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Semiannual Report to
THE ATOMIC ENERGY COMMISSION

MARCH 1967

LEON O. JACOBSON, M.D.
Editor

MARGOT DOYLE, Ph.D.
Associate Editor

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Printed in the U.S.A.

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Price: Printed copy \$3.00; Microfiche \$0.65

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ESTROGEN PHARMACOLOGY. IV. STUDIES ON THE STRUCTURAL BASIS FOR ESTROGEN-INDUCED IMPAIRMENT OF LIVER FUNCTION*

By

T. F. Gallagher, Jr.,[†] M. N. Mueller,[‡] and A. Kappas

The natural hormone estradiol, and its metabolite estriol, impair hepatic disposal of the dye sulfobromophthalein (BSP) in man, as shown in previous studies from this laboratory.¹ This impairment is characterized by depression of the secretory transport maximum (T_m) for BSP^{2,3} to levels which may be as low as those seen in patients with the Dubin-Johnson syndrome;³ the presence of increased amounts of conjugated dye components in plasma during estrogen as compared with control periods; and the absence by light microscopy, of significant accompanying morphological changes in the liver.⁴

This study, dealing with the influence of estrogens on BSP metabolism in the rat, was undertaken to determine whether this experimental species was susceptible to the estrogen effect described in man; and if so, to devise a test system which would permit an exploration of certain aspects of the structural basis for this estrogen action. The results indicate that estrogens do impair BSP metabolism in the rat via a mechanism which does not involve the conjugation process; that the effect extends to other steroids derived from the *in vivo* metabolic transformation of estradiol in man; and that certain structural characteristics of the estratriene steroid nucleus have an important influence on the capacity of natural estrogens to impair the excretion of BSP by the liver.

METHODS AND RESULTS

Steroid solutions were prepared as previously described⁴ and administered by subcutaneous injection. Steroid concentrations were such as to require 0.2 ml of vehicle for each amount injected; control animals received equivalent amounts of solvent vehicle alone. Ready absorption of steroid was indicated by the rapid, consistent development of appropriate, classical hormone effects in this study, as well as other studies in humans utilizing the same (water-soluble) solvent vehicle. Rats were purchased from Sprague-Dawley, Inc., Madison, Wisconsin, housed four to six in a cage in controlled temperature animal rooms and fed the Rockland-Rat/Mouse (complete) diet and water *ad libitum*.

The indicated amounts of BSP and its congener, phenol-3,6-dibromophthalein disulfonate, were injected in 0.5 ml saline into a tail vein; the animals were killed by decapitation (without preceding anesthesia) at appropriate intervals; and the plasma dye concentrations measured on the same day by the usual means.⁴ Chromatographic analysis of free and conjugated dye components in rat plasma pools was done as described previously for humans;⁴⁻⁷ in these analyses all conjugated dye fractions in a given pool were eluted and assayed together. Hepatic content of

* This paper appeared in *Medicine*, 45:471, 1966.

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BSP-glutathione conjugating enzyme was measured by the procedure of Combes and Stakelum;⁸ hepatic content of reduced glutathione (GSH) was measured in liver homogenates by application of the method of Beutler, Duron, and Kelly,⁹ through the courtesy of Dr. Paul Carson, Department of Medicine, The University of Chicago.

Many characteristics of BSP metabolism in the rat have been described in studies from other laboratories.^{6,10-15} In this study exploratory experiments were done to determine whether estrogens impaired BSP metabolism in this species and to establish the conditions of timing, dosage (BSP, steroids, etc.) appropriate to the development of a reasonably simple and reproducible test system which would permit a systematic examination of the structural basis for this hormonal action. These exploratory studies confirmed that intravenously administered BSP in the rat underwent rapid initial and slower late plasma removal; and that plasma concentration of BSP 30 to 40 minutes after dye injection was dependent in this species, as in humans, in part on the body weight of the animal and the amount of dye injected.¹⁶

An example of the plasma disappearance curve of BSP in control rats is shown in Figure 1A. In this experiment, plasma disappearance of 10 mg of BSP was studied in control animals

Figure 1. Plasma disappearance curves of BSP in control (A) and estradiol-treated (B) rats. The single exponentials derived from the data in these experiments are graphically compared in C.

weighing 140 to 160 g at intervals of 2 to 40 minutes after dye injection. The points indicate mean plasma dye levels, and the brackets, the range of values for at least 6 rats at each time interval. Similar exploratory experiments indicated that natural estrogens significantly impaired BSP disposal in the rat; that the effect was dose related; and that it developed quickly after these hormones were administered and receded rapidly after they were discontinued. Figure 2 shows the results of a study in which 3 groups of rats (140 to 160 g) received 0.1, 0.5 or 1.0 mg estradiol

Figure 2. Plasma BSP—mg per cent at 40'. The range of plasma BSP values in rats injected with 10 mg of dye, and pre-treated with 0.1, 0.5, and 1.0 mg of estradiol per day for 5 days.

each day for five consecutive days. Twenty-four hours later all animals, and those in a control group, were injected with 10 mg of BSP and the plasma dye level determined at 40 minutes. Figure 3 shows the results of an experiment in which a group of rats received 1.0 mg estradiol per day for ten consecutive days after which all animals were injected with 10 mg of BSP and plasma BSP concentration determined 40 minutes later. Figure 4 shows the results of an experiment in which the rate of onset of the estrogen effect was studied by estimation of the plasma BSP concentration (40 minutes after injection of 10 mg BSP into 140 to 160 g rats) in rats treated with 1.0 mg estradiol per day for 1, 2, 3, 4, 6, 8, 10, or 20 consecutive days. Control groups were studied at 0, 5, 10, and 20 days. Three hormone treated groups were withdrawn from the treatment schedule at 10 days in order to study the rate of functional recovery from the estrogen effect. All plasma BSP values in control animals fell within the shaded area at the bottom of the figure. The points on the curve indicate the mean plasma BSP value of groups of at least 6 animals at each interval.

Figure 3. Plasma BSP—mg per cent at 40'. Typical effect of pre-treatment of rats with estradiol 1.0 mg per day for 10 days, on plasma dye levels 40' after injection of 10 mg of BSP.

Figure 1B shows the results of an experiment in which the plasma disappearance of 10 mg of BSP was studied at intervals of 2 to 40 minutes in rats of fixed weight range (140 to 160 g), pretreated with estradiol in the amount of 1.0 mg per day for 10 consecutive days. The points indicate the mean plasma dye concentration and the brackets, the range of values, in groups of at least 6 animals. The plasma disappearance curve in control animals under the same test con-

Figure 4. Demonstration of the rate of onset of, and recovery from, the estrogen effect on BSP metabolism in the rat. The shaded area represents the maximum range of plasma BSP values observed in control-vehicle treated animals injected with 10 mg of dye. The points on the solid curve up to 10 days represent the mean plasma BSP of similarly injected groups of animals treated continuously with 1.0 mg estradiol per day. The points at 20 days on the solid curve represent the mean plasma BSP in a group of rats continuously treated for an additional 10 days; the points on the dashed curve represent the mean plasma BSP values in groups of rats whose estradiol treatment was discontinued at 10 days.

ditions is shown in Figure 1A. It is possible that the data plotted in Figures 1A and 1B differ not only in the parameters that best fit them to a given mathematical model but also in the models themselves. However, the standard deviations of the data plotted in Figure 1B are too large to permit one to establish the latter difference, so we have compared both sets of data by calculating the parameters that best fit each to a mathematical model that appears to be appropriate to the set in Figure 1A. A non-linear regression fits the data plotted in Figure 1A very well to the sum of two exponentials (on which the disappearance curve shown is based), but, since the slower time component accounts for less than 1.0 per cent of the sulfobromophthalein, approximation by a single exponential is considered to be adequate. Of these two types of curves, however, only the single exponential fit is made reliably to the data in Figure 1B, so it is for this fit that parameters are compared for both sets of data. The exponential factor that relates to the slope is known, at the 95 per cent confidence level to be 0.073 ± 0.012 for the data in Figure 1B, and 0.124 ± 0.041 for the data in Figure 1A. The magnitude of this difference in slope may be appreciated by observing the exponentials in Figure 1C.

A chromatographic analysis for free and conjugated dye components was made on 5 individual plasma pools obtained from rats treated for 10 days with hormone in the test system graphically demonstrated in Figure 4. Conjugated BSP compressed on the average, 26 per cent (range 13-46 per cent) of the circulating dye components in these estrogen treated animals. A direct comparison between the amounts of conjugated BSP in plasma of control and estrogen treated animals was not possible because of the low BSP content of control plasma under test conditions.

The left chart in Figure 5 shows the activity of the BSP-glutathione conjugating enzyme in liver homogenates of 12 control and 11 estrogen treated animals. Five of the latter were treated with 1.0 mg estradiol per day for 10 days; 6 received the same amount of steroid for 27 consecutive

Figure 5. Comparison of hepatic BSP-GSH conjugating activity and hepatic GSH content. The chart on the left shows a comparison of the hepatic BSP-GSH conjugating enzyme activities in livers of control and of estradiol-treated rats (1.0 mg per day for 10 days in 5 animals; 1.0 mg per day for 27 days in 6 animals). The chart on the right shows a comparison of GSH content in the livers of control and estradiol-treated animals (1.0 mg per day for 10 days).

utive days. Liver weights in control animals averaged 6.3 g (range 5.4 to 7.5 g); and in estrogen treated animals averaged 7.0 g (range 6.0 to 8.1 g). The right chart in Figure 6 shows the hepatic content of the substrate reduced glutathione (GSH) in the livers of 8 control animals and 6 animals treated with estradiol, 1.0 mg per day for 10 days.

Phenol-3,6-dibromophthalein disulfonate is a chemical analogue of BSP which lacks bromine atoms at carbons 4 and 5 of the phthalein nucleus. Javitt studied the metabolism of this substance in the rat¹⁷ and showed that it is excreted from the hepatic cell into the bile entirely in the unconjugated form; moreover, it does not undergo conjugation with glutathione in vitro. Plasma levels of this dye 40 minutes after injection of 10 mg into 12 control and 11 estrogen (1.0 mg estradiol per day for 10 days) treated animals (140 to 160 g) were examined; the results shown in

PHENOL-3, 6-DIBROMOPHTHALEIN DISULPHONATE

Figure 6. Plasma levels of phenol-3,6-dibromophthalein disulphonate. Plasma levels of phenol-3,6-dibromophthalein disulphonate in control and estradiol-treated rats (1.0 mg per day for 10 days) 40' after injection of 10 mg of the dye. The dots represent dye plasma concentrations of individual animals in each group.

Figure 6 indicate that plasma disappearance of the congener is also delayed by estrogens, despite the fact that it is not conjugated during hepatic transit.

Utilizing the observations described above, a test system was devised for assessing the effects of steroids on BSP disposal in rats by estimation of the plasma BSP concentration 40 minutes after intravenous injection of 10 mg of dye into Sprague-Dawley rats weighing 140 to 160 g pretreated with 1.0 mg per day of the test steroid for 10 consecutive days. Animals were fed ad libitum and although no significant sex difference was noted in respect to the estrogen effect, only virgin female animals were used in the test. Parallel control groups were examined in each test. Under the conditions described, plasma BSP concentrations ranged from 0.3 to 1.1 mg per cent with a mean of 0.7 ± 0.2 mg per cent (± 1 S.D.) in control animals treated with solvent vehicle for 10 days. In test animals, plasma BSP values in excess of 1.5 mg per cent were there-

fore considered to reflect impaired BSP metabolism. Figure 4 shows the results of a typical test with estradiol. The compounds tested and the number of animals and the mean and range of BSP values in each test are shown in Table 1.

DISCUSSION

There are significant species differences in the metabolism of steroids and wide individual variations in responses to hormones¹⁸ and in the activity of physiologic processes such as those concerned with hepatic disposal of various substances. Despite these limitations and those imposed by the large excretory capacity of the rat liver for BSP,¹⁰ the test system described generally permitted a clear distinction to be made between animals whose dye metabolism was normal and those whose BSP disposal was significantly delayed by the test compounds.

The only natural steroids which regularly impaired hepatic disposal of BSP in this test system were C18 compounds of phenolic A ring type. Estradiol¹⁹ is the structural prototype of this group of hormonal steroids. The amount of this compound required to impair BSP disposal in humans has previously been shown to be well within the amount of total estrogens produced daily during normal pregnancy.^{4,20} In this study, the amount required for the same effect in rats can be inferred from Figure 2, to be between 0.5 and 2.5 mg administered over several days. Although a direct comparison of activities was not made, estradiol would, on this basis, appear to be at least as potent in depressing hepatic capacity for disposal of BSP as such natural substances as icterogenin and other pentacyclic plant triterpine acids.^{10,21,22}

This action of estradiol extends to a variety of metabolites produced during the *in vivo* metabolism of this hormone in man, including such compounds as its oxidation product estrone (II) and its trihydroxy derivative estriol (IV). The activities of compounds III, IV, V, VII, X, XI, and XV are somewhat variable; nevertheless the fact that all, including the 17-deoxysteroid, impair BSP metabolism to some extent emphasizes that neither the type, configuration, presence nor absence of specific oxygenated substituents in the C or D rings of the estratriene nucleus appear to be essential requirements for this biological activity. Insertion of a double bond between carbons 6 and 7 (compounds XIII, XIV), in otherwise active steroids, diminishes, or eliminates activity. Insertion of an additional double bond at carbons 8 and 9 (compound XII, equilenin) restores activity. The reason for this striking effect is not clear; it may be due in part to ready hydroxylation²³ (and thus presumably inactivation) of the reactive C6-7 double bond in the compounds XIII and XIV in the rat, a metabolic transformation which may be hindered by the naphthol ring structure of equilenin (XII).

Hydroxylation of the steroid nucleus at the C2 position (XXI, XXII, XXIII) diminishes the capacity of estrogens to impair hepatic disposal of BSP. Methoxylation (XXIV, XXV) at this position has the same effect. Methylation at this, or the C1 position (XVII, XVIII, XIX) appears to abolish this activity entirely; it is of interest that additional methylation at the C17 position (XX) restores activity. This finding is not surprising in view of the previously recognized effect of C17 alkylation on the capacity of neutral steroids to induce BSP retention and icterus in man; nevertheless the failure of 17 α -methyltestosterone (XXX) to produce BSP retention in this test suggests that with respect to this biologic action, a phenolic A ring structure may confer as much potency to C18 steroids, as may a 17-alkyl radical to the neutral (C19, C21) series of steroid compounds. This is supported by the failure of the C17 alkylated, estrene derivative (non-phenolic A ring), compound XXVIII, to delay BSP disposal as compared with the potent ac-

Table 1

THE EFFECTS OF VARIOUS NATURAL AND SYNTHETIC STEROIDS ON
BSP METABOLISM IN THE RAT

Limited availability of certain compounds accounts for the small number of animals tested in some experiments. The term "active" refers to the relative ability of these steroids to impair hepatic disposal of BSP in the test system described. Plasma BSP concentration in excess of 1.5 mg% 40' after I.V. injection of 10 mg of dye is considered abnormal.

No.	Compound	Animals tested	Plasma BSP range	Plasma BSP mean	Comment
			mg%	mg%	
I	Estradiol	39	0.6 - 16.2	5.0	Active
II	Estrone	22	0.7 - 21.4	10.9	Active
III	α -estradiol	8	0.5 - 4.6	1.7	Active
IV	Estriol	26	2.4 - 22.5	10.5	Active
V	Epi-estriol	8	0.5 - 10.8	4.0	Active
VI	Estradiol-3-acetate	10	0.8 - 8.0	3.4	Active
VII	Estradiol-17-acetate	10	0.7 - 16.9	6.7	Active
VIII	Estradiol-3-methyl-ether	11	0.8 - 14.0	4.8	Active
IX	Mestranol	12	0.9 - 8.2	2.8	Active
X	11 β -hydroxyestrone	5	1.2 - 3.9	2.4	Active
XI	16-ketoestrone	6	0.8 - 4.6	1.6	Active
XII	Equilenin	10	0.8 - 20.4	7.9	Active
XIII	6-dehydroestrone	26	0.2 - 1.0	0.5	Inactive
XIV	6-dehydroestradiol	6	0.6 - 4.2	1.3	One value above 1.5 mg%
XV	17-deoxyestrone	14	1.3 - 10.4	6.2	Active
XVI	3-deoxyestrone	6	0.5 - 0.8	0.7	Inactive
XVII	1-methyl estrone	10	0.3 - 1.3	0.7	Inactive
XVIII	1-methyl estrone-3-acetate	8	0.4 - 0.8	0.5	Inactive
XIX	2-methyl-estrone	3	0.6 - 0.8	0.7	Inactive
XX	2,17 α -dimethyl estradiol	5	0.5 - 13.3	4.7	Active
XXI	2-hydroxyestrone	5	0.4 - 5.0	1.5	One value above 1.5 mg%
XXII	2-hydroxyestradiol	4	0.5 - 0.9	0.7	Inactive
XXIII	2-hydroxyestriol	6	0.6 - 1.9	1.7	Four values above 1.5 mg%
XXIV	2-methoxyestrone	6	0.5 - 1.0	0.8	Inactive
XXV	2-methoxyestradiol	6	0.5 - 4.5	1.5	One value above 1.5 mg%
XXVI	Diethylstilbestrol	7	2.1 - 7.9	5.1	Active
XXVII	Norethynodrel	12	0.6 - 1.2	0.7	Inactive
XXVIII	17 α -ethinyl-4-estrene-17 β -ol, acetate, 3-one	14	0.5 - 1.0	0.7	Inactive
XXIX	Norethandrolone	12	0.3 - 1.0	0.5	Inactive
XXX	Methyltestosterone	5	0.5 - 0.9	0.7	Inactive
XXXI	Testosterone	9	0.4 - 0.8	0.6	Inactive
XXXII	Androsterone	8	0.5 - 0.7	0.6	Inactive
XXXIII	Etiocolanolone	9	0.4 - 0.8	0.5	Inactive
XXXIV	Pregnanediol	9	0.4 - 0.7	0.5	Inactive
XXXV	Progesterone	15	0.4 - 0.8	0.6	Inactive
XXXVI	Corticosterone	8	0.5 - 0.8	0.6	Inactive
XXXVII	Cortisol	7	0.3 - 0.6	0.5	Inactive
XXXVIII	Cholic acid	9	0.3 - 0.5	0.4	Inactive
XXXIX	Lithocholic acid	8	0.3 - 0.7	0.5	Inactive
XL	Taurocholic acid	9	0.3 - 0.7	0.5	Inactive
XLI	Taurolithocholic acid	9	0.3 - 0.6	0.5	Inactive
XLII	Glycolithocholic acid	8	0.3 - 0.8	0.5	Inactive

activity of the C17-alkylated (phenolic A ring) estrene compound (XX).

Chemical substitutions on the oxygen group at C3 (VI, VIII, IX) do not abolish the capacity of estrogens to impair BSP excretion, although the oxygen function itself at this position appears essential (XVI) for this activity. This consideration does not apply to synthetic C18 3-deoxy steroids in which alkyl substitution at C17 for example¹⁷ may confer some ability to impair liver function. The synthetic non-steroid estrogen diethylstilbestrol (XXVI) also impairs BSP metabolism, as do compounds VIII and IX, both of which are used as the estrogenic constituents of certain ovulation suppressing agents.

It is of interest that hepatic dysfunction, particularly excessive BSP retention, has been reported²⁴⁻³³ in a number of patients using these agents. Kleiner, Dresch, and Arias³⁴ have suggested that this is probably due to the progestational component in these steroid mixtures. Eisalo, Adlerkreutz, and their associates^{24,26,27} however, believe it is the estrogen which impairs liver function; and in studies in which the hepatic effects of the progestational and estrogenic components were examined separately, reported that the progestin was innocuous,²⁶ except when administered in very large amounts or for prolonged periods of time.²⁷ The estrogen however, alone, or in combination with the progestin, induced a greater frequency in impairment of BSP metabolism in normal subjects and provoked icterus in patients with a previous history of recurrent jaundice of pregnancy.^{19,24,26,27,30} The latter effect is reminiscent of the observations of Bearn, Kunkel, and Slater³⁵ who had earlier demonstrated the inimical effects of estrogens on hepatic function in patients with primary liver disease. In this investigation, natural (XXXV) and synthetic (XXVII) progestins did not alone impair BSP metabolism (or at least were considerably less potent in this regard than the estrogens) in the test system described; suggesting that the estrogenic component may contribute significantly to the hepatic dysfunction associated with the clinical use of ovulation suppressing agents. These considerations do not exclude further and additive deleterious effects of the progestin itself on liver function particularly since several of the synthetic 19-nor progestins used in these mixtures are alkylated at carbon 17. However, it may be difficult to document with certainty a separate ability of these compounds to impair liver function because of the metabolic transformation of such 19-nor progestins to phenolic A-ring estrogenic steroids in man.³⁶ It is evident in any case from the present study that the capacity of C18 steroids to impair BSP metabolism is structurally determined and does not necessarily correlate directly with the classical estrogenic potency of these compounds. An assessment of the relative roles of "progestins" or "estrogens" in the etiology of hepatic dysfunction caused by contraceptive or other steroid mixtures therefore cannot always be made in relation to the classical hormonal actions of these steroids on sexual tissues but should be based primarily on the molecular characteristics of the individual steroid components in question.

A variety of natural steroids of neutral (compounds XXXI through XXXVII) as well as acidic (compounds XXXVIII through XLII) type were shown in these experiments not to delay hepatic disposal of BSP in the rat. However, certain steroid (bile) acids have in other studies been shown to impair the metabolism of BSP in dogs.³⁷ The inactivity of the bile acids in these experiments with rats therefore may be due in part to their rapid transformation (inactivation) via metabolic pathways peculiar to this species²³ as well as to their relatively lesser potency, as compared with estrogens, for impairing BSP disposal in the test system described. The latter explanation probably accounts for the failure of certain synthetic steroids (XXIX and XXX), which occasionally produce BSP retention when given in large amounts to man, to impair dye disposal

in these experiments. In this respect, it should be emphasized that the structure-activity correlations reported in this study, as in related types of investigations in one man or experimental animals, should be interpreted only in terms of the structural characteristics of the administered compounds. It is evident that the biological potency of the steroids described here may be significantly affected by the particular characteristics of their in vivo pathways of metabolism, or as we have observed in another context,¹⁸ by their in vivo conversion to biologically active metabolites.

Several lines of evidence from the present study support the view¹⁹ that the mechanism of the estrogen action on hepatic disposal of BSP does not involve the conjugation reaction of this dye. For example, significant amounts of conjugated BSP were found in the plasma of estrogen treated rats as described above (Methods and Results). Although a direct comparison of plasma levels of BSP conjugates in control and estrogen treated animals was not possible, the presence of such conjugates attests per se to entry of some dye into hepatic cells and the apparent intactness of the BSP-conjugating process. Direct estimation of the latter process in liver homogenates confirmed that the activity of the BSP-GSH conjugating enzyme system was unimpaired in estrogen treated animals; moreover hepatic content of GSH in such animals was not different from that of controls. Finally, the estrogen-induced delay in plasma disappearance of phenol-3,6-dibromophthalein disulfonate, despite the fact that it is not conjugated during hepatic transit,¹⁷ excludes a major effect of these hormones on dye conjugation and suggests that the impairment in BSP disposal induced by estrogens involves a decrease in the net rate of hepatic removal from plasma of both free, as well as the conjugated form of the dye. Recent studies from this laboratory³⁸ confirm that the net hepatic removal from the circulation of both free as well as conjugated BSP is similarly impaired by these hormones in man.

ACKNOWLEDGMENTS

Computations relating to certain of these data were accomplished through the kind assistance of Dr. Carol Newton and the Biological Sciences Computation Center, University of Chicago under USPHS Grant FR-00013 from the Division of Research Facilities and Resources of the National Institutes of Health.

We wish to express our appreciation to Dr. H. A. Brown Dunning, Jr., Hynson, Westcott and Dunning, Inc., for a gift of phenol-3,6-dibromophthalein disulfonate, and to the following for generously providing samples of the various steroids used in this study: Professors Elwood Jensen and Josef Fried, The University of Chicago; Drs. T. F. Gallagher and Jack Fishman, The Institute for Steroid Research, Montefiore Hospital; Drs. John Babcock and John Pike, The Upjohn Company; Dr. Oscar Wintersteiner, The Squibb Institute for Medical Research; Dr. A. Zaffaroni, Syntex, S. A.; and Dr. J. K. McGowan, G. D. Searle and Company.

We are indebted to Miss Evelyn Damgaard and Miss Elizabeth Laves for invaluable technical help; and to Mrs. G. LaPinska and Mrs. F. J. Skozen for assistance in preparation of the manuscript.

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JUVENILE GOUT WITH OVERPRODUCTION OF URIC ACID: BIOCHEMICAL EVIDENCE FOR A DISTINCT TYPE OF PRIMARY GOUT*

By

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In recent years several reports,¹⁻³ have called attention to a disorder occurring in the pediatric age group which is characterized by marked overproduction of uric acid. The clinical features include hematuria and uric acid stone disease, and often, but not always, mental retardation, neurologic signs, and self-mutilation. Intravenous injection of glycine-¹⁴C into such patients results in a rapid incorporation¹ of isotope into urinary uric acid, signifying the presence of a shunt pathway whereby precursor is incorporated into uric acid without prior incorporation into nucleic acid purines.

Adult patients with gout characterized by overproduction of uric acid exhibit a similar, but less marked pattern of incorporation of glycine into uric acid.^{4,5} Recently, it has been shown that in the adult type of gout the shunt pathway can be abolished by administration of the synthetic purines azathioprine (Imuran)⁵ and allopurinol (Zyloprim).⁶

This communication reports on uric acid dynamics in a case of juvenile gout, and the failure of azathioprine and allopurinol to suppress de novo purine biosynthesis. The patient was a 13-year-old boy who had had recurrent uric acid stone disease and hematuria since the age of 3. Except for mild choreiform movements in the outstretched arms and hands, and an area of self-induced alopecia, he presented no neurologic signs.

The size of the miscible pool of uric acid and its rate of turnover were determined after intravenous injection of uric acid-2-¹⁴C.⁵ The incorporation of intravenously administered glycine-1-¹⁴C was determined thrice; first during a period without treatment, and subsequently during treatment with azathioprine (4 mg per kg b.w. per day) and allopurinol (400 mg per day). Glycine-1-¹⁴C was injected 96 h after treatment with azathioprine had begun, and on the twelfth day of allopurinol treatment.

Table 1 summarizes the results of the studies. The daily production of uric acid was markedly enhanced to 2,396 mg per day, which, when corrected for body weight, represents a value of 42.8 mg per kg body weight. The normal endogenous production of uric acid is about 10 mg per kg daily. In contrast to the situation in adult gout with overproduction of uric acid, azathioprine did not reduce either the plasma uric acid level or the urinary excretion of uric acid. Allopurinol produced dramatic reduction in both plasma and urinary uric acid, but simultaneously, there was an increase in the precursors of uric acid so that the total excretion of urinary oxypurines remained constant.

The strikingly accentuated incorporation of glycine into urinary uric acid may be compared with a normal incorporation of 0.1 to 0.15 per cent of the injected dose after seven days. The concentration of ¹⁴C in urinary uric acid during the three glycine incorporation studies is de-

* This paper appeared in *Nature*, 213:1122, 1967.

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Table 1

SUMMARY OF DATA ON URIC ACID METABOLISM IN A CASE OF JUVENILE GOUT,
DURING A CONTROL PERIOD, AND DURING PERIODS OF TREATMENT
WITH AZATHIOPRINE AND ALLOPURINOL

	Control study	Azathioprine study	Allopurinol study
Miscible pool of uric acid (mg)	2,695		
Turnover of uric acid (mg/day)	2,396		
Mean plasma uric acid (mg %)	12.45	13.06	5.15
Mean urinary uric acid (mg/day)	1,741	1,778	344
Mean urinary uric acid formed from precursors (mg/day)	123		1,787
Mean urinary hypoxanthine (mg/day)	65		780
Mean urinary xanthine (mg/day)	38		767
Cumulative recovery of glycine- ^{14}C in urinary uric acid, 7 days (% of dose)	1.350	1.370	.238

Figure 1. The concentration of ^{14}C in urinary uric acid following intravenous injection of 100 μc of glycine-1- ^{14}C at zero time into a patient with primary juvenile gout, during a control period, and during treatment with azathioprine and allopurinol. The stippled curve on the left depicts the normal glycine incorporation pattern.

picted in Figure 1. Although the maximum isotope concentration occurred later when azathioprine and allopurinol were given than in the control study, the shunt pathway was not suppressed by these compounds, and the cumulative recovery of ^{14}C in uric acid and its precursors was remarkably similar in all three studies. With allopurinol, the cumulative recovery of ^{14}C in urinary uric acid was decreased, but when this figure was corrected for the simultaneous increase in hypoxanthine and xanthine the incorporation of glycine- ^{14}C into all oxypurines was comparable to the control value.

There is growing evidence for the view that purine biosynthesis is controlled by feedback inhibition. Wyngaarden and associates⁷ have shown that glutamine phosphoribosylpyrophosphate amidotransferase, the synthesizing enzyme that catalyzes the first reaction in purine biosynthesis, has at least two separate regulatory sites, one for adenylyl and one for guanylyl ribonucleotides. The results of the studies presented here indicate that there is a distinction between the metabolic pathways in juvenile and adult patients with overproduction of uric acid. They imply that in juvenile gout glutamine phosphoribosylpyrophosphate amidotransferase is insensitive to its normal allosteric feedback inhibition; it is conceivable that this may be related to structural alterations in those subunits of the enzyme concerned with the binding of normal inhibitors.

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THE EFFECTS OF PENICILLAMINE THERAPY ON URIC ACID METABOLISM IN WILSON'S DISEASE*

By

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Hypouricemia is a significant manifestation of Wilson's disease. Its occurrence has been commented upon several times in the clinical literature dealing with this disorder, but its actual incidence and its relation to the activity of the disease or to its therapy have not been examined in detail. Certain aspects of uric acid metabolism in Wilson's disease were studied in the laboratories of Talbott¹ and Wintrobe² during the 1950's. The miscible pools of uric acid were smaller than normal in two patients, and following intravenous loading with uric acid, several other patients showed cumulative uric acid excretions that were greater and more rapid than normal. These results were interpreted as reflecting inability of the renal tubule to reabsorb uric acid efficiently in this disorder.

The present study was undertaken to reexamine uric acid metabolism in Wilson's disease using ¹⁴C-labeled uric acid; and especially to study the effects on uric acid dynamics of treatment with the copper-chelating agent, penicillamine.

Three patients with Wilson's disease having significant hepatic and/or cerebral involvement were studied before and after prolonged therapy with penicillamine. In addition, the acute effects of penicillamine on the renal clearance of uric acid were studied in two healthy control individuals.

The semi-logarithmic plots of the isotope dilution curves in the three patients before and after treatment are shown in Figure 1. Subject M.H. was a teen-age girl who presented with acute onset of neurologic symptoms. Subject C.H. was a younger asymptomatic sister of M.H. who, on family search, was discovered to have the disease. Both had major abnormalities of liver function, and cirrhosis and increased copper content on liver biopsy. Subject E.Z., studied through the courtesy of Drs. Schulman and Reilly,[†] was an unrelated adult with significant hepatic and neurological complications of the disorder.

The intravenous injection of labeled uric acid, and the subsequent determination of the rate at which the isotope content of urinary urate is diluted (see Figure 1), permit us to calculate the total miscible pool of uric acid in the body and the quantity of newly formed urate introduced into this pool daily.³

The urate dilution curve of a normal subject is shown in the left chart for comparison purposes. The isotope dilution curve for patient M.H. during the pre-treatment period, as shown by the open circles, is characterized by a very high isotope concentration at zero time, indicating a small pool, and a much more rapid isotope dilution in urinary uric acid as compared with the normal. Following nearly two years of treatment with penicillamine the isotope dilution curve, indicated by the closed circles, was significantly altered toward normal. The urinary uric acid

* This paper appeared in Trans. Assoc. Amer. Physic., 79:157, 1966.

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Figure 1. Semilogarithmic plots of the isotope concentration in urinary uric acid versus time for three patients with Wilson's disease, before and after treatment with penicillamine.

dilution curves of patients C.H. and E.Z. showed similar characteristics before and after treatment with penicillamine. The change in the dilution curve in the asymptomatic patient C.H. was not as pronounced as that in her sister, but her treatment period was shorter and the severity of her disease, as reflected in deranged urate metabolism and other physiologic indices, was also not as pronounced as in patient M.H. The alteration in the post-treatment isotope dilution curve of patient E.Z. was striking, and undoubtedly reflects the effects of the much longer duration of his treatment.

Figure 2 shows the rate of excretion of uric acid- ^{14}C and the cumulative urinary recovery of injected ^{14}C -labeled uric acid in these three patients, and compares them with a typical normal excretion curve of labeled urate, shown in the left chart. Under normal circumstances, approximately two-thirds of the uric acid produced is excreted in the urine—the remainder being disposed of via the gastrointestinal tract.³ In subject M.H., the extremely rapid appearance of uric acid- ^{14}C in urine and the almost quantitative recovery of injected uric acid in the pre-treatment period shown in the uppermost curve on the left, are evident. The sequential changes toward normal that follow penicillamine therapy in this pattern of urate disposition are also clear. These curves depict the lessening in both the rate of excretion, as well as the total urinary recovery of injected uric acid which developed concurrently with the clinical remission.

The changes in patient C.H. were similar but much less pronounced, probably for the reasons indicated earlier. The changes in the uric acid excretion curve following prolonged penicillamine treatment in patient E.Z. were dramatic, and brought these indices of urate metabolism very close to the normal range.

Figure 2. Cumulative urinary recovery of uric acid- ^{14}C injected intravenously into three patients with Wilson's disease, before and after prolonged treatment with penicillamine.

Table 1 presents quantitative data on a variety of parameters of urate metabolism before and after penicillamine therapy in these three subjects. The pre-treatment findings were similar in each patient and were characterized, as seen in columns 1, 2, and 3, by low plasma levels of uric acid; slightly enhanced total urate excretion; and, especially in patients M.H. and E.Z., markedly increased renal uric acid clearance. Column 4 shows the strikingly reduced size of the pre-treatment miscible pool of uric acid in these patients. The values may be compared with an expected normal pool size of approximately 700 to 1,200 mg.

Although the daily turnover rate of uric acid shown in column 6 was, during the pre-treatment period, several times higher than normal, this relation only reflects the small size of the miscible pool in these subjects. In fact, as shown in column 5, the daily production of uric acid in these patients was entirely normal relative to body weight. Thus the disturbances in urate metabolism in Wilson's disease are not attributable to abnormal endogenous formation of uric acid.

In these subjects, renal disposal of uric acid constituted a much larger fraction of total elimination than normal during the pre-treatment period, as shown in the data in columns 7 and 8.

Prolonged penicillamine therapy induced a dramatic and sustained clinical remission in each patient; and concurrent with this remission, the abnormalities in uric acid metabolism reverted toward normal.

Thus the plasma urate increased substantially, as did the total body pool of uric acid in each patient; in subject E.Z. this increase was nearly 3-fold, bringing the pool size close to, if not

Table 1

SUMMARY OF DATA ON URIC ACID METABOLISM IN THREE PATIENTS
WITH WILSON'S DISEASE, BEFORE AND AFTER TREATMENT
WITH PENICILLAMINE

	M.H. ♀		C.H. ♀		E.Z. ♂	
	Control 1963	R _x 1965	Control 1963	R _x 1965	Control 1959	R _x 1965
1 Plasma uric acid, mg %	.88	2.98	2.40	3.36	2.21	3.67
2 Urine uric acid, mg/day	430	471	328	368	690	536
3 Uric acid clearance, cc/min	34	14	13	10	24	11
4 Uric acid pool, mg	217	534	372	523	370	1000
5 Daily turnover, mg	477	554	413	476	720	813
6 Daily turnover, % of pool	220	104	111	91	195	81
7 Urine uric acid, % of turnover	90	85	80	77	96	66
8 Recovery of uric acid- ¹⁴ C, % of dose	96	83	78	75	87	74
9 Body weight, kg	51	50	34	41	70	69

Figure 3. Absence of effect of penicillamine on plasma and urinary uric acid in two normal subjects maintained on a constant diet.

within, the normal range. As expected, urate turnover was not altered; but the renal clearance of uric acid decreased, and markedly so, in patients M.H. and E.Z. Further, both the rate of appearance and the cumulative recovery of injected uric acid in the urine diminished toward the normal range.

This progression toward normal of the deranged urate metabolism in Wilson's disease following penicillamine therapy cannot be attributed to a direct effect of this agent upon the renal handling of uric acid, since all studies were conducted during periods when the drugs had been temporarily discontinued. Furthermore, as shown in Figure 3, equivalent amounts of penicillamine administered to normal subjects in short-term experiments did not affect either plasma uric acid levels or urate excretion.

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EFFECT OF MAGNESIUM DEFICIENCY ON THE LOCATION OF THE INTESTINAL ABSORPTION OF MAGNESIUM IN RATS*

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The absorption of magnesium is increased in young rats fed a diet low in magnesium.¹ In the following studies, ²⁸Mg was used to determine whether this alteration is primarily associated with a local or general enhancement in the uptake of magnesium from the alimentary tract.

METHODS

Non-fasting male albino rats (Sprague-Dawley strain) weighing 90 to 110 g at the beginning of the experiments were housed in individual metabolism cages that provided separation of the urine and the feces. The animals were fed either a low magnesium or a magnesium supplemented (control) diet¹ and distilled water ad libitum.

Magnesium-28 as ²⁸MgCl₂ with a specific activity of 16 to 20 μ C of ²⁸Mg-²⁸Al per mg of stable magnesium at the time of administration was obtained from Brookhaven National Laboratory. Detailed descriptions of the equipment and methods have been presented elsewhere.^{1,2} In all the experiments, each animal received approximately 2 μ C of ²⁸Mg in 0.5 ml of 5 per cent dextrose in water containing an additional 3 mg of magnesium as MgCl₂·6H₂O. The extra magnesium was added to compensate, in part, for the much greater dilution of specific activity of the ²⁸Mg which would otherwise occur in the bowel of the control rats due to ingested dietary magnesium. Except for net plasma counts lower than 100 cpm, the per cent probable error of the net counting rate was never greater than 3 per cent and usually 2 per cent or less.

Unless otherwise noted, the animals were anesthetized with ether during the administration of ²⁸Mg and operative procedures. All blood samples were obtained by aortic exsanguination using heparin as an anticoagulant. Plasma magnesium levels were determined in duplicate on a Beckman B spectrophotometer by the titan yellow method.^{3,4}

Except for the multiple comparison analysis of Scheffe,⁵ statistical methods were taken from Snedecor.⁶

Experiment 1. The absorption of ²⁸Mg after direct injection through the bowel wall into the gastric or colonic lumen. Two studies of identical design were performed on a total of 51 animals, and the results were combined.

The rats were divided into 4 groups and placed on the magnesium supplemented diet. After an initial period of adjustment, 2 of the groups were transferred to the low magnesium diet. Four days later, 0.5 ml of the solution containing the ²⁸Mg was injected directly into either the gastric or cecal lumen of each animal using a No. 28 needle through an abdominal incision. (Preliminary studies in which carmine red dye was used as an indicator showed that there was no loss through the puncture site.) During injection into the cecum, the ileum was gently compressed at the ileocecal junction to prevent any initial large reflux of the isotope. The abdomen was closed with sutures and metal clips, and feces were collected over the next 44 hours. Although

* This paper appeared in Proc. Soc. Exptl. Biol. Med., 123:836, 1966.

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plasma magnesium levels were not measured in this part of the study, animals in many previous experiments¹ fed the same magnesium supplemented ration remained clinically and, in terms of plasma electrolyte levels, chemically normal. When normal rats were transferred to the low magnesium diet, hypomagnesemia developed within 16 hours; by 33 to 38 hours at the latest, the absorption of ²⁸Mg plus 0.2 to 5.2 mg of carrier rose to levels comparable to those observed after 14 days of magnesium deprivation; and peripheral vasodilatation—a diagnostic feature of hypomagnesemia if it develops during the initial phase of magnesium deficiency—appeared regularly on the fourth to fifth day. It may be assumed, therefore, that these changes also occurred in the present experiment, especially since most of the rats on the low magnesium diet developed mild vasodilatation by the fourth day.

Experiment 2. The absorption of ²⁸Mg in control and deficient rats after the administration of the radioisotope by gavage. Thirty-eight rats were divided into 2 groups; one group was placed on the control, and the other on the low magnesium diet. Peripheral vasodilatation appeared in all the deficient animals by the fifth day. On the sixth day, each rat received 0.5 ml of the solution containing the ²⁸Mg plus 3.1 mg of carrier. Under light nembutal anesthesia, single control and hypomagnesemic rats were killed by aortic exsanguination at the following approximate times after injection: 3/4, 1, 1-1/2, 2, 2-1/2, 3-1/4, 3-3/4, 4-1/4, 4-3/4, 5-1/4, 6, 7, 8-1/4, 9-1/4, and 10-1/2 hours. In addition, two rats from each group were killed at 24 hours; and the feces of the remaining animals were collected for a total period of 94 hours. A 2.0 ml aliquot of heparinized plasma was saved for counting and, in some cases, for magnesium determinations. The entire bowel of an animal was removed and divided into the following sections: colon, distal 30 cm of ileum, and remaining proximal bowel including the stomach. The intestinal segments and the feces passed per anum were placed in individual Erlenmeyer flasks containing about 10 ml of water. Because the low magnesium diet is constipating, the formed fecal pellets in the distal colon from the 24-hour deficient rats were included with the stool collections. The contents of the urinary bladder were added to previously voided urine, and the remaining carcass saved in thin cellophane bags.

In addition to 4 standards (equal in volume to those administered to the rats) in Erlenmeyer flasks, a fifth standard was placed in a lucite container with an internal volume approximately equal to that of the carcass of a 120 g rat. After an initial assay of the radioactivity in each standard, 2 were diluted to 10 ml to serve as standards for the bowel sections and feces; and one to 50 ml for the urines. The carcass standard in the lucite holder was diluted to about 120 ml. To minimize any differences in counting geometry, the tissue, fecal and urinary samples along with their respective standards were counted inside the previously described 6 x 6 x 6 foot iron cell about one foot beneath the center of a square formed by four 5 x 5 inch NaI crystals. The activity in each carcass was assayed by a method used for total-body counts of live animals.² The plasma samples were counted in a separate well-crystal. The per cent of administered dose recovered in each specimen = $(\text{net cpm in the sample at } T_N \div \text{net cpm in the standard } T_N) (\text{net cpm in the standard } T_O \div \text{mean net cpm in all standards } T_O) \times 100$.

Results. The absorption of ²⁸Mg after direct injection into the bowel. As shown in Table 1, magnesium was absorbed in the colon. In both dietary groups, 15 to 16 per cent more of the initial dose was taken up after direct injection into the stomach than into the colon ($P < 0.01$ by Scheffe analysis); and, regardless of the site of administration, the magnesium deficient animals absorbed 24 to 25 per cent more ²⁸Mg than the controls ($P < 0.01$).

Table 1

THE ABSORPTION OF ^{28}Mg PLUS 3 mg OF CARRIER MAGNESIUM INJECTED
DIRECTLY INTO THE LUMEN OF THE BOWEL OF NORMAL
AND MAGNESIUM DEFICIENT RATS^a

Site of injection	Control		Magnesium deficient	
	Number of animals	Initial dose absorbed (%) (Mean $\pm$ S.E.M.)	Number of animals	Initial dose absorbed (%) (Mean $\pm$ S.E.M.)
Stomach	11	57.5 $\pm$ 3.39	13	82.5 $\pm$ 1.35
Caecum	14	42.3 $\pm$ 2.19	13	66.5 $\pm$ 2.52

^aBased on the 0 - 44 hour recovery of ^{28}Mg in the feces, uncorrected for endogenous excretion.

The absorption of ^{28}Mg administered by gavage. The average recovery of administered radioactivity per rat was 100.1 ± 0.45 per cent (mean $\pm$ S.E.M.). For comparison, each set of data was expressed as per cent of the total activity recovered in the animal from which it was taken. The amount of isotope absorbed was calculated to be equal to that found in the carcass and urine, and the results in Tables 2 and 3 are arranged in order of increasing absorption. The correlation of ^{28}Mg absorption with the location of radioactivity in the contents of the various segments of bowel from individual rats killed at different times after administration of the isotope is assumed to represent the intestinal uptake of magnesium that would normally occur in a single animal as the bolus of ^{28}Mg passed through the gut.

In the normal rats (Table 2), the amount of radioisotope absorbed appeared to increase abruptly as the radioactivity in the distal ileum rose (rats 4 through 6) and, again, after large amounts of ^{28}Mg had moved into the colon (rat 7 through 10). Based on a mean uptake of 46.6 ± 1.9 per cent (mean $\pm$ S.E.M.) for the group, * at least 10 per cent of the initial dose was absorbed before the isotope reached the colon (rats 1 through 4), and 19 per cent was absorbed in the colon (rats 11 through 17). In rats 2 through 5, the plasma magnesium was 1.84 ± 0.08 mEq per liter (mean $\pm$ S.D.).

In the magnesium deficient rats, the mean plasma magnesium was 0.69 ± 0.10 mEq per liter (rats 1, 3, 4, and 5). As shown in Table 3, at least 21 per cent of the radioisotope was absorbed before reaching the colon (rats 1 through 6); and, based on a mean absorption for the group of 72.8 ± 1.38 per cent (mean $\pm$ S.E.M.), * 35 per cent was absorbed in the colon (rat 11). It is doubtful that any significant difference in the relative distribution of absorption of magnesium occurred as the result of magnesium deficiency.

DISCUSSION

Magnesium is absorbed throughout the bowel of young rats fed a stock laboratory diet. Quantitatively, the most important site of absorption in vivo appears to be the colon.⁷ These results

*The mean absorption for the group was calculated from the results in animals 16 and 17 and the recovery of isotope from the feces of the two animals which were followed for 94 hours. The latter values were corrected for intestinal excretion of the absorbed ^{28}Mg by a previously described method.¹ This correction amounted to 3.4 per cent of the initial dose in the control and 5.2 per cent in the deficient rats.

Table 2

THE ABSORPTION OF ^{28}Mg PLUS 3 mg OF CARRIER MAGNESIUM ADMINISTERED BY GAVAGE
TO YOUNG RATS FED THE CONTROL DIET

Rat No.	Time in hours after injection	Radioactivity absorbed ^a (%)	% of recovered radioactivity found in:						Plasma radioactivity (cpm per 2 ml)
			Urine	Carcass	Proximal gut ^{b,c}	Terminal 30 cm of small bowel ^c	Colon ^c	Feces passed per anum ^d	
1	3/4	1.8	0.8	1.0	98.2	0.0	0.0	0.0	80
2	1-1/2	4.6	0.6	4.0	85.4	10.3	0.1	0.0	955
3	1	4.8	0.0	4.8	85.5	10.3	0.0	0.0	1048
4	2	10.1	1.8	8.3	46.5	43.2	0.2	0.0	1479
5	2-1/2	14.9	2.2	12.7	11.5	60.8	12.8	0.0	1770
6	3-1/4	15.4	2.8	12.6	4.7	28.4	51.5	0.0	1370
7	4-1/4	21.4	5.4	16.0	3.1	10.5	65.1	0.0	1500
8	8-1/4	22.5	3.3	19.2	1.6	0.5	75.1	0.3	1044
9	3-3/4	22.9	4.3	18.6	3.4	31.6	41.5	0.0	1974
10	4-3/4	24.7	6.5	18.2	13.2	7.2	54.9	0.0	1849
11	7	27.5	8.7	18.8	1.6	0.7	71.0	0.0	1044
12	5-1/4	27.8	6.2	21.6	6.1	8.2	58.7	0.0	1744
13	9-1/4	28.3	9.0	19.3	2.1	1.9	67.8	0.0	1528
14	6	28.5	6.7	21.8	3.3	3.0	65.2	0.0	1398
15	10-1/2	30.0	10.3	19.7	1.3	1.0	67.2	0.0	1343
16	24	42.0	18.2	23.8	e	e	16.6	41.4	f
17	24	46.7	18.4	28.3	e	e	12.5	40.7	f

^aEqual to the per cent of recovered ^{28}Mg in the carcass plus the urine.

^bEntire intestinal tract from the esophagus to the terminal 30 cm of small bowel.

^cIncludes intestinal wall and luminal contents.

^dThe figure 0.0 indicates that no feces were passed during the indicated interval or that the activity was < 0.1%.

^eIncluded with recovered activity in the carcass.

^fCount not taken.

Table 3

THE ABSORPTION OF ^{28}Mg PLUS 3 mg OF CARRIER MAGNESIUM ADMINISTERED BY GAVAGE
TO YOUNG MAGNESIUM DEFICIENT RATS

Rat No.	Time in hours after injection	Radioactivity absorbed ^a (%)	% of recovered radioactivity found in:						Plasma radioactivity (cpm per 2 ml)
			Urine	Carcass	Proximal gut ^{b,c}	Terminal 30 cm of small bowel ^c	Colon ^c	Feces passed per anum ^d	
1	1	1.6	0.0	1.6	95.8	2.7	0.0	0.0	361
2	3/4	1.9	0.0	1.9	98.1	0.0	0.0	0.0	45
3	1-1/2	9.6	0.0	9.6	42.5	47.5	0.2	0.0	1403
4	2-1/2	10.9	0.0	10.9	47.0	41.8	0.3	0.0	1366
5	2	15.5	0.0	15.5	40.4	43.9	0.5	0.0	1674
6	3-1/4	20.7	0.8	19.9	15.2	63.6	0.5	0.0	2618
7	3-3/4	22.5	1.9	20.6	6.0	7.8	63.6	0.0	g
8	4-1/4	30.0	2.8	27.2	13.8	49.5	6.6	0.0	1885
9	4-3/4	32.0	2.5	29.5	4.9	13.3	49.6	0.0	2148
10	5-1/4	34.1	1.3	32.8	5.2	11.9	48.8	0.0	1855
11	7	37.9	1.1	36.8	4.5	3.4	53.2	0.0	1836
12	6	38.5	3.3	35.2	(4.6) ^e	2.2	54.7	0.0	1661
13	8-1/4	42.2	2.9	39.3	4.9	1.6	51.3	0.0	1317
14	10-1/2	43.7	3.4	40.3	4.4	2.1	49.8	0.0	1081
15	9-1/4	53.1	6.5	46.5	5.1	1.7	40.1	0.0	1158
16	24	69.1	5.8	63.3	f	f	4.4	26.5 ^h	g
17	24	72.2	5.3	66.9	f	f	2.8	24.9 ^h	g

^aEqual to the per cent of recovered ^{28}Mg in the carcass plus the urine.

^bEntire intestinal tract from the esophagus to the terminal 30 cm of small bowel.

^cIncludes intestinal wall and luminal contents.

^dThe figure 0.0 indicates that no feces were passed during the indicated time interval or that the activity was < 0.1%.

^eSpecimen lost. Activity based on an assumed recovery of 100% of the initial dose.

^fIncluded in recovered activity in the carcass.

^gCount not taken.

^hSee text for details.

are confirmed in the present studies in both the control and magnesium deficient animals. Previous reports indicate that the small intestine is the major site of uptake for dietary magnesium,⁸⁻¹² although there is evidence substantiating absorption in the large bowel.¹³⁻¹⁷ Species differences in the rate at which food traverses the alimentary tract make comparisons and interpretations of these data difficult. Such variations alter the duration of contact between luminal magnesium and the absorptive surfaces in any given segment of the bowel, and, assuming that the mineral enters the mucosal cells in an ionic form, modify the interaction of magnesium with those intraluminal factors that may change the chemical state of magnesium, rendering it unavailable for absorption. In the rat, food moves relatively rapidly through the small intestine and then pools in the proximal colon for several hours, and this may represent a partial explanation for the distribution of absorption of ²⁸Mg which was observed in these animals.

The enhanced uptake of magnesium in the hypomagnesemic rat appears to be distributed throughout the gut so that the normal pattern of absorption is basically unchanged. At the present time, no relevant data are available to explain the mechanisms underlying this specific alteration.

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RESTORATION OF THE IMMUNE RESPONSE BY CIRCULATING LYMPHOCYTES*

By

D. C. Cannon[†] and R. W. Wissler

Intravenous antigen injection results in marked hyperplasia of the spleen, which has been described in various species, but is particularly well shown in the rat following a single injection of a particulate antigen such as typhoid vaccine.¹⁻³ The cellular proliferative pattern in the rat spleen is characterized by large, actively mitotic, pyroninophilic cells in the red pulp, the proliferation of which has been correlated with antibody formation.⁴⁻⁶ The fluorescent antibody technique has demonstrated that similar cells in the rabbit actually contain antibody.⁷ Various investigations have indicated that these pyroninophilic or antibody-forming cells originate from reticuloendothelial cells,⁸ from non-phagocytic primitive reticular cells,⁹ from plasmablasts,¹⁰ or from small lymphocytes.¹¹ It has been established that particulate antigen rapidly localizes in phagocytic cells of the splenic red pulp, but these phagocytic cells do not appear to give rise to the antibody-forming cells.¹ Morphological considerations have helped to support the theory that the antibody-forming cells arise from primitive reticular cells with limited, if any, phagocytic ability.^{3,12}

Recently there has been renewed interest in the role of the lymphocyte in the initial events of the immune response. Gowans and co-workers^{13,14} were able to abolish or markedly suppress the immune response of rats to a first injection of antigen by utilizing a thoracic duct fistula to deplete the animals of lymphocytes. They also found that thoracic duct lymphocytes restored the hemolysin response in irradiated rats.¹³ Langevoort,¹¹ furthermore, has presented morphological findings which he interprets as indicating that the red pulp pyroninophilic cells arise directly from white pulp lymphocytes in the rabbit spleen.

In view of these recent reports, a re-examination of the previous concepts about cellular interactions in the early stages of the immune response has been undertaken. The major question considered is as follows: Can circulating lymphocytes influence the development of red pulp hyperplasia and the related humoral antibody response following primary antigen stimulation? An experimental model was devised which allowed evaluation of the ability of circulating autologous lymphocytes to restore immunological reactivity to the spleen whose capabilities in this respect had been greatly suppressed by irradiation. The rat spleen was exteriorized with its vascular pedicle intact and subjected to irradiation while the body was protected by lead shielding. A dose of x-ray was chosen which would result in marked atrophy of lymphoid tissue and inhibition of humoral antibody formation in unshielded control animals. Twenty-four hours later the spleen was again exteriorized. This time the spleen was shielded while the remainder of the body was irradiated with the same x-ray dose as previously. In this way all cells of the body were exposed

* This report is taken from a paper that appeared in Arch. Path., 83:188, 1967.

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to an identical dose of radiation except for those cells that migrated from other tissues of the body to the spleen during the twenty-four hours between irradiations. It was reasoned that if the spleen participates in the recirculating lymphocyte pool as described by Gowans,^{15,16} it should be repopulated with lymphocytes following the first irradiation. The ability of these lymphocytes to restore immunological function to the spleen could then be evaluated by administering antigen after the second irradiation.

MATERIALS AND METHODS

Sprague-Dawley albino male rats, 8 to 9 weeks old and weighing 250 to 310 g, were maintained on laboratory chow and water ad libitum. All surgery was performed under general anesthesia obtained by intraperitoneal chloral hydrate using clean, but not sterile techniques. All animals were subjected to the same surgical manipulations whether they were irradiated or not. The spleen was exteriorized through a left subcostal incision. During irradiation of the spleen alone the rat was confined in a one-half inch thick lead cylinder whose longitudinally bisected halves were joined by hinges. The spleen was exposed by passing its pedicle through a notch on the free margin of one half of the cylinder. Total-body irradiation of the rat with spleen shielding has been described in detail by others.¹⁷ X-rays were generated in a 250 kV G.E. Maxitron unit at 30 Ma with filters of 0.5 mm copper and 1.0 mm aluminum. The half-value layer in copper of the filtered beam was 1.4 mm. Exposures were monitored with a Victoreen condensor meter equipped with a 250 roentgen (R) chamber. The animals were irradiated in groups of three or four on a board revolving in a plane perpendicular to the beam at a rate of 3.5 rpm. The target distance was 75 cm and the average dose rate varied from 54 to 56 R per minute. A single dose of 600 R was administered.

Formalin-killed Salmonella typhi (strain T-29) vaccine was used as antigen. The general methods of antigen preparation and of agglutinin determinations were those described by Cannon et al.^{18,19} Prior to use, the stock vaccine was washed and diluted with normal saline to a standard optical density. A single 0.5 cc dose of the diluted vaccine was injected intravenously into a lateral tail vein. Serum samples were obtained by incision of a tail vein. For agglutinin determinations serial two-fold dilutions of serum were made from an initial 1:20 dilution in the first tube.

Animals were killed by exsanguination under ether anesthesia. Portions of spleen, thymus, and mediastinal, mesenteric, and peripancreatic lymph nodes were fixed in Carnoy's solution. Paraffin sections, cut at 6 μ , were stained with methyl green-pyronin according to a modification of the Unna method⁴ except that a 1.5 minute staining was employed. Femoral bone marrow was fixed in Helly's solution, sectioned, and stained with eosin-azure.

RESULTS

Seventy-five rats were randomly assigned to four experimental groups: (1) The first group, representing the normal immune response, was sham-operated on each of two days prior to antigen injection. (2) The second group received total-body irradiation two days before antigen injection and was sham-operated one day prior to antigen. (3) The third group received irradiation of the exposed spleen with body shielding two days before antigen injection and total-body irradiation with spleen shielding one day before antigen. (4) The fourth group was treated identically to group 3 but received saline instead of antigen. Serum samples for agglutinin determi-

nation were taken from five animals of each of the first three groups immediately prior to antigen injection and at 4, 7, 10, and 17 days. Three animals from each of the four groups were autopsied immediately prior to antigen injection and at 1, 3, 5, and 8 days for histology.

Agglutinin titers. The individual and mean serum agglutinin titers in the three experimental groups which received antigen are shown in Table 1 and the geometric mean titers are com-

Table 1
AGGLUTININ RESPONSE OF VARIOUS TREATMENT GROUPS

Group	Animal No.	Agglutinin titer ^a on day:			
		4	7	10	17
1	1	7	8	6	6
	2	7	7	6	6
	3	6	6	5	5
	4	5	5	6	5
	5	5	7	4	5
	Mean	6.0	6.6	5.4	5.4
2	6	0	0	0	0
	7	0	0	0	0
	8	0	0	0	0
	9	0	0	0	0
	10	0	0	0	0
	Mean	0	0	0	0
3	11	2	6	6	8
	12	1	1	2	5
	13	2	6	5	6
	14	1	4	5	7
	15	2	5	5	7
	Mean	1.6	4.4	4.6	6.6

^aAgglutinin titers are expressed as the last tube showing agglutination. Serial twofold dilutions of serum were made from an initial 1:20 dilution in tube 1. A tube value of 0 was arbitrarily assigned to samples in which no agglutination occurred in tube 1. The actual serum dilution corresponding to a titer thus expressed can be calculated from the formula 10×2^n where n = the tube number.

Group 1—sham-operated normal rats. Group 2—600 R total-body irradiation two days before antigen injection. Group 3—irradiation of the exposed spleen with body shielding two days prior to antigen injection followed by total-body irradiation with spleen shielding one day prior to antigen. 600 R on each occasion.

pared in Figure 1. These results show that considerable protection of antibody formation was obtained by using the double shielding procedure with irradiation as in group 3. The agglutinin response of group 3 differed significantly ($p < 0.001$) from that of the normal control, group 1, only by its lower value on day 4. During the time of the experiment, the unshielded, irradiated animals formed no detectable serum agglutinin.

Histology of the spleen. Sham operation did not significantly alter the normal histology of the spleen, as was evident in the unirradiated animals autopsied immediately prior to antigen injection (Figure 2a). Stimulation of this group with typhoid vaccine resulted in morphological

Figure 1. Agglutinin response following a single intravenous injection of typhoid vaccine. Each point represents the geometric mean titer for a group of five animals. A titer of 10 was arbitrarily assigned to animals whose sera did not show agglutination at the lowest dilution tested. Treatment of the various groups: Group 1—sham-operated normal rats; Group 2—600 R total-body x-irradiation two days before antigen; Group 3—600 R x-irradiation of the exposed spleen with body shielding followed in one day by 600 R total-body x-irradiation with spleen shielding. Typhoid vaccine injected one day after the second dose of irradiation.

changes in the spleen similar to those that have been described in detail by others.¹⁻³ One day after antigen, white pulp changes were prominent, consisting of enlargement of the follicles with loss of architecture of the germinal centers due to dispersal of the component cells throughout the follicles. Red pulp changes were limited to a slightly increased mitotic rate of the large pyroninophilic cells and infiltration of polymorphonuclear neutrophils in the sinusoids. By day 3, large germinal centers with an active mitotic rate had reappeared in the greatly hypertrophied lymphoid follicles, a pattern that was maintained throughout the interval studied. Red pulp hyperplasia, which was well-developed by day 3, was even more prominent on day 5 as shown in Figure 2b. At this time aggregates of lymphocytes, which have been termed small "dark" cells,³ were numerous in the red pulp. By day 8 there was a striking decline in the red pulp population of large pyroninophilic cells concomitant with a further increase in small "dark" cells.

The spleens of the animals irradiated without shielding were severely atrophic two days after irradiation, i.e., immediately prior to antigen injection (Figure 3a). The effects of irradiation on the spleen have been described elsewhere.²⁰ Particularly noticeable was the depletion of lymphocytes, only a few of which remained in the barren follicular stroma. Polymorphonuclear neutrophilic leukocytes were exceptionally numerous in the sinusoids in the first few days after

Figure 2. Spleens of sham-operated rats receiving intravenous typhoid vaccine. (a) Immediately prior to antigen injection. (b) Five days after antigen. The marked red pulp hyperplasia is typical. Methyl green-pyronin stain, X152.

a

b

Figure 3. Spleens of rats subjected to 600 R total-body x-irradiation two days prior to receiving intravenous typhoid vaccine. (a) Immediately prior to antigen injection. (b) Five days after antigen. Antigen stimulation results in only minimal histologic alteration of the severely atrophic spleens within this time period. Methyl green-pyronin stain, X152.

irradiation. The first histological evidence of a response to antigen was not observed until five days after injection, when small clusters of pyroninophilic cells appeared in the periarteriole sheaths (Figure 3b). These cells were somewhat more numerous by day 8, at which time a few small "dark" cells were also present.

The two groups of animals (Groups 3 and 4) irradiated with the double shielding procedure showed a remarkable degree of cellular repopulation of the spleen within one day after completion of irradiation, i.e., immediately prior to antigen injection (Figure 4a). The cellular repopulation was particularly prominent in the white pulp. The group injected with antigen (Group 3) developed a marked red pulp hyperplasia which, although somewhat delayed in development, was almost indistinguishable from that of the immunized unirradiated animals on day 5 (Figure 4b) and thereafter. Numerous lymphocytes appeared later in the red pulp in close association with the newly proliferated pyroninophilic cells, as in the typical response to typhoid vaccine. In contrast to the prominence of red pulp hyperplasia later in the immune response, the earliest distinct changes following antigen administration were in the white pulp. Immediately prior to antigen injection, the white pulp follicles were composed of a rather uniform population of lymphocytes, the germinal centers having been destroyed by irradiation (Figure 5a). One day after antigen, however, large pyroninophilic cells had become conspicuous in the repopulated follicles and many were in mitosis (Figure 5b). In addition, many of the "lymphocytes" exhibited an increased amount of cytoplasm. Other cells had enlarged nuclei with alteration of the clumped chromatin to produce a diffuse reticular or vesicular pattern accompanied by prominent nucleoli. These cells appeared to be morphologically intermediate between lymphocytes and the large pyroninophilic cells found normally in the germinal centers or in the red pulp following immunization. By day 3, when distinct red pulp hyperplasia was present, germinal centers had reappeared. At subsequent intervals the white pulp mass remained significantly less than in the unirradiated animals.

The animals subjected to the double irradiation-shielding procedure and injected with saline (Group 4) exhibited a slight red pulp hyperplasia which was appreciably less than in those that received antigen. This mild hyperplasia has been correlated with irradiation effects on the gastrointestinal tract.²¹ Also in contrast to the animals receiving antigen, the germinal centers did not reappear until day 5 or later.

Histology of other tissues. The unirradiated animals showed no morphological changes in the lymph nodes, thymus, or bone marrow which could be correlated with antigen injection. The changes in these tissues following irradiation were essentially as those described in detail elsewhere.²⁰

The lymph nodes of the irradiated, unshielded animals had minimal regeneration three days after antigen, i.e., five days after completion of irradiation. The severe atrophy of the cortex, with absence of germinal centers, persisted throughout the period of study.

In contrast, the lymph nodes in the animals subjected to the double irradiation-shielding procedure were rapidly repopulated by lymphocytes. Germinal centers, which were absent immediately prior to antigen injection, were regenerating by day 3. On day 8, the lymph nodes had an advanced degree of cellular restoration, a slightly decreased number of lymphocytes in the cortex being the only difference from the unirradiated animals. Regeneration was somewhat less rapid in the animals treated with the double irradiation-shielding procedure and subsequently injected with saline.

a

b

Figure 4. Spleens of rats subjected to 600 R x-irradiation of the exposed spleen with body shielding followed in one day by 600 R total-body x-irradiation with spleen shielding. Typhoid vaccine administered intravenously one day after the second dose of irradiation. (a) Immediately prior to antigen injection. Lymphocyte repopulation is particularly prominent in the white pulp. (b) Five days after antigen. The well developed red pulp hyperplasia approximates that of the unirradiated animals (Figure 2). Methyl green-pyronin stain, X152.

Figure 5. Changes in the white pulp follicles of rats subjected to 600 R x-irradiation with the double shielding procedure. (a) One day after completion of irradiation and immediately prior to intravenous typhoid vaccine. The follicle consists almost entirely of "small" lymphocytes. (b) One day after antigen injection. Numerous large pyroninophilic cells and apparent transition forms between lymphocytes and large pyroninophilic cells are present. Two mitoses are indicated by arrows. Methyl green-pyronin stain, X920.

The thymus was identical in irradiated animals regardless of shielding procedures or antigen administration. Regeneration was first evident about five days after antigen and was progressing actively by eight days.

Bone marrow regeneration was distinctly more rapid in irradiated animals subjected to the double shielding procedure than in irradiated unshielded animals. In the two shielded groups no difference could be detected between antigen-stimulated and saline-injected animals. Normal cellularity was not completely restored by day 8.

COMMENT

The results indicate that substantial protection of lymphoid cellularity and antibody-form-

ing capacity in the rat is obtained by using the double shielding procedure during irradiation. This phenomenon can be explained in terms of the recirculating lymphocyte pool as described by Gowans.^{15,16} Following irradiation of the exposed spleen with body shielding lymphocytes from the blood repopulate the spleen, localizing chiefly in the white pulp. After subsequent irradiation with spleen shielding some of these lymphocytes re-enter the blood to be redistributed to the irradiated lymph nodes and bone marrow. The irradiated thymus is not repopulated by the recirculating lymphocytes, the histological pattern being identical whether or not shielding is employed. This is in agreement with the results of Gesner and Gowans²² who found considerable repopulation of the lymph nodes and splenic white pulp, but not of the thymus, following injection of isologous thoracic duct lymphocytes into irradiated mice. Jacobson et al.,²³ however, described repopulation of the mouse thymus following 950 R total-body irradiation with Peyer's patch shielding. This repopulation of the thymus apparently did not occur until six days after irradiation, which is the approximate time at which we noted regeneration of the thymus. It seems likely, therefore, that the phenomenon described was actually an accelerated regeneration of intrinsic thymic cells. The thymus thus seems to be unique among the lymphoid tissues studied in the rat in that it does not appear to receive cells from the recirculating lymphocyte pool.

The double shielding procedure largely protects the ability to produce antibody in response to an intravenous particulate antigen after irradiation. Compared with unirradiated animals, the only significant difference in the circulating agglutinin levels of animals subjected to irradiation with the double shielding procedure was a slower rise to peak titer. This would seem to indicate that irradiation inhibits a cell type or perhaps a cellular function that is important in the early stages of the immune response and that is not restored by the repopulating lymphocytes. Studies of the antibody response of the rat in terms of the type of immunoglobulin formed have shown that 19S antibody reaches a peak four days after a single injection of sheep erythrocytes and then rapidly declines.^{24,25} On the other hand, 7S antibody is maintained at high titers from 8 to 28 days. Serum antibody in the present investigation was not characterized as 7S or 19S globulin, but formation of the latter may have been selectively inhibited in the irradiated animals subjected to double shielding. Such inhibition of 19S immunoglobulin formation coupled with essentially normal formation of 7S immunoglobulin might explain the delayed rise to an essentially normal peak titer. The results may have important implications in relation to the type and location of the cells forming each of these globulins.

Clearly, repopulation of the irradiated spleen restored, to a remarkable degree, its ability to respond to intravenous particulate antigen injection with red pulp hyperplasia. There are several ways in which the repopulating cells might have accomplished this restoration.

The first possibility is that when stimulated with antigen, some of the repopulating lymphocytes developed directly into the large pyroninophilic antibody-forming cells. In discussing the function of "lymphocytes" one is constantly confronted with the difficulties inherent in current cell classifications, which are based on morphological criteria. It has been asserted that markedly different functions may be performed by morphologically identical "lymphocytes."²⁶ Such morphological designations as "small," "medium," and "large" should be used therefore with some reservations.

Objections to the proposal of a lymphocytic origin of the antibody-forming cells are largely based on two factors: (1) The lack of observed mitotic activity of the circulating or tissue "small"

lymphocytes, and (2) the morphological differences between the lymphocyte and plasma cell lineages, particularly as revealed by electron microscopy. On the other hand, abundant data have existed for many years indicating that the lymphocyte can, under many conditions of inflammation develop into a macrophage.²⁷⁻²⁹ Whether the cells with this capability are "small" lymphocytes, or are in reality "medium" lymphocytes or monocytes from the blood, has been the subject of much debate and considerable conflicting evidence.³⁰

The belief that "small" lymphocytes have an extremely low mitotic index or do not divide at all was supported by initial experiments in which the "small" lymphocytes failed to incorporate tritium thymidine in vitro with short periods of incubation.³¹ Furthermore, although labeled "large" lymphocytes are present immediately after in vivo administration of tritium thymidine, labeled "small" lymphocytes do not appear until after a characteristic maturation delay.^{32,33} In addition, the evidence for a relatively long life span of many circulating lymphocytes would suggest little further differentiation or division. Little et al.,³⁴ for instance, utilized a continuous infusion of tritium thymidine and measured the disappearance rate of unlabeled lymphoid cells. By this method it was established that some circulating lymphocytes do persist for more than 100 days without undergoing mitosis. More recently, however, MacKinney, et al.³⁵ reported significantly different results following prolonged culture of human peripheral blood in vitro. In such preparations, divisions were observed after 40 hours of incubation and the index of mitosis increased to 1 per cent per hour at 70 hours. During the same 70-hour interval, the number of cells incorporating tritium thymidine increased from less than 0.5 per cent at the onset to 45 per cent. By using colchicine and variations in the labeling procedure, they concluded that the lymphocytes in the original culture were the cells responsible for the observed mitotic activity. Unfortunately some of the data necessary to validate this conclusion were not presented in the publication.

Morphological differences between the lymphocyte and plasma cell series have long been apparent from ordinary histological studies. Ultrastructural studies have emphasized additional differences, particularly the abundant endoplasmic reticulum of the plasma cell and its paucity in most lymphocytes.³⁶ The failure to identify transitional cell types between the two series has further supported the belief that they are independent.

A considerable body of experimental data has now accumulated, however, which indicates that at least some lymphocytes can transform into large pyroninophilic cells resembling antibody-forming cells. For instance, Gowans et al.³⁷ studied the graft-versus-host reaction of labeled parental strain rat lymphocytes collected by thoracic duct fistula after multiple doses of tritium thymidine and then transferred into F-1 hybrids. Twenty-four hours after transfer, many labeled pyroninophilic cells as well as labeled mitoses appeared in the spleens of recipients. A further investigation, also by Gowans and co-workers,¹³ largely eliminated the possibility of transfer of labeled DNA to explain these findings. Unlabeled rat lymphocytes were collected as before, incubated until the suspensions contained 99.5 per cent "small" lymphocytes, and then transferred into irradiated mice. Tritium thymidine administered 48 hours later resulted in heavy labeling of many cells in mitosis as well as large pyroninophilic cells in the splenic white pulp. Virtually all of the cells in mitosis were determined by chromosome analysis to be of rat origin.

The lymphocyte's potential for morphological metamorphosis was shown in another way by Holub.³⁸ Using gradient centrifugation of thoracic duct lymph, he prepared suspensions report-

edly containing 99.98 per cent "small" lymphocytes. Within 24 hours after homotransplantation of such suspensions in diffusion chambers, a significant fraction of the cells exhibited morphological changes of increased pyroninophilic cytoplasm and nucleolar hypertrophy. If homotransplanted with protein antigen, such cultures resulted in the formation of "plasmacytoid" cells which contained specific antibody demonstrable by the fluorescent antibody technique. Holub, like Braunsteiner, had previously²⁶ emphasized the possibility that metabolic differences exist in what appear to be morphologically identical lymphocytes.

The present findings provide histological evidence that the antibody-forming cells arise by metamorphosis of lymphocytes. Thus, following irradiation and immediately prior to antigen injection, the white pulp of rats subjected to the double shielding procedure consisted of a rather uniform field of lymphocytes. One day after antigen injection numerous large pyroninophilic cells, some in mitosis, were present in the white pulp. Many cells similar to lymphocytes, and interspersed among them, exhibited features of nuclear alteration, nucleolar enlargement, and increase in cytoplasm. On subsequent days the pyroninophilic cells populated the red pulp. It seems likely that they migrated from the white pulp, but one cannot completely rule out their local development from primitive cells in the red pulp. This histological evidence for the origin of the large antibody-forming cells from lymphocytes is similar to that obtained by Langevoort in his study of the rabbit spleen following antigen stimulation.¹¹ Neither study furnishes direct or complete evidence of the lymphocytic origin of the antibody-forming cell following the primary antigenic stimulus. In both studies, the cells originating from lymphocytes may be limited to 7S globulin formation.

The second possible explanation for the present results is that the repopulating lymphocytes might function by transferring some essential protein or nucleic acid product to the irradiated but surviving primitive spleen cells which resemble the early forms of the antibody-forming cells. This explanation thus embodies Ranvier's³⁹ concept of the lymphocyte as a mobile unicellular gland. The importance of transfer of vital substances from normal to irradiated cells has not been adequately evaluated. The existence of a humoral agent was hypothesized by Jacobson⁴⁰ to explain the protection of the immune response observed in rabbits subjected to irradiation with various shielding procedures. Lack of success in substantiating the existence of such a humoral agent has led others⁴¹ to favor the cellular hypothesis which asserts that any such protection is dependent on intact viable cells. On the other hand, the investigations of Jaroslow and Taliaferro^{42,43} on the restoration of the hemolysin-forming capacity of irradiated rabbits would seem to support the feasibility of a humoral factor. Their investigations revealed that various preparations from yeast or HeLa cells could completely restore the rate of rise and peak titer of the hemolysin response although the induction period was not restored to normal. Enzymatically-degraded preparations of deoxyribonucleic acid or ribonucleic acid were partially restorative. The mechanism of restoration in their investigations has not been elucidated. Nevertheless the result with degraded nucleic acids is of particular interest in view of recent investigations which demonstrate the transfer of nucleic acid metabolites from lymphocytes to other cells.⁴⁴⁻⁴⁶ The possible importance of the transfer of nucleic acid metabolites or other vital products from the lymphocytes to the irradiated spleen cells has not been ruled out in the design of the present double shielding procedure. It must be stated, however, that there is at the present time no strong evidence to support such a mechanism in radiation recovery.

The third possible explanation for the present results is that some other "blast" cell type

with the potential to differentiate into antibody-forming cells has participated in the repopulation of the spleen. Bond et al.,^{31,47} for instance, have demonstrated that in human peripheral blood there are cells capable of incorporating tritium thymidine in vitro and therefore presumably capable of further mitotic activity. These cells, including large mononuclear cells, lymphocyte-like cells, and "basket" cells, were present in relatively small numbers, in the order of 0.02 to 0.06 per cent of all leukocytes, i.e., about 4 cells/cu mm. If this result were applicable to other species, the blood stream of the rat would contain approximately 75,000 such cells at any given time. The function of these cells has not been established, but it seems that one would have to assume a remarkably selective localization of them in the irradiated rat spleen to explain the present findings. However, it must be admitted that such cells may have superior survival and colonizing powers.

The present results do not provide a definite answer to the question of how the migrating cells restored immunological activity to the spleen. Available information would seem to favor the explanation that the restoration resulted from transformation of lymphocytes to antibody-forming cells following appropriate antigenic stimulation.

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SPLEEN CELL MIGRATION IN THE IMMUNE RESPONSE OF THE RAT^{*}

By

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Studies of the rat spleen have established a well-defined pattern of cellular proliferation resulting from a single intravenous injection of particulate antigen.¹⁻³ Large pyroninophilic cells, the "antibody-forming" cells, proliferate actively in the red pulp to attain maximum hyperplasia on the fourth day after antigen. Subsequently these cells rapidly disappear, and by eight or ten days the spleen has largely regained its prestimulation pattern except for a slight increase in plasma cells.

There are several possible explanations for the rapid disappearance of the "antibody-forming" cells. An obvious possibility, namely, cellular death and dissolution within the spleen itself, has not been supported by morphological observations. A second possibility is maturation into some other cell type. The small residual increase in plasma cells is inadequate to account for the fate of the large number of cells that proliferate in response to antigen. At the peak of red pulp hyperplasia, however, cells resembling lymphocytes appear in the red pulp and temporarily increase in numbers as the pyroninophilic cells disappear. Wissler et al.³ proposed that most of the antibody-forming cells "shed their cytoplasm" to yield 19S (IgM) immune globulin and to give rise to these lymphocytic cells or small "dark" cells which then enter the blood stream. It was furthermore suggested that the small number of antibody-forming cells which do not undergo this metamorphosis will mature into plasma cells and remain in the spleen. A third but similar explanation is that of Langevoort⁴ who concluded from purely morphological studies of the rabbit spleen that many of the large pyroninophilic cells enter the blood directly. Migration of immune engendered cells into the blood during the immune response was supported by the observation that small numbers of circulating lymphocytes exhibit specific adherence for the bacteria used in intravenous immunization.² It has been postulated that the widespread dispersal of these immune engendered lymphoid cells from the spleen may be an important factor in the heightened and more generalized antibody response that occurs upon a second or challenging dose of antigen.⁵

In previous papers we have described a method of following migration of spleen cells during the course of the immune response.^{6,7} This method uses tritium thymidine to label those spleen cells which are rapidly proliferating in response to a previous injection of antigen. Labeling of cells in extrasplenic sites is effectively eliminated by total-body irradiation with spleen shielding prior to administration of tritium thymidine. Initial results have indicated a significant migration of morphologically diverse spleen cells into the blood from the third to fourth day after antigen.⁷ The present study uses this experimental method to investigate further the fate of these migrating cells.

^{*} This report is taken from a paper that will appear in Arch. Pathology, August 1967.

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MATERIALS AND METHODS

Eight- to ten-week-old Sprague-Dawley albino male rats, weighing 220 to 320 g, were maintained on laboratory chow and water ad libitum. The method of total-body irradiation with spleen shielding has been described by others.⁸ Clean but not sterile surgical technique was used with general anesthesia obtained by intraperitoneal chloral hydrate. All experimental animals were subjected to the same surgical manipulations.

X-rays were generated with a 250 kV machine operating at 30 ma. Filters of 0.5 mm copper and 1.0 mm aluminum were utilized. The filtered beam had a half-value layer of copper of 1.4 mm. Exposures were monitored with a Victoreen condensor equipped with a 250-roentgen (R) chamber. Groups of four animals were irradiated on a board revolving in a plane perpendicular to the beam at three and one-half rpm. The target distance was 75 cm and the dose rate averaged 55 R per minute. A single dose of 900 R was administered.

A vaccine prepared from formalin-killed Salmonella typhi organisms (strain T-29) was used as antigen. The detailed methods of antigen preparation and of agglutinin determinations have been described elsewhere.^{9,10} The stock vaccine was washed twice and diluted with normal saline to a standard optical density immediately prior to use. A single 1.0 ml dose of diluted vaccine was injected intravenously. Serum samples for agglutinin determinations were obtained by incision of a lateral tail vein. Serial twofold dilutions were prepared from an initial 1:20 dilution. Agglutinin titers are expressed as the last tube showing agglutination.

Animals were injected intraperitoneally with tritium thymidine, specific activity 0.36 curies per millimole, at a dose of 0.25 microcuries per g body weight. Animals were killed by exsanguination under ether anesthesia. Bone marrow smears and samples of thymus, spleen, and mediastinal, mesenteric, and peripancreatic lymph nodes were fixed in Carnoy's solution. Tissues were embedded in paraffin and cut at 6 μ . Autoradiography was performed with Kodak Autoradiographic Stripping Plate AR 10 according to the method of Pelc.¹¹ After 30 days exposure, autoradiographs were stained with methyl green-pyronin using a variation of the Unna method.¹² Further modification included a one and one-half minute staining followed by a dilute hydrochloric acid rinse.

RESULTS

Thirty-six rats were randomly distributed among four groups for histologic study: (1) The first group received antigen and 48 hours later was subjected to total-body irradiation with spleen shielding. Tritium thymidine was injected 18 hours after irradiation. Three animals were autopsied on the third day after antigen, i.e., six hours after tritium thymidine, and on the fifth and seventh days. Representative tissues were taken for histology and autoradiography and serum samples for agglutinin determinations. (2) The second group was treated similarly but received saline instead of antigen. (3) The third group, which also received antigen, was treated identically, but irradiated without spleen shielding. (4) The fourth group served as an unirradiated, antigen-injected control for splenic histology. Sham-operation was performed on this group 48 hours after antigen injection; tritium thymidine was not administered. Autopsies were performed at the same times as in the previous groups.

Twelve additional animals were assigned to groups 1, 3, and 4 for determination of the agglutinin titer response. Serum samples were taken from each animal prior to antigen and on days 3, 5, 7, 12, 18, 24, and 30.

The entire experiment was repeated according to this same plan. Results of both experiments were comparable.

Agglutinin titers. Animals subjected to irradiation with spleen shielding had an agglutinin response that did not differ significantly from that of unirradiated animals, as shown in Figure 1. Total-body irradiation without shielding completely suppressed the appearance of circulating agglutinins within the first seven days, which was the limit of survival for this group. Agglutinin titers of animals autopsied for histology was within the experimental range for the comparably treated groups as recorded in Figure 1.

Splenic histology. The spleens of the irradiated, spleen-shielded animals had histologic changes from antigen stimulation which were similar to those in unirradiated, antigen-injected animals. The single distinct difference was a decrease in the number of lymphocytes in the

Figure 1. The effect of total-body irradiation with and without spleen shielding on the agglutinin response. A single dose of typhoid vaccine was injected intravenously. Each point represents the geometric mean titer for a group of four rats. A titer of 10 was arbitrarily assigned to animals whose sera did not show agglutination at the lowest dilution tested. Treatment of the various groups: Group 1 - 900 R x-irradiation with spleen shielding two days after antigen, Group 3 - 900 R total-body x-irradiation two days after antigen. All members of this group died between days 7-12. Group 4 - sham-operated normal rats.

white pulp. Three days after antigen, i.e., one day after irradiation, autoradiographs revealed heavy labeling of the hyperplastic antibody-forming cells in the red pulp (Figure 2a) and also the large pyroninophilic cells of the germinal centers. The small lymphocytes of the white pulp were not labeled at this time. On day 5, many labeled cells remained in the same distribution as previously. The intensity of label was decreased, as would be expected in a mitotically active cell population. Most of the small "dark" cells that appeared in abundance in the red pulp by this time were labeled with about the same intensity as the remaining large pyroninophilic cells. A few labeled lymphocytes were also present in the white pulp. On day 7, the number of labeled cells in the red pulp and the intensity of individual cell-labeling were greatly diminished. There were more labeled lymphocytes in the follicles.

The spleens of the irradiated, spleen-shielded animals that received saline exhibited a normal histologic pattern except for a decreased number of lymphocytes in the white pulp. Autoradiographs on day 3 revealed labeling of frequent large pyroninophilic cells in the germinal centers and in the small aggregates of the red pulp (Figure 2b). The few small "dark" cells which are normally present in the red pulp also had a high index of labeling. The total number of labeled cells in this group of animals, both in the germinal centers and in the red pulp, was much lower than in the antigen-stimulated group. The lymphocytes of the white pulp were not labeled on day 3. At subsequent times the number of labeled cells rapidly diminished. By day 7, a few labeled lymphocytes appeared in the white pulp follicles but in very small numbers as compared to the corresponding antigen-stimulated group.

The spleens of the antigen-stimulated animals that were irradiated without spleen shielding had severe atrophy with almost complete loss of lymphocytes on day 3. Polymorphonuclear leukocytes were exceptionally numerous in the sinusoids. Autoradiographs revealed rare labeled cells, chiefly large pyroninophilic cells, in both the red and white pulp and stromal connective tissue cells (Figure 2c). There was no histologic evidence of regeneration by day 7.

Lymph node histology. All the lymph nodes of any given animal exhibited essentially the same response to experimental treatment. On day 3, one day after irradiation, there was considerable lymphocytic repopulation of the lymph nodes of the spleen-shielded groups (Figures 3a and 3b) as contrasted to the severe atrophy in the group that was irradiated without shielding (Figure 3c). Approximately the same number of unlabeled lymphocytes, chiefly "small" lymphocytes, was present in the lymph nodes of both spleen-shielded groups, indicating that the repopulation with unlabeled lymphocytes is independent of antigen stimulation. Regeneration of lymph node architecture, as distinguished from lymphocytic repopulation, similarly was stimulated by spleen-shielding: It was completely absent from the unshielded animals by day 7. Regeneration in the spleen-shielded animals was also enhanced by antigen stimulation, which resulted in more numerous germinal centers and greater cellularity of the medullary cords by day 7.

Autoradiographs revealed that the migration of labeled cells to the lymph nodes was greatly influenced by both antigen stimulation and spleen shielding. Lymph nodes of the antigen-stimulated, spleen-shielded group contained numerous labeled cells, for the most part large pyroninophilic cells and larger lymphocytes, by day 3 (Figure 3a). These labeled cells were present in both the cortex and subcapsular sinus (Figure 4). By day 7, the labeled cells included rather frequent "small lymphocytes in the cortex and especially in the follicles. The lymph nodes of the spleen-shielded, saline-injected animals had distinctly fewer labeled lymphocytes and large pyroninophilic cells on day 3 (Figure 3b). A few labeled "small" lymphocytes remained in the lymph node

a

b

c

Figure 2. Autoradiographs of the splenic red pulp. Typhoid vaccine or saline was administered intravenously three days previously followed in two days by 900 R x-irradiation with or without spleen shielding. (a) Group 1 - antigen, x-irradiation with spleen shielding. The hyperplastic large pyroninophilic cells are heavily labeled. (b) Group 2 - saline, x-irradiation with spleen shielding. Small clusters of large pyroninophilic cells and a single cluster of small "dark" cells (left lower corner) are labeled. (c) Group 3 - antigen, x-irradiation without spleen shielding. Rare labeled cells (arrow) are present. The sinusoids contain many neutrophils. Methyl green-pyronin stain; X680.

a

b

c

Figure 3. Autoradiographs of lymph nodes. Typhoid vaccine or saline was administered intravenously three days previously followed in two days by 900 R x-irradiation with or without spleen shielding. (a) Group 1 - antigen, x-irradiation with spleen shielding. Numerous heavily labeled cells are present throughout the cortex, which is well populated with lymphocytes. (b) Group 2 - saline, x-irradiation with spleen shielding. Comparatively few labeled cells are present (arrows). Unlabeled lymphocytes are about as numerous as in group 1. (c) Group 3 - antigen, x-irradiation without spleen shielding. Severe atrophy is evident. Only rare labeled cells (arrow) are present. Methyl green-pyronin stain; X424.

Figure 4. Autoradiograph of lymph node. This animal received typhoid vaccine three days previously followed in two days by x-irradiation with spleen shielding. Numerous large pyroninophilic cells and larger lymphocytes in the cortex and subcapsular sinus are labeled. Methyl green-pyronin stain; X850.

cortex of this group by day 7. The lymph nodes of the animals irradiated without spleen shielding had only rare labeled cells on day 3 (Figure 3c) and afterwards.

Bone marrow histology. In contrast to the severe atrophy in animals subjected to irradiation without spleen shielding, the marrow of spleen-shielded animals contained numerous lymphocytes on day 3. A few lymphocytes and rare large pyroninophilic cells were labeled at this time. Labeled cells appeared to be somewhat more numerous in the antigen-stimulated animals as compared to the saline-injected controls, but quantitative comparison could not be established because of the relatively large variations between individual smears and the small number of labeled cells involved. Although the total number of lymphocytes increased on days 5 and 7, the proportion of labeled cells rapidly disappeared.

A few hematopoietic cells, including "blast" cells, were labeled in the marrow of the two groups of spleen-shielded animals. These labeled cells were distinctly fewer in the group of animals subjected to total-body irradiation without shielding. This difference suggested the migration of labeled hematopoietic cells to the marrow from the shielded spleen, which in the rat normally contains hematopoietic tissue. Marrow regeneration was well established by day 7 in the spleen-shielded animals in contrast to the persisting severe atrophy in the unshielded group.

Thymic histology. The histology of the thymus was the same in all irradiated animals regardless of spleen-shielding or antigen administration. The severe atrophy that was evident on day 3 persisted throughout the period of study. There was no migration of lymphocytes or other labeled cells into the thymus.

COMMENT

Spleen shielding during irradiation results in marked protection of the antibody-forming capacity, as was originally demonstrated in the rabbit by Jacobson et al.,¹³ and later confirmed in the rat by Wissler et al.⁸ This protection of the antibody-forming mechanism is correlated with preservation of the splenic histologic reaction to antigen,¹ which is confirmed in the present investigation.

The present autoradiographic study provides evidence on the genesis of the lymphocytic cells, the small "dark" cells, which appear in large numbers in the red pulp at the peak of immune-induced splenic hyperplasia. The heavy labeling of these cells on day 5 clearly indicates their origin from actively dividing precursors. The only heavily labeled cells that were present in large numbers in the red pulp on day 3 and could therefore qualify as precursors of the small "dark" cells were the large pyroninophilic or "antibody-forming" cells of the red pulp. Other reports of similar studies in spleen-shielded, irradiated rats have also indicated the metamorphosis of large pyroninophilic cells into the small "dark" cells.^{6,7,14} In these previous studies, labeled spleen cells which migrated into the blood at the peak of immune-induced hyperplasia included a spectrum of cells resembling small lymphocytes at one extreme and large pyroninophilic cells at the other. The autoradiographic evidence presented here thus substantiates the genesis of the small "dark" cells from "antibody-forming" cells which was first proposed by Wissler et al.³

Insofar as the present results with the irradiated, spleen-shielded rat are applicable to the intact animal, it may be concluded that antigen-stimulated cells from the spleen enter the blood as early as the second to third day after antigen. The cells that migrate early in the immune response are chiefly large pyroninophilic cells, the migration of which was previously suggested on purely morphological grounds by Langevoort.⁴ It seems likely that some of the migrating spleen cells, including both the large pyroninophilic cells and the small "dark" cells at the peak of red pulp hyperplasia, are represented in the group of blood lymphocytes described by Braunsteiner and Pakesch.¹⁵ The cells which they described appeared as "medium" lymphocytes by conventional histological preparation but on ultrastructural investigation were found to possess a prominent endoplasmic reticulum similar to that of plasma cells.

The cells that proliferate and migrate from the spleen after antigen stimulation are distributed to the lymph nodes and bone marrow, chiefly the former. Because of the rapid decrease in labeled DNA which results from cell division, the course of most of the labeled cells could not be followed over an extended period of time. Some of these cells presumably transformed into labeled "small" lymphocytes, which were observed in the lymph node cortices and the splenic follicles. It is possible that other migrating cells transformed into large pyroninophilic cells in the germinal centers of the lymph nodes and spleen or into plasma cells in the medullary cords of the lymph nodes. This could explain the more rapid regeneration of lymph node germinal centers and medullary cords in the spleen-shielded animals given antigen as compared with those given saline.

The migration of immune engendered lymphoid cells from the spleen to the lymph nodes, bone marrow, and later to the splenic follicles provides a morphological basis for some of the concepts proposed by Wissler et al.^{3,5} These migrating cells may indeed play an important role in later immune reactions. For instance, Gowans and McGregor¹⁶ have recently reported experiments in which thoracic duct lymphocytes were collected from rats previously stimulated with

typhoid antigen and transferred into normal recipient rats. Upon challenge of the recipient rats with typhoid antigen, a brisk anamnestic type of response was elicited. Thoracic duct lymphocytes from immunized donors might well include cells similar to the labeled cells that migrated from the spleen in the present investigation.

A second type of cell migration not related to previous antigen stimulation, occurred from the shielded spleen following irradiation. This migration involved the rapid redistribution of unlabeled lymphocytes from the spleen to the lymph nodes and bone marrow but not the thymus. That these lymphocytes largely originated in the splenic white pulp is evidenced by the significantly diminished population of white pulp lymphocytes observed in the spleen-shielded animals following irradiation. Gesner and Gowans¹⁷ similarly found definite repopulation of lymph nodes and the splenic white pulp but not of the thymus following injection of isologous thoracic duct lymphocytes into irradiated mice. The redistribution of lymphocytes observed in the present investigation is thus to be expected from the concept of the recirculating lymphocyte pool as originally described by Gowans.^{18,19}

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RIBONUCLEIC ACID SULFUR-TRANSFERASE ACTIVITY IN EXTRACTS FROM ESCHERICHIA COLI*

By

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The identification of sulfur-containing nucleotides as minor constituents of soluble ribonucleic acid (sRNA) in various organisms has prompted an investigation into the mechanism by which these thionucleotides are formed. Recent reports from this laboratory¹ and from another group² have described the transfer of ³⁵S from cysteine to RNA, in vitro, catalyzed by soluble extracts of Escherichia coli. Optimal incorporation requires the addition of ATP, Mg⁺⁺ and sRNA to the reaction system. The use of ¹⁴C-cysteine rather than ³⁵S-cysteine gives rise to no significant labeling of RNA, indicating that only the sulfur moiety of cysteine is transferred in the reaction. Alkaline hydrolysis and chromatography of the ³⁵S-labeled RNA indicate the existence of several reaction products; a major one was tentatively identified as 4-thio UMP, which is a known component of E. coli sRNA.³ These studies were carried out with extracts from E. coli Q13, but extracts from commercially available E. coli B cells show a similar activity and were used in the work described below.

In our initial study, we observed that of the various natural and synthetic nucleic acids tested as sulfur acceptors in vitro, only sRNA was active. Comparisons of the acceptor abilities of different RNA preparations, and of the labeled products obtained from them, were made difficult because of the relatively high level of endogenous ³⁵S-acceptor (presumably sRNA) in the crude enzyme extract. This difficulty has now been largely overcome by treatment of the extract with DEAE cellulose; the enzymatic activity can be adsorbed onto the anion exchanger from 0.02 M Tris buffer and eluted at high ionic strength (0.3 M phosphate buffer, pH 7.5). The protein recovered after precipitation with ammonium sulfate (80 per cent of saturation at 0°) contains most of the original activity and has a 280/260 mμ absorbance ratio of about 1.5. This enzyme preparation shows a nearly absolute dependence on the addition of sRNA for sulfur-transferase activity. The present report describes some of the properties of this "DEAE-enzyme" with respect to its transferase activity and also compares the labeled products that are formed when different sRNA preparations are used as sulfur acceptors.

PROPERTIES OF THE TRANSFERASE REACTION

When untreated E. coli B sRNA is used as acceptor, the "DEAE-enzyme" requires ATP and magnesium ion for transferase activity, the requirement for ATP being almost absolute (Table 1). In the absence of added sRNA no significant amount of ³⁵S is transferred from cysteine to the RNA fraction. Pyridoxal phosphate was included in the complete reaction mixture because it had been reported to be a cofactor requirement for transferase activity.² Table 1, however, shows

* This paper was presented at a Cold Spring Harbor Symposium on Quantitative Biology, June 1966, and appeared in the Proceedings of this meeting. The work was supported in part by funds from the Joseph and Helen Regenstein Foundation.

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Table 1

REQUIREMENTS FOR TRANSFER OF LABEL FROM ^{35}S -CYSTEINE TO RNA

Reaction system	Labeled substrate	Counts in RNA fraction
		(cpm)
Complete (zero time)	^{35}S -cysteine	93
Complete		766
Complete (heated enzyme)		200
Omit enzyme		165
Omit sRNA		119
Omit ATP		175
Omit Mg^{++}		331
Omit Mg^{++} , add EDTA (2.5 μmoles)		161
Omit pyridoxal phosphate		712
Complete + actinomycin D (10 μg)		780
Complete + DNase (10 μg)		678
Complete + RNase (11 μg)		97
Complete (zero time)	^{14}C -cysteine	122
Complete		98

The complete system (0.50 ml) contained Tris-HCl buffer (pH 8.5), 50 μmoles ; ATP, 0.8 μmoles ; MgCl_2 , 1.6 μmoles ; 2-mercaptoethanol, 5 μmoles ; pyridoxal phosphate, 0.02 μmoles ; *E. coli* B sRNA (untreated), 265 μg ; ^{35}S -cysteine (approximately 8×10^6 cpm per μmole), 0.1 μmoles ; and "DEAE-enzyme," 1.45 mg of protein. The mixtures were incubated for 15 minutes at 37° and then assayed for label incorporation into the RNA fraction as described previously.¹ The ribonuclease was a mixture of pancreatic (10 μg) and T_1 (1 μg). Where indicated, ^{35}S -cysteine was replaced by ^{14}C -cysteine having a similar specific activity.

that its omission does not significantly reduce the extent of label incorporated in the system described here. Although the presence of actinomycin D and pancreatic DNase have no measurable effect on the extent of ^{35}S -transfer, pancreatic RNase completely abolishes the formation of labeled production. When ^{14}C -cysteine is used in place of the ^{35}S -substrate, no labeled product is formed.

The activity of various natural and synthetic ribopolynucleotides as ^{35}S -acceptors is shown in Table 2. All of the sRNA preparations demonstrate acceptor activity with the enzyme employed, and, in agreement with our earlier observations, prior treatment of the sRNA with NaIO_4 increases the incorporation of label into the RNA fraction. The ribohomopolymers of adenylic, uridylic and cytidylic acids were without any activity.

The kinetics of the reaction catalyzed by the "DEAE-enzyme" are shown in Figure 1. The incorporation of ^{35}S into the RNA fraction is fairly linear with time for the first 30 minutes. With further incubation, the rate of product formation is reduced.

Figure 2 shows ^{35}S -transfer as a function of the amount of sRNA added to the system. At low sRNA concentration the incorporation is linear with respect to the amount of acceptor added. For the amount of enzyme used in this experiment, saturation with *E. coli* B sRNA appears

Table 2
THE ABILITY OF VARIOUS RIBONUCLEIC ACIDS
TO ACCEPT ^{35}S

Type of RNA added	Counts in RNA fraction
	(cpm)
None	78
<u>E. coli</u> B sRNA	684
<u>E. coli</u> B sRNA (NaIO_4 -treated)	858
<u>E. coli</u> K12 sRNA	390
<u>E. coli</u> K12 sRNA (NaIO_4 -treated)	921
Yeast sRNA	664
Yeast sRNA (NaIO_4 -treated)	1035
Poly A	72
Poly U	88
Poly C	127

The system and conditions used were as described for Table 1. The RNA's (255 μg each) were all of commercial origin and the treatment of sRNA with NaIO_4 was as previously reported.¹

Figure 1: Kinetic of ^{35}S incorporation into RNA. The complete system (0.50 ml) was as described for Table 1 except that the reactions were stopped after various times of incubation.

Figure 2. ^{35}S incorporation into RNA as a function of sRNA concentration. The complete system (0.5 ml) was as shown for Table 1 except that different amounts of *E. coli* B sRNA were used. The results have not been corrected for "zero time" control values.

to occur with about 0.5 mg of the acceptor. Figure 3 shows that the incorporation of ^{35}S into the RNA fraction is linear with protein concentration up to at least 1.5 mg per 0.5 ml of reaction mixture.

CHROMATOGRAPHIC ANALYSES OF THE ^{35}S -RNA PRODUCTS

Large quantities of product were synthesized by using 10 ml reaction mixtures with non-saturating amounts of *E. coli* B and yeast sRNA (untreated or treated with NaIO_4) as acceptors. The ^{35}S -labeled RNA was isolated from the reaction mixture as previously described and hydrolyzed with alkali for chromatographic analysis. Table 3 shows the amount of ^{35}S transferred into the RNA fraction in each case. As before, the acceptors treated with periodate gave higher ^{35}S incorporations than the corresponding untreated sRNA's. In the absence of added sRNA, a significant amount of ^{35}S was found in the RNA fraction. This baseline may consist largely of ^{35}S -cysteine, incompletely washed out due to the large amount of protein present. DEAE-urea chromatography of this baseline material after alkaline digestion showed that all the radioactivity appeared in the region of the major nucleotides where cysteine and its oxidation products

Figure 3. ^{35}S incorporation into RNA as a function of enzyme concentration. The complete system (0.5 ml) was as shown for Table 1 except that different amounts of enzyme were used. The results have not been corrected for "zero time" control values.

Table 3
LARGE SCALE REACTIONS WITH E. coli B AND
YEAST sRNA ACCEPTORS

sRNA acceptor	^{35}S incorporated into RNA fraction
	(cpm)
None	4,175
<u>E. coli</u> B (untreated)	18,770
<u>E. coli</u> B (NaIO_4 -treated)	37,500
Yeast (untreated)	19,370
Yeast (NaIO_4 -treated)	32,250

The reaction systems were of 10 ml volume. The conditions and concentrations of reagents were as shown for Table 1.

had previously been found to elute, but none appeared in the 4-thio UMP region. However, the possibility that some of the counts represent thionucleotides formed on surviving endogenous acceptor RNA cannot be entirely excluded.

Figure 4 represents the chromatographic elution pattern obtained after alkaline digestion of the E. coli B sRNA reaction products. With untreated sRNA (Figure 4A), two minor radioactive peaks (I and II) elute close to CMP and UMP respectively, while the major peak (III), which represents 50 per cent of the counts applied, is coincident with the well separated 4-thio UMP marker added prior to chromatography. Peaks I and II, which may partly correspond to the base-

Figure 4. Chromatographic analysis of alkali-hydrolyzed enzymatic products. The labeled products obtained in Table 3 with the two types of *E. coli* B sRNAs as acceptors were hydrolyzed in alkali and chromatographed on DEAE cellulose as previously reported.

line seen above in the absence of added RNA, may include cysteine and its derivatives, but may also represent other thionucleotides.⁴

With periodate-treated *E. coli* B sRNA as acceptor (Figure 4B), alkaline hydrolysis of the product yielded three major radioactive peaks which eluted in the same positions as those described above, plus several minor "anomalous" peaks. It is apparent that there is a difference between the chromatographic profiles of the hydrolyzed labeled reaction products when untreated sRNA and periodate-treated sRNA are used as sulfur acceptors. Although twice as many to-

tal counts had been incorporated into the reaction product and applied to the chromatogram in Figure 4B, the amount of ^{35}S found in the 4-thio UMP region was approximately the same as in Figure 4A. The increased incorporation of sulfur which occurs when NaIO_4 -sRNA is used as acceptor is accounted for in the increased amount of radioactivity found in peaks I and II, in such minor peaks as IV, and in the increased amount of "background" counts throughout the entire profile.

A comparison of the chromatographic profiles for the hydrolyzed products obtained with untreated and NaIO_4 -treated yeast sRNA gave very similar results. With untreated yeast sRNA, approximately 45 per cent of the radioactivity applied was found to coincide with 4-thio UMP; peaks I and II contained 20 per cent of the total counts. With NaIO_4 -treated yeast sRNA, although twice as much labeled RNA material was formed, the hydrolyzed product contained approximately the same amount of labeled 4-thio UMP, while peaks I and II contained considerably more radioactivity.

DISCUSSION

The properties of the "DEAE-enzyme" preparation used in this study may be summarized as follows. The enzyme catalyzes the transfer of sulfur from cysteine into the RNA fraction in the presence of a divalent metal ion, ATP, and sRNA, the requirement for the last two factors being nearly absolute. All the evidence points to a reaction occurring at the polynucleotide level. So far, no requirement for pyridoxal phosphate or any other additional co-factor has been detected; the enzyme preparation, however, is still at a relatively low state of purification. Within certain limits, the transfer reaction is linear with time, and proportional to the amount of sRNA acceptor and enzyme present. Commercial (stripped) *E. coli* B and K 12 sRNA and yeast sRNA have comparable activities as sulfur acceptors; poly A, poly C, and poly U are inactive, and earlier experience with the cruder system suggests that *E. coli* ribosomal RNA and DNA are also inactive. It might have been expected that in the homologous system in which both sRNA and enzyme were derived from *E. coli* B no transfer of sulfur would occur since all the transferase-specific sites on the sRNA molecule would already be occupied. It is possible however that sRNA molecules which are not fully thiolated exist in vivo, or are produced in the course of sRNA isolation. Moreover, the possibility of an enzyme-catalyzed exchange between cysteine- ^{35}S and RNA-sulfur has not as yet been excluded.

The ^{35}S -reaction product, when hydrolyzed to mononucleotides and chromatographed on DEAE cellulose, gave several radioactive peaks. When either *E. coli* B or yeast sRNA was used as acceptor, most of the hydrolyzed radioactivity chromatographed with 4-thio UMP. When NaIO_4 -treated sRNA was the acceptor, twice as much total radioactivity was incorporated into RNA under the same reaction conditions. The amount of labeled 4-thio UMP isolated after hydrolysis however, was no greater than with the sRNA acceptors that had not been subjected to periodate treatment. These results suggest that periodate treatment of sRNA does not increase the number of sites available for 4-thio UMP labeling, although spectrophotometric analysis of the sRNA shows that NaIO_4 treatment causes the 335 m μ absorption peak of 4-thio UMP to disappear. It had seemed possible to us that the latter effect might correspond to the conversion of 4-thiol to 4-hydroxyl function (as happens with periodate treatment of 4-thiouridine)³ and hence might yield sites suitable for re-thiolation. The observed lack of effect is capable of several interpretations. The increased amount of ^{35}S incorporated into the RNA fraction when NaIO_4 -

sRNA is used as acceptor is accounted for by increased labeling of peaks eluted early in the chromatography, and by a general appearance of anomalous radioactive fractions. A better understanding of the periodate effect must await further elucidation of the products formed, and of the chemistry of periodate-treated sRNA. Meanwhile, the present system, in which untreated sRNA is used as acceptor, provides a possible approach to elucidation of the biosynthetic mechanism for formation of 4-thiouridylic acid.

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STUDIES OF THE EFFECT OF PROTEOLYTIC ENZYMES ON RIBOSOMES AND POLYSOMES FROM RETICULOCYTES AND RAT LIVER*

By

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The fundamental unit of protein synthesis is generally considered to be an aggregate of ribonucleoprotein particles held in position by messenger RNA. It has been shown, for instance, that mild treatment with ribonuclease results in dissociation of reticulocyte polysomes; that the strand linking individual ribosomes to one another stains positively with uranyl acetate in electron micrographs; and that RNA with messenger activity can be isolated from polysomes upon their dissociation.¹⁻⁵ However, in a study of ribosomal particles from rat heart muscle, Rabinowitz et al.⁶ found that these aggregates are only partially broken down by ribonuclease treatment and are also partially broken down by incubation with chymotrypsin or trypsin. When ribonuclease and proteolytic enzyme are used simultaneously nearly all of the "heavy" material is converted to 70 - 80 S particles. Furthermore, ribosomal clusters involved in the synthesis of collagen, and γ -globulin are not totally broken down to monomers by RNase alone but require proteolytic digestion in addition.^{7,8} It was therefore suggested that in some instances ribosomal particles may be held together by more than messenger RNA alone. A detailed study of the behavior of well characterized polysomes from reticulocytes and rat liver under conditions of mild proteolysis was therefore undertaken.

MATERIALS AND METHODS

Escherichia coli strain B was grown in a medium supplemented with 0.2 per cent dextrose and 0.2 per cent casamino acids as described by Gilbert.⁹ The cells were harvested in the exponential phase (optical density 550 m μ approximately 0.4) and ribosomes were isolated and prepared according to the method of Nirenberg and Matthaei.¹⁰ Reticulocyte polyribosomes were obtained from rabbits made anemic by intraperitoneal injections of a neutral 2.5 per cent solution of phenylhydrazine for 5 days. The blood was obtained by cardiac puncture, lysed in a hypotonic medium and subjected to differential centrifugation for the isolation of polysomes according to the method of Arnstein et al.⁵ Reticulocyte polysomes were suspended in a medium that contained 0.25 M sucrose, 0.025 M KCl, 0.001 M MgCl₂, 0.05 M Tris-HCl, pH 7.6 (Medium A₄). In general, such preparations contained about two-thirds of the ribosomes in the form of aggregates. These ribosomes were similar in amino acid incorporating ability and sedimentation characteristics to those described by Arnstein et al.⁵ Polysomes from rat liver were prepared from deoxycholate treated post-mitochondrial supernatant as described by Wettstein et al.⁴ In addition, liver ribosomal dimers and monomers were isolated on a sucrose density gradient with a 2 M sucrose "step" at the bottom of the tube (Wettstein et al.⁴) or by preincubation of ribosomes ob-

*This report is taken from a paper that appeared in *Biochemistry*, 5:2674, 1966. The work was supported in part by grants from the National Institutes of Health, U. S. Public Health Service #HE-04442 and the Chicago Health Association.

tained by the method of Tashiro et al.¹¹ in the amino acid incorporating system described by Nirenberg and Matthaei.¹⁰

Ribosomes were analyzed on linear sucrose density gradients. For *E. coli* and liver ribosomes the gradients were prepared with 15 per cent and 30 per cent sucrose solutions containing 0.005 M MgCl_2 ; 0.05 M Tris-HCl, pH 7.6; 0.05 M KCl. For reticulocyte ribosomes the gradients were made with 15 per cent sucrose and 30 per cent sucrose but the solutions contained 0.0015 M MgCl_2 ; 0.01 M Tris-HCl, pH 7.6; 0.01 M KCl. On the top of each gradient 0.2 ml of ribosomal suspension containing 2-5 A_{260} units was carefully layered and the gradients were spun at 30,000 rpm for 2 hours at 4° in the SW-39 rotor of the Model L 2 Spinco ultracentrifuge. In some instances the gradients were run at 39,000 rpm for 1 hour. The gradients were analyzed by running the contents through a Gilford multiple absorbance recorder (Model 2000).

The cell free system for ^{14}C L-phenylalanine incorporation into *E. coli* ribosomes was according to the method of Nirenberg and Matthaei.¹⁰ Each tube contained in a final volume of 0.1 ml the following: 2 $A_{260\text{m}\mu}$ of ribonucleoprotein 5×10^4 cpm ^{14}C L-phenylalanine; 5 μ moles Tris-HCl buffer, pH 7.6; 1.5 μ moles MgCl_2 ; 5 μ moles KCl; 0.75 μ moles mercaptoethanol; 0.1 μ moles ATP; 0.2 μ moles phosphoenol pyruvate; 0.025 μ moles GTP; 2.5 μ g pyruvate kinase (Boehringer); 0.1 mg *E. coli* 105,000 x g supernatant protein; and 20 μ g poly-U when indicated. For reticulocyte ribosomes the cell free system of Arnstein et al. was used.⁵ The system is similar to the one described above except that the MgCl_2 concentration used was 8 mM; 10 mM glutathione was substituted for mercaptoethanol and 1 mg of 'pH 5' enzymes replaced the 105,000 x g supernatant. Each tube was assayed in duplicate; the incubations were carried out for 5'-15' at 35°. Ribosomes were also labeled with ^{14}C leucine (specific radioactivity 41.8 $\mu\text{C}/\mu$ moles) in intact reticulocytes by the method of Borsook et al.¹² In experiments involving cell free systems the amount of radioactivity incorporated into the ribosomes was assayed as follows: The reaction mixture was treated with 10 per cent TCA containing 0.1 per cent ^{12}C L-phenylalanine at the end of the incubation, and 1 mg of bovine serum albumin was added. The precipitate was washed on Millipore membranes with cold and hot (90°) TCA. The filter and the protein were then dissolved in Bray's solution¹³ and counted in a Tricarb liquid scintillation counter with approximately 49 per cent efficiency. Radioactivity in aliquots from sucrose density gradients was determined by the filter paper method of Mans and Novelli.¹⁴

Trypsin, α -chymotrypsin, and soybean trypsin inhibitor were obtained from Worthington Biochemical Corp. Estimates of peptide release following mild proteolysis of ribosomes were made by the quantitative ninhydrin method of Spies.¹⁵ Polyuridylic acid was purchased from Miles Chemical Co. and ^{14}C L-phenylalanine (specific activity 333 mC/mM) from New England Nuclear Corp.

RESULTS

Proteolytic digestion of ribosomes was carried out at zero degrees for 30' with trypsin or chymotrypsin; in the case of trypsin the reaction was terminated by adding a 5-fold excess by weight of soybean inhibitor. The details of the procedure are given in the legends to Figures 1 and 3. Suitable controls were run to show that soybean trypsin inhibitor had no dispersive effect on the ribosomal aggregates, or in any way impaired their ability to synthesize protein. We have detected no activation of latent ribonuclease upon the proteolytic breakdown of ribosomes (Table 1a; Zak et al.¹⁶). Under the conditions of our incubation (30' at 0°) neither trypsin nor soybean

Figure 1. Effect of trypsin on the sedimentation profile of rat liver polysomes. The polysomes were prepared by the method of Wettstein et al.⁴ Samples were incubated with 2 and 10 μ g trypsin/ A_{260} RNP respectively in a medium containing 1×10^{-3} M $MgCl_2$, 5×10^{-2} M KCl and 5×10^{-2} M Tris-HCl, pH 7.5, for 30' at 0°. The reaction was terminated by the addition of a 5-fold excess (w/w) of soybean trypsin inhibitor. A 0.2 ml aliquot containing 4 A_{260} of RNP, pre-incubated as described above, was layered on the top of a linear 5 to 20% sucrose density gradient as described in Methods. The centrifugal run was for 1 hour at 39,000 rpm in the SW-39 rotor of the Model L-2 Spinco ultracentrifuge.

trypsin inhibitor (SBTI) had any ribonuclease activity; and both failed to inhibit the breakdown of RNA by ribonuclease (Table 1b). Furthermore, when the ribosomes were centrifuged for 2 hours at 151,000 $\times$ g after mild tryptic digestion at 0° the supernatant solution showed no significant release of material reacting with ninhydrin (Table 2). It is estimated from four such experiments that the amount of amino acid released does not exceed 1 per cent of the amino acids present in the ribosomal protein.

Figures 1, 2, and 3 show that in general the effect of both trypsin and chymotrypsin is to cause dissociation of the ribosomal aggregates into monomers. Liver ribosomal dimers are not dissociated on incubation with 1 μ g RNAase/ml at zero degrees for 30', but treatment with trypsin at a concentration of 5 μ g per A_{260} ribosome at zero degrees for 30' leads to breakdown of the dimer (Figure 4). At double this concentration of trypsin the dimeric structure is almost completely broken down and some subunits are also seen (Figure 4).

Figure 1 shows the behavior of rat liver ribosomal aggregates submitted to mild proteolysis. At a concentration of 2 μ g trypsin per A_{260} ribosome a considerable dissociation of the heavier particles has already occurred, and with 10 μ g trypsin per A_{260} ribosome the ribosomes are almost completely in the monomer form, although a small shoulder representing dimers remains.

Reticulocyte ribosomal aggregates also break down chiefly into monomers under conditions

Table 1a

ASSAY FOR LATENT RIBONUCLEASE ACTIVITY

Additions	CPM/0.2 ml aliquot
³² P-RNA, Medium A ₄	298
³² P-RNA, trypsin	297
³² P-RNA, SBTI	288
³² P-RNA, reticulocyte polysomes	250
³² P-RNA, reticulocyte polysomes, trypsin. SBTI at end of incubation	233
³² P-RNA, reticulocyte polysomes, SBTI and trypsin	235
³² P-RNA, <u>E. coli</u> ribosomes	337
³² P-RNA, <u>E. coli</u> ribosomes, trypsin. SBTI at end of incubation	387
³² P-RNA, <u>E. coli</u> ribosomes, SBTI and trypsin	372

The incubation mixture contained in a final volume of 0.2 ml: 2.5 A₂₆₀ units of reticulocyte polysomes of E. coli ribosomes (where indicated); trypsin 5 μ g, 1×10^{-3} M MgCl₂, 5×10^{-2} M Tris-HCl, pH 7.6, 5×10^{-2} M KCl and 0.25 M sucrose. Each tube contained 1.5 μ g RNA from rat liver (sp. act. 4397 cpm/ μ g) and the incubation was carried out for 30' at 0°. Ten micrograms of SBTI were added either at the beginning or the end of the incubation as shown above. The reaction was terminated by the addition of 0.2 N HClO₄. One milligram of yeast RNA was added to the mixture which was then spun at 2500 rpm x 10' and 0.2 ml aliquots of the supernatant solution counted in a dioxane based scintillation mixture.

Table 1b

EFFECT OF TRYPSIN AND SBTI ON RNase ACTIVITY

Additions	CPM/0.2 ml aliquot
³² P-RNA, RNase (1 μ g)	1519
³² P-RNA, reticulocyte polysomes, trypsin, RNase. SBTI at end of incubation	1616
³² P-RNA, reticulocyte polysomes, SBTI, trypsin, RNase	1558
³² P-RNA, trypsin, RNase (1 μ g)	1445
³² P-RNA, SBTI, RNase (1 μ g)	1501

Conditions for incubation and counting of radioactivity as in Table 1a.

Table 2

ASSAY FOR RELEASE OF MATERIAL REACTING WITH NINHYDRIN

Additions	A ₅₇₀ (ninhydrin reaction)
Reticulocyte polysomes	0.031
Reticulocyte polysomes, SBTl, trypsin 2 μ g/A ₂₆₀ (8 μ g/ml)	0.109
Reticulocyte polysomes, trypsin, incubate 30', then SBTl	0.112
<u>E. coli</u> ribosomes	0.100
<u>E. coli</u> ribosomes, SBTl, trypsin 2 μ g/A ₂₆₀ (8 μ g/ml)	0.159
<u>E. coli</u> ribosomes, trypsin, incubate 30', then SBTl	0.169

The incubation mixture contained in a final volume of 0.25 ml the following: Two and five-tenths A₂₆₀ units of reticulocyte polysomes or E. coli ribosomes, 1×10^{-3} M MgCl₂, 5×10^{-2} M Tris-HCl, pH 7.6, 5×10^{-2} M KCl and 0.25 M sucrose. Trypsin was added at a concentration of 2 μ g/A₂₆₀ ribosome (8 μ g/ml) and 10 μ g SBTl where indicated. After incubation for 30' at 0° the volume of the mixture was increased to 1.6 ml with cold medium A₄ and the contents centrifuged at 50,000 rpm in the Ti-50 head of the Spinco L-2 ultracentrifuge for 2 hours at 0°. One-tenth ml aliquots of the supernatant solution were treated with ninhydrin reagent (Spies,¹⁵).

of mild proteolytic attack. Figure 2 shows the effect of 10 μ g α -chymotrypsin per A₂₆₀ ribosome at zero degrees for 30'. The ribosomes were labeled first in intact reticulocytes with ¹⁴C L-phenylalanine. Whereas the label is chiefly associated with the polysome peak in the control preparation, chymotrypsin causes the label to be present mainly in the monomer peak.

Similarly, trypsin causes a considerable breakdown of reticulocyte polysomes (Figure 3). There is a marked flattening of the polysome peak with commensurate increase in the height of the monomer peak. We have also observed some degree of subunit formation at higher concentrations of trypsin.

Figure 5 compares the effect of varying amounts of trypsin on the response of E. coli monomers and reticulocyte polysomes to poly-U directed polyphenylalanine synthesis. It can be readily seen that in both cases the response is more sensitive in the presence of poly-U than in its absence. It is also notable that this effect is manifest at as low a dose as 0.1 μ g trypsin per A₂₆₀ ribosome, a level at which there is no subunit formation. A similar observation has been made by Kaji and Kaji with respect to E. coli ribosomes.¹⁷

Furthermore, pretreatment of reticulocyte polyribosomes with small amounts of trypsin for varying intervals shows that the poly-U directed stimulation of polyphenylalanine synthesis is reduced within a few minutes of exposure to the proteolytic enzyme (Table 3).

DISCUSSION

Although there is evidence to suggest that in some instances ribosomal aggregates are held together not only by a messenger RNA strand but also by nascent peptide chains, it is clear that the complete dissociation of reticulocyte polysomes and liver ribosomal dimers by trypsin is due to structural alterations in ribosomal protein itself. The further breakdown of monomers to subunits under relatively high concentrations of proteolytic enzymes additionally supports the

Figure 2. Effect of α -chymotrypsin on the sedimentation profile of reticulocyte polysomes. The ribosomes were labeled in intact reticulocytes by incubating 5 ml of packed reticulocytes in a medium containing: $10 \mu\text{C}$ ^{14}C L-leucine (sp. activity $41.8 \mu\text{C}/\text{ml}$), 2×10^{-4} moles of each of the remaining nineteen ^{12}C L-amino acids, 5×10^{-2} M Tris HCl, pH 7.6, 2.5×10^{-3} M KCl, 5×10^{-3} M MgCl_2 , and 20 mg of glucose in a final volume of 7.5 ml for 15' at 37° (Borsook et al.¹²). The incubation was terminated by chilling the flask in ice and by the addition of 4×10^{-2} moles of ^{12}C L-leucine. Polysomes were then isolated by the method of Arnstein et al.⁵ and an aliquot was incubated with $20 \mu\text{g}$ of α -chymotrypsin/ A_{260} RNP for 30' at 0° . 4.6 A_{260} units of the treated ribosomes and untreated controls were layered on 5 to 20% sucrose density gradients, and spun in the SW-39 rotor of the Spinco L-2 ultracentrifuge at 30,000 rpm for 130 minutes at 0° . The left hand panel shows the profile of the control sample; on the right is shown the profile after treatment with the proteolytic enzyme. Ten drop fractions were collected on rectangular strips ($3\text{-}1/4'' \times 1''$) of Whatman 3MM paper and the radioactivity assayed by the method of Mans and Novelli.¹⁴

conclusion that the primary effect is on the protein moiety of the ribosome. Under the conditions of our assay system there was no evidence for activation of latent ribonuclease. Hence it is safe to conclude that the effects observed were due to mild proteolytic damage of the polysomes leading to their disaggregation.

In an earlier communication (Zak et al.¹⁶) we showed that trypsin treatment of ribosomes from *E. coli* and reticulocytes causes marked reduction in their ability to bind poly-U, and to a lesser extent, an impairment in the binding of charged sRNA. Pretreatment of ribosomes with proteolytic enzymes affects their capacity to synthesize protein; synthesis of new peptide chains under the direction of natural messenger RNA is less affected than that involving an artificial messenger such as poly-U (Figure 5). These findings suggest that tryptic digestion affects the binding of poly-U to ribosomes, presumably by damaging ribosomal protein, and that release of natural mRNA from polysomes is also likely. It is also possible that aggregation of ribo-

Figure 3. Dissociation of reticulocyte polysomes by trypsin. 4 A₂₆₀ units of polysomes were incubated for 30' at 0° in the presence of trypsin (10 µg trypsin/A₂₆₀ RNP). The reaction was carried out in a medium containing 1 x 10⁻³ M MgCl₂, 2.5 x 10⁻² M KCl, 5 x 10⁻² M Tris-HCl, pH 7.6, and 0.25 M sucrose (Medium A₄). The incubation was terminated by the addition of a 5-fold excess of soybean trypsin inhibitor. An aliquot (3 A₂₆₀ units) was analyzed by the sucrose density gradient method as described in Methods. The centrifugal run was for 130 minutes at 30,000 rpm and 0° in the Spinco SW-39 rotor.

Table 3

EFFECT OF PRETREATMENT WITH TRYPSIN ON THE RESPONSE OF
RETICULOCYTE POLYSOMES TO POLYURIDYLIC ACID

Duration of pretreatment with trypsin (5 µg/A ₂₆₀)	Complete system minus poly-U (cpm/A ₂₆₀)	Per cent control	Complete system plus poly-U (cpm/A ₂₆₀)	Per cent control
0' (control)	2514.0		6321.4	
2'	1332.0	52.9	1449.0	22.9
5'	980.7	38.9	1005.4	15.9
10'	504.0	20.0	615.5	9.7
15'	388.7	15.4	569.4	9.0

Tubes containing 2 A₂₆₀ units of reticulocyte polysomes were pre-incubated in the presence of trypsin at a concentration of 5 µg/A₂₆₀ for 0'-15' at 0°. The reaction was run in duplicate and at the end of the pretreatment, a 5-fold excess of soybean trypsin inhibitor was added. A second incubation was then carried out in a complete amino-acid incorporating system containing 1 µc ¹⁴C L-phenylalanine, 19 remaining unlabeled amino acids, and other factors as described in the text.

Figure 4. Dissociation of liver ribosomal dimers by trypsin. The dimers were obtained by sucrose density gradient fractionation (Wettstein et al.⁴) of liver ribosomes prepared by the method of Tashiro and Siekevitz.¹¹ Samples were incubated with 1 μ g RNAase/A260, 5 μ g trypsin/A260, 10 μ g/A260 respectively for 30' at 0° in a medium containing 1×10^{-3} M $MgCl_2$, 5×10^{-2} M Tris-HCl, pH 7.6, and 5×10^{-2} M KCl. The tryptic digestion was stopped as usual and 4 A260 units were analyzed on linear sucrose density gradients as mentioned in the text. The gradients were run at 0° for 1 hour at 39,000 rpm in the SW-39 rotor of the Spinco L-2 ultracentrifuge. The sedimentation values are relative to that of *E. coli* monomers run under similar conditions.

comes into a polysomal structure renders them less susceptible to the effects of tryptic digestion.

Aepinus observed that liver polysomes are broken down with trypsin but not with chymotrypsin.¹⁸ He therefore concluded that the proteins involved in the stability of polysomes must be basic in nature. We have noted no such difference between the effects of trypsin and chymotrypsin (see Figures 1 and 2) and cannot substantiate this claim. Our observations on the effects of proteolytic enzymes on ribosomal aggregates from heart muscle (Rabinowitz et al.⁶) are

Figure 5. Effect of trypsin on the incorporation of ^{14}C L-phenylalanine in *Escherichia coli* monomers and reticulocyte polysomes in the presence and absence of poly-U. Both *E. coli* monomers and reticulocyte polysomes were pre-incubated in tubes containing 2 A₂₆₀ units of ribosomes and trypsin at varying concentrations ranging from 0.1 μg trypsin to 2.5 μg trypsin/A₂₆₀ ribosome for 30' at 0°. The final volume of the incubation mixture was 0.2 ml and was identical in composition to that mentioned in Figure 1. The reaction was stopped with soybean trypsin inhibitor. Control samples were incubated in the presence of trypsin inhibitor alone. The ribosomes, thus pre-treated, were incubated for a second time in a complete amino acid incorporating system as mentioned in the text. The incubations were carried out for 5' at 37°. Poly-U was added at a concentration of 10 μg /A₂₆₀ ribosome.

- ▲ ▲ trypsin treated ribosome plus poly-U.
- ● trypsin treated ribosomes.

(Control values (100%) are as follows: 2165 cpm/A₂₆₀ reticulocyte ribosomes; 12,219 cpm/A₂₆₀ reticulocyte ribosomes plus poly-U; 187 cpm/A₂₆₀ *E. coli* ribosomes; 2397 cpm/A₂₆₀ *E. coli* ribosomes plus poly-U.)

also different from those of Breuer et al.¹⁹ who noted no breakdown of heart muscle polysomes with chymotrypsin as judged by electron microscopy. Furthermore, unlike Wilson and Hoagland,²⁰ we have been able to dissociate rat liver ribosomal aggregates with proteolytic enzymes. These effects are obtained with proteolytic enzymes of very high purity (Worthington 2 X crystallized) with no detectable ribonuclease activity. Soybean trypsin inhibitor, added in excess, completely abolishes the effects of trypsin on ribosomes, and DFP-chymotrypsin when substituted for chymotrypsin produces no effect on ribosomal integrity. It is of interest that although liver ribosomal dimers are insensitive to ribonuclease, chymotrypsin or trypsin produces dissociation of dimers into monomers (Figure 4). Here again, a relatively mild disturbance in the structure of ribosomal protein results in a dislocation of the bonds holding the monomers together.

We conclude that mild proteolysis of ribosomal aggregates from different tissues leads to damage of ribosomal protein with consequent disaggregation of ribosomes and their breakdown

into monomers and subunits. The binding of mRNA and sRNA is affected and hence an adverse effect on the capacity of the ribosome to synthesize protein obtains.

ACKNOWLEDGMENT

We are indebted to Margaret Scott and Mei-Hwa Chen Tu for their excellent technical assistance.

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THE PROTECTIVE EFFECT OF PREVIOUS IRRADIATION*

By

C. W. Gurney[†] and W. Fried[‡]

The explosive recovery of the primitive marrow cells responsive to erythropoietin, beginning on the 4th or 5th day after 150R,¹ and reaching supra-normal levels by day 7,² has led us to postulate the existence of, and seek ways to demonstrate a homeostatic mechanism that is directed at maintaining the number of these cells constant. We have recently presented results of experiments which we believe support this concept.³ The present communication describes one of the models we have employed in an examination of the kinetics of primitive marrow cells in the period following radiation damage.

Erythropoiesis can be virtually eliminated in mice by producing a plethoric state.^{4,5} A quantitative erythropoietic response may be obtained in these plethoric animals by injecting erythropoietin and following reticulocyte formation⁶ or incorporation of radioiron into newly formed red cells.⁷ Since this erythropoietic effect may predictably be impaired by irradiation,⁸ we have concluded that the response to erythropoietin is directly related to the number of viable, responsive erythropoietin-sensitive cells.

Polycythemic mice given a challenging dose of erythropoietin several days (t_2) after a large dose of radiation (R_2), administered with one leg shielded, show a decreased response to erythropoietin when compared with non-irradiated control animals (see Figure 1). However, mice given a smaller dose of irradiation (R_1), administered with the same leg shielded several days (t_1) before receiving R_2 , may respond to the erythropoietin distinctly more effectively than mice receiving only R_2 . We shall refer to this phenomenon as the protective effect of previous irradiation. We attempt to arrive at a quantitative expression of this protective effect by determining the ratio of the response in animals receiving both R_1 and R_2 , minus the response in animals receiving only R_2 , to the response in animals receiving only R_2 . With proper selection of the variables, twice-irradiated mice may yield a response to erythropoietin which is even greater than that of non-irradiated animals.

We have sequentially explored the variables R_1 , R_2 , t_1 , and t_2 (Figure 1) by assigning arbitrary values to any 3 of these parameters, and recording the results obtained when a number of different values of the 4th parameter are employed. While these explorations are not yet complete, it appears that R_1 of 300 to 400R, t_1 of 8 to 10 days, R_2 of 900R, and t_2 of 5 days are close to optimal values, and that a protective effect of 4 or more may be observed with proper selection of these variables.

We have considered the possibility that the protective effect of previous irradiation is a reflection of that timing of R_2 which corresponds to the overshoot of recovery of cell numbers fol-

* This paper was presented at a Bone Marrow Conference in San Francisco on December 3, 1965 and appears in *Exptl. Hematol.*, 9:52, 1966.

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Figure 1.

lowing R_1 , a phenomenon previously described by Alexanian et al.² The following experiment, however, suggests that such an explanation is insufficient to account for the protective effect observed under the conditions here employed. With R_1 100R (right leg shielded), t_1 7 days, R_2 600R (right leg shielded), t_2 5 days, the average radioiron incorporation obtained in response to erythropoietin was 17.8 per cent. If an $R_1 = 0$ was substituted in this experiment, radioiron incorporation following erythropoietin was 7.4 per cent. Hence the protective effect was 1.4. With R_1 100R (right leg shielded), t_1 7 days, R_2 600R (left leg shielded), t_2 5 days, the average radioiron incorporation was 6.4 per cent and there was no protection. Were the protection dependent upon R_2 's being given at a time when the primitive hematopoietic cells throughout the animal's body were supra-normal in number because of recovery from R_1 , we would anticipate that protective effects would be observable when either hind leg was shielded at the time of R_2 .

Although these experiments have been designed in reference to the postulation that a humoral regulation of primitive hematopoietic cell numbers exists, and the results obtained are consistent with this postulate, it may very well be that some other mechanism accounts for the results obtained. Such protection has also been observed in non-polycythemic mice, employing as a model endogenous colonization of the spleen from the protected leg.³

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THE EFFECT OF ^{89}Sr ON THE STEM CELL COMPARTMENT OF THE SPLEEN*

By

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Jacobson and coworkers have shown that if spleens of mice are protected with lead during whole body exposure to 600 R x-radiation, compensatory ectopic blood formation occurs in the protected spleens.¹ Furthermore, repopulation of hematopoietic tissue in areas depopulated by doses of radiation as high as 1025 R occurred in mice in whom the spleen was shielded during irradiation.² This repopulation has since been shown to be due to colonization by hematopoietic cells originating in the shielded spleen.³

Jacobson and coworkers have also shown that after injecting ^{89}Sr , a bone-seeking isotope, hematopoietic tissue in the bone marrow disappears for a prolonged period but a compensatory increase in the amount of erythropoietic tissue in the spleen and other ectopic sites prevents the appearance of anemia.⁴

The nature of this compensatory increase in hematopoietic tissue in the spleen after destruction elsewhere is not completely understood. At least two different possibilities might explain this phenomenon.

First, it is conceivable that the stem cells in the spleen, which were spared radiation damage, can respond to the existing stimuli for erythropoietic proliferation, by an increased tendency toward differentiation. This would result in a larger erythropoietic cell compartment, in the face of a constant, or even decreased stem cell compartment. We have recently shown evidence that mice whose stem cell compartment has been depleted by x-irradiation respond poorly to erythropoietin, and that the recovery of the ability to respond to erythropoietin lags behind the recovery of the CFU (colony forming units).⁵ If this is so, it is unlikely that the relatively few stem cells in the spleen will be able to compensate for the body's need for erythropoiesis.

A second possibility is that the number of stem cells in the spleen increases in response to depletion of stem cells elsewhere. The increased number of stem cells may then respond to the physiological demands for erythropoiesis by giving rise to an increased number of erythrocytic cells. McCulloch and Till have provided a method for estimating the size of the stem cell compartment by their observation that cell suspensions, when injected into lethally irradiated mice, produce macroscopic spleen colonies in proportion to the number of stem cells in the injected suspensions.⁶

Using this method of estimating the number of hemopoietic stem cells, we studied the effect of ^{89}Sr induced destruction of stem cells in the bone marrow on the number of stem cells remaining in the spleen. The results of these experiments will be presented here.

* This report is taken from a paper that appeared in *Radiation Research*, 29:50, 1966.

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METHODS

Fifty 8- to 10-week-old CF No. 1 female mice were given intraperitoneal injections of ^{89}Sr at the rate of $2\ \mu\text{c/g}$ of body weight, on day 1. Fifty control animals received the same volume of normal saline. On the fourth and thirteenth days of the experiment, groups of 6 control and 6 experimental mice were sacrificed, and cells from their spleens and femoral bone marrows were assayed for CFU. Three weeks later (day 21), the survivors of the experimental group received an additional injection of $6\ \mu\text{c}$ of ^{89}Sr per gram of body weight, and the controls received an equivalent volume of saline. The numbers of CFU in the spleens and bone marrow of subgroups of these mice were determined on the twenty-fourth, thirty-ninth, and fifty-third days of the experiment.

Cell suspensions from the spleens were prepared as follows: The mice were killed by transection of the cervical cord, and the spleens were removed immediately and transferred to a 1-cc tuberculin syringe. The spleen cells were then ejected into a 5-cc volume of chilled normal saline and the suspension was agitated without foaming until no visible tissue aggregates remained. The cells from the spleens of 6 mice prepared in this manner were pooled. The suspension was then filtered through two layers of standard 4×4 cm surgical gauze and the number of nucleated cells was counted by the standard technique employing a hemacytometer and white cell pipette. The cell suspension was diluted to a concentration of 2.5×10^8 cells/cc with normal saline.

For bone marrow cell preparations, one femur was cleanly dissected from each of 6 mice. The head of the femur was removed and a 23-gauge needle attached to a 1-cc tuberculin syringe was inserted into the distal end of the bone. The proximal end was then immersed in 5-cc of chilled normal saline which was flushed repeatedly through the marrow cavity. Marrow cells from 6 femurs were suspended in 5 cc of chilled saline, and a nucleated cell count was made. The cell suspension was then diluted to a concentration of 5×10^5 cells/cc. Seven to 8×10^7 nucleated cells were obtained from the 6 femurs of mice injected with saline, whereas only $1-2 \times 10^7$ nucleated cells were obtained from the femurs of ^{89}Sr -treated mice.

CFU were assayed by the method of McCulloch and Till⁶ as follows: Recipient mice received 900 R total-body x-radiation from a 250-keV maxitron machine with 0.25 mm Cu and 1.0 mm Al filtration; dose rate factor 57 R minute; target distance 81 cm. On the same day, shortly after irradiation, the mice received 5×10^5 spleen cells from either the saline- or strontium-treated groups in a volume of 0.2 cc saline, or 10^5 bone marrow cells from normal mice or 10^6 bone marrow cells from ^{89}Sr -treated mice. A larger number of bone marrow cells from ^{89}Sr -treated mice was used because it was anticipated that the number of CFU in the bone marrows of these animals had been decreased. To facilitate comparison, the results are expressed in terms of the calculated number of CFU/ 10^5 cells. Eight days later, the mice were sacrificed, their spleens were removed and placed in Bouins solution, and the number of macroscopic nodules on each spleen was counted. When more than 40 colonies/spleen were present and discrete colonies could no longer be easily recognized or counted, the spleens were considered to be diffusely colonized.

RESULTS

CFU Produced by Injection of Spleen Cells

Figure 1 shows the number of CFU per 5×10^5 spleen cells from saline controls, from mice given $2 \mu\text{c}$ of ^{89}Sr , and from mice given first $2 \mu\text{c}$ and later, $6 \mu\text{c}$ of ^{89}Sr . The times indicated on the abscissa represent the days of an experiment beginning with the day on which the original dose of ^{89}Sr was given. Each point represents the number of colony-forming units in one recipient mouse, and the short horizontal lines show the mean values for the group. Four days after receiving ^{89}Sr , the average number of CFU in the spleens of strontium-treated mice was below normal. Nine days later, on the thirteenth day of the experiment, the average number of CFU in the experimental animals was more than twice normal. After the second dose of ^{89}Sr (day 21), the average number of spleen CFU again fell below normal (day 24), and 18 days after the second injection (day 39), the number of spleen CFU was again more than twice that in control spleens. Thirty-two days after receiving the second dose of ^{89}Sr (day 53), the cells from spleens of the twice-treated group produced diffuse colonization in irradiated recipients.

Figure 1. Effect of ^{89}Sr on CFU in the spleen.

CFU Produced After Injection of Bone Marrow Cells

Figure 2 shows the results of the assay of CFU from the bone marrow of control and ^{89}Sr -treated mice expressed as numbers of CFU per 10^5 bone marrow cells. It is apparent that within 4 days after receiving ^{89}Sr , the concentration of CFU in the femoral marrow fell to a very low level and remained there throughout the experiment.

Figure 2. Effect of ^{89}Sr on CFU in the femoral marrow.

DISCUSSION

Within the first 3 days after the intraperitoneal injection of ^{89}Sr , 54 per cent of the injected dose localizes in the bone marrow and 45 per cent is excreted.⁵ Thus sufficient radiation is directed to the bone marrow to cause depletion of its hematopoietic tissue. The amount of isotope in the spleen is 0.22 per cent of the injected dose per gram in the first 24 hours and declines to 0.02 per cent per gram from 3 days to 3 months after injection.⁴ Anemia does not supervene because there is a marked increase of erythropoietic tissue in the spleen⁴ and other extramedullary sites.

There are several possible explanations for this phenomenon. In exploring the problem, we assumed that the concentration of CFU is a valid indicator of the concentration of stem cells within any sample of hematopoietic tissue. In addition, we assumed that all types of cells were quantitatively harvested from the spleens of both treated and control mice. First, as yet poorly understood mechanisms of regulation may respond to the radiation-induced depletion of the hematopoietic cells in the bone marrow in such a manner as to cause stem cells in extramedullary sites to differentiate more rapidly. If the rate of proliferation of the extramedullary pool of stem cells were to keep pace with the rate at which these cells were drawn off for differentiation into blood cell precursors, normal hematopoietic demands could be sustained in extramedullary sites without any increase in stem cell numbers. Second, it is possible that radiation damage of cells in the bone marrow somehow stimulates stem cells in extramedullary sites to proliferate at an increased rate, after which the expanded stem cell compartment in these areas responds to normal, or perhaps increased, levels of erythropoietin at an increased rate.

The experiments reported here were designed to study the role of irradiation-induced de-

pletion of the stem cells in the bone marrow on proliferation of the stem cells in the spleen. Within 3 days after injection of ^{89}Sr , the stem cell population of the femoral marrow fell to a very low level and remained there for more than a month. Shortly after receiving ^{89}Sr , the stem cell population of the spleen fell slightly. This may have been a result of radiation damage to the spleen produced by ^{89}Sr before this isotope was cleared from the blood by bone uptake or excretion. Thereafter, however, the concentration of stem cells in the spleen increased to well over twice normal and remained at that high level for at least a month, the total duration of the study.

The results are therefore consistent with the concept that stem cells in the spleen respond to changes in the stem cell compartment elsewhere; namely, the bone marrow. Since the stem cell population in the bone marrow is markedly decreased, it appears unlikely to us that increased migration of cells from the bone marrow to the spleen can account for the supranormal numbers of CFU found in the spleen. It is conceivable that stem cells leaving the spleen at an increased rate to colonize depleted sites elsewhere, may somehow stimulate the rate of stem cell proliferation in the spleen. This presumably would occur secondary to the decreased local density of stem cells resulting from the increased rate of emigration. Such a mechanism would not, however, satisfactorily explain the two to threefold increases in the concentration and number of stem cells in the spleen which we observed, for if the stimulus to increased proliferation came from a decreased local density of stem cells, one would expect the rate of proliferation to again decrease before the cell density exceeded normal.

It is, on the other hand, possible that the increased rate of migration of stem cells from the spleen stimulates the remaining stem cells to proliferate faster via some mechanism other than a change in cell density. We suggest, however, that the most likely explanation is that those cells in the spleen which are spared radiation damage respond to some humoral message activated as a consequence of destruction of stem cells elsewhere (in this case, in the bone marrow). This humoral message may take the form of a stimulatory factor, or may simply represent a decrease in some inhibitory factor. Another alternative hypothesis is that many cells in the spleen which are dormant and incapable of forming colonies before strontium and administration, are somehow activated by administration of the bone-seeking isotope, but before transplantation.

We have recently described experiments which indicate that the stem cells in a shielded femur proliferate after the stem cell population elsewhere has been destroyed by x-radiation.⁷ Considering those experiments and the present study together, it seems probable that a stimulator or inhibitor may be concerned with regulation of the stem cell compartment.

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PROGRESS IN THE PURIFICATION OF ERYTHROPOIETIN*

By

E. Goldwasser and C. K. H. Kung

In our last publication¹ on the purification of sheep plasma erythropoietin (EPO) we reported a potency of about 450 units/mg of protein: since that time we have purified the hormone by another factor of about thirteen.

The material used for the earlier purification was a side fraction (0.67 saturated ammonium sulfate supernate) from the sheep plasma erythropoietin produced in the large scale program undertaken by the United States Public Health Service in collaboration with the University of Chicago and Armour and Company. Experiments with that fraction indicated that potencies in the range of 400-500 could be achieved, but quantities of the best fraction were insufficient to carry the fractionation any further. In part, this was due to the extensive losses of activity in the calcium phosphate gel adsorption step. More recently, we have developed methods for reproducibly obtaining fractions of anemic sheep plasma with potencies of about 6,500 units/mg protein, and this communication presents some of our results.

MATERIALS AND METHODS

The starting erythropoietin fraction was the Step III material provided by the Hematology Study Section of the United States Public Health Service. Except where otherwise stated, assays were done by the fasted rat method.² The standard used in each assay was anemic sheep plasma (6.8 units/ml) that had been standardized with reference to both cobaltous chloride and to Standard A. Protein concentrations were usually determined by the absorbancy at 278 m μ in a Zeiss PMQII spectrophotometer. At that wave length, the absorbancy of a 1 per cent solution of mixed plasma glycoproteins is approximately 10, so that for rough analysis we used one absorbancy unit as equivalent to a concentration of 1 mg/ml. When protein concentrations were very low, they were calculated from the absorbancy at 194 m μ determined with a Perkin-Elmer Model 202 spectrophotometer. Because of the inherent danger of stray light artifacts at the short wave length, determinations of A₁₉₄ at various concentrations of protein (both crystalline beef serum albumin and highly purified sheep orosomucoid) were carried out. The data in Figure 1 show that the curve goes through the origin, indicating that there is no stray light problem, that the A₁₉₄ is proportional to the protein concentration, that the nature of the protein is not important, and that a few micrograms per ml of protein can be determined by this method.

Calcium phosphate gel was prepared by the method of Tsuboi and Hudson,³ and the other reagents used were the best possible commercial grades.

RESULTS AND DISCUSSION

One of the major disadvantages in our earlier fractionation method involved the calcium phosphate gel adsorption step. Although the purification effected by this method was appreciable,

* This report is taken from a paper that was presented at the New York Academy of Sciences Conference on Erythropoietin in June 1966 and appears in the Proceedings of that Conference.

Figure 1. Absorbancy of orosomucoid and bovine plasma albumin at 194 mμ.

the yield was small, and if too much gel was used the erythropoietin activity was adsorbed, and in many cases little or no activity could be eluted.¹ This loss of activity was, in fact, more apparent than real. Data in Table 1 demonstrate that addition of serum albumin to the assay aliquots prevents the loss of an appreciable amount of activity. This protection of the erythropoietin activity appears to be the result of diminished surface denaturation of the hormone in very

Table 1
PROTECTION OF ERYTHROPOIETIN ACTIVITY
BY PROTEIN

Addition	Units recovered
None	6.9
Albumin, 5 mg/ml	18.0
Orosomucoid, 5 mg/ml	18.6
Gelatin, 1 mg/ml	18.0

dilute solution, since orosomucoid and gelatin also had the same effect. This expedient enabled us to recover substantial amounts of activity from calcium phosphate gels at very high potency.

Step III erythropoietin (2.7 u/mg) prepared by DEAE-cellulose and Amberlite XE-97 adsorption was further fractionated with ammonium sulfate to yield a Step IV fraction in 57 per cent yield with a potency of 15. The dialyzed Step IV fraction was dissolved in 0.005 M acetate buffer pH 4.5, and chromatographed on a sulfoethyl Sephadex (SES) column (2.2 cm x 34.4 cm) equilibrated with the same buffer. Elution was done in a stepwise fashion as indicated in Table 2. The major portion of the activity was located in fraction SES-5 eluted with the 0.015 M acetate pH 5.0 buffer. The potency of this fraction was about 130 and recovery at this step was 52 per cent, making the yield from Step III 30 per cent.

Table 2
CHROMATOGRAPHY OF STEP IV ERYTHROPOIETIN ON
SULFOETHYL-SEPHADEX

Fraction	Buffer	Units/mg	% recovery
Step IV	0.005 M acetate, pH 4.5	19.2	100
SES-1	0.005 M acetate, pH 4.5	4.5	7.8
SES-2	0.005 M acetate, pH 4.5	12.8	7.8
SES-3	0.01 M acetate, pH 4.5	0	0
SES-4	0.015 M acetate, pH 5.0	23.5	3.1
SES-5	0.015 M acetate, pH 5.0	133	52
SES-6	0.02 M acetate, pH 5.6	18.4	8

The SES-5 fraction was dialyzed against de-ionized water until the conductivity was $< 100 \mu$ mho cm^{-1} before adsorption on gel. During the handling of this fraction there was an appreciable decline in potency. In Table 3, data from a typical gel adsorption experiment are collected; it can be seen that the calcium phosphate gel eluate 2B (CaP-2B) eluted with 0.0005 M phosphate buffer at pH 7.4 had the highest potency—about 6,500 units per mg protein. The recovery of activity from Step III was 6.3 per cent, and the yield from the original anemic plasma was about 2.2 per cent. Since the original plasma had a potency of about 0.007, the overall purification factor to the CaP-2B fraction is approximately 930,000. A summary of the purification processes is presented in Table 4.

Table 3
CALCIUM PHOSPHATE GEL PURIFICATION OF ERYTHROPOIETIN

Fraction	Buffer	Units/mg	% recovery
SES-5	-	79	100
Sup-2	-	435	28.6
CaP-1A	0.0005 M phosphate, pH 7.4	1,430	17.8
CaP-2B	0.0005 M phosphate, pH 7.4	6,500	37.5

Table 4

SUMMARY OF PURIFICATION OF ERYTHROPOIETIN

Fraction	Units/mg	% recovery	Purification factor
Anemic plasma	0.007	100	-
Step III	2.7	35	388
Step IV	19	20	2,700
SES-5	133	10.4	19,000
CaP-2B	6,500	2.2	930,000

Because of inadequate supplies of starting material (anemic sheep plasma and/or Step III) we have not yet been able to accumulate enough of the CaP-2B fraction to do any tests for heterogeneity. During the gel phosphate procedure however, we have occasionally obtained fractions with potencies in the region of 8,000 units/mg. If this can be confirmed, we must conclude that the CaP-2B fraction is not pure erythropoietin. The heterogeneity may be due to the presence of some erythropoietin that had been inactivated during the purification processes, or to the presence of a protein unrelated to erythropoietin.

We have also been attempting to purify human urinary erythropoietin, but with much less success in obtaining very high potency fractions. As starting material we have used material precipitated from urine of anemic patients by addition of three volumes of acetone. The dried precipitate was extracted with 0.14 M NaCl, dialyzed, lyophilized and then fractionated by a reverse ammonium sulfate gradient dissolution method. The active fraction from the ammonium sulfate technique was then further fractionated with CaP gel to yield a fraction with a potency of about 300 units/mg protein. This material is, at best, only about 5 per cent pure.

Because of the minute amount of plasma fraction CaP-2B obtained, it is impossible to do any direct determinations of its chemical and physical properties. We have been forced, therefore, to rely on biological assay as a means of studying the nature of the hormone. What meager information exists from other laboratories and our own can be summarized as follows:

- 1) Erythropoietin is a sialic acid containing protein;
- 2) Serine and/or threonine hydroxyls do not seem to be required for biological activity;
- 3) Sulfhydryl groups do not seem to be required for biological activity;
- 4) Phenolic hydroxyls and/or free amino groups may be required for biological activity;
- 5) The molecular weight is probably in the region of 50,000-60,000.

The data in support of points (2) and (4) have come, in the main, from the work of Lowy and Borsook.⁴ We have confirmed the inactivation of erythropoietin by fluorescein isothiocyanate, and have found that p-chloromercuribenzoate⁵ does not inactivate it (point 3).

The question of molecular size has been studied by Rosse et al.,⁶ using inactivation by ionizing radiation. They concluded that erythropoietin was an elongated (axial ratio 10:1) molecule of molecular weight 27,000. Hodgson, on the other hand, found a target size of 68,000 by γ -ray inactivation.⁷ We have measured the sedimentation coefficient of erythropoietin by bioassay, using the moving-partition separation cell⁸ in the analytical ultracentrifuge, and found a value of 5-6 S. A sedimentation coefficient this large is not compatible with a molecular weight of 27,000,

but is compatible with a molecular weight of 60-70,000. The elution volume for erythropoietin activity using gel filtration on Sephadex G-200 is also suggestive of the larger molecular size. It is clear that much work remains to be done before this relatively simple problem is solved.

Several laboratories have presented data showing that removal of sialic acid by either sialidase⁹ or dilute acid¹⁰ results in the complete loss of erythropoietin activity determined by bioassay. If the de-sialated erythropoietin is assayed by a marrow cell culture method,¹¹ however, there is no loss of activity (Table 5).¹² We consider the difference between the two groups

Table 5
EFFECT OF SIALIDASE ON ERYTHROPOIETIN ACTIVITY

		Units recovered
Assayed in vivo	Control	11.4
	Sialidase treated	0
Assayed in vitro	Control	0.04
	Sialidase treated	0.05

assayed in vitro to be of doubtful significance. We do not yet understand this phenomenon but think it may relate to the binding of erythropoietin by a carrier substance. Attempts to restore activity to de-sialated erythropoietin by adding suspected carrier glycoproteins have been uniformly without success, suggesting that sialic acid is an intrinsic part of the erythropoietin molecule but is not required either for interaction with the target cells in the marrow, or for action within the cell. Sialic acid may be required in the whole animal for binding to a carrier, or may serve to protect erythropoietin from degradation in, or excretion by, the assay animal. If this is the case, we should not expect the de-sialated erythropoietin to be active (except perhaps in massive amounts) in whole animals, but to be effective in cell cultures. It would also be impossible to restore in vivo activity to de-sialated erythropoietin, short of re-forming the specific covalent bonds that had been broken.

The obvious need at present is a supply of starting material large enough to yield an adequate quantity of erythropoietin, at the highest purity obtainable, for further study. If we want, for example, 10 mg of material with a potency of 6,500, we shall need about 3,250,000 units as anemic plasma. Present methods in use at the Connaught Laboratories of the University of Toronto¹³ yield about 10,000 units per sheep so that a herd of 325 sheep must be processed to produce the desired amount. Although the problem is not impossible of solution, these figures indicate that it will be some time before the final purification and characterization are achieved. Urine from anemic patients may prove to be the only feasible large scale source despite what appears to be a lower stability for the hormone found in urine. Using the same end point and assuming a 2 per cent yield, we calculate that 3,250 L of urine would be required, assuming that 1 unit per ml could be obtained. This would represent about 3 years' daily collection from a single patient, or one month's collection from 36 patients, which does not seem to be an impossible goal. Whatever the source, it is abundantly clear that much work needs to be done before we can begin to study the chemical and structural properties of this hormone and the relationship of these properties to its striking role in red cell development.

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APPLICATION OF THE IN VITRO ERYTHROPOIETIN SYSTEM TO THE STUDY OF HUMAN BONE MARROW DISEASE: POLYCYTHEMIA VERA*

By

S. B. Krantz†

Previous reports from this laboratory have described the development of a cell culture system for the study of bone marrow in vitro.¹⁻³ We have recently shown that these methods can be used to study the increased hemoglobin synthesis that follows the introduction of erythropoietin into cultures of normal human marrow.⁴ These results suggest that the method may prove valuable for intensive biochemical investigations of many of the common clinical disorders of the blood.

Since erythropoietin is believed to regulate erythropoiesis by initiating the differentiation of primitive stem cells into erythroblasts,⁵⁻⁸ the system provides an especially good model for studying proliferative diseases of the red cell in which this control mechanism appears to have malfunctioned. Such a disease is polycythemia vera in which, while there is an overproduction of which cells and platelets as well as red cells, the latter may be singled out for study. The present paper reports some preliminary experiments with polycythemia vera marrow which show that this marrow does not respond in a normal way to the in vitro introduction of erythropoietin and that this relative lack of response to the hormone is not due to a plasma factor but to an intrinsic marrow cell defect.

MATERIALS AND METHODS

The bone marrow cell suspension was prepared as previously described^{1,4} and was also filtered through a sterile stainless steel mesh to remove loose bone particles and thereby produce a more uniform suspension. The dispersed cells were added to 35 x 10 mm tissue culture dishes (Falcon Plastics) in 0.8 ml of a medium which consisted of 60 per cent NCTC 109, 20 per cent newborn calf serum, and 20 per cent human AB plasma or serologically compatible polycythemia vera plasma. ⁵⁹Fe was added in 0.1 ml of homologous AB plasma as a source of transferrin, and radioactive heme was extracted and counted as previously indicated.¹ In order to compare sequential experiments in which the plasma and marrow unlabeled iron might vary, the data are expressed as per cent of control in the dose response experiments, or per cent of initial 4-hour rate in the time course experiments. Sheep plasma erythropoietin (Step IV, lot R 103214A) was used throughout and was obtained from the United States Public Health Service Study Section on Hematology.

RESULTS

When human marrow is placed into this in vitro system and treated with erythropoietin, the rate of heme synthesis is maintained within 70 per cent of the initial rate for up to 70 hours

*This report is taken from a paper that was presented at the New York Academy of Sciences Conference on Erythropoietin in June 1966, and appears in the Proceedings of that Conference.

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while the control rate falls progressively (Figure 1). A dose response experiment with normal human marrow shows a 500 per cent stimulation of heme synthesis above the controls (Figure 2). These data provide a clear baseline for comparison of the response of diseased bone marrow to the hormone.

Figure 1. Effect of erythropoietin on normal human marrow in vitro. The marrow was obtained from a 43-year-old female (hematocrit 41 per cent). Twenty-eight hundred nucleated cells per mm^3 and 108,000 red blood cells (RBC) per mm^3 were present in each plate with 20% homologous AB plasma. (o) erythropoietin, 0.3 units per ml. (●) no added erythropoietin. Each point is the mean of duplicates and indicates the middle of a 4-hour incubation period with $0.49 \mu\text{c}$ of ^{59}Fe per ml.

The effect of erythropoietin on the rate of heme synthesis by polycythemia vera marrow incubated in 20 per cent of the patient's own plasma is shown in Figure 3. Here the control rate of heme synthesis also falls progressively, but the erythropoietin produces a much smaller stimulation than occurs with the normal marrow.

Table 1 shows the results obtained when polycythemia vera marrow and normal marrow, incubated in neighboring dishes, are treated simultaneously with erythropoietin and then tested for heme synthesis. A marked difference in the two responses is again evident, showing that the response is not simply due to variations in material or conditions of incubation.

In order to find out whether this comparative lack of response to erythropoietin might be related to a different dose requirement of the hormone in this disease, a dose response experiment was performed on polycythemia vera marrow (Figure 4). This shows a graded but relatively small response to increasing amounts of the hormone, the maximum response at high concentrations being only 127 per cent of the control. The fall in heme synthesis with very large

Figure 2. Erythropoietin dose response curve with normal human marrow in vitro. The marrow was obtained from a 25-year-old male (hematocrit 43%). Four hundred and eight nucleated cells per mm^3 and 100,800 RBC per mm^3 were present in each plate with 20% homologous AB plasma. After 46 hours incubation, 0.55 μc ^{59}Fe was added and the cultures were terminated 24 hours later. Each point is the mean of triplicates.

doses of the Step IV erythropoietin preparation has been noted before with rat marrow, and is probably related to a contaminant in the preparation. This experiment demonstrates that polycythemia vera marrow follows a similar dose response pattern as normal marrow, the response being much smaller for all doses of the hormone.

The lack of a normal response of polycythemia vera marrow to erythropoietin could be due to a difference within the cell itself or to the presence of an inhibitor in the patient's plasma. This latter possibility was tested by incubating normal human marrow in polycythemia vera plasma. Samples of the same marrow tested with erythropoietin in normal human plasma (Figure 1) were treated with the hormone in polycythemia vera plasma. Figure 5 shows that a similar response was obtained: addition of the hormone produced a normal marked increase in heme synthesis despite the presence of the polycythemia vera plasma. No inhibitor of the hormone, therefore, is present in the polycythemic plasma.

DISCUSSION

These experiments demonstrate that polycythemia vera is characterized by a relative lack of marrow response to erythropoietin and that this characteristic is not due to an environmental or plasma defect but to an intrinsic marrow cell defect. Other investigators have already clearly demonstrated by animal assays that subnormal levels of erythropoietin are present in the blood and urine of polycythemia vera patients.⁹⁻¹¹ Theoretically, erythropoietin could still

Figure 3. Effect of erythropoietin on polycythemia vera marrow. The marrow was obtained from a 69-year-old female who had polycythemia vera for 10 years. When first seen the white blood count (WBC) and RBC were elevated; the marrow was 100% cellular and splenomegaly was present. At the time of marrow aspiration, no suppressive drugs had been administered for 4 years and the WBC was 6,300 and RBC 6.05 million cells per mm^3 . The hemoglobin was 16.3 g %. Each plate had 4,000 nucleated cells per mm^3 and 280,000 RBC per mm^3 in 20% of the patient's own plasma. (o) erythropoietin, 0.3 units per ml. (●) no added erythropoietin. Each point is the mean of duplicates and indicates the middle of a 4-hour incubation with $0.99 \mu\text{c } ^{59}\text{Fe}$ per ml.

cause the overproduction of red cells in this disease if the marrow were hypersensitive to low doses of the hormone. The experiments reported here now show that no such hypersensitivity exists.

It might still be maintained that erythropoietin is consumed at a higher rate by the polycythemic marrow, thus maintaining a low blood level, or that the animal assays for the hormone are not valid for a newly mutated erythropoietin that is active only on human marrow. The former hypothesis can be discarded because of recent studies which show that hyperactive marrows do not decrease the blood half life of injected hormone more than normal marrows do.^{12,13} Again, it has been shown that normal rat marrow in vitro does not consume much of the erythropoietin present.² The data supplied by this in vitro method now make it unlikely that a new, species-specific erythropoietin is present, since when polycythemia vera plasma is assayed on human marrow in vitro it does not stimulate heme synthesis beyond the levels observed with normal plasma. Indeed, the rate of heme synthesis is usually lower than with normal plasma.¹⁴ Thus erythropoietin is clearly not the cause of the overproduction of red cells in this disease.

Erythropoietin normally acts on primitive stem cells to promote differentiation into erythroblasts,⁵⁻⁸ and this is believed to occur through the synthesis of new messenger RNA.³ If erythropoietin production in the mouse is suppressed by hypertransfusion, then further differentiation ceases and no normoblasts can be found in the marrow.⁵ Similarly, removal of the erythropoietin in vitro by transferring the cells to normal medium stops a further increase in

Figure 4. Erythropoietin dose response curve with polycythemia vera marrow. The marrow was obtained from a 66-year-old male who had polycythemia vera for 11 years. When this marrow was aspirated he had been treated only with phlebotomy for 1 year and had a RBC of 7.65 million cells per mm^3 ; WBC was 12,900 cells per mm^3 and platelet count was 955,100 cells per mm^3 . Each plate had 2,100 nucleated cells per mm^3 and 372,000 RBC per mm^3 in 20% of the patient's own plasma. After 46 hours of incubation $0.95 \mu\text{c}$ ^{59}Fe was added and the cultures were terminated 24 hours later. Each point is the mean of triplicates.

Table 1
EFFECT OF ERYTHROPOIETIN ON NORMAL MARROW
AND POLYCYTHEMIA VERA MARROW

Sample	Treatment	Heme (cpm)
Normal marrow	Control	282
	Erythropoietin (0.3 units per ml)	842
Polycythemia vera marrow	Control	332
	Erythropoietin (0.3 units per ml)	388

The polycythemic marrow was obtained from a 65-year-old male who had polycythemia vera treated only with phlebotomy for 1.5 years. When first seen the hemoglobin was 20.6 g % and the RBC was 7.06 million cells per mm^3 . The bone marrow was 90% cellular with clumps of megakaryocytes. Each plate had 1100 nucleated cells per mm^3 and 148,000 RBC per mm^3 in 20% of the patient's own plasma.

The normal marrow was obtained from a 44-year-old male who had tuberculosis but whose blood counts were normal. At the time of this marrow aspiration his WBC was 7500 cells per mm^3 with a normal differential count and the hematocrit was 41%. Each plate had 1600 nucleated cells per mm^3 and 440,000 RBC per mm^3 in 20% of the above polycythemic patient's plasma. Both series were incubated simultaneously for 67 hours and then $0.62 \mu\text{c}$ ^{59}Fe was added for 8 hours before terminating the cultures. Each set is the mean of triplicates.

Figure 5. Erythropoietin effect on normal human marrow incubated in 20% polycythemia vera plasma. The marrow is a replicate specimen of the one described in Figure 1 and cell counts are the same. (○) erythropoietin, 0.3 units per ml. (●) no added erythropoietin. The polycythemic plasma was obtained from a 68-year-old male who had polycythemia vera for 3 years. When first seen the hemoglobin was 19.2 g % and the hematocrit 68%. The bone marrow was 90% cellular and palpable splenomegaly was present. When this plasma was obtained he had been treated only with phlebotomy for 6 months and had a hemoglobin of 16.4 g %, hematocrit of 59% and RBC of 7.43 million cells per mm³. Each point is the mean of duplicates and indicates the middle of a 4-hour incubation with 0.49 μ c ⁵⁹Fe per ml.

hemoglobin synthesis above the controls.² Thus normally the stem cells are inhibited from further differentiation in the absence of the hormone. This inhibitor acts like the "repressor" described by Jacob and Monod¹⁵ and erythropoietin may be considered as analogous to the "de-repressor." An absence or malfunction of the repressor would produce an uncontrolled differentiation not very responsive to the de-repressor. Thus polycythemia vera is best seen as a disease with an intrinsic defect in the specific stem cell repressor that is acted on by erythropoietin. Further investigation must now center on the repressor mechanism that ordinarily prevents this red cell overproduction unless the switch is turned on by erythropoietin.

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ISOTOPE DOSIMETRY IN NUCLEAR MEDICINE*

By

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Although the use of all pharmaceutical preparations comes under the authority of the Food and Drug Administration, in some specialized areas this responsibility is delegated at the present time e.g., to the National Institutes of Health for blood products and to the Atomic Energy Commission for radiopharmaceuticals. The ultimate plan appears to be that these delegated functions should return to the FDA itself as growth in personnel and technical capabilities permits. Meanwhile, the Atomic Energy Commission controls the use of radiopharmaceuticals through its licensing division. This office was originally set up to control the distribution of by-product materials, that is, reactor-produced radioisotopes. Individual licenses issued by this agency are reviewed by a national medical advisory committee. Broad licenses are issued to medical centers, which in turn control individual projects through local committees. Under its existing regulations, the Atomic Energy Commission does not license the use of accelerator-produced isotopes, and the FDA retains direct responsibility for radiopharmaceuticals containing nuclides so produced.

The general guide lines of the Atomic Energy Commission licensing division appear to follow those of the FDA for use of new pharmaceuticals. The user of a radiopharmaceutical must present evidence of his competence, of the safety and efficacy of the material he proposes to administer to patients as compared with other pharmaceutical products, and in particular that he is able to give thoughtful consideration to the question of radiation dosage caused by the agent to the patient, to the physician and his helpers, and to the general public. Because many physicians have not had training in this area, the applicant is required to submit a calculation of the radiation dosage to be expected. Conversations with various members of the Atomic Energy Commission's Advisory Committee on the medical use of radioisotopes have revealed a number of instances where there were fundamental misconceptions of even the basic nature of radiation dosage, e.g., "the dose to both kidneys is 10 rads, making a dose to each kidney of 5 rads," and frequent examples where the dose calculations furnished by the manufacturer of a radiopharmaceutical were blindly copied word for word into the application. It was with the hope of introducing some illumination into this situation that the present article was undertaken.

Absorbed radiation dosage as opposed to exposure dosage is expressed in terms of the energy absorbed from ionizing radiations per unit mass of tissue. The unit in current use is the rad, which is defined as 100 ergs absorbed per gram. It is thus an intensive rather than an extensive measure since it is independent of the total mass of tissue involved. This unit is at best a sort of common point of departure. Different tissues have greatly varying sensitivities to radiation. In general, rapidly dividing cells such as the epithelium of the intestinal crypts and bone marrow are most sensitive, while other tissues, e.g., muscle and liver, show relatively little biological effect. The genetic effects from gonadal irradiation which we all fear have not as yet

* This paper appeared in the Year Book of Nuclear Medicine, 1965/66 series, ed. by J. Quinn, Year Book Medical Publishers.

been firmly documented in viable human offspring, although temporary or permanent changes in menstruation or spermatogenesis are familiar at relatively high radiation dosage, as are the effects on skin in chronic radiation exposure. The effects of chronic and acute radiation in producing leukemia in humans are at the moment under intensive investigation, and a fairly convincing case has been made for the induction of dysplasia and neoplasia of the thyroid in children exposed to radiation dosage in the 100 to 1000 rad range. The lethal effect of total body irradiation in this range is similarly well known. To what extent the information gathered from exposure to external radiation sources can be compared to that for internal emitters is a matter of controversy. This is related to the geography of the energy deposition. In the case of external sources, the biological effect of radiation is dependent in a number of ways on the dose rate and the ionization density along the path of the radiation or the linear energy transfer (LET). With internal emitters, an additional factor, the location of the source, becomes critical in a number of ways. While the radiations from iodine-125 localized in the colloid of the thyroid follicles are very largely absorbed without reaching living cells at all, it is hard to imagine a more biologically effective localization than that of tritiated thymidine in the chromosomes of dividing cells. The ^{131}I β radiation dosage surrounding particles of aggregated albumin in the lungs is very high to the immediately adjacent endothelium cells, but very modest averaged over the volume of the lung. If we had complete knowledge of an individual situation, it might be possible to make an evaluation of the radiation dosage somewhat as follows:

Effective radiation dosage = energy absorbed (rads) x effects of linear energy transfer x effects of nature of radiation x effects of dose rate x tissue sensitivity x mass of tissue affected.

This quantity would be summed over the whole organ or body. Such an expression is

$\text{rem(Roentgen equivalent man)} = \text{rads} \times \text{RBE(Relative biological effectiveness)}$.

Our knowledge at the present time is much too fragmentary to attempt anything of the sort in a formal way, but all of the factors known, estimated, or guessed at must enter into the decision of whether the information gained from a test using a radiopharmaceutical is justified in view of the radiation dosage incurred. The prevailing compromise is to calculate the radiation dosage in rads to the target organ, or organ which receives the largest radiation dosage; the total body dose using the most reasonable assumptions possible; and the dose to the bone marrow, gonads, intestine and urinary tract, if there is substantial reason to believe that it may be high.

Even in calculation of the simple rad dosage to an organ there is in many cases considerable uncertainty. In consequence, where necessary parameters are unknown, or known only approximately, it has been customary to use "pessimistic assumptions," so that the calculated radiation dosage represents an upper limit. Actually, what the physician desires to know is whether his patient is receiving 200 rads or 200 millirads or 0.200 millirads. In this dose range of 10^6 an error of a factor of two may appear inconsequential, although it certainly seems desirable to know if possible whether this error lies in the biological or physical parameters.

In practical clinical isotope dosimetry, a number of the factors discussed above may usually be ignored. Since we are dealing almost exclusively with β , γ emitters, the effects of linear energy transfer are not important. Except for bone, the density of tissue is usually assumed to be 1.00. The dose from β and β -like radiations which are absorbed close to their point of origin are considered separately from the more penetrating γ and x-ray radiation. It should be

pointed out that the distinction between β -like and γ -like radiation is not rigid. For instance, the x- and γ -radiations from ^{125}I which have a narrow beam HVL of 1.9 cm must clearly be considered γ -like when calculating the thyroid dose. In the total body dose or liver dose assuming uniform distribution, however, a correct value is obtained for the central core of the body or organ assuming that the radiation is β -like (completely absorbed), with somewhat lower values at the periphery due to escape of the radiation from the surface. This constitutes a pessimistic but reasonable assumption.

The classical expressions of Marinelli et al.¹ are as follows:

$$D_{\beta} = 73.8 C_0^{\rho} \bar{E}_{\beta} T_{\text{eff}}$$

$$D_{\gamma} = 0.0346 C_0^{\rho} \Gamma \bar{g} T_{\text{eff}}$$

In these expressions, D_{β} and D_{γ} represent the dose in rads from β and γ radiations respectively. C_0 is the initial concentration of the isotope in microcuries per gram. $\bar{E}_{\beta}$ is the mean energy of the β -like radiations in MeV. Γ is the γ -ray dose rate constant, e.g., r per millicurie hour at 1 cm. More properly expressed in rads this becomes

$$\Gamma \text{ rad} = \Gamma r \times \frac{\text{ergs/gm roentgen}}{100}^2$$

T_{eff} is the effective half-time in days of the radiation source in the tissue, and $\bar{g}$ a geometrical factor. The effective half-time is composed of the physical half-life T_p and the biological half-time T_b as follows:

$$\frac{1}{T_{\text{eff}}} = \frac{1}{T_p} + \frac{1}{T_b} \quad \text{or} \quad T_{\text{eff}} = \frac{T_p T_b}{T_p + T_b}$$

The two constants 73.8 and 0.0346 are conversion factors, so that the product appears in rads.

The major errors in practical dosage calculations in most cases lie in the uncertainties in the biological parameters C_0 and T_{eff} . Frequent pessimistic assumptions which must be made are that the entire injected dose of isotope is concentrated in a particular organ (for instance, ^{198}Au colloid in the liver); that the entire dose is evenly distributed throughout the body (^{131}I albumin); or that all the radioactive material remains in the body without excretion, so that $T_{\text{eff}} = T_p$. Even the most fragmentary information about concentrations or excretion is an improvement over this. In some cases where the organ concentration is well known, the biological disappearance is no longer a simple curve, and one portion of the injected activity may disappear rapidly, while another fraction disappears much more slowly: The localization of neohydrin in the kidney is a good example.

The geometrical factor $\bar{g}$ is a number with the dimensions of centimeters which is proportional to the average radiation dose in the volume of tissue under consideration. The value of $\bar{g}$ is determined by the size and shape of the mass of tissue; by the attenuation of the gamma radiation; by the scatter and dose build-up surrounding the radiation source; and by the isotope distribution, usually assumed to be uniform. For γ energies of 100 keV or more, the attenuation of the radiation by passage through tissue is more or less balanced by dose build-up, and analytical expressions exist for $\bar{g}$ for some simple geometrical shapes; e.g., for the sphere: $\bar{g} = 3\pi r$, where r is the radius of the sphere in centimeters. These assumptions are approximately valid up to $r = 10$ cm. Tabulated values of $\bar{g}$ under these conditions are available³ for a variety of use-

ful shapes and sizes which are fairly accurate in this energy range. For γ energies below 100 keV, these simplifying assumptions lead to errors of almost a factor of 2 low in the 70-80 keV range because of the effects of multiple scattering and dose build-up; and a factor of 2 high in the 20-30 keV range because of the rapid rise in the γ -ray absorption coefficient with decreasing energy, which results in a disproportionate increase of Γ without a corresponding decrease in $\bar{g}$ when calculated in the usual way. More sophisticated methods for calculation are available which are applicable in this region. Actual measured dose gradients may be used with machine methods for numerical integration to obtain dose distributions in a mass of tissue of any shape or volume. Another approach is to calculate again by computer methods the fraction of the emitted gamma energy from a uniformly distributed source which will be absorbed in a solid of given shape. The gamma dose is then

$$D_{\gamma} = 73.0 \times E_{\gamma} \times \text{Absorbed Fraction} \times T_{\text{eff}}$$

thus using a formulation similar to that for D_{β} . Tabulations are also available for absorbed fractions for a useful variety of shapes.⁴

Frequently, more than one β and/or γ component is emitted by a single radionuclide. The various β components may be lumped together if a value for $\bar{E}_{\beta}$ is available. Although this number is often unknown, the maximum β energies and abundances are usually known. Since $\bar{E}_{\beta}$, the average beta energy, is roughly one-third of the maximum β energy, one may calculate approximately $\bar{E}_{\beta}$ under these circumstances. When more than one gamma is present, the values for Γ may be lumped if the γ energies are all over 100 keV. When using the absorbed dose method, however, each gamma must be considered separately and the results summed.

The dosimetry of isotopes which decay by electron capture or which exhibit internal conversion of the emitted gamma radiation has some special problems. Conversion electrons have the energy of the converted γ minus the binding energy of the particular converted electron. These electrons of course behave like β radiation. The fluorescent x-rays which appear following either electron capture or internal conversion may be β -like or gamma-like depending on the energy of radiation and geometry of the organ. The total energy available for producing ionizing radiation is equal to the binding energy of the electron captured or converted. For practical purposes only the K fluorescent x-rays are γ -like, and the fraction of the total energy present as K photons subtracted from the total available energy for ionization gives the amount of β -like energy which remains.

Special calculations are necessary in such situations as the exposure of the intestinal mucosa to a source in the intestinal content. For β radiation this is very simple. The dose at the surface of a volume containing a uniformly distributed β emitter is exactly $1/2 D_{\beta}$, the dose in the interior of the volume. This is clear since if two such volumes are placed in contact, the touching surface points become interior points with half the dose contributed from each side. The surface dose from a gamma emitter in the same situation may be calculated somewhat less exactly by assuming a model in which the bowel is simulated by a string of spheres with the source distributed as point sources at the centers of these spheres. The dose at the surfaces of the spheres is thus easily calculated from the source strength, Γ , and radius of sphere.

Approximate calculation of such special situations as the dose to the ovaries caused by radioactivity in the urine in the bladder may be carried out in a similar way. Model dose calculations covering a variety of situations are available.⁵

This article is obviously not intended to serve as a short cut to dosimetry for the hurried physician, but we hope that it may permit the reader to attack the standard reference books with an increased insight and orientation when he is forced to carry out dosimetric calculations.

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PROTOCOL FOR THE DOSIMETRY OF HIGH ENERGY ELECTRONS*

By

The Subcommittee on Radiation Dosimetry[†]
of the
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In the various medical applications of high energy electrons, it is essential to have an accurate knowledge of the dose delivered at specified points in the patient. The increasing number of high energy electron beam installations in the United States makes it highly desirable that standard methods for the measurement of output and of absorbed dose be explicitly described in order to facilitate uniformity of dosimetry. In the past, conventions have been developed for the determination of these parameters by the few institutions in this country using electrons clinically. The Subcommittee on Radiation Dosimetry (SCRAD) of the American Association of Physicists in Medicine (AAPM) has evaluated these conventions and has also studied other methods for achieving standardized dosimetry. This protocol presents recommendations of SCRAD for a uniform dosimetry for high energy electron beams.

When high energy x-rays and electrons were first used for therapy, it was necessary to establish standard methods for monitoring of output, measurement of absorbed dose, and procedures for the correlation of output with the dose delivered in patient treatment. The conventions adopted for output and dose measurement were necessarily specialized (NCRP 1955,¹ Laughlin²) since the Roentgen was not defined above 3 MeV and was not applicable to electrons. These conventions have served very adequately.

As an absolute laboratory standard for the calibration of dosimetric quantities, the calorimetric method was developed. Although it was applied initially to high energy x-rays, it has also been applied to x-rays of all energies, as well as to electrons. Details of the design of calorimeters for the measurement of intensity, energy fluence, and output, as well as those designed for the measurement of absorbed dose, have been described in the literature (Milvy et al.,³ Genna et al.,⁴ Bewley⁵). Specific applications of this method have been made to electron beam dosimetry, usually to calibrate other dosimeters (Geisselsolder et al.,⁶ Bewley,⁵ Bradshaw⁷). Although field calorimeters have been developed, this method is still not generally available, and therefore will not be further described here.

There are several special problems of importance for the dosimetry of electrons. Electrons

* This report appeared in *Phys. Med. Biol.*, 11:505, 1966.

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are absorbed quite differently from x-rays, are easily scattered, change drastically in energy with depth in the patient, etc. A particular problem of importance is the dependence of their absorption upon the physical density of the absorbing medium, as well as upon their velocity. The rate of energy loss of electrons depends strongly on the intensity distribution of their associated electric field; the effect of which in the medium is modified by the molecular polarization produced in the immediate vicinity. At relativistic energies, Lorentz contraction of this field increases the importance of more distant interactions. The effect of the shielding by the polarized molecules then depends strongly upon the condensed or gaseous state of the medium. This effect was predicted by Fermi⁸ and the theory extended by Sternheimer.⁹ The effect was demonstrated by extrapolated range measurements in water by Laughlin and Beattie,¹⁰ by energy loss measurements in metals by Goldwasser et al.,¹¹ and specifically for dosimetry in liquid and gaseous media by Zsula et al.¹²

In order to obtain uniformity of dosimetry between high energy radiation installations at different institutions, dosimeters have on many occasions been carried between institutions, both in this country and abroad. Some intercomparison meetings have been reported (Johns et al.,¹³ Sinclair, et al.,¹⁴ Kretschko et al.¹⁵). These intercomparisons have been based most frequently on the response of such dosimeters as the Baldwin-Farmer Substandard Dosimeter, Victoreen thimble chambers, and the Fricke Ferrous Sulphate dosimeter, all exposed under conditions specified as to surrounding material, depth, field size, reference calibration, electron energy, etc. In order to facilitate more periodic intercomparisons, the feasibility of the use of mailed dosimeters has been examined. This was undertaken as an extension of the personal courier intercomparison and to assist the Scientific Committee of the AAPM in its consideration of methods for more general periodic comparison of high energy dosimetry. Both irradiation and control samples of the Fricke ferrous sulphate solution and of lithium fluoride dosimeters have been handled and irradiated under different conditions. The effect of different procedures of mailing and measurement has been studied (Laughlin et al.¹⁶) and the results of the preliminary intercomparisons have been reported (Pinkerton et al.¹⁷).

In general, the dosimetry of high energy x-rays and electrons, as developed over the past several years, is on an absolute basis and its special dosimetry problems are well understood. Methods for uniform standardization of widely scattered installations have been developed. This protocol is intended to formalize the experience gained in the dosimetry of electrons in the energy range 5 MeV to 50 MeV.

RECOMMENDATIONS FOR ELECTRON BEAM DOSIMETRY

Some of the recommendations contained in the text apply directly to calibration and intercomparison, while others apply more generally to preferred methods of electron beam handling (i.e., scattering, collimation, etc.). Those which are directly pertinent to dosimetry procedures are briefly summarized here.

1. Field size specification. Field size is defined by the dimensions of the plane figure described by the intersection of the 90 per cent isodose surface with the plane passing through, and normal to, the central axis at the position of the relative dose maximum, which is normalized to 100 per cent.

2. Energy calibration. For the calibration of electron beam energy, the electron-disinte-

gration threshold method is recommended as being the most generally available of the reliable methods.

3. Beam monitoring. The electron beam should be continuously monitored by a transmission detector which is broad enough to include the entire beam. The monitoring system should indicate both dose rate and integrated dose. The monitor should preferably be calibrated once each week, and not less than once each month.

4. Absorbed dose calibration. The Fricke Ferrous Sulphate Dosimeter should be used for absorbed dose measurements, with a 'G' value of 15.4 molecules oxidized per 100 electron volts. The measurement should be carried out in a tissue-equivalent phantom similar to that employed for the isodose distributions in use at each institution.

5. 'Output' convention. A useful method of expressing monitor units is in terms of 'output.' The convention for its measurement is the reading of a thimble chamber placed at the maximum of the dose distribution in a polystyrene Intercomparison Phantom. The cobalt-60 calibration factor is applied to the chamber. The Farmer Secondary Standard Dosimeter (Farmer¹⁸) is recommended for the thimble chamber.

6. Intercomparison Phantom. A specific phantom is defined for the 'output' convention and for intercomparison measurements of either absorbed dose or of 'output.' The phantom consists of a block of polystyrene 25 cm x 25 cm in cross-section, and with a depth dependent on the highest electron energy with which it will be used, according to the relation:

$$\text{phantom depth} = \frac{\text{highest electron energy (MeV)}}{2 \text{ (MeV/cm)}} + 1 \text{ cm.}$$

7. Intercomparison. It is recommended that institutions engaged in electron beam therapy carry out absorbed dose intercomparisons periodically. These are carried out with either the Fricke Ferrous Sulphate Dosimeter or a thermoluminescent dosimeter located at specified depths in the Intercomparison Phantom. 'Output' intercomparison can be carried out with the recommended thimble chamber in the same phantom.

RECOMMENDATIONS AND BACKGROUND

Beam Geometry

Field Intensity Distribution

(a) Scattering. Scattering material may be introduced in the path of the beam close to the accelerator tube window in order to cause the beam to diverge, so that the electrons will be spread over a larger useful area, and also to obtain more "flattened" isodose surfaces. High atomic number material is more efficient for scattering relative to the amount of energy loss and for this reason foils of gold or lead have been preferably employed. The amount and composition of the scatterer has only a limited effect on the shape of the central axis depth dose curve for the same emergent energy. The minimum thickness of material that is necessary should be employed.

(b) Scanning. An alternative to a scattering foil for enlarging the electron beam field sizes has been the use of a small pencil beam of electrons that scans the tumour (Carpender et al.,¹⁹ Wachsmann et al.²⁰). In this system of moving field therapy, the beam is guided by means of magnetic fields, and is capable of scanning arbitrary field sizes and shapes in one mode of operation. Alternatively, the beam can move over a circular arc up to 360° about the patient, while

also carrying out translational motion perpendicular to the arc. In either mode of operation both uniform or line irradiations are obtainable. A simple template determines the range of movement of the magnet, thus precluding the need for collimators and cones for delineating the field.

Method of Collimation

Collimation may be achieved by the use of physical beam-defining apertures of either the fixed or variable type, or by the use of a pencil beam which is deflected over the area of the desired field. The material of the collimator or diaphragm system has a limited effect on the overall three-dimensional dose distribution (Laughlin,^{2,21} Loevinger et al.,²² Turano et al.²³). The effect is more pronounced for small field sizes where the scattered contribution from the collimator is more important. Intermediate or high atomic number material is preferable in order to minimize large angle scattering. However, the use of transparent low atomic number materials may be clinically useful and is entirely acceptable. The design and material of the collimators affect the dose distribution at the edge of the beam (Bradshaw and Maysent²⁴). This effect is deliberately made use of in treatment planning. It is, of course, essential that the three-dimensional distribution of dose be known in all cases.

Specification of Field Size

The field size which is determined by the combination of the various pertinent parameters, including electron energy, collimator size, scattering material, distance, etc., can be significantly expressed only in terms of the dose distribution achieved in tissue-equivalent material. For the purposes of calibration and intercomparison, it is recommended that field size be defined by the dimensions of the plane figure described by the intersection of the 90 per cent isodose surface and the plane that passes through, and is normal to, the central axis at the location of the relative dose maximum. Inasmuch as the field size produced by a given aperture is a function of the electron energy as well as of the scatterer employed, each institution will have its own system for labeling its collimators.

Energy Calibration

The energy of the electrons should be specified after passing through any foils or other material that would be present during patient irradiation or other use. Methods for energy calibration are: magnetic deflection, electron-disintegration thresholds, and range measurements.

Magnetic Deflection

The primary energy calibration of electrons is by magnetic methods. If the radius of curvature of the path of an electron and the magnetic field are known, the momentum and, in turn, the energy of the electron is determined. In betatrons or synchrotrons, which inherently make use of a changing magnetic field for electron acceleration or guidance, a fixed search coil in the field is used to pick up a signal. A time integral of this signal is then related to the magnetic field (Penfold and Garwin²⁵). The radius of curvature of the path of the electron is related to the distance from the magnetic centre of the betatron to the search coil. For the linear accelerator, which does not have such an electron-guide magnet, an external spectrometer magnet is used (Panofsky and McIntyre²⁶). In situations where a magnet deflecting and scanning system is used in conjunction with the linear accelerator, the deflecting analyser magnet can be used to calibrate the energy of the electron (Skaggs et al.²⁷). The magnetic field in this case is measured

most accurately by a proton nuclear resonance gaussmeter and the "floating-wire" technique. Magnetic field calibration methods have been described by Cranberg²⁸ and by Germain.²⁹

Electron-disintegration Thresholds

Energy calibrations are most conveniently achieved by nuclear electron or photon reactions. A basic difficulty in using neutron separation energy values in light elements as energy standards arises from the presence of the discrete resonances in the photon absorption cross-section. The position of the observed reaction threshold may be determined by the strengths, widths, and relative spacings of these resonances in the vicinity of the nuclear separation energy.

In the determination of the carbon threshold, it is important that the specimen contain no oxygen. Since photonuclear cross-sections are much larger than electro-nuclear cross-sections, more radioactivity can be obtained with the interposition of a 2 mm-thick lead foil on the beam side of the specimen foil. The bremsstrahlung, produced in the lead foil, which is above the threshold energy activates the specimen foil. The background activity produced in copper foils by thermal neutrons is reduced by enclosing all sides of the specimen foil with cadmium, except that side on which the electrons are incident. A shielded end-window Geiger-Müller (G-M) counting arrangement is often used for determination of the activity of the specimen foils. The oxygen threshold at 32.5 MeV can be difficult to measure since the threshold at 15.6 MeV will simultaneously be present, and since the half-life of ^{14}O is only 72 seconds. However ^{15}O has no γ -ray while ^{14}O emits a 2.3 MeV γ -ray which is measured with scintillation crystal techniques (Breuer and Pohlit,³⁰ O'Connell et al.³¹).

A specific suggested procedure is as follows: several identical foils are prepared and numbered. An arrangement is made for mounting the foil in the beam in such a way that it can be removed easily and another placed at exactly the same location. Since the activation rate is the same as the half-life for disintegration, the time of exposure should be chosen to be at least three half-lives of the isotope. After exposure, the foil is put into a shielded sample changer and the number of disintegrations in a given time is counted. Several more runs are made at different energies, exposing the foil for the same time, keeping the output as constant as possible. The square root of the net number of counts (total number minus background) when plotted against the reading of the panel energy meter will give a nearly straight line. The point at which this plot intersects the energy axis will indicate the panel meter reading which corresponds to the threshold energy for the given nuclide. It is usually effective to commence about 3 MeV above the threshold energy and descend to it in 0.5 MeV, or smaller, steps. The time intervals of irradiation and counting, and the length of time between the end of the irradiation and the beginning of the counting is most conveniently made the same for each determination. It is usually most efficient to count for the same time interval as that for which the foil is irradiated. When a scatterer is placed in the path of the electron beam, the amount of energy loss may be checked experimentally by exposing foils with the scatterer in and out of the beam, or it may be calculated. Copper foils should be thoroughly cleaned, so that no appreciable oxide is present. Carbon, being porous, will always contain oxygen if used in a pure form; this problem can be solved by using polystyrene foil.

For most of those radionuclides in Table 1 which are positron emitters it is possible to make use of the 0.51 MeV annihilation gamma-rays in the threshold counting determinations.

Table 1

Reaction	Threshold (MeV)	Decay particle and maximum energy (MeV)		Activity half-life
$^{181}\text{Ta} (e; n, e') ^{180}\text{Ta}$	7.6	β^-	0.7	8.2 hours
$^{63}\text{Cu} (e; n, e') ^{62}\text{Cu}$	10.8	β^+	2.9	10.5 min
$^{16}\text{O} (e; n, e') ^{15}\text{O}$	15.6	β^+	1.7	2.0 min
$^{12}\text{C} (e; n, e') ^{11}\text{C}$	18.8	β^+	0.99	20.0 min
$^{16}\text{O} (e; 2n, e') ^{14}\text{O}$	32.5	β^+	1.8	72.0 sec

The activated sample is placed between two NaI crystal scintillation detectors. The output of each of these detectors is fed through its own spectrometer into a coincidence circuit gated for simultaneous pulses. This coincidence count method greatly reduces the background and increases the precision near the threshold over that obtainable with G-M counting.

Figure 1. The result of this calibration, carried out as described in the text, was that a panel-meter reading of 10.5 corresponded to an electron energy of 10.8 MeV. The accuracy of the energy calibration in this instance is ± 0.2 MeV which is acceptable, provided the dose distribution data are obtained under similar conditions.

Range Measurements

Range measurements can also be employed to estimate the electron energy. Since this method depends somewhat on the energy spectrum of the electron beam, it is desirable that results obtained by this method be checked by either of the first two methods for a similar ma-

chine operating under similar conditions. The most convenient range measurement method is to utilize central axis depth dose measurements in a phantom. These have been made in several ways, including the use of film in both water and tissue-equivalent phantoms, the use of cavity ionization chambers in water, and the use of cavity ionization chambers in solid phantoms. Range measurement methods were originally described in nuclear physics procedures, where the number of electrons reaching a certain depth was counted rather than the ionization produced by them. It has been shown that the cavity ionization dose distribution is not significantly different from the number plot (Laughlin and Beattie¹⁰).

If the number of electrons, or the relative ionization in water, is plotted as a function of depth, the practical (or extrapolated) range R_p can be determined by extrapolation of the decreasing portion of the curve. The point at which the electron curve becomes indistinguishable from the background gives the maximum range R_o ; the point where the extrapolation of the linear region of the curves cuts the abscissa is called R_a . The difference between R_p and R_a is very small at energies below 25 MeV but becomes appreciable above 40 MeV. Figure 2 shows the relationship between the various R's for a typical curve. Several authors (Loevinger et al.,²²

Figure 2. Representation of the calibration of electron energy by range measurement. Taken from Katz and Penfold³⁴ and Loevinger et al.²²

Markus^{32,33}) have discussed the relationship between practical range and the energy, and the following mean range-energy relation in water is proposed:

$$R_p = 0.52 T - 0.3 \text{ cm water}, \quad 5 < T < 50$$

where T is the kinetic energy in MeV, for sufficiently large beam diameters. The required field diameter is about $2.5 R_p$ at 5 MeV, falling to about R_p at 15 to 20 MeV (Markus³²). Above 20 MeV the required field diameter is about 10 cm.

Energy Calibration Check

It is desirable to have a relatively convenient method of checking the calibration of the elec-

tron beam energy. A cavity ionization measurement made at a fixed depth in a phantom (described below) with a thimble ionization chamber provides such a rapid check (Almond³⁵). The ratio of the ionization at a fixed depth to the ionization measured at the depth of maximum cavity ionization can be plotted versus energy to yield a straight line. It is suggested that the top of the phantom calibration block be reversed to make this measurement (see Figure 3). For high energies it may be necessary to drill an additional hole in the base of the calibration block at a greater depth than is indicated on the drawing. Unoccupied holes should be filled with polystyrene plugs when not occupied by a thimble chamber. Any serious deviation from the normal ratio value at a fixed energy and fixed depth would indicate that there has been a change in the energy of the beam. It is emphasized that the ratio measurement is not a substitute for the energy calibration obtained initially by disintegration-threshold or magnetic deflection methods.

Figure 3. Polystyrene Intercomparison Phantom for absorbed dose and 'output' measurements.

Monitoring

The electron beam should be continuously monitored by passage of the beam through a transmission detector. The integrated monitor current determines the total exposure. It is important that the monitor include the entire beam, and not only a sample portion of the beam, since the intensity distribution of the electron beam may not be uniform. The plates of the monitor should be kept as thin as possible and certainly should not exceed the thickness of the scatterer. The design of the integrator circuit is important for accuracy of control. A well designed circuit will make possible the display of both dose rate and also the integrated total dose delivered.

In the event that an ionization chamber is employed, it is important that maximum ion collection efficiency for the high intensity pulses of short duration characteristic of accelerators be achieved (Boag³⁶). Saturation of the collection current should be specifically checked. If the chamber is not sealed, a convenient procedure for correction for temperature and pressure should be established.

A secondary emission monitor may alternatively be employed. It avoids the problems of lack of saturation and dependence on temperature and pressure, but the secondary electron current is much smaller, is energy dependent, and requires higher amplification.

'Output' Convention

The measurement and calibration of absorbed dose is discussed in a subsequent section. For the specification of patient treatment the measurement or calculation of absorbed dose at pertinent points is required. Although the determination of the absorbed dose is of primary importance, it has been found useful to have a method of measuring a quantity identified here as 'output.'

Significance of 'Output'

One of the earliest methods for quantitating patient exposure to x-radiation was to measure the 'output' of the x-ray generator with an ionization chamber. As the effect of the construction of the chamber and operating parameters on the ionization measurements became recognized, standard methods for measuring output were promulgated. The work of many investigators on ionization chamber design and operation culminated in the definition of the Roentgen as the unit of x-ray exposure. There are now several national physical laboratories equipped to measure the Roentgen, and the specification of x-ray exposure-rate in terms of R per minute has been found useful. Although the patient treatment must be specified in terms of the absorbed dose at specific points, it has been useful to compare and indicate the output of various x-ray sources by means of exposure-rate. The success of the Roentgen is due to the ease and accuracy of ionization measurements, as well as to the availability of secondary standards that may be calibrated at national laboratories. Its success is also due to the fact that it provides a convenient basis for the quantitative expression of the output of x-ray sources. For the same reasons that apply to the standardization of x-ray machines, it has proved useful to have a somewhat similar method for the standardization of the output of electron beam generators. For several years such a concept has served to maintain uniformity in the dosimetry of electron beam treatment in widely separated installations.

Definition of Electron Beam 'Output'

Output is determined from the reading of a thimble-type cavity ionization chamber exposed in a polystyrene phantom (specified below) at the position of the maximum in the depth dose distribution. It is specified that the chamber have applied to it the calibration factor appropriate for cobalt-60 gamma-rays. Although the results obtained from this measurement are generally expressed as 'r' per monitor unit, the output is expressed as 'r' per minute. It is recognized that output is a general term that ordinarily does not have a precise definition. The definition given here is arbitrary but has the virtue of having successfully served an essential purpose over a considerable period of time. Furthermore, every radiation therapy installation should have a calibrated thimble chamber. This output convention has proved useful for electron beams because it is convenient, reproducible, and readily intercompared.

Phantom Specification

It is recommended that the phantom consist of two blocks of polystyrene with cross-sectional dimensions of 25 cm by 25 cm and a depth given by the following relation:

$$\text{depth of phantom (cm)} = \frac{\text{Highest electron energy (MeV)}}{2 \text{ (MeV/cm)}} + 1 \text{ cm.}$$

A hole into which the thimble ionization chamber snugly fits should be drilled at a depth of 1.5 cm extending beyond the centre of the block, the axis of the hole being contained in a plane parallel to one face of the block. As indicated in Figure 3, it is recommended that the phantom consist of two blocks, one 5 cm deep and the other, 'back-up' block, sufficiently deep to provide the remaining depth necessary. With the chamber hole drilled at the indicated depth in the front block, the chamber can be placed at a depth of either 1.5 cm or 3.5 cm, depending on the orientation of the block. It is further recommended that for electron energies from 10 MeV to 25 MeV the chamber be used at the 1.5 cm depth, and that for energies from 25 MeV to 50 MeV the chamber be used at the 3.5 cm depth. For energies below 10 MeV, it is recommended that a hole be drilled in another block, presumably the back-up block, at a depth of 1.0 cm for energies down to 5 MeV. Because of the size and weight of the phantom, it should preferably be stored and used on a cart.

Cavity Ionization Chamber

The Baldwin-Farmer Substandard Dosimeter and the Victoreen 100 R thimble chamber are the most commonly used chambers for this purpose, although other chambers of comparable specifications may be used. From considerations of size, convenience and minimal stem effect, the use of the Farmer Secondary Standard Dosimeter is recommended. It is important that the amount of stem effect be checked on any chamber employed.

Chamber Calibration

As indicated in the definition, the chamber should have applied to it the calibration factor for cobalt-60 gamma-rays. The chamber should be calibrated at the National Bureau of Standards or by intercomparison with a chamber calibrated at the National Bureau of Standards. Constancy checks with a suitable radium or other constant radiation source should be made once a month, and the voltage sensitivity of the electrometer should also be checked once a month. Since the Baldwin-Farmer chamber has a small volume and exhibits no appreciable stem effect when irradiated with high energy x-rays or electrons, and does not require removal from the phantom for reading, it would appear to be the instrument of choice.

Field Size

Electron beams should be calibrated for output for all field sizes employed. For intercomparison purposes, it is recommended that the field size be at least 7 cm by 7 cm. As noted above this refers to the dimensions of the plane figure defined by the intersection of the 90 per cent isodose surface with the plane passing through, and normal to, the central axis at the position of the maximum in the depth dose distribution. For calibration purposes, these depths are taken to be 1.5 cm or 3.5 cm, as specified above.

Calibration Frequency

Calibration of the output should be performed simultaneously with absorbed dose calibration for different field sizes and energies. Thereafter, the output calibration could be made at regular intervals, preferably each week, and not less than monthly. The output calibration is convenient and serves as a constancy check of the electron beam absorbed dose calibration.

Absorbed Dose Calibration

The purpose of the absorbed dose calibration is to make it possible to convert the isodose contours on the patients' treatment plans into rads in tissue. The absorbed dose can be related to the output measurement described above, in which case it can be expressed as rads/'r.'

Physical Beam Parameters

The physical parameter (scatterer, monitor arrangement, collimator or aperture setting, etc.) should be identical with those used in treatment or experiment. The electron beam energies and field size should be the same as those employed for irradiation purposes.

Phantom

The phantom should be a tank of water with dimensions of at least 30 cm by 30 cm by 30 cm. Water is recommended because most isodose surfaces used in treatment planning refer to water. If treatment planning is based on isodose surfaces determined in some other tissue-equivalent medium, that phantom should be used. Measurements should be made at the depth of the maximum dose on the central axis since this is the most reliable normalization point. These measurements may be made at other depths as well, and at other positions, in order to normalize the isodose distributions more accurately.

Measurement Methods

The absorbed dose microcalorimeter system, referred to above, is the absolute method for this purpose but its limited availability at the present time precludes recommendation of it for general use.

(a) Fricke Ferrous Sulphate Dosimeter. A commonly used and acceptable secondary standard for the measurement of absorbed dose is the Fricke ferrous sulphate solution. Irradiation of this solution produces a change in optical density due to the oxidation of ferrous to ferric sulphate. The mechanism of this reaction is well understood and, when used with care, it has good reproducibility and accuracy. Solutions are made with distilled water, sulphuric acid, and CP grade reagents, and contain millimolar quantities of sodium chloride and ferrous ion. The solution is ordinarily placed in a polystyrene wafer with inside dimensions of 4 cm diameter and a depth of 5 mm. The polystyrene wafers must be pre-irradiated and contain distilled water or ferrous sulphate to prevent contamination of the dosimeter solution. These irradiations are carried out until a constant blank reading is obtained from a ferrous sulphate solution contained in the wafer for a period of time equal to the irradiation time. These conditions are usually obtained by pre-irradiation doses of about 200 kilorads. After irradiation the dosimeter solution is placed in a quartz cuvette of volume 1 cc and its optical density determined by means of a spectrophotometer. The molar extinction coefficient of ferric ion in 0.8 N sulphuric acid is 2196 at 305 millimicrons and 25° C (I.C.R.U.³⁷). Great care must be exercised to avoid contamination of the solution during storage, irradiation, shipping, and measurement. Comparison measurements indicate that irradiation of the ferrous sulphate within the quartz cell produces results within 1/2 of 1 per cent of those obtained in polystyrene cavities, and this simplification is recommended if difficulty is experienced with polystyrene cavities. As indicated above, the container of either polystyrene or quartz is placed in the water phantom at the position of the maximum on the central axis for calibration of this point. Further details on Fricke ferrous sulphate dosimetry are available in the literature (Weiss et al.,³⁸ I.C.R.U.³⁷). There is some range in

the 'G' values for this dosimeter for high energy electrons. Recent reviews and determinations on this subject have been made by Pettersson and Hettinger (to be published), Almond (to be published), and Pinkerton.³⁹ A value of 15.5 molecules per 100 eV is recommended for use at present.

(b) Cavity ionization. Absorbed dose may be estimated from the conversion of ionization measured in a cavity into the dose absorbed in the cavity walls by means of the Bragg-Gray relation (I.C.R.U.,³⁷ Gray⁴⁰). The depth of the ionization chamber should be small in the direction of the greatest variation in the dose distribution, and the volume of the chamber must be precisely known. Extrapolation chambers have been successfully used to extrapolate the ionization current to zero wall thickness (Rase and Pohlitz⁴¹). If the walls are so thin that they make a negligible contribution to the total secondary electron emission which ionizes the air within the chamber, the material of which the wall is composed is not important. In the case of extrapolation chambers, the material is normally polystyrene, carbon, or other plastic material of low atomic number. Its composition must be sufficiently well known, so that its stopping power can be computed for various electron energies. Conversion of the measured cavity ionization J_m into the energy absorbed in the surrounding wall material E_m is carried out by the Bragg-Gray relation:

$$E_m = J_m w s_m$$

where w is the average energy in ergs expended by the electrons crossing the cavity per ion pair produced, and s_m is the ratio of the effective mass stopping power in the medium to that of the cavity gas for electrons. Values of w for air and s_m relative to air, for energies up to 1.5 MeV and for different materials, are listed in NBS Handbook 85. Stopping powers to 10 MeV are tabulated in National Bureau of Standards Monograph 1, "Energy Dissipation by Fast Electrons," September, 1959. The value of "33.7 eV/ion pair" for the value of w in air is recommended. The stopping power ratio and correction for the polarization effect should be averaged over the electron spectrum at the point of interest. The electron spectra measured at different depths in absorbers, as reported by Harder⁴² and by Epp et al.,⁴³ are useful for this purpose.

(c) Calibrated dosimeters. If the above methods are not available a dosimeter calibrated elsewhere should be employed. Such dosimeters as the Farmer Secondary Standard Dosimeter, the Fricke ferrous sulphate dosimeter solution, and thermoluminescence dosimeters, have been successfully used in this manner. Their use under the conditions specified in this protocol makes it possible for an institution equipped to make dosimeter calibrations to provide meaningful calibrations to other institutions.

Ratio of Absorbed Dose to Output

The ratio of the absorbed dose at a specified point in the water phantom as measured by one of the methods outlined in this section, to the output as measured by the method in the subsection on output determines the normalization constant: rads per 'r' or rads per monitor unit.

INTERCOMPARISON

As the number of institutions using high energy electrons increases, it is recommended that intercomparison efforts be expanded to include all such institutions. The intercomparison should measure absorbed dose, and use of the Fricke ferrous sulphate dosimeter solution, or a thermo-

luminescent dosimeter in the phantom under the conditions specified above, is recommended for this purpose. Measurement of output can also be useful for intercomparison purposes but is not a substitute for the intercomparison of absorbed dose determination. For intercomparison purposes the irradiation of the dosimeter is to be carried out in the same polystyrene Intercomparison Phantom described above. The minimum field size employed for this purpose should have widths (or a diameter) of at least 7 cm in a plane bounded by the 90 per cent isodose contour at the depth of the dosimeter in the polystyrene Intercomparison Phantom.

It has been demonstrated, primarily in interchanges between the Memorial Hospital and the M. D. Anderson Hospital, that both the Fricke ferrous sulphate dosimeter solution and the encapsulated lithium fluoride thermoluminescence system can be suitably prepared and used for intercomparison by mail. The use of calcium fluoride also appears attractive. The AAPM has recommended that its Sub-Committee on Radiation Dosimetry (SCRAD) attempt to facilitate and expand these intercomparison measurements as well as to encourage the establishment of a national calibration service.

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THE ACRH BRAIN SCANNING SYSTEM*

By

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The feasibility of using radioisotope scanning systems to detect a variety of brain lesions has been recognized for a number of years, and the use of this procedure on a routine basis has increased gradually. The fact that several well established methods for locating intracranial lesions already exist, e.g., angiography, pneumoencephalography and electroencephalography, has not hindered this development, since each of these methods has well-known limitations and undesirable features. The lack of widespread enthusiastic acceptance of brain scanning is due more to the cumbersome and time-consuming nature of the procedure. To keep the radiation dosage to the patient below the maximum permissible level, only relatively small quantities of iodine-131—the first and most commonly used isotope—can be used. In addition, collimated detectors for the 364 keV γ -rays from this isotope are relatively inefficient. As a result, the time required for brain scanning is long, usually one to three hours if scans are to include more than one view of the head. Furthermore, the spatial resolution is exceedingly poor by X-ray standards.

We took up the problem of brain scanning at a time when several approaches to the imaging of distributions of high energy photons had already been introduced. The pioneering work of Curtis and Cassen¹ had stimulated the development of what are now regarded as "conventional scanners" consisting of single scintillation detectors responding to single photons; Brownell² had successfully demonstrated the use of a "positron scanner" consisting of two detectors that responded to coincident photons from positron annihilation; Anger³ had succeeded in developing a stationary scintillation detector or " γ -ray camera" that viewed the entire region of interest simultaneously. Each of these approaches required serious consideration since each approach was capable of further development. This fact has subsequently been shown by the introduction of a highly efficient hemispherical collimator by Cassen;⁴ the ingenious use of conventional detectors in a novel scanning mode producing cross section images, by Kuhl;⁵ the adaptation of the camera system to positron emitters by Anger;⁶ the development of a digital version of the Anger camera by Bender and Blau;⁷ and the recent use of image intensifiers by Kellershohn⁸ and Ter-Pogossian.⁹

This paper describes a multi-detector brain scanner that represents our attempt to improve systems of the conventional, rectilinear moving detector type. Any attempt to improve the brain scanning procedure must consider both physical and biological parameters. On the physical side, a theoretical study^{10,11} of detector sensitivity and tumor contrast as function of γ energy indicates that, under typical conditions, γ 's in the energy range of 100-200 keV are optimum[†] for detecting midline tumors. On the biological side, the isotope must be taken up in different concentrations by the tumor and normal tissues, and the radiation dosage to the patient should be minimal.

* This paper appeared in J. Nuclear Medicine, 8:1, 1967.

† The term "optimum" is used in the mathematical sense to refer to that γ energy which, for a specified set of assumed physical conditions, maximizes a certain criterion called the "normalized figure of merit," a quantity inversely proportional to the time required to resolve the tumor and normal count rates statistically.¹⁰ For a different criterion or a different set of physical

These considerations stimulated the search for a more suitable γ emitter, which led to the introduction of technetium-99m.¹¹ This isotope is produced by the β^- decay of molybdenum-99 from which it is easily separated and prepared for injection.^{13,14} The metastable ^{99m}Tc subsequently decays by an isomeric transition, with the emission of a 140 keV γ -ray, to Tc, which is essentially stable (2.2×10^5 year half-life). Since the half-life of ^{99m}Tc is short (6 hours) and the material is essentially a pure γ emitter (only about 7 per cent of the 140 keV γ 's are internally converted), the radiation dosage per injected μCi is very low compared with that from ^{131}I .

The ACRH brain scanner, shown in Figure 1, was a concurrent development. Briefly, the system consists of four collimated scintillation detectors, arranged in opposing pairs, which simultaneously scan the patient's head. Pulses from these detectors are fed to four pulse height analyzers adjusted to accept only photopeak pulses for recording. The recording system consists of four light projectors which move with the detectors and produce a bell-shaped spot on film for each selected photopeak pulse. Count rate meters monitor each channel, but are not used for contrast enhancement or background erase.

When used with a 10 mCi intravenous injection of ^{99m}Tc the ACRH brain scanner can produce simultaneous lateral photoscans of conventional quality in approximately one minute, although the scanning time is usually extended to about 10 minutes to produce pictures of superior quality. This quantity of ^{99m}Tc as pertechnetate produces a whole-body radiation dosage of approximately 100 mr, the critical organ being the stomach, which receives approximately 3 rads. Although spatial resolution is still poor by X-ray standards, this system employs collimators having a smaller radius of view than is ordinarily used for brain scanning. The ACRH brain scanner has been used with ^{99m}Tc since August, 1963. Some of its features were first demonstrated on a prototype small-animal scanner.¹⁵

THE IDEAL IMAGING SYSTEM

Since the distribution of radioactive material in the brain is a volume distribution, an ideal imaging system would produce a three-dimensional image. As yet, such systems have not been devised, although the cross section scanner of Kuhl⁵ has demonstrated their feasibility and Harper¹⁶ has discussed a possible realization. A (hypothetical) ideal system of the general type described here would produce an exact image of an attenuated normal projection of the volume distribution onto a plane. This can be described as follows: If $\rho(x,y,z)$ specifies the concentration of γ activity, * an attenuated normal projection onto the (X-Y) plane is given by

$$(x,y) = \int_0^\infty \rho(x,y,z)e^{-\mu z} dz \quad (1)$$

conditions, a different "optimum" energy would be obtained. The study referred to above compared systems designed for a 2" x 2" NaI(Tl) crystal, with lead collimators having a 4" focal length, 1" diameter of view, to detect a 1" diameter midline tumor having an uptake ratio of 2:1. For equal numbers of γ 's produced per $\text{cm}^3\text{-sec}$ in the tissue, it appeared¹¹ that the optimum γ energy is 100 keV. This study did not deal with scattered radiation. When scatter is taken into account, the optimum γ energy is somewhat higher.¹²

* In this formulation, ρ has dimensions of $[\frac{\gamma\text{'s produced}}{\text{sec-cm}^3}]$ and can be expressed in terms of terms of the tracer efficiency, $k[\frac{\gamma\text{'s produced}}{\text{sec-cm}^3\text{-rad}}]$, and the permitted radiation dosage to the whole body or to a critical organ, $d[\text{rads}]$; that is, $\rho = kd$. The projection of ρ , namely $\sigma(x,y)$, has dimensions of $[\frac{\gamma\text{'s produced}}{\text{sec-cm}^2}]$.

Figure 1. The ACRH brain scanner consists of four scintillation detectors arranged in opposing pairs, each detector having an associated pulse height analyzer and pulsed light projector. Scan information is recorded on two sheets of film, one for each side of the head.

where z is the depth below the surface of the head, and μ is the attenuation coefficient in the tissue. In practice, we do not observe a perfect image of the projected distribution; rather, the observed image differs from $\rho(x,y)$ in a variety of ways which may be described as artifacts due to the limitations of the scanning system.

ARTIFACTS AND THEIR REDUCTION

Our approach to designing the ACRH brain scanner might be described as an attempt to minimize artifacts inherent in systems of this type, without increasing the scanning time, radiation dosage, etc. Certain aspects of this approach are described in more detail in reference 17. The following artifacts were considered:

Statistical structure. When a collimated scintillation detector scans a source characterized by its equivalent sheet distribution $\sigma(x,y)$, the true mean count rate due to properly collimated γ 's is given approximately* by

$$C(x,y) = G\eta\psi\sigma(x,y) \quad (2)$$

where $G(\text{cm}^2)$ is the geometrical efficiency† of the collimator for responding to activity on a sheet distribution, η is the photopeak crystal efficiency, and ψ is the "window efficiency," or the fraction of photopeak pulses selected by the pulse height analyzer.

If $t \left[\frac{\text{sec}}{\text{cm}^2} \right]$ is the time spent by the detector per unit area, the true mean count rate gives rise to the true mean image‡ of the source defined by

$$N(x,y) = C(x,y)t \quad (3)$$

where $N(x,y) \left[\frac{\text{counts}}{\text{cm}^2} \right]$ is the true mean number of γ 's which would be detected per unit area, if the scan could be repeated many times.

On this basis, the ideal image of a uniform source [$\sigma(x,y) = \text{constant}$] is a uniform image [$N(x,y) = \text{constant}$]. In practice, the observed number of counts per unit area is not constant, but varies randomly to produce a non-uniform or "structured" image. If N is the true mean number of counts per unit area, then the observed number, being Poisson distributed, will vary randomly about N with a standard deviation $\sigma_N = \sqrt{N}$, or a fractional standard error $\epsilon = \frac{\sigma_N}{N} = \frac{1}{\sqrt{N}}$. The magnitude of ϵ can be used as a measure of this random structure which approaches 0 as N approaches ∞ .

[Rossman²⁰ has described a related, but more complex structure, called quantum mottle, which depends not only on N but also on the shape of the recorded spots.] The origin of this structure is therefore described as statistical fluctuation which can be reduced only by increasing the number of properly collimated γ 's recorded per unit area. Despite the use of high scan speeds, this artifact is reduced in the ACRH system in the following ways:

- 1) High count rates result from the choice of ^{99m}Tc which can be administered in large quantities.
- 2) Four detectors scan the head simultaneously (see Figure 1).

The axes of the upper and lower detectors are separated by 4-1/2" so that a 9" region is scanned when the detectors index vertically 4-1/2". A selector switch enables the operator to

*Equation (2) holds precisely only when $\sigma(x,y)$ is uniform over the collimator field of view; thus, for a highly non-uniform source, equation (2) holds precisely only when the width of the field of view is "infinitely small." The effect of finite spatial resolution of $C(x,y)$ is discussed on page 118.

†In previous papers^{10,11,18} the symbol E has been used to denote this geometrical property of the collimator, which is similar to Brownell's¹⁹ "slab efficiency," $G_s \cong g_0 d^2$.

‡In what follows, we use the term "image" to refer to either $C(x,y)$ or $N(x,y)$ since they differ only by a constant factor t .

perform the scan with either the lower detectors, the upper detectors, or all four.

Two sets of detectors have been constructed for this system. The original set consisted of NaI(Tl) crystals, 3-5/8" by 1-3/4", integrally mounted on EMI9531B photomultiplier tubes. (See Figure 2.) This crystal thickness was considered adequate for γ 's up to ^{131}I radiation (364 keV). The new detectors are similar, except for the crystal thickness, which is 1/2". This is adequate for γ 's below 200 keV.

Figure 2. Each detector consists of a NaI(Tl) crystal and photomultiplier tube, integrally mounted (A). Electrical contact to concentric rings is made by spring-loaded electrodes (B) which can retract 1" to allow for collimators (C) of varying thickness. A light bulb (photodiode) is mounted in the center hole of each collimator on the patient's left (right).

- 3) Detector sensitivity can be maximized by making the septa of focused collimators just thick enough to reduce unwanted septal penetration to a negligible level. (See Figures 3 and 4.) For a given penetration fraction, the required septum thickness increases with γ energy, so that focused collimators designed for 140 keV ($^{99\text{m}}\text{Tc}$) are about twice as efficient as similar collimators designed for 364 keV (^{131}I).
- 4) An interrupted light beam system is used to minimize the time spent in scanning beyond the patient's head so that the detectors are over the region of interest during a large fraction of the scanning time.

In the center holes of the collimators on the patient's left are small focused light bulbs whose beams indicate the positions of the collimator axes and aid in positioning the patient. In the center holes of the opposite collimators are IN2175 photodiodes which produce signals when the light path is closed by scanning beyond the patient's head. This system is used to reverse the direction of scan so as to minimize the time spent in scanning beyond the head. In addition, this system is used to record an outline of the head on the film. This is accomplished by four light projectors (see Figure 5) consisting of single Amperex 6977's which are pulsed by signals from the photodiodes to produce small, intense outline marks on the film. These projectors may also be pulsed manually by a push button switch to produce "land marks" on the film.

Figure 3. Collimator designed for brain scanning with ^{99m}Tc . $D=2.33''$, $F=3''$, $L=4''$, $R=.34''$; geometrical efficiency $G = 2.87 \times 10^{-3} \text{ cm}^2$. Despite thin septa, penetration is negligible for 140 keV γ -rays.

Reduced contrast. When the distribution of activity is not uniform [$\sigma(x,y) \neq \text{constant}$], the true mean count rate gives rise to structures in the image which are neither as prominent nor as sharply outlined as the corresponding structures in the object. This can be formulated quantitatively as follows: Let σ_t be the source strength over a tumor region that is small compared to the entire source, the latter being otherwise normal with a uniform strength σ_o . The lack of uniformity in the tumor and normal regions could be measured by the arithmetic difference ($\sigma_t - \sigma_o \neq 0$); the fractional difference ($\frac{\sigma_t - \sigma_o}{\sigma_o} \neq 0$); the ratio ($\frac{\sigma_t}{\sigma_o} \neq 1$); or by a somewhat less familiar quantity called the contrast or modulation, and defined by

$$m_o = \frac{\sigma_t - \sigma_o}{\sigma_t + \sigma_o}. \quad (4)$$

Using the latter measure, this "object structure" gives rise to "image structure" which is also measured by its contrast or modulation defined by

$$m_i = \frac{C_t - C_o}{C_t + C_o}. \quad (5)$$

We can go one step further and introduce a quantity called the modulation transfer function (MTF) defined by

$$m_i = (\text{MTF})m_o \quad \text{or} \quad \text{MTF} = \frac{m_i}{m_o} \quad (6)$$

Figure 4. Collimator designed for ^{203}Hg (279 keV). If sensitivity is associated with exposure density within the collimator field of view, the upper figure illustrates the "geometrical" response to a point source. The spatial frequency response (MTF) is plotted below for several γ energies. The decrease in MTF with increasing γ energy is equivalent to a decrease in contrast, due to increasing septal penetration.

The MTF measures the degree to which contrast or modulation in the object is transferred to the image by the scanning system.

If the detector responded only to properly collimated γ rays, and, in addition, had perfect spatial resolution, then equation (2) could be used to compute the count rates in equation (5). Under these conditions the image contrast would be a perfect replica of the object contrast; that is, $m_i = m_o$ and $\text{MTF} = 1$.

Equation (2), however, should be interpreted as describing a theoretical ideal not realized by actual systems, which always transmit object contrast to the image imperfectly; thus, for actual systems, $m_i < m_o$ or $\text{MTF} < 1$.^{*} The phenomena responsible for contrast reduction can be separated into two categories: (1) uncollimated radiation, which reduces contrast uniformly over large areas, and (2) finite spatial resolution of the collimator, which results in local smoothing and greatly reduces image contrast for small structures.

1) Detector response to uncollimated radiation.

- a) Background or noise: Even when $\sigma(x,y) = 0$, the observed count rate is not zero.

^{*}The image may be recorded by a nonlinear system using circuits for contrast enhancement so that the contrast in the recorded image may exceed m_o . Circuits of this type are built into this scanner for research purposes, but are not used for patient scanning.

Figure 5. If the distribution of radioactivity (Object) and the output (Image) are described in terms of their space frequency components, the scanner (Imaging System) can be described in terms of its frequency response or Modulation Transfer Function. The MTF can be used as a generalized definition of spatial resolution.

This is due to a combination of cosmic radiation, nearby radioactive sources, natural radioactivity in the materials of the radiation detectors, etc.

In the brain scanner, the background count rate is reduced by the use of NaI(Tl) crystals that are just thick enough to achieve high photopeak efficiency for the γ energy used. Background is further reduced by lead shields for these crystals, and pulse height analyzers that select only the useful portion of the pulse amplitude spectrum. Shields of 2" lead (for the original 1-3/4" crystals) have been replaced by much lighter, 1/2" shields (for the 1/2" crystals) which are adequate for γ 's below 200 keV.

b) Septum penetration: Since most of the γ 's that enter the detector by penetrating the collimator septa originate outside the geometrical field of view, they are essentially uncollimated and do not contribute to the formation of a structured image. For this reason, the penetration fraction, P , is one of the most important quantities considered in collimator design. It is controlled by the choice of septum thickness for a specific γ energy, collimator material, crystal diameter, radius of view, and focal length.¹⁸ If the septa are too thick, collimator efficiency is needlessly reduced; if too thin, excessive penetration reduces contrast. In all collimators designed for ^{131}I , ^{203}Hg , and $^{99\text{m}}\text{Tc}$, the penetration fraction has been kept below 0.1. Figures 3 and 4 show collimators designed for $^{99\text{m}}\text{Tc}$ and ^{203}Hg respectively. Figure 4 shows marked contrast (MTF) reduction for ^{131}I due to septum penetration.

c) Scattered radiation: Gammas that enter the detector from outside the collimator field of view, by Compton scattering in the tissue, also reduce image contrast. Since scattered photons have less energy than the unscattered γ -rays, some of the scattered radiation can be rejected by use of pulse height analyzers (PHA's). Because scintillation detectors have imper-

fect energy resolution, the pulse amplitude spectra due to scattered and unscattered photons overlap. As a consequence, not all scattered photons can be rejected by PHA's without seriously reducing the counting efficiency for unscattered γ -rays. A base line discriminator setting is needed which achieves an optimum compromise between rejection of scattered photons and the recording of unscattered γ -rays. If the optimum base line setting is defined as that setting which maximizes the above mentioned figure of merit,^{10,12} then the optimum setting is a function of the γ energy, the energy resolution of the detector, and the source size. For example, the optimum setting for a uniform distribution of ^{99m}Tc in a brain phantom 16 cm in diameter is approximately 125 keV for a detector having 16 per cent resolution at 140 keV.¹²

Taking the above responses into account, the total response of the detector can be thought of as the sum of responses to collimated and uncollimated radiation, and equation (2) can be modified as follows: When the detector is over a normal region where the source strength is σ_0 , the total count rate can be written

$$C_0 = G\eta\psi\sigma_0(1 + P + S) + B \quad (7)$$

where P and S are the penetration and scatter fractions,²¹ respectively, and B is the background count rate. When the detector is over a tumor region, where the source strength is σ_t , the situation is somewhat more complex. In this case, we assume that the tumor is large enough to cover the geometrical field of view uniformly, but not much larger. Thus the response to properly collimated γ 's is $G\eta\psi\sigma_t$. Since, however, most of the γ 's which enter the detector by septum penetration or by scattering in the source originate outside the collimator field of view, where the source strength is σ_0 , the count rate due to penetration and scatter is still approximately $G\eta\psi\sigma_0(P+S)$. The total count rate over the tumor region is therefore

$$C_t = G\eta\psi[\sigma_t + \sigma_0(P + S)] + B \quad (8)$$

When equations (7) and (8) are used in equation (5) to compute the image contrast, that quantity is reduced to

$$m_i = \frac{G\eta\psi(\sigma_t - \sigma_0)}{G\eta\psi[\sigma_t + \sigma_0 + 2\sigma_0(P+S)] + 2B} \quad (9)$$

From this equation it is clear that image contrast is reduced by all three forms of uncollimated radiation; therefore, in general, $m_i < m_0$ or $\text{MTF} < 1$.

2) Finite resolution of the collimator.

Even when we assume that background, penetration, and scattered radiation are all negligible, the image contrast is still, in general, less than the object contrast to a degree that depends on the size of the object structure relative to the width of the collimator field of view. For structures that are very large compared to the field of view (this means that the source, $\sigma(x,y)$, is essentially uniform over the field of view), equation (2) is a very good approximation, and the image contrast is very nearly equal to the object contrast. On the other hand, when structures in the object are much smaller than the width of the field of view (this means that the source, $\sigma(x,y)$, is non-uniform within the field of view), equation (2) no longer holds and image contrast is much smaller than object contrast. As a result, the image is a smoothed version of the object, and very small structures may be lost entirely. (If P, S, and B are not zero, then the contrast is, of course, even further reduced.)

To compute the geometrical response to small structures, equation (2) must be replaced by a more general formulation that takes into account the collimator sensitivity to activity at each point within the field of view. The function describing this sensitivity is called the point source response function (abbreviated psrf), $p(x,y,z)$, and is proportional to the solid angle of view, $\Omega(x,y,z)$, through the collimator from the point (x,y,z) at which a γ -ray is emitted. This solid angle is greatest for points on the collimator axis and decreases to zero at the periphery of the field of view. The shape of this function changes with distance from the collimator. Near the collimator face it tends to be uniform or flat-topped, while at the focal distance it tends to be quite peaked and almost conical. At intermediate distances it is bell-shaped and approximately Gaussian.

The total response to an arbitrary source, $\rho(x,y,z)$, is then found by summing the product of the sensitivity function over the source distribution, taking exponential attenuation with depth, z' , into account

$$C(x,y) = \eta \psi \int_V p(x'-x, y'-y, z'+z_0) \rho(x', y', z') e^{-\mu z'} dx' dy' dz'. \quad (10)$$

Here, z_0 is the clearance between the collimator face and the tissue surface. While this formulation is general enough for most applications, it is too complex to be very useful.

If we put aside the problem of computing $C(x,y)$ precisely, the description of collimator response by $p(x,y,z)$ can be simplified conceptually by considering the average magnitude of this function (at a given z) to represent the collimator sensitivity, and the shape of the function to determine the spatial resolution. The description can be further simplified mathematically by defining the spatial resolution as the full width of $p(x,y,z)$ at its half-maximum height. This amounts to replacing the two-dimensional function $p(x,y)$ at each distance z , by two numbers that denote the sensitivity and spatial resolution. While this simplification eliminates the possibility of computing $C(x,y)$ precisely, it would appear that we can still compare the quality of collimators in terms of this definition of resolution; thus, we would expect to observe the best scans from the collimator with the smallest value of spatial resolution, for a given sensitivity. For this reason, this simplification of $p(x,y,z)$ appears to be useful.

A similar situation occurs in optics where the point spread function of a lens is analogous to the psrf of a collimator. Here too, equations analogous to equation (10) are too complex for routine computation and comparison of images produced by different lenses. To establish criteria for lens quality, several approaches have been taken. In keeping with the interest (among astronomers) in separating double stars, Lord Rayleigh²² introduced the resolution concept called resolving power, which was defined in terms of the width of the point spread function or the Airy diffraction pattern.²³ It was, however, recognized early that the lens which resolved point sources best did not always produce the best image of distributed objects. Accordingly, Foucault²⁴ introduced, as test objects, line patterns consisting of sets of lines having a width equal to the spacing between them. The resolution or resolving power of a lens was then defined in terms of the smallest line spacing that could be seen in the image; e.g., a lens might have a resolving power of 100 lines/mm. Some form of this test is still the most widely used method for evaluating lenses.²⁵

Although this criterion of lens quality is based on the response of a lens to a distributed source of illumination, it is nevertheless invalid to conclude that, if lens A has higher resolving power than lens B, then lens A will form a better image of an arbitrary object. This is because

this measure of resolving power tells us something about how the lens will reproduce the small details or fine structure in the object, but nothing about how the large, coarse structure will be reproduced. In short, the intuitive notion that the lens which resolves small structures best will certainly resolve large structures best is not valid. In general, objects viewed by optical systems contain a wide range of structure sizes. Therefore it appears that a definition of resolution based on a measure of lens response as a function of structure size would be more useful than a single number to describe lens performance. In recent years, this generalization of resolving power has been intensively developed in optics and reviewed by Perrin.²⁶ Since an almost identical formulation can be applied to scanning systems, this approach will be briefly described. [Here, $\sigma(x,y)$ is used to specify either the intensity distribution of light or radioactivity in the object, while $C(x,y)$ specifies either the light intensity or count rate forming the image.]

To clarify this alternative formulation of spatial resolution, we will draw an analogy between optical systems (or scanning systems) and the more familiar sound reproducing system.

It is well known among audiophiles, as well as engineers, that any sound, from a single note on the piano to the incredibly complex output of a full symphony orchestra, can in principle be described in terms of its Fourier spectrum; that is, the amplitude, phase, and frequency of the sine and cosine waves that must be superimposed to produce the sound. Somewhat less familiar is the fact that a two-dimensional object,*, such as $\sigma(x,y)$, can be analyzed in terms of its spatial frequency components, measured in cycles/cm (sound is analyzed in terms of its temporal frequency components, measured in cycles/second). The fine structural details of $\sigma(x,y)$ require high frequency sine and cosine waves for their representation while the large, coarse structures require low frequency, long wave length components.

To continue the analogy, the engineer must take into account the range of frequencies contained in the music that the audio system is to reproduce, and design a system having uniform or flat response over this range. Similarly, if the optical (or scanning) system is to form a perfect image of $\sigma(x,y)$, the system must respond to all frequencies in $\sigma(x,y)$ equally well; that is, the response must be flat over the band of frequencies required to represent the object, $\sigma(x,y)$, if its image, $C(x,y)$, is to be accurate.

This can be related to the discussion of contrast or modulation if σ_t and σ_o in equation (4) are now thought of as the maximum and minimum amplitudes associated with a sinusoidal distribution of radioactivity having frequency ν , and C_t and C_o in equation (5) are the corresponding count rates due to this frequency component. (See Figure 5.) The $MTF(\nu)$ is then just the spatial frequency response of the optical system (or the collimator-detector-pulse height analyzer of the scanning system); it tells us what fraction of the object contrast is transmitted by the system to appear as image contrast. It is important to note that the $MTF(\nu)$ is not a single number, but a function of space frequency, ν . This means that we have in the $MTF(\nu)$ a measure of the detector response to simple structures (sinusoidal distributions) of all sizes. The response to a complex structure is simply the sum of responses to its frequency components.

In view of this consideration, it would be desirable to design imaging systems so as to make the $MTF(\nu)$ as near 1 as possible for the entire range of frequencies contained in $\sigma(x,y)$. In practice, this is never achieved for scanning systems because the MTF can be made large only by

* We assume that the structures in $\sigma(x,y)$ are oriented in no special way, so that the frequency spectra along X and Y axes (in any orientation about the Z axis) are essentially the same; thus, the spectral analysis of $\sigma(x,y)$ need be treated in only one dimension.

making the collimator field of view small;* unfortunately, this is accompanied by a sharp decrease in collimator efficiency, and therefore by an increase in random, statistical structure. For this reason, it is always necessary to compromise between the magnitude of the MTF and counting efficiency (or between resolution and sensitivity).

Although the $MTF(\nu)$ is more complex than the usual definition of resolution, it has the advantage that, once it is determined, we can find the image, $C(x,y)$, of a given object, $\sigma(x,y)$, by summing the responses to the frequency components in the object. In principle, it is possible to compute the MTF from the theoretical point source response function, $p(x,y,z) = \Omega(x,y,z)/4\pi$. Since $\Omega(x,y,z)$ is a rather complex function for a multichannel focused collimator, it is more convenient to determine the MTF experimentally.

There are two simple methods for doing this. The most direct and intuitively clear is to observe (1), the response to a sinusoidal source of radioactivity, such as the one scanned for Figure 7. In such a source $\sigma_0 = 0$, so that $m = 1$ in equation (4). In this special case, the $MTF = m_i = \frac{C_t - C_o}{C_t + C_o}$. This is analogous to measuring the frequency response of an audio amplifier by observing its response to a sine wave input signal. Alternatively (2), if the line spread function, $L(x)$ (the response to a line source of radioactivity parallel to the Y-axis) is observed, the MTF can be found²⁶⁻²⁸ by taking the Fourier transform of $L(x)$.

If a source of low energy γ -rays is used in either of these procedures, the resultant MTF reflects, primarily, the smoothing effect due to the finite width of the collimator field of view. If γ 's of higher energy are used, so that septum penetration is not negligible, then the resultant MTF reflects the effects of both geometrical and penetration responses. (See Figure 4.) If, in addition, the measurements of C_t and C_o are made with the source imbedded in a scattering medium, the effect of scattered radiation on MTF is also observed. Thus, by proper choice of γ energy and surrounding medium, the MTF can be used as a measure of the reduction in contrast due to a combination of background, penetration, scatter and finite collimator resolution. Since, in the special case when $m_o = 1$, as in method (1) above, the $MTF = m_i$, the relationship between MTF and P, S, and B is given by equation (9).

In terms of its complexity and generally, the $MTF(\nu, z)$ of a detector stands between the point source response function, $p(x,y,z)$, and the resolving power measured by any single number. While this level of analysis has recently been seen as a "breakthrough" in optics, there is some doubt, at this time, whether the added complexity is justified, for radioisotope imaging systems, by the increased generality of the description. Although it is generally agreed that any single number used to measure spatial resolution at a given distance must be somewhat less adequate than a one-dimensional function such as the $MTF(\nu)$, Brownell²⁹ has pointed out that the psrf's of properly designed focused collimators have approximately Gaussian shapes (and in any case are less bizarre than the corresponding point spread functions of some lenses), and may therefore be replaced by a single number that measures the resolution.

Spot structure. There is quite general agreement that every collimated γ -ray should be recorded by the imaging system, but there is little consensus as to how this should be done.

* For $MTF \approx 1$ at frequency ν , it is necessary that the collimator field of view be small compared to one-half wave length; e.g., for $\nu = 1$ cycle/cm, $\frac{\lambda}{2} = .5$ cm and the diameter of view must be much smaller than .5 cm for MTF to be near 1 at this spatial frequency.

Most systems produce some kind of mark or spot on recording paper or film for each detected γ : for example, tiny dots, discs, circles, crescents, lines, rectangles, triangles, asterisks, letters, numerals, etc. Clearly none of these marks bears any intrinsic relation to the distribution of activity or the imaging properties of the system, and in this sense they introduce artificial "spot structure."

This artifact is reduced by recording a bell-shaped spot on film, which tends to duplicate the position information given by the collimator. (See Figure 6.) In contrast, the recording of a tiny dot at a position corresponding to the collimator axis amounts to replacing $p(x,y,z)$ by its most probable value, and thereby introducing distortion.

Figure 6. For each detector, there is (1) a pulsed light projector which produces a bell-shaped spot on film for each detected γ -ray and (2) an outline mark projector. The size of the projected bell-shaped spot can be controlled.

Experiments have been performed in which γ pulses from a single detector were recorded on film by two pulsed light projectors producing spots of different sizes. (See Figure 7.) Subjective evaluations of these photoscans tend strongly to favor 1/2" diameter bell-shaped spots over smaller spots that are either bell-shaped or sharply defined. Larger spots have not yet been tried. Thus, we have not carried this notion so far as to produce bell-shaped spots that are as large as the collimator field of view (which was 3/4" diameter for the photoscans shown in Figure 7). A further rationale for doing so has been discussed by Morgan.³⁰ If a tiny dot is recorded for each detected γ -ray, then the high frequency response (and the bandwidth) of the record-

Figure 7. Here, a sunburst pattern containing radioactivity in which the concentration at a fixed radius varies sinusoidally, is scanned by a single detector. Each detected γ -ray pulses two spot projectors which produce bell-shaped spots of 1/2" diameter (Left) and 1/4" diameter (Right). The 1/2" spots reduce the high frequency response of the recording system to more nearly match that of the detector.

ing system greatly exceeds that of the collimated detector. Since the noise in a system increases with bandwidth, high frequency noise is introduced and recorded by such a system. If, on the other hand, the bandwidth of the recording system is matched to that of the detector, a minimum of noise is introduced by the recording system. This match can be achieved, approximately, by recording a spot that resembles in size and shape the point source response function of the collimator. (Since the shape of the psrf varies with distance from the collimator, an average shape might be used.)

Morgan and Tuddenham³¹ have pointed out that a mismatch of bandwidths of the detector and recorder is especially serious because the bandwidth of the human eye is even greater than that of the recorder; consequently, recorder noise might disturb the visualization of important structures in the photoscan. This can be overcome to some extent by viewing the photoscan from a distance so great that the eye cannot resolve the high frequency noise.

To summarize this point of view, we can say first, that the spatial frequency response, or $MTF(\nu)$, of the collimated detector should be made as close to 1 as possible over the band of frequencies contained in the structures of $\sigma(x,y)$ which are to be detected; second, that the band-pass of the recording system should be matched to that of the detector; and third, that the resultant image should be so viewed that the structures to be detected appear on the retina under optimal conditions for visual perception [one such condition being that the structure have a fundamental frequency of approximately 10 cycles, or line pairs, per millimeter on the retina³²].

In addition to these considerations, the smooth image (see Figure 8) that is formed by overlapping bell-shaped spots is ideal for contrast enhancement on closed circuit television,³³ for

a

b

Figure 8a. Simultaneous lateral scans made in 85 sec showing a midline lesion. Scan was begun 10 minutes after intravenous injection of 10 mCi of ^{99m}Tc as $(\text{TcO}_4)^-$. Scan speed, 2" per sec; index 1/4". Such rapid scans are made only under emergency conditions.

Figure 8b. Routine simultaneous lateral scans made in 10 minutes show the midline lesion in somewhat more detail. Scan was begun immediately after completion of 8a. Scan speed, 0.9" per sec; index 1/16". Collimators illustrated in Figure 3 were used.

isocount contour plotting^{34,35} (see Figure 9) or for similar manipulations with a flying spot scanner.³⁶

In the brain scanner, for each detector there is a pulsed light projector (see Figure 6), resembling a slide projector in miniature, which produces a spot on film for each γ pulse selected by the associated PHA. In each projector, the light source consists of three Amperex 6977 triode indicator tubes operated in parallel. The green phosphor on the plate of this tube appears

Figure 9. The count rate at any point on the brain scan can be determined by matching the film density at that point to a density on the calibrated wedge of known count rates. Quantitative measurement of count rates may be facilitated by plotting isocount contour lines of the brain scan and calibrated wedge.

to have a decay time of approximately 5μ sec as measured with a photomultiplier having an S11 photocathode. Hence, the light per pulse is essentially constant over a very wide range of count rates. Different pulse lengths may be selected to provide a useful range of film densities. The slide that is projected is a disc which is nearly transparent at the center and opaque at the edges, its density varying smoothly between these extremes. When this is projected, the light intensity on the film has a bell-shaped distribution. The diameter of this projected image can be set at $1/2''$, $3/8''$, or $1/4''$ by choice of the objective lens position relative to the slide and film. The four light projectors are oriented with respect to the two sheets of film in the same manner as the four scintillation detectors are oriented with respect to the patient's head. The projectors travel with the scan mechanism by means of a rigid mechanical connection.

Line structure. Perhaps the most prominent artifact in photoscans (as in television) is line structure caused by the relatively large spacing between scan lines. To reduce this artifact, we have used a line spacing of $1/16''$ in most scans, with five multiples of this value available. When a $1/2''$ bell-shaped spot is recorded for each γ -ray, no line structure is evident. However, reduced line spacing tends, indirectly, to aggravate the following two artifacts:

Variable line spacing. If the space between scan lines varies, the number of counts recorded per unit area varies inversely, introducing an apparent variation in the concentration of activity. If the line spacing is so close that the spacing variation is not evident, there is danger of interpreting a variation in the number of counts per unit area due to spacing variation as a variation in concentration.

In the ACRH brain scanner, the line spacing is determined by the rotation of the indexing screw, rather than by the time during which the indexing motor is turned on. Thus, variations in the loading of the indexing motor do not affect the line spacing. In addition, the accumulation of spacing errors is minimized.

In this system, ball screws are used for both X and Y motions, and one complete rotation of the index screw produces a vertical travel of $1/4''$. A cam with four detents, mounted on the index screw, operates a microswitch to determine the $1/16''$ line spacing.

Scalloping. Another consequence of reduced line spacing is the need for greater scan speed if the same area is to be covered in the same time. If the recording system employs any form of integrating circuitry that has a response time much greater than the mean time between pulses (such as count rate meter circuitry used for background erase or contrast enhancement), then structures detected in the object are displaced in the image in the direction of scan motion. Since the direction of motion changes on alternate lines, this displacement alternates, creating scalloping. Inasmuch as the displacement is proportional to scan speed, scalloping is aggravated by increased speed. This artifact is eliminated in the ACRH scanner since no integrating circuitry is used in the recording system. When the low energy detectors and light weight shields were installed, scan speeds were doubled, so that speeds of 2, 1.3, .9, .6, and .4 inches per second are available. Scan speed is monitored by a generator-tachometer.

Variable scan speed. A variation in scan speed is indistinguishable from a change in count rate. For this reason, it is essential that the fractional variation in scan speed be negligible compared to the minimum fractional change to be detected in the count rate. In the present system, scan speed is held constant $\pm 1\%$ by means of a feedback system that incorporates a magnetic amplifier. Even so, some structure due to variations in scan speed can be detected when regularly spaced timing pulses are recorded.

Non-equilibrium. If the distribution of activity changes during the scan period, the image does not represent an equilibrium state. This is especially noticeable in the ACRH system where the first scan line made by the upper detectors is adjacent to the last line of the lower detectors. Since the time between these lines is usually about 10 minutes, any change in the distribution during this time is apparent. It has been found that this artifact is negligible when the scan is begun about 10 minutes after injection of ^{99m}Tc as pertechnetate.

Patient motion. A common cause of distortion in the image is motion of the patient during the scan. This is less likely to occur if the scan time is short and the patient is comfortable. In the present system, the patient reclines on a modified operating table (see Figure 1) which can be contoured in a variety of ways and which has padded arm and back rests that are adjustable.

In addition, the head is held in a comfortable sling of flexible, transparent plastic sheet which is supported by two rods that can be rotated to elevate and turn the head.

To summarize, we have attempted to reduce scanning artifacts wherever possible. This goal has necessitated the elimination of any form of data manipulation that depends on circuitry having a time lag, such as a count rate meter. It is felt that these manipulations (contrast enhancement, background erase, etc.) are best performed on the undistorted* photoscan by means of closed circuit television or a flying spot scanning system.

SPECIAL FEATURES

Calibration system. Ordinarily, no quantitative information is available from a photoscan, and in order to obtain such information, it is necessary to have some way of relating film density to count rate. For this purpose, it is convenient to record on the photoscan a wide range of known count rates under the same conditions (scan speed, index width, spot intensity, etc.) used to perform the scan. This can be accomplished by using a simple random count generator consisting of a radioactive source and detector. The mean count rate can be varied by introducing a calibrated absorber between the source and detector. The calibration system installed in the brain scanner (see Figure 10) consists of a ^{204}Tl source and a Geiger tube detector. (Thallium-204 emits a .765 MeV β^- -ray, no γ -rays, and has a 4.1 year half-life.)

Figure 10. The calibration wedge generator consists of a line source of ^{204}Tl and a Geiger tube separated by a step wedge absorber to produce random count rates which cover the range of 100 to 9500 counts per minute in 26 steps of 20% increase as the absorber is moved through the 12" horizontal scan motion.

*A form of background erase that does not make use of circuitry having a time lag is actually available, and used, in this system. By using a suitably short pulse length to the light projectors, it is possible to require any number of pulses to be present before the film density exceeds the fog level. This technique makes use of the non-linear response of the film rather than a time dependent non-linearity in the electronic circuitry.

The source is contained in a polyethylene tube located behind a slit collimator that has an adjustable width for calibration and to compensate for decay. The Geiger tube is mounted behind a slit collimator of fixed width. The effective slit length, hence the count rate, is determined by the position of an absorber, which is attached to the scan mechanism and moves horizontally between the source and detector collimators. The .765 MeV β^- -rays are completely absorbed in a 1/32" Al sheet which is backed by a 1/16" Pb sheet to attenuate low energy bremsstrahlung produced in the Al. Another Al sheet covers the lead to provide a better working surface. This sandwich of Al-Pb-Al sheets is held together by epoxy. The height of this absorber changes in 26 steps, each altering the count rate by 20 per cent with respect to adjacent steps. As the scanner moves between its limits of horizontal motion (12"), the count rate changes from 100 counts per minute to 9,500 counts per minute during the 26 steps of 20 per cent increase. Pulses from this system are fed to the spot projectors to produce a calibrated wedge for each detector channel. The count rate at any point on the patient's head can then be determined by matching the film density at that point with the corresponding film density on the calibrated step wedge. Photoscans and calibrated step wedges are recorded on two sheets of Royal Blue X-ray film. The film is held in X-ray cassettes in which the aluminum entrance windows have been replaced by 1/8" sheets of tempered glass and removable, optically opaque, cover slides. The film is processed in a Kodak X-O-Mat to insure uniformity of development.

Patient and scan information recording system. For each scan, the operator fills out an information form that identifies the patient and specifies the conditions under which the scan was performed. (Quantity, chemical form, and time of injected activity, scan time, speed, index, collimator, spot size, are recorded, as well as other data and remarks that might be relevant to the interpretation of the photoscan.) A dual channel optical system records a reduced but readable image of this form on each sheet of film and simultaneously marks one film LEFT, the other RIGHT, corresponding to the left and right sides of the patient's head. This system has virtually eliminated the problem of labeling and identifying the film.

Film format. While the photoscans are recorded on 14" x 17" film, the horizontal motion is limited to 12", leaving a two-inch strip of film to record the information form described above. The vertical motion is also limited to 12", but since the upper and lower detectors are separated by 4-1/2", the total area covered is 12" by 16-1/2". The upper and lower 1" strips are used to record the calibration step wedges for the upper and lower detectors, respectively. This leaves an area 12" by 14-1/2" in which the brain scan can be recorded.

Scan control system. Two modes of scanning are possible, namely, Profile Scanning and Rectangular Scanning. In profile scanning, the vertical indexing and horizontal scan reversal are accomplished by the interrupted light beam mentioned earlier. In rectangular scanning, adjustable limit bars define the region to be scanned. Microswitches, actuated by a control rod carried by the detectors and operating within this rectangle, index the detectors vertically and reverse the horizontal scan direction. Panel lights indicate failure of the microswitches, in which case the system will scan another .080" before actuating a "backup" microswitch that reverses the scan direction.

Electronic circuits. The circuits comprising the ACRH brain scanner employ semiconductor components wherever possible. Silicon controlled rectifiers are used as power switches in the horizontal and vertical drive systems to eliminate switch contact noise. All circuits for the detection, analysis, and recording of γ -ray signals, including those used for calibration, are

transistorized. These circuits are built into small plug-in units and have proven to be remarkably stable and trouble-free.

Modifications under way. While maintaining a certain skepticism concerning the efficacy of a "Universal Scanner," the success of ^{99m}Tc in applications other than brain scanning,¹¹⁻¹⁴ has led us to modify the ACRH scanner for more general use with low energy isotopes. In addition to the modifications of the detectors and shields which have already been mentioned, these units have been set on movable carriages that permit the distance between collimator faces to be adjusted from 8" to 14-1/2". A sliding couch is under construction which will allow the head, neck or torso of the patient (lying on his side) to be placed between the detectors. In this way we hope to use the system for thyroid scanning (with one detector), for kidney scanning (with two detectors), and for lung and liver scanning (with all four detectors).

RESULTS AND CONCLUSIONS

Between August, 1963 and the present date, this system has been used with ^{99m}Tc to scan approximately 800 patients. The clinical procedure and findings have been reported in detail elsewhere.³⁷ Figure 11 summarizes an analysis of the first 182 scans.

Category	Definite	Probable	Total
Positive	63	5	68
Negative	78	10	88
False positive	2	4	6
False negative	8	2	10
Technically unsatisfactory			<u>10</u>
			182

Figure 11. Classification of 182 brain scans.

Although the present system was designed primarily for brain scanning, it is felt that many of the techniques incorporated in this device will be helpful in other scanning applications. Modifications of this system are being made which will allow us to investigate these possibilities.

ACKNOWLEDGMENT

We are especially grateful to John J. Stupka, Charles R. Brebach, and Bernard W. Fromes for innumerable ideas and complete cooperation in every aspect of the mechanical design and construction, and to Alexander Sinila for his essential contribution to the detector transport design. In addition, Rosa Lee Seslar, Violet Stark, Vivian Reynolds, and Joan Anderson performed the brain scans with great care.

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