	5	10	0	0		226	4	950
	8	3845		100	68	30	25	25	15	10	0	148	2	850
128	Controls	0	100	66	0							150	3	10,000
	1	900	100	70	2	0						140	3	3,500
	2	1800		100	30	17	4	4				136	3	1,400
	3	2695		100	73	51	43	35	33	8	0	248	3	580

[a] Inocula in nearest logs.

[b] Geometric mean of each group at time of challenge.

tality until the third week of irradiation, by which time they had accumulated 1470 r and their average white counts were 1600.

No 4-week mice were challenged.

Among the mice irradiated from 5 to 8 weeks increased susceptibility to infection was not manifested by increased mortality from challenge with 10^7 Pseudomonas, but rather by an increasing number of deaths from smaller and smaller inocula.

Deaths from these smaller inocula tended to occur later than those from the large inocula. To illustrate this point, the results of a typical experiment are presented in detail in Table 4.

Table 4

RESULTS OF AN ILLUSTRATIVE CHALLENGE INOCULATION, SHOWING TIME OF DEATH

(69-r series)

Irradiation		Inocula[a]	Deaths (among 10 mice) by days after inoculation:						
Weeks	Total dose (r)		1	2	3	4	5	6	9
-	-	10^9	10						
		10^8	4						
		10^7	0						
6	2925	10^9	10						
		10^8	10						
		10^7	2		1	1			1
7	3410	10^9	10						
		10^8	10						
		10^7	4	4					
		10^6	1	2		3			
8	3845	10^8	10						
		10^7	6	1					
		10^6	1			1			
		10^5		1		1		1	
		10^4		2		2			

[a]Approximate numbers of Ps. aeruginosa.

The 128-r series. In this series, the mortalities from the lower inocula were more numerous, particularly at 3 weeks. But even then, when the mice had accumulated 2700 r and their average white counts were reduced to 580, the mortality from inoculation with 10^7 Pseudomonas averaged only 73% in three challenges.

HEMATOLOGICAL OBSERVATIONS DURING AND AFTER IRRADIATION

Leukocyte counts. During irradiation the leukocyte counts decreased at different rates, depending on the daily dose of radiation (Figure 1). In the 34-r and 69-r series there was a marked reduction in the rate of decline after the third week of irradiation. Miller and Sacher have made a similar observation in studies on mice subjected to chronic γ-radiation ([4], personal communication). The leukocyte counts during the post-irradiation period were followed in only two series (Figure 2). Each curve is a composite of the groups indicated, since their separate curves were nearly identical.

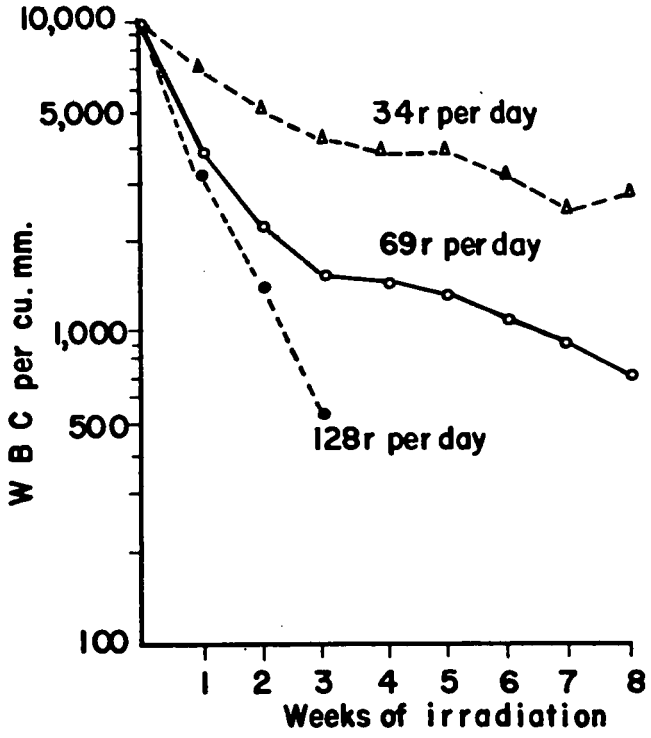


Figure 1. Leukocyte counts during exposure.

In the 128-r series the counts continued to fall for a week after irradiation had been discontinued. It was during this week that a considerable number of deaths occurred among these mice. The counts in this series, starting from a lower minimum, increased more rapidly postirradiation and reached a higher level than those in the 69-r series.

The steeper rise in the 128-r series may possibly reflect a more vigorous response to the greater damage suffered by the hematopoietic system. It seems more likely, however, that it is due to the elimination by death of the more radiosensitive individuals. By the end of the 9- to 10-week period of observation the counts in both series were still below the preirradiation level.

Incidence of leukemia. During the second month after irradiation very high leukocyte counts (above 100,000) were found to have developed in 2 mice in the 69-r series and in 3

mice in the 128-r series—roughly 1% and 1.5%, respectively, of the mice under observation at that time. The diagnosis of leukemia was based on the white counts and very marked splenomegaly, but was not confirmed by histopathological examination. The counts on these mice were deleted from the data.

Differentials and hematocrit determinations. Table 5 lists the percentages of polymorphonuclear heterophiles and lymphocytes. There was no marked change during or after irradiation. The other types of leukocytes have been omitted from the table. They were never numerous and became very scarce during periods of leukopenia.

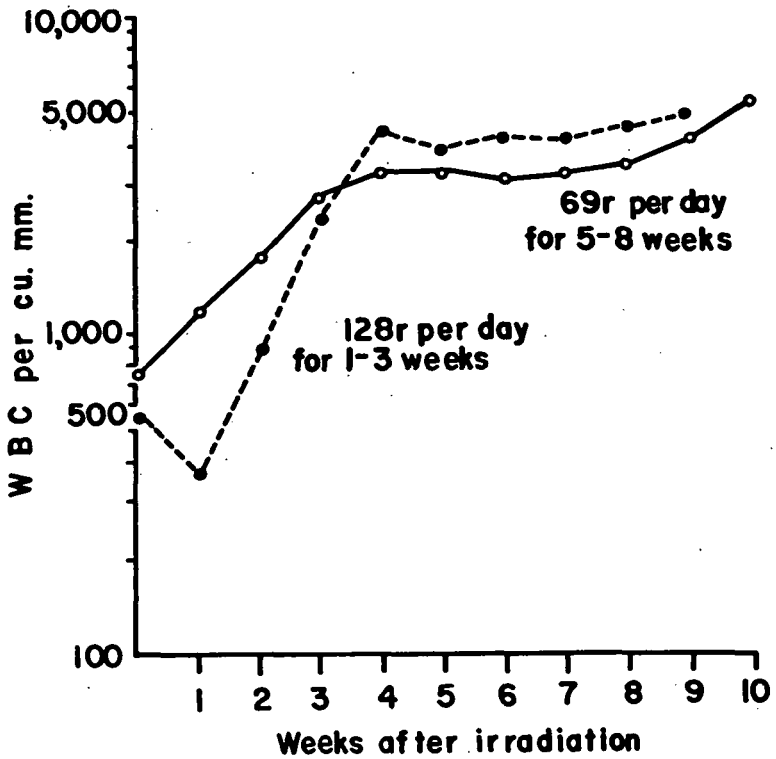


Figure 2. Leukocyte counts after exposure.

The hematocrit determinations showed that the volume of packed red cells fell more rapidly and to a lower level in the 128-r series than in the 69-r series and rose more rapidly during the postirradiation period. In neither series did it quite reach the normal level by the end of the observation period.

DISCUSSION

Several investigators have determined the LD_{50} and survival time of small animals exposed more or less continuously to γ-radiation at different dose rates. Thomson and Tourtellotte[5] found that the LD_{50} of mice exposed to 144 r/day was 1658 r accumulated over 11.5 days and was 2760 r in 24 days at an exposure rate of 115 r/day. Sacher[6] also

Table 5

DIFFERENTIAL LEUKOCYTE COUNTS (HETEROPHILE AND LYMPHOCYTE) AND RED CELL VOLUMES DURING AND AFTER EXPOSURE

Weeks	Differential counts (%)				Red cell volume (%)	
	69 r/day		128 r/day		69 r/day	128 r/day
	Het.	Lymph.	Het.	Lymph.		
(Normal)	25	70	25	70	48	48
			During exposure			
1	33	62	32	65	45	47
2	28	65	39	52	44	42
3	28	67	30	67	44	31
4	29	66			43	
5	33	63			44	
6	28	70			40	
7	24	68			40	
8	26	68			40	
			After exposure			
1	23	71	18	79	43	30
2	24	72	22	75	46	35
3	32	63	45	51	44	46
4	32	60	39	55	44	49
5	32	61	28	70	40	47
6	34	62	32	61	41	47
7	25	69	24	68	46	47
8	26	67	28	66	42	45
9	26	69	24	71	43	45
10	24	70			42	

found that the average accumulated dose to death of CF-1 female mice increased as the daily dosage was decreased from 200 to 40 r/day.

The data here presented also show that it was the rate of γ-irradiation rather than the total amount accumulated which determined the degree or severity of injury as reflected by susceptibility to a bacterial infection, development of leukopenia and anemia, and occurrence of spontaneous deaths during and immediately after exposure.

The results of the challenge inoculations with Pseudomonas aeruginosa showed, for instance, that 2695 r accumulated over a 3-week period increased susceptibility much more than did 2925 r spread over twice this length of time. And 2140 r accumulated over 9 weeks caused practically no change in susceptibility. Increased susceptibility, when it occurred, did not manifest itself as a progressive decrease in the numbers of Pseudomonas required to kill 100%, but rather as an increase in mortalities among the mice receiving smaller and smaller inocula. This distribution of lethality among the challenged mice is presumed to result from differences among individual mice in their natural ability to withstand radiation injury.

The results of these challenge inoculations were compared with those made with the same strain of Pseudomonas aeruginosa on the same kind of mice (10-week-old CF-1 females) during the second week after a single exposure to 500 r of X-radiation, a dose very seldom lethal for unchallenged mice. This comparison emphasizes the importance of dose rate, because 3410 r (69-r series) accumulated over a period of 7 weeks increased susceptibility to this bacterial infection less than did 500 r given as a single acute exposure. At the time of challenge the average white count was 950 in the chronically irradiated and 750 in the acutely irradiated mice.

Stoner and Hale have reported some interesting studies in which continuous low-dose and acute γ-radiation were compared for their effectiveness in: (1) depressing the secondary antitoxin response in mice previously immunized to tetanus toxoid[7] and (2) increasing susceptibility of mice to anaphylactic shock.[8] In general, continuous radiation (at the rate of 4 rep per hour) was much less effective than an equivalent amount given in a single acute exposure. Nevertheless, their experiments on antitoxin production showed that at levels below 450 rep antitoxin production was depressed more by continuous than by acute radiation. The secondary stimulus (tetanus toxoid) was injected 1 to 2 hours after irradiation was completed, however—i.e., at different intervals after irradiation was begun. This time factor, in the case of the continuously irradiated mice, is a critical variable which, as the authors point out, may account for their surprising results.

SUMMARY

CF-1 female mice 10 weeks old were exposed continuously to γ-radiation (from a cobalt-60 source) at three dose levels: approximately 34, 72 to 67, and 130 to 127 r/day. Leukocyte counts (total and differential) and red cell volumes were followed, and at intervals susceptibility to bacterial infection was determined by intraperitoneal challenge with graded inocula of Pseudomonas aeruginosa.

Increased susceptibility to this infection was related to the rate of irradiation rather than the total amount accumulated. At the lowest dose rate (34 r/day) the accumulation of 2140 r during 9 weeks caused practically no increase in susceptibility to this experimental infection.

At the next higher dose rate (72 to 67 r/day) susceptibility began to increase after the third week. It was manifested principally by deaths among mice receiving smaller and smaller inocula of the test microorganism.

At the highest dose rate used (130 to 127 r/day) susceptibility increased more rapidly.

The development of leukopenia and anemia was also related to the rate of irradiation rather than the total amount accumulated.

Note Added in Proof

Nelson and Becker[9] have recently reported observations on the bactericidal activity of peritoneal exudate cells of some mice in the 128-r series. They found that this property had declined moderately after two weeks of exposure (1800 r) and markedly after the

third week (2695 r) to a level roughly corresponding to that resulting from a single acute exposure to 500 r.

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INHIBITION OF THE SODIUM-RETAINING INFLUENCE OF ALDOSTERONE BY PROGESTERONE*†‡

By

Richard L. Landau and Kathleen Lugibihl

Progesterone is a natriuretic and chloruretic agent in man.[1-3] In subjects with intact adrenocortical function a course of 50 mg per day intramuscularly induced a prompt rise in urinary sodium excretion which was sustained for from two to six days and produced a net loss of sodium of about 70 to 150 milliequivalents. In each of these subjects urinary sodium excretion dropped below control levels immediately after progesterone was discontinued, and in the ensuing days the amount of sodium and chloride which had been dissipated during the first days of progesterone administration was regained. This natriuretic influence was detectable when the dosage of progesterone was as little as 12 to 15 mg per day. When the dosage was increased to 150-300 mg daily, the loss of sodium was as much as 396 milliequivalents in seven days.

These effects of progesterone are dependent upon the presence of functioning adrenal cortices. When progesterone was given to adrenal-deficient patients who were receiving constant replacement amounts of cortisone acetate and desoxycorticosterone acetate, the rise in urinary sodium excretion was greater and the duration of the effect was longer than when the same dosage was administered to other subjects. Despite the substantially greater loss of sodium incurred by subjects with treated Addison's disease, there was never any retention of salt after progesterone was discontinued. In 1 case of Addison's disease, progesterone failed to affect sodium excretion when it was given while the patient was treated only with added salt. Very shortly after treatment with cortisone and desoxycorticosterone had been instituted in this patient, a repetition of progesterone therapy induced intense natriuresis and chloruresis. From these results it was concluded that the sodium-dissipating influence of progesterone was probably due to the competitive inhibition at a renal level of the salt-retaining influence of administered desoxycorticosterone and cortisone. It was similarly suggested that the natriuresis induced in normal subjects could be explained by inhibition of aldosterone, the adrenal steroid hormone with the greatest salt-retaining activity.[4]

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†Progesterone was generously supplied by Dr. Edward Henderson of the Schering Corporation, Bloomfield, New Jersey. Aldosterone was obtained from the Ciba Pharmaceutical Products, Inc., Summit, New Jersey, on the basis of a request made to the Endocrinology Study Section, National Institutes of Health (Dr. R. T. Hill).

‡Reprinted from J. Clin. Endocrinol. & Metab., 18:1237 (1958).

In the studies to be reported here we have endeavored to secure evidence to support the view that the salt-retaining action of aldosterone may be inhibited by progesterone.

PROTOCOLS

M. A. (Figure 1) was a 46-year-old woman with Addison's disease. She was healthy otherwise and there were no signs of tuberculous or other infection of the adrenal glands.

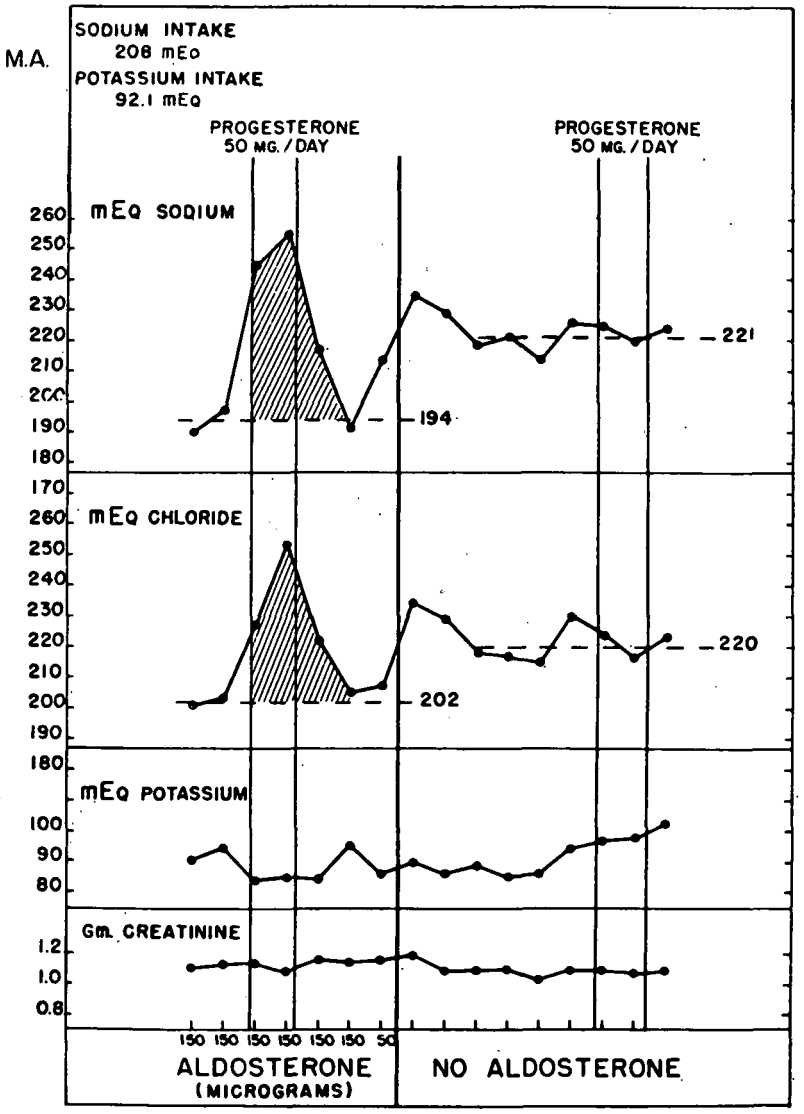

Figure 1. (M.A., a 46-year-old woman with Addison's disease.) The effects of progesterone on the urinary excretion of sodium, chloride, potassium and creatinine. Throughout these observations she received 50 mg of cortisone acetate per day intramuscularly. The heavy vertical line separates the period of aldosterone therapy from that without aldosterone. The indicated dosages of aldosterone represent the amounts of biologically active d-aldosterone. Broken horizontal lines indicate averages of control values. Oblique hatching indicates significant shifts.

During the study she received 25 mg of cortisone acetate intramuscularly twice a day. She also required desoxycorticosterone acetate for satisfactory management of the adrenal deficiency.

L. K. was a 32-year-old man with Addison's disease apparently due to idiopathic atrophy of the adrenal cortices. During the study he received 12.5 mg of cortisone acetate orally three times a day, except as noted on the experimental days. His total salt intake was 280 milliequivalents per day. Serum electrolyte concentrations did not vary significantly throughout the studies.

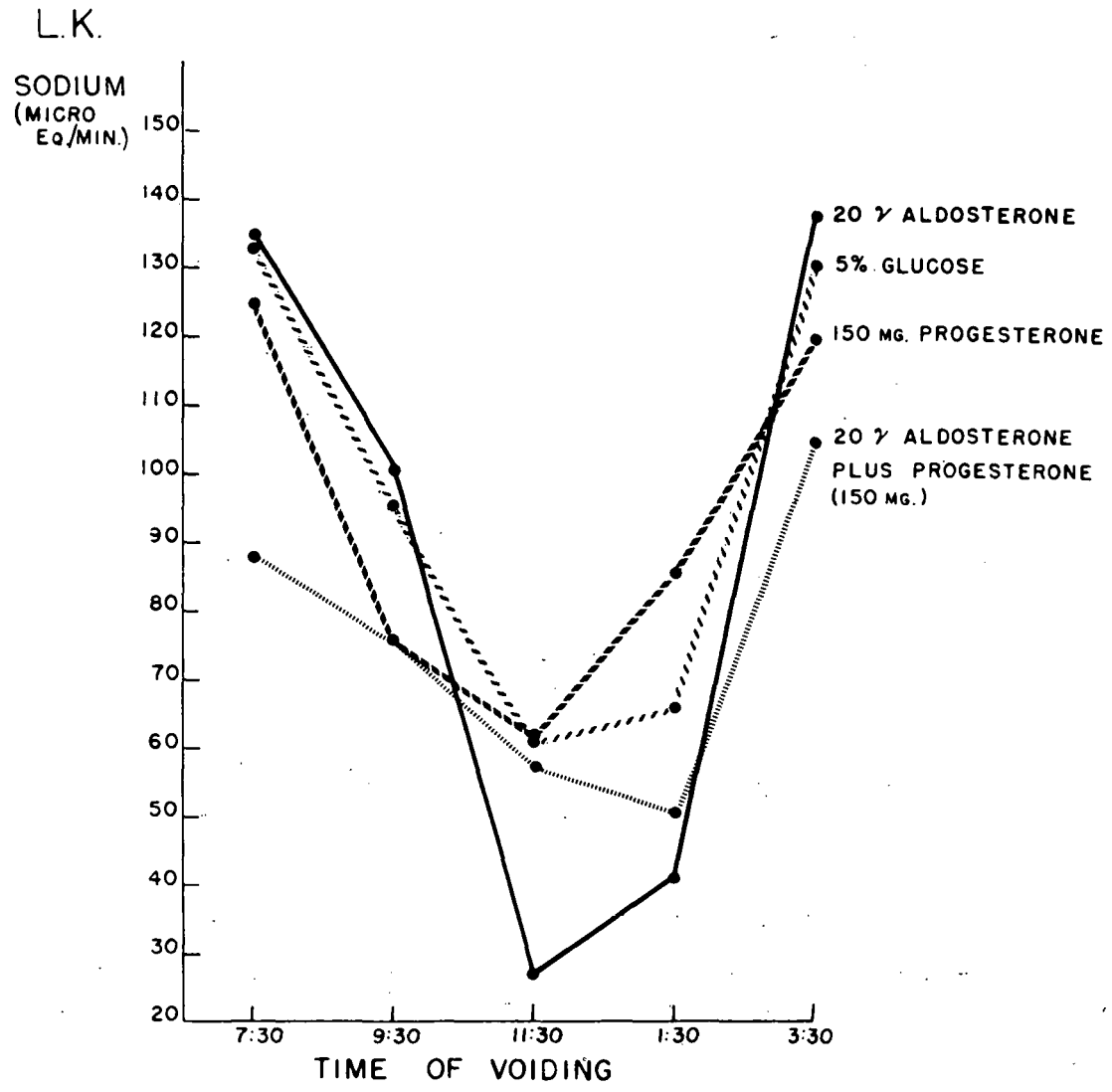

Figure 2. (L.K., a 32-year-old man with Addison's disease.) The rate of sodium excretion through four experimental days. The values plotted above the times of voiding actually represent the rate of excretion of sodium during the previous two-hour interval, except for the rate at 7:30, which represents the period beginning at 10:30 P.M. the night before. The 20-microgram dosage of aldosterone was the indicated quantity of the biologically active d-aldosterone.

D. E. was a 49-year-old woman with metastatic breast cancer who had been adrenalectomized nine months previously. She was in good physical condition. During the study she received 25 mg of cortisone acetate orally twice a day, except as noted on the experimental days. Her total salt intake was 117 milliequivalents per day. Serum electrolyte concentrations did not vary significantly throughout the studies.

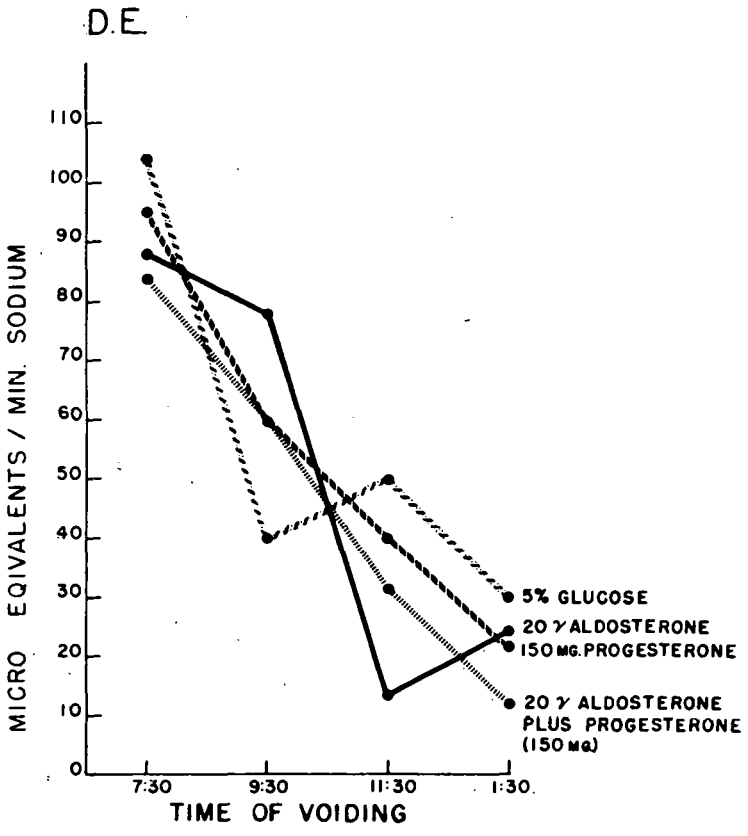

Figure 3. (D.E., a 49-year-old adrenalectomized woman with breast cancer.) The rate of sodium excretion through four experimental days. For key to charting, see legend of Figure 2.

EXPERIMENTAL DESIGN AND METHODS

In the first study (Figure 1) conventional balance techniques were employed. Patient M.A., a woman with Addison's disease (see protocols), was given a constant adequate diet; 24-hour urine collections were made and checked for completeness by means of creatinine estimations. After reasonable control of the adrenal deficiency had been secured by administration of 50 mg of cortisone acetate and 1 mg of desoxycorticosterone acetate per day, the desoxycorticosterone acetate was replaced by 100 micrograms of d,l-aldosterone-21-monoacetate in sesame oil given intramuscularly every eight hours (equivalent to approximately 150 micrograms of biologically active d-aldosterone[5] each day). Dosages recorded in Figure 1 and subsequently indicate the quantities of the biologically active isomer.

After two days of this program, 50 mg per day of progesterone was given intramuscularly for two days; three days thereafter, aldosterone was discontinued. Seven days later the two-day course of progesterone was repeated.

In the acute studies carried out on the cortisone-treated adrenal-deficient patients, L.K. (Figure 2) and D.E. (Figure 3), the effect of a small intravenous dose of aldosterone on electrolyte excretion was determined by administering the hormone under carefully controlled conditions, twelve to eighteen hours after the last dose of oral cortisone. Since the effect of aldosterone would be superimposed on a phase of the diurnal variation in sodium, potassium and chloride excretion prevailing under these conditions, its delineation would require comparison with observations on an identical day in which no aldosterone was given. The influence of progesterone on the effect of aldosterone was determined on a third day by pre-treating the subjects with intramuscular progesterone; the effect of progesterone alone was evaluated on a fourth experimental day which was identical with the third except that no aldosterone was given.

Both L.K. and D.E. were given constant maintenance diets and treated with oral cortisone acetate and added salt (see protocols). Twenty-four hour urine collections were made, and evidence of metabolic equilibration was secured before any studies were carried out. Each of the experimental days required breaking the constant regimen; the studies were always separated by two or three days of the constant control program. The day prior to the study, the 24-hour urine collection was broken at 10:30 P.M.; the 10:30 P.M. to 7:30 A.M. collection served as a pre-treatment control urine for the experimental day. At 7:40-7:45 A.M. on the day of the study, an intravenous infusion of 150 ml of 5 per cent glucose was started and carefully timed to require one hour for its delivery. The patients were supine during this infusion but thereafter were allowed to be ambulant. The morning doses of cortisone acetate and sodium chloride, as well as breakfast, were omitted. The patients drank 200 ml of water every hour from 8:00 A.M. through 1:30 P.M. At 1:30 lunch was served (the same for each experimental day). Precisely timed two-hour urine collections were made throughout the period of study. These, as well as the ones from 10:30 P.M. to 7:30 A.M., were analyzed for sodium, chloride and potassium. At 3:30 P.M. the experiment was terminated, and the cortisone acetate and salt which had been withheld were given. The aldosterone, dissolved in ethanol (200 micrograms per ml of d,l-aldosterone-21-monoacetate), was added to the 150 ml of 5 per cent glucose on the days on which it was to be given. Progesterone dissolved in sesame oil and 20 per cent benzyl-benzoate was given intramuscularly in divided dosage—100 mg at 5:30 A.M. and 50 mg at 7:30 A.M.

Urinary chloride and creatinine were determined as noted previously.[6] Sodium and potassium measurements were made using the Perkin-Elmer internally standardized flame photometer. Diluted urine was loaded with a gross excess of potassium when measuring sodium, and a similar excess of sodium when potassium was determined.

RESULTS

When progesterone was given to Patient M.A. (Figure 1) while her adrenal deficiency

was being treated with cortisone and aldosterone, the usual effect on sodium and chloride excretion was observed. Urinary sodium rose sharply from the baseline of 194 milliequivalents per day to a peak of 255 milliequivalents and then dropped to the control level on the second post-treatment day. The elevation noted on the third day after treatment was associated with the lowering of aldosterone dosage to a single injection of 50 micrograms. The sodium loss induced by progesterone totaled 135 milliequivalents. Chloride excretion followed a course which closely paralleled that of sodium but, as usual under these circumstances, the total loss was less (95 milliequivalents). Urinary potassium excretion was probably uninfluenced by progesterone.

Following the discontinuance of aldosterone, the sodium and chloride balances shifted from slightly positive to negative and a new baseline was assumed. Urinary sodium then averaged 220 milliequivalents per day with a daily intake of 208 milliequivalents. During this period the serum sodium concentration dropped from 134 to 123 milliequivalents per liter; and that of chloride, from 93 to 85 milliequivalents per liter. Urinary sodium and chloride did not deviate from their newly established baselines when the administration of progesterone was repeated. The excretion of potassium which had begun to increase slightly before progesterone was given was also uninfluenced by it.

This experiment confirms the previously reported study in which it was shown that the saluretic action of progesterone was observed only while the organism was under the influence of salt-retaining corticoids. Both cortisone and desoxycorticosterone were given in that instance,[1] but the latter undoubtedly provided most of the sodium-retaining activity. In Patient M.A. (Figure 1) the cortisone acetate had virtually no salt-retaining action, and it is clear that progesterone was only salt-dissipating when given while the patient was receiving the powerful sodium-retaining hormone, aldosterone.

The acute experiments in Patients L.K. and D.E. (Figures 2 and 3) were carried out because in M.A. (Figure 1) and in the previously reported study,[1] when no aldosterone or no desoxycorticosterone and cortisone were given to the subjects, the failure of progesterone to be a sodium-dissipating agent may not have been due primarily to the fact that there was no sodium-retaining corticoid present to be inhibited or antagonized. In both subjects there was a mild to moderate negative sodium balance. The serum concentrations of sodium and of chloride had declined, and the patients' general condition, though reasonably good, was deteriorating. These factors could also be offered as explanation for the failure of progesterone to act as a natriuretic. It was hoped that the single-day studies might provide added evidence that the sodium-retaining influence of aldosterone is indeed antagonized by progesterone. This would be demonstrated if the aldosterone effect were blocked by adding progesterone, and on a subsequent day progesterone alone were shown to have no direct influence on sodium excretion.

Under the conditions of these studies the pattern of sodium excretion during control days (5 per cent glucose infusion) was very similar in Patients L.K. (Figure 2) and D.E. (Figure 3). The rate of sodium excretion was relatively high during the overnight period (7:30 A.M. voiding), and then dropped progressively until 11:30 A.M. in L.K. and until 1:30

P.M. in D.E. The rate increased slightly from 11:30 to 1:30 in L.K., and then sharply during the next two hours. In D.E. there was an abrupt return to the overnight rate of sodium excretion from 1:30 to 3:30 (data not shown). The addition of 20 micrograms of aldosterone to the glucose infusion caused a very significant drop in the rate of sodium output during the 9:30 to 11:30 A.M. period. When aldosterone was given to Patient L.K. (Figure 2) the rate of sodium excretion was 27 microequivalents per minute as compared with 61 microequivalents per minute on the control day. In Patient D.E. (Figure 3) the drop at this point was from 49 microequivalents on the control day to only 13 microequivalents per minute. A similar suppression of the rate of sodium excretion by aldosterone was noted on 4 other experimental days in the same subjects and in 2 studies in a third adrenal-deficient patient. The suppression of urinary sodium excretion from two to four hours after the start of the one-hour intravenous infusion of aldosterone would thus appear to be the characteristic response.

When the subjects were pre-treated with intramuscular progesterone (with or without aldosterone) the excretory rate for sodium during the critical 9:30-11:30 A.M. interval compared closely with the rates observed on control days. In Patient L.K. these 3 rates were almost identical. In Patient D.E. the values were not grouped so closely, but the rates of excretion when progesterone was injected and when progesterone plus aldosterone was given were more than twice as great as that observed when aldosterone alone was administered. Larger amounts of aldosterone (the equivalent of 50 micrograms of d-aldosterone in L.K. and 40 micrograms in D.E.) lowered the sodium excretion to about the same level as did administration of 20 micrograms, but the same dosage of progesterone did not abolish or significantly lessen these aldosterone responses.

Potassium excretion was not systematically affected by any of the hormonal treatments. Urinary chloride tended to vary in parallel with the rate of sodium excretion but, not too surprisingly, since aldosterone primarily promotes sodium retention, the suppression of chloride from 9:30 to 11:30 A.M. was less than that of sodium, and its nullification by progesterone was not uniform.

These acute studies thus confirm the impression gained from the balance study in Patient M.A. (Figure 1) that the natriuretic influence of progesterone was due to the peripheral antagonism of aldosterone. When given prior to the infusion of aldosterone, progesterone prevented the anticipated suppression of urinary sodium. Since there was no evidence that progesterone had a primary natriuretic action, abolition of the aldosterone effect must have been due to a blocking of the influence of aldosterone, presumably in the kidney tubules. The failure of progesterone to be salt-dissipating in the absence of aldosterone in Patient M.A. can accordingly be explained on the basis of absence of sodium-retaining hormone which could be antagonized, rather than on the basis of unresponsiveness due to the systemic effects of loss of sodium and chloride from the body.

DISCUSSION

The capacity of progesterone to inhibit the sodium-retaining influence of aldosterone

and desoxycorticosterone is probably dependent upon some close structural similarities of these compounds. When a change as small as the introduction of an hydroxyl group (as in 17- α -hydroxyprogesterone) is made, the ability to inhibit salt-retaining corticoids is lost. Several synthetic progestational compounds which, from the chemical point of view, are less closely related to progesterone also failed to elicit a natriuretic response.[7] On the other hand the unusual synthetic compounds, 3-(3-oxo-17- β -hydroxy-4-androsten-17 α -yl)propionic acid, γ -lactone and its 19-nor analog, have recently been shown to be sodium-dissipating in rats[8] as well as man,[9] apparently by virtue of a similar inhibition of aldosterone. In rats there was a suggestion that the relationship between these blocking agents (including progesterone) and aldosterone followed the law of mass action. In the studies reported here, a 20-microgram dose of d-aldosterone was inhibited by 150 mg of progesterone, whereas 40- to 50-microgram doses were not. Such a blockade of aldosterone by progesterone, as well as the apparent chemical similarities, might suggest that progesterone is a relatively weak salt-retaining steroid itself. As a matter of fact, such an action has been demonstrated in dogs[10] and suggested in rodents, in which progesterone in large amounts supports the life of adrenalectomized animals.[11,12] However, in man, no salt-retaining activity has ever been described.

The antagonism of progesterone to the typical sodium-retentive influence of aldosterone permits the assumption that such a blockade of the effects of endogenous aldosterone accounts for the sodium-dissipating effects of progesterone in normal persons. The more limited losses of salt and the prompt post-treatment recovery of lost sodium in these subjects is probably explained by a compensatory increase in the secretion of aldosterone. A similar adrenal response to the progesterone secreted during the normal menstrual cycle and during pregnancy would be anticipated. The greatly increased excretion of aldosterone noted during normal pregnancy[13] probably reflects its enhanced secretion, in part at least, in response to the large amount of progesterone secreted by the placenta and the corpus luteum of pregnancy.

It has been suggested that in some circumstances the adrenal cortex may secrete a sodium-dissipating agent.[14] In the light of the findings reported here, it is conceivable that this presently unidentified secretion may be progesterone.

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A STUDY OF THE LABILITY OF CHOLESTEROL IN HUMAN ATHEROSCLEROTIC PLAQUES*

By

R. G. Gould,† R. J. Jones,† and R. W. Wissler

It has been demonstrated that dietary cholesterol mixes with and becomes indistinguishable from the cholesterol of endogenous origin in the liver-blood pool. The cholesterol in this pool equilibrates with that in all tissues studied in experimental animals, except brain, but at widely varying rates. To what extent the cholesterol present in human atherosclerotic plaques is still capable of equilibration with blood cholesterol, will be an indication of the reversibility of the atherogenic process.

Tritium-labeled cholesterol was fed to a group of patients with poor prognoses (including some with myocardial infarction) at a constant daily dosage after a priming dose ten times as large. This regimen was found to give almost constant plasma cholesterol specific activity values. The cholesterol concentration and specific activity were determined in samples of plasma, liver, the coronary, iliac and pulmonary arteries, samples of several parts of the aorta, and of other tissues from all patients coming to autopsy. Results were generally in agreement with previous animal studies in showing most rapid equilibration of liver-blood cholesterol with spleen, intestine, lung, heart, adrenal, and kidney; less rapid with skeletal muscle and fat; and the least rapid with arteries. The more severely atherosclerotic samples of arterial tissue showed, in general, slower rates of equilibration, but even those with cholesterol concentrations over ten times the normal value gave, in one case, 8 per cent equilibration in 4 days (mean of 3 samples) and, in a second case, 11 per cent in 14 days (mean of 3 samples).

One patient who took cholesterol-H^3 daily for 2 months and who died 5 months after the last dose, showed essentially equal specific activity values in serum, liver, spleen, and thoracic aorta and 75 per cent of this value in abdominal aorta. Atherosclerosis score was 2+ in thoracic and 3+ in abdominal aorta. These results suggest that the cholesterol in human atherosclerotic lesions is interchangeable with blood cholesterol but at a very slow rate.

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STUDIES ON PYROGENIC STEROIDS IN MAN*

By

Attallah Kappas, William Soybel,† David K. Fukushima,† and T. F. Gallagher†

This report deals with the capacity of a number of steroid hormone metabolites to provoke fever in man. These pyrogenic steroids belong to a class of compounds previously considered to be devoid of biological activity; they share the basic steroid nucleus characteristic of 5 β, or pregnane compounds; they are derived from endogenous adrenal and gonadal hormones; and they represent the first pure substances, of known chemical structure and of physiologic origin, having consistent pyrogenic activity in man.

The chemical prototype for this thermogenic activity is the metabolite etiocholanolone.[1,2,3] This 19 carbon steroid originates from several precursors including androgenic hormones produced by the testes and adrenal cortex. Figure 1 demonstrates the pyrogenic

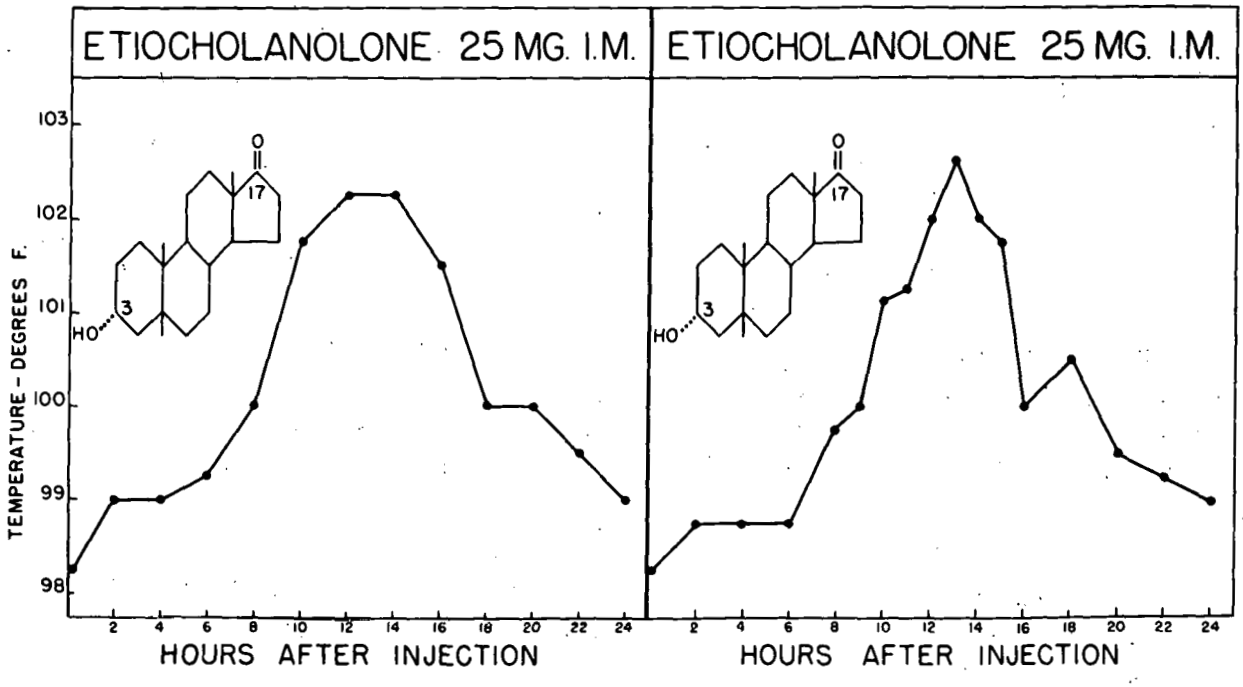

Figure 1. Temperature response to intramuscular injection of etiocholanolone in man.

behavior of this compound in two adult men, each of whom received 25 mg by intramuscular injection. The responses shown are typical. Temperature elevation begins 4 to 8 hours

*Reprinted from Trans. Assoc. Am. Phys., 72:54 (1959).

†Sloan-Kettering Institute for Cancer Research, New York City, New York.

after injection, reaches a peak at approximately 12 hours and generally subsides within 24 hours. This temperature elevation may range up to 6°F. or more, depending on dose. There is no sex difference in this reaction. The response is consistent and reproducible. Repeated injections do not produce tolerance to the steroid pyrogen. A local inflammatory reaction may be produced which is manifest in some subjects by a mild ache at the site of injection; in others, there may be pain, swelling and heat with larger doses. A systemic reaction consisting of transient chills, headache, malaise, myalgia and arthralgia may occur. This steroid does not produce a fever in rabbits according to standard pyrogen assay. In Figure 1 and the following figures only representative examples of pyrogenic responses are shown.

The pyrogenic action of etiocholanolone can be changed by certain alterations of the molecule which are known to occur in vivo. A typical thermogenic response to etiocholanolone is shown in the upper left hand corner of Figure 2 for comparative purposes. In the lower left hand corner the pyrogenic response to etiocholandione is shown. The arrow indicates that the alcohol group at carbon 3 of etiocholanolone is oxidized to a ketone, and this conversion removes a large part of the pyrogenic action of the parent steroid. The chart in the lower right hand corner of Figure 2 demonstrates that complete elimination of the fever-producing activity of etiocholanolone results from its esterification to form the acetate derivative. The in vivo counterpart of this chemical alteration is conjugation of the free steroid with glucuronic acid and this process undoubtedly accounts for the fact that etiocholanolone which is produced endogenously is not pyrogenic in man under normal circumstances.

The steroid in the upper right hand chart is of interest. The arrow indicates the addition of an oxygen to the etiocholanolone molecule. This substance does not represent a metabolite of etiocholanolone however, rather it is derived in vivo from hydrocortisone. It has a significant degree of pyrogenicity and presents the interesting paradox of a fever-suppressing hormone being metabolized in part to a fever-producing end product.

The pyrogenic effect of the 19 carbon compound etiocholanolone extends to 21 carbon steroids having the 5β configuration as well. Two examples of these related C 21 steroids are pregnanolone and pregnanediol. These two compounds are intensely pyrogenic as shown in Figure 3. It is our impression that pregnanolone is more potent in this regard than either pregnanediol or etiocholanolone. The two compounds shown in this figure are the principal metabolites of progesterone, a hormone which has been shown by Buxton[4] and Davis[5] and associates to have a slight temperature-elevating activity of its own. Consideration should be given the possibility, however, that the slight fever-producing capacity of progesterone may be mediated through transformation to metabolites of the type shown in Figure 3 and that the physiologic temperature elevation which occurs during the luteal phase of the menstrual cycle and during pregnancy may possibly be attributed to the latter substances.

The capacity of pregnanolone to produce fever is changed by the same chemical alterations shown to influence the activity of etiocholanolone. A typical thermogenic re-

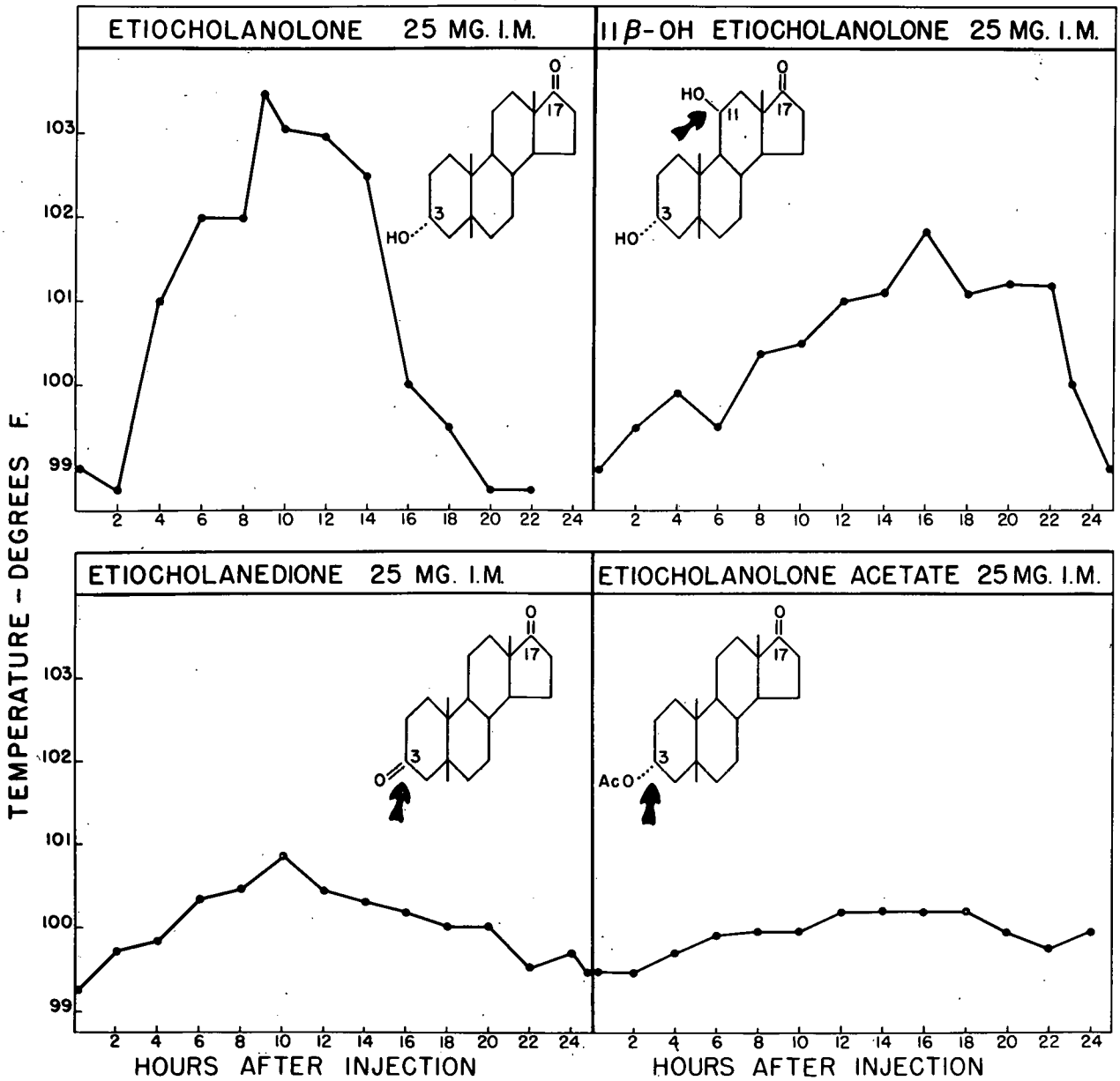

Figure 2. Relation of structure to pyrogenic action of C19 steroids.

sponse to pregnanolone is shown in the upper left chart of Figure 4. In the lower left corner, conversion of the alcohol group at carbon 3 to a ketone, indicated by the arrow, diminishes considerably the pyrogenic activity of the parent steroid. Esterification of the alcohol group at carbon 3, indicated in the lower right chart completely eliminates pyrogenic activity, as previously shown for etiocholanolone.

The compound in the upper right corner of Figure 4 having an oxygen function at carbon 11 is not a metabolite of pregnanolone but is an intermediate in the synthesis of hydrocortisone. The pyrogenic activity of this 11-oxygenated compound shown together with the

similar activity of 11-hydroxyetiocholanolone implies that an oxygen function at carbon 11, while characteristic of adrenal corticoids, is not the structural feature that in itself is significant in their ability to suppress fever. These observations are further evidence of the interesting paradox that an antipyretic steroid, hydrocortisone, has both precursors and metabolites that are pyrogenic.

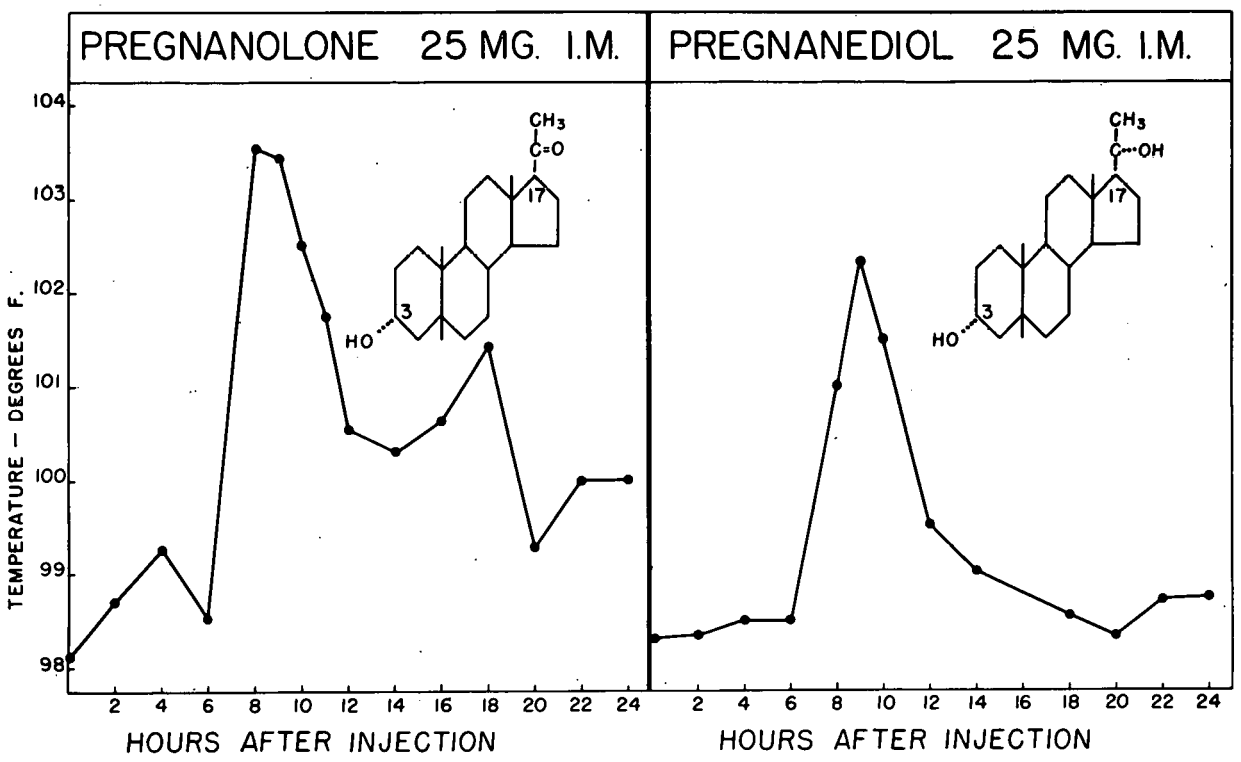

Figure 3. Pyrogenic effect of C21 pregnane (5β-H) steroids in man.

Further observations of the influence of structure on the pyrogenic activity of steroid metabolites are shown in Figure 5.

The compound on the left has an alcohol group at carbon 21 as indicated by the arrow. In most subjects this steroid is inert when administered intramuscularly or intravenously. In an occasional person, however, it is intensely pyrogenic as shown. The explanation for the variable response to this compound is not apparent.

The steroid in the middle and that at the right are both characterized by having an alcohol group incorporated at carbon 17 as indicated by the arrow. This chemical substituent eliminates the pyrogenic capacity of this class of compounds. Intravenous studies with these steroids confirm their inertness.

The means by which these pyrogenic steroids provoke fever in man are not clear. The following observations may be considered in any conjectures regarding mode of action: first, these steroids are effective pyrogens by the intravenous route as indicated by the representative studies in Figure 6; second, a minimum period of intravenous injection may

be necessary to obtain this effect. For example, infusion of 25 mg of 11-ketopregnanolone in 30 minutes does not provoke fever in some subjects. Reinfusion of this amount of steroid over 2 to 3 hours in these subjects, however, generally provokes an intense pyrogenic

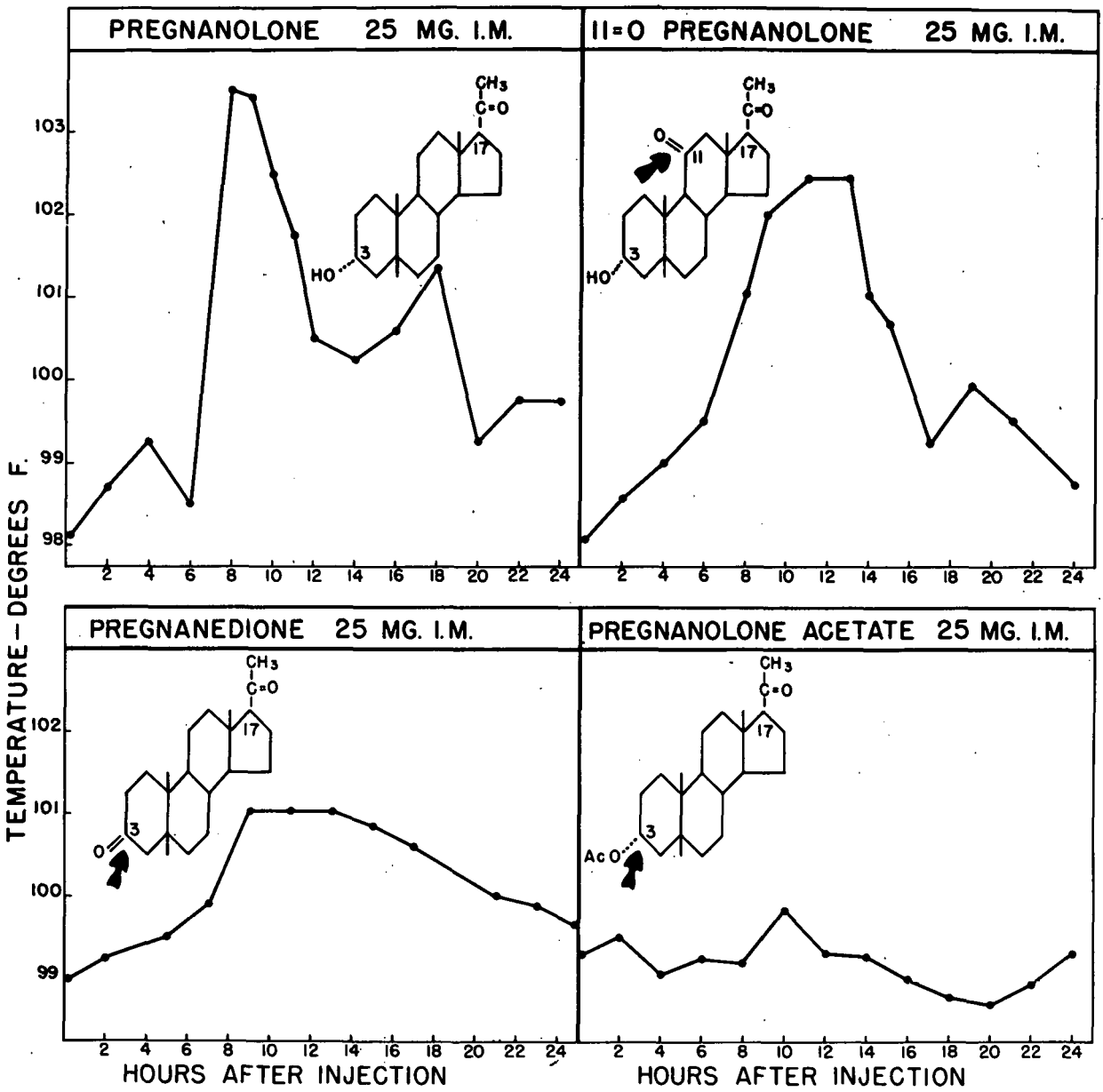

Figure 4. Relation of structure to pyrogenic action of C21 steroids.

reaction; third, no significant alteration in administered steroid pyrogen apparently takes place. For example, etiocholanolone is recovered unchanged and nearly quantitatively following its injection into man;[3] fourth, the half-life of intravenously administered steroid,

in the form of free compound circulating in the blood, is well under 60 minutes. Thus, during the period of development and peak of fever in these subjects, the free steroid pyrogen cannot be identified in the plasma; fifth, the fever that follows the intravenous injection of steroid pyrogen takes as long to develop and reach a peak as that following intramuscular injection (Figure 6); and finally, no transferable pyrogen in man has been demonstrated in amounts of plasma up to 300 ml, taken during the development and peak of fever.

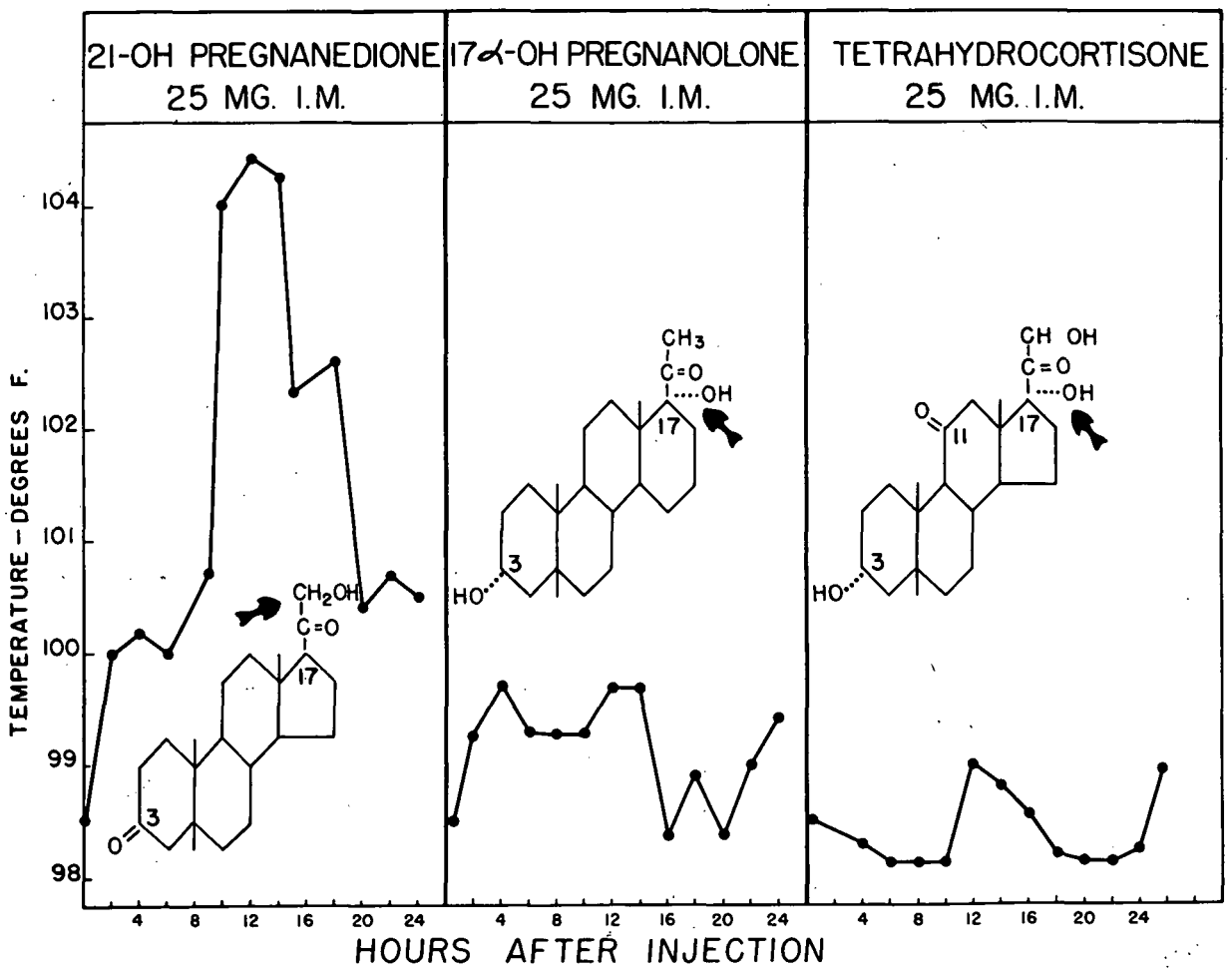

Figure 5. Temperature response to 21-hydroxy and 17α-hydroxy steroids injection I.M.

In summary, these observations are of interest in the following respects: the pyrogenic capacity we have previously noted in etiocholanolone extends to a variety of pregnane metabolites. A new form of biological activity is thus established for this class of compounds. Since these compounds are transformation products of hormones, the probable existence of as yet unrecognized biological activity among other steroid hormone meta-

bolites is implied. The hypocholesterolemic effect of androsterone noted by Hellman and colleagues in studies from the Sloan-Kettering Institute[6] supports this conjecture. The endogenous origin of these steroid pyrogens naturally stimulates speculation regarding their

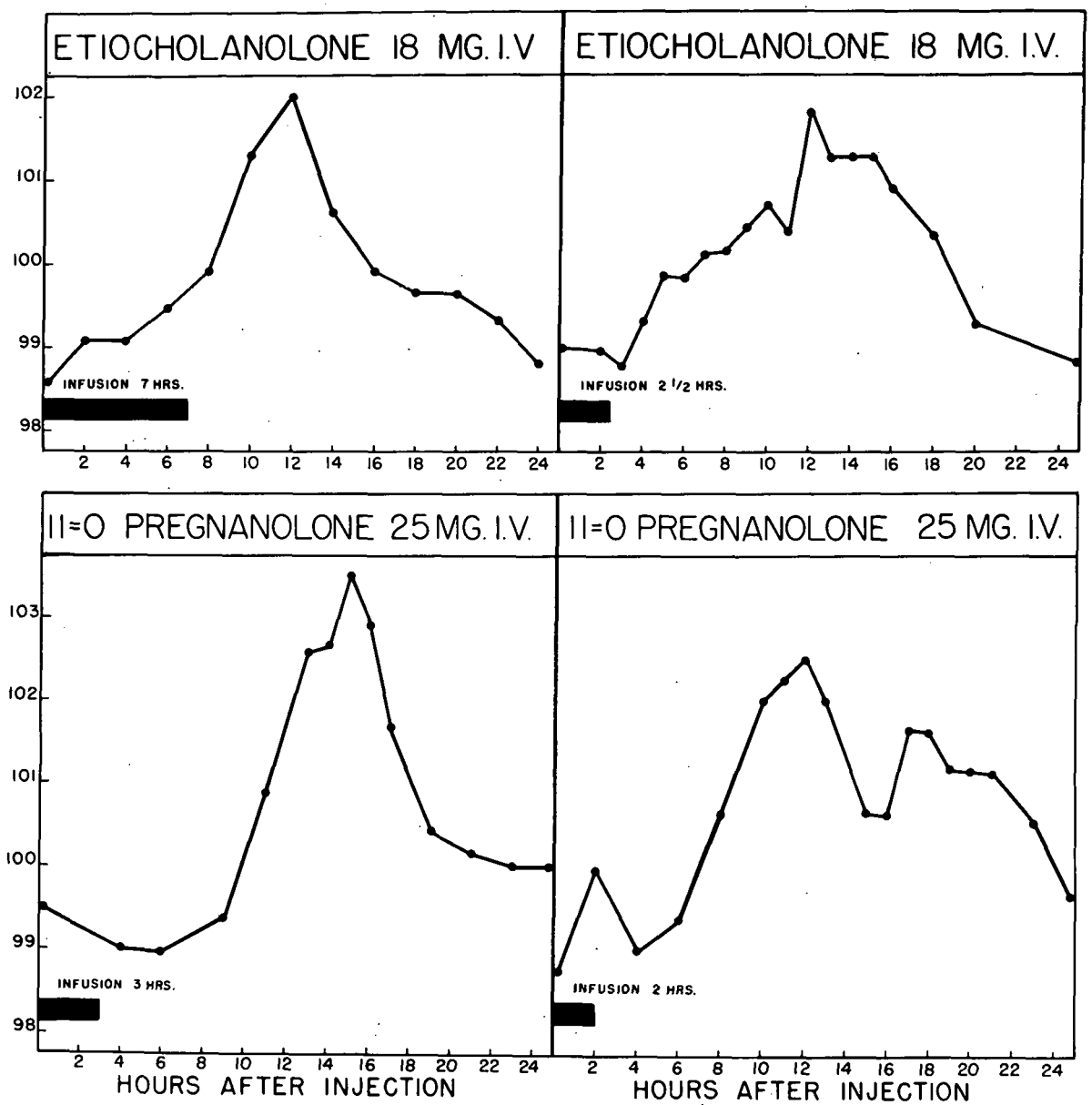

Figure 6. Temperature response to intravenous administration of steroid pyrogens.

possible participation in the pathogenesis of fever in man under certain circumstances. This participation would probably originate in some defect in steroid conjugate formation, as suggested by Heckel[7] and recently demonstrated in a highly interesting study of the re-

lationship of etiocholanolone to periodic fever, by Bondy, Cohn, Herrmann, and Crispell.[8] Finally, the established chemical structure of these pyrogens, their availability as pure compounds through chemical synthesis and the consistency of the fever which they provoke, all combine to provide a safe and useful experimental tool for the study of the pathogenesis of fever directly in man.

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FAILURE OF HUMAN PLACENTAL TISSUE HOMOGENATES TO CONVERT PROGESTERONE TO ESTROGENS

By

P. M. Ejarque, E. J. Plotz, and M. E. Davis

The conversion of androgens to estrogens has been shown to occur in vitro[1-4] and in vivo.[5-7] Ryan,[8] localized the aromatizing activity in the microsome fraction of the placenta when the incubation was performed in air with added reduced triphosphopyridine nucleotide and C-19 precursors. No aromatization could be found in the microsome fraction when the precursor was a C-21 steroid.

This study was undertaken to determine whether human placental tissue homogenate is capable of converting progesterone to estrogens or not.

METHODS AND RESULTS

A term placenta was obtained immediately after expulsion, freed of membranes, and washed with cool tap water. After trituration with a meat-grinder 450 g of tissue were obtained. Three 50-g portions of ground tissue were combined with pH 7.0 buffer (0.25 M sucrose, 0.05 M phosphate, 0.04 M nicotinamide) using 1 part buffer to 3 parts tissue, homogenized in a Waring Blender for two minutes, and transferred to 125 ml Erlenmeyer flask. One flask contained 291.05 μg of 4-C^{14} progesterone* in 1 ml of propylene glycol, a second flask contained 300 μg of androstene-3,17-dione in 1 ml of propylene glycol and the third flask served as a control; 5,400 μM of TPNH were added to each flask and the incubation was carried out at 37°C for one hour in a Dubnoff incubator. The gas phase was air. Ryan's[8] technique was followed to obtain the phenolic fraction. The distribution of radioactivity as assayed by gas flow and combustion-line technique is given in Table 1.

Table 1

DISTRIBUTION OF RADIOACTIVITY IN VARIOUS STEROID FRACTIONS

Fractions	μc	Per cent of original dose
Heptane	0.019	0.24
Phenolic	0.476	6.13
Aqueous base	0.055	0.70
Neutral	1.63	21.00
Extracted tissue	4.50	71.00

*Specific Activity 26.71 (μc/mg (purified by paper chromatography).

The phenolic fraction from each of the three flasks was run in benzene-methanol-water (2:1:1)[9] for 24 hours. The overflow was collected and run in the petroleum-ether-benzene-methanol-water system (33:66:80:20)[10] for 3 hours.

A ferric chloride positive band with the same R_f as estrone was found in the paper from the androstenedione incubation. A similar band with an R_f between those of estrone and estradiol was found in the paper from the 4-C^{14} progesterone incubation. A radioactive peak was found in the same area. The paper eluate contained 125 μg of estrogenic material determined by the Brown's modification[11] of the Kober color reaction. One milligram each of authentic estrone and estradiol were added.

The combined carriers and unknown were chromatographed in petroleum-ether-benzene-methanol-water (33:66:80:20) for 3 hours. Two well-separated bands were found. These had the same R_f's as authentic estrone and estradiol respectively and showed a positive ferric chloride reaction.

Purification of estradiol. The estradiol was eluted, assayed colorimetrically, and radioassayed in the gas flow counter. The results recorded in Table 2 are corrected for the addition of 50 mg of standard estradiol. The diacetate was made, recrystallized twice from methanol, dried, weighed, radioassayed by the scintillation technique, and a sample removed for infrared analysis.

The sample was dried under vacuum and the scintillation phosphor (DPO) was removed by washing with petroleum ether. To hydrolize the ester linkage 2 ml of 5 per cent KOH in absolute ethanol were added and the pH adjusted to 7.0. The mixture was extracted five times with 20 ml diethyl ether, the ether was pooled, washed twice with 10 ml water and dried over Na_2SO_4. The ether fractions were then removed by distillation.

The estradiol crystallized from methanol was dried, weighed, radioassayed and a sample removed for infrared analysis. The results are recorded in Table 2.

Purification of estrone. To the eluate from the band having the same R_f as authentic estrone, 50 mg of estrone and 50 mg of estradiol were added as carrier. After a Girard's separation, 44.5 mg of estradiol and 46 mg of estrone were recovered. An aliquot of each was assayed for radioactivity by the scintillation technique (Table 3). The estradiol did not contain any radioactivity.

The acetate of the estrone fraction was prepared and after two crystallizations from methanol the material was dried, weighed, radioassayed and the infrared spectrum determined (Table 3).

The toluene and DPO was freed as before, the hydrolysis was performed as above, and after decolorization by charcoal, and two crystallizations from methanol, the weight, U.V. spectra of the phosphoric[12] complex and radioassays were obtained (Table 3).

SUMMARY

An homogenate of term placenta did not convert progesterone to estrone or estradiol. Thus it seems to be likely that the placenta does not have the enzyme system which removes the side-chain of C-21 compounds.

Table 2

DETERMINATION OF RADIOACTIVITY DURING PURIFICATION PROCESS OF ESTRADIOL

Compound	Total radio-activity CPM	Total radio-activity DPM*	Weight of compound Mg	Free estradiol Mg	Specific activity DPM/mg	Remarks
Estradiol†	3055	5024	51.250	51.250	98.0	
Diacetate of estradiol‡	1220.7	2142.3	51.90	39.707	53.9	Infrared spectrogram identical with standard
Estradiol‡	0.0	0.0	11.1	11.100	0.0	Infrared spectrogram identical with standard

*A factor obtained by using a known amount of radioactivity and of steroid was used to correct for the quenching effects of estradiol (0.45 per cent quenching effects) and estradiol diacetate (3.24 per cent).

†Counted by gas flow technique.

‡Counted by liquid scintillation technique.

Table 3

DETERMINATION OF RADIOACTIVITY DURING PURIFICATION PROCESS OF ESTRONE

Compound	Total radio-activity CPM	Total radio-activity DPM*	Weight of compound Mg	Free estrone Mg	Specific activity DPM/mg	Remarks
Estrone	3329	5813	46	46	126.3	
Estrone acetate	193.63	324.99	22.09	18.55	17.51	Infrared spectrogram identical with standard
Estrone	4.31	7.24	4.01	4.01	1.83	U.V. spectrogram in 100 per cent phosphoric acid identical with standard

*The quenching effects were calculated in the last two radioassays with the same amount of nonradioactive material as the unknown; 2.87 per cent of quenching was found for the acetate of estrone and 0.63 per cent of quenching for the estrone.

Counted on a liquid scintillation counter.

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A MAMMALIAN SYSTEM FOR THE INCORPORATION OF CYTIDINE TRIPHOSPHATE INTO RNA*

By

S. B. Weiss and L. Gladstone

The biosynthesis of DNA† by bacterial and mammalian enzymes has been shown to require the four deoxynucleoside triphosphates.[1,2] However, the synthesis of RNA by bacterial polynucleotide phosphorylase may utilize one or more of the four ribonucleoside diphosphates to form simple or mixed polynucleotides.[3] A mammalian preparation that incorporates cytidine-P^{32}-P-P into RNA and requires the participation of the three other ribonucleoside triphosphates is described in this report.

CMP-5'-P^{32} was prepared by a modified procedure for the phosphorylation of 2', 3'-benzylidene-0-cytidine.[4] Labeled CTP was prepared from P^{32}-CMP by a cytidylate kinase isolated from brewers' yeast. Enzyme was prepared from a 20 per cent rat liver homogenate in 0.25 molar sucrose, centrifuged for six minutes at 600 x g, and the sediment washed twice in sucrose. Microscopic examination revealed a high concentration of intact nuclei and a few whole cells.

When P^{32}-CTP was incubated with the 600 x g preparation in the presence of all the ribonucleoside triphosphates, a significant amount of radioactivity was incorporated into the acid-insoluble, salt-extractable material. Omission of any one of the triphosphates resulted in a reduction of 85 per cent or more of P^{32}-CTP incorporation (Table 1). Desoxyribonuclease depresses the incorporation slightly, whereas ribonuclease causes a marked reduction. In another experiment, a fivefold excess of deoxy-CTP included in the reaction mixture did not reduce the incorporation of P^{32}-CTP.

When P^{32}-labeled RNA (70,000 total cpm), formed by this system, was isolated and hydrolyzed with alkali, all the mononucleotides, separated by chromatography on Dowex-1-Cl, were labeled. The distribution of radioactivity was as follows: 2'(3')-CMP, 22,890 cpm; 2'(3')-AMP, 8,680 cpm; 2'(3')-GMP, 8,800 cpm; 2'(3')-UMP, 21,940 cpm.

The uniform distribution of radioactivity in the mononucleotides, formed after hydrolysis, suggests strongly that P^{32}-CTP is incorporated into the interpolynucleotide linkages of RNA rather than terminally as reported previously.[5-7] No requirement for a primer has as yet been demonstrated. The requirement for Mg^{++} and the four ribonucleoside triphosphates, the latter being more than twice as effective as the corresponding di-

*Based on a paper appearing in the Journal of the American Chemical Society, 81: 4118, 1959.

†Abbreviations: DNA, deoxyribonucleic acid; RNA, ribonucleic acid; CTP, ATP, UTP, and GTP for the tri- and 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; Pi, inorganic phosphate.

Table 1

REQUIREMENTS FOR P^{32}-CYTIDINE TRIPHOSPHATE INCORPORATION INTO RNA*

Reaction mixture	Radioactivity RNA (total cpm)
Complete	3872
Omit ATP	636
Omit UTP	280
Omit GTP	104
Omit ATP, UTP, and GTP	60
Complete + 20 γ ribonuclease	828
Complete + 20 γ desoxyribonuclease	2940
Complete + 10 μM inorganic pyrophosphate	100
Complete in 100 μM Pi buffer, pH 7.5 (no Tris)	4030
Complete; ADP, UDP, GDP in place of ATP, UTP, GTP	1620
Complete; AMP, UMP, GMP in place of ATP, UTP, GTP	128

*The complete system contained 10 μM $MgCl_2$, 100 μM Tris·HCl, pH 8.0, 0.1 μM P^{32}-CTP (16.8×10^6 cpm/μM, 0.1 μM ATP, 0.1 μM UTP, 0.1 μM GTP, 100 μM KCl, 40 μM NaF, 10 μM cysteine, and 10 - 12 mg of twice washed nuclei (dry weight), in a total volume of 2.0 ml. After incubation at 37° for 12 minutes, 5 ml of cold 5 per cent TCA was added. The acid-insoluble material was washed 3 times with 5 per cent TCA, 2 times with ethanol-ether (3:1), and extracted 3 times with 2 ml of 10 per cent NaCl at 100°, pH 8.0, with 2 mg of yeast RNA added. The extracts were combined and precipitated twice with 2 volumes of ethanol. The residue was dissolved in 4 ml of water, and 1.0 ml was dried and assayed in a windowless flow counter.

phosphates, as well as the inhibition by pyrophosphate but not by inorganic phosphate, suggest that the reaction mechanism involved here resembles that described for the formation of DNA rather than that for the formation of bacterial[3] or mammalian nuclei[8] polynucleotide phosphorylase.

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