ACRH-4

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

Semiannual Report to
THE ATOMIC ENERGY COMMISSION

SEPTEMBER 1955

LEON O. JACOBSON, M.D.
Editor

ANTREEN PFAU
Associate Editor

OPERATED BY THE UNIVERSITY OF CHICAGO
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I

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II

FOREWORD

The semiannual reports of the Argonne Cancer Research Hospital consist for the most part of representations of papers that have been published or submitted for publication in the scientific literature, which is outside the province of the Atomic Energy Commission. Hence, each of these reports brings together a picture of some of the investigations that have been conducted in the Hospital and are considered to be completed units of research that are ready for critical evaluation.

In a few instances, papers are included that describe work that is of particular interest to the Atomic Energy Commission but which the authors wish to withhold from widespread circulation until the project has been completed or related to other research that is planned or in progress.

Leon O. Jacobson
Editor

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THE ASSOCIATION OF IRRADIATION WITH CANCER OF THE THYROID IN CHILDREN AND ADOLESCENTS*

By

D. E. Clark

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INTRODUCTION

The possibility that irradiation might be an etiological factor in carcinoma of the thyroid in children and adolescents was suggested by Duffy and Fitzgerald,[1] who found that 10 of the 28 patients, 18 years of age or younger, had been subjected to radiation to the thymus gland some time between the 4th and 16th month of life. Simpson, Hempelmann, and Fuller[2] studied the frequency of neoplasms in 1408 children that had received roentgen-ray therapy to the thymic area and found that the number of cases of cancer of the thyroid was significantly higher in the treated group than in either the untreated siblings or the general population.

Short courses of low voltage roentgen rays have been used widely to treat children for benign conditions about the head, neck, and upper thorax other than thymic enlargement. A search of the literature was made to learn whether such treatments had been correlated with the subsequent development of carcinoma of the thyroid, but no references could be found. For this reason, a careful survey was made of cases of carcinoma of the thyroid in patients who were 15 years of age or under that the author had treated or seen to learn whether any had been irradiated previously about the neck.

RESULTS OF SURVEY

Thirteen cases† of carcinoma of the thyroid have been seen in children and adolescents, all within the past 6 years. Ten have been treated and are being followed; 3 were seen in consultation but were treated elsewhere. All of the patients had been subjected previously to irradiation. Three had been irradiated on the upper chest for an enlarged thymus; 3 on the neck for cervical adenitis; 5 on the head and neck for enlarged tonsils and adenoids; 1 on the face and anterior chest for sinusitis and peribronchitis; and 1 on the anterior upper chest for pertussis (Table 1).

Of the 13 cases, 12 were girls and 1 was a boy. The predominance of the neoplastic condition in girls is in accordance with the findings of others.

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* Text of paper presented at the International Conference on the Peaceful Uses of Atomic Energy, August 8-20, 1955, Geneva, Switzerland, and based on paper that appears in the Journal of the American Medical Association, 159:1007 (1955).

† Two cases that are not included in the series are known by correspondence. One, a 14-year old girl, had been X-irradiated on the upper chest in early childhood for a chronic postpneumonic cough. The other, a 7-year-old girl, had been irradiated in the thymic region in infancy.

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Table 1

CASES OF CARCINOMA OF THE THYROID IN CHILDREN AND ADOLESCENTS WHO HAD RECEIVED IRRADIATION*

Patient	Sex	Age when irradiated	Age when carcinoma was diagnosed (yrs.)	Time from irradiation to diagnosis (yrs.)	Area irradiated	Treatments (no.)	Total exposure (r)	Size of portal (cm.)	Reason for irradiation
K.D.	F	2 mos.	7	7	chest	1	210	5 x 5	enlarged thymus
J.S.	F	3 mos.	7	7	chest	4	400	--	enlarged thymus
K.S.	F	8 mos.	8	7	ant. upper chest	2	200	--	enlarged thymus
M.N.	F	5 yrs.	11	6	neck	4	200	10 x 10	cervical adenitis
S.L.	F	4 yrs.	12	8	right upper neck	3	300	3 x 3	cervical adenitis
M.K.	F	6 yrs.	15	9	neck	3	--	--	cervical adenitis
J.M.	F	10 mos.	4	3	head and neck	3	600	8 x 10	enlarged tonsils and adenoids
D.S.	F	3 yrs.	8	5	head and neck	3	--	--	enlarged tonsils
M.B.	F	3 yrs.	10	7	head and neck	3	624	6 x 8	enlarged tonsils
P.W.	M	4 and 6 yrs.	14	8 or 10	right and left nasopharynx	6	725	8 x 10	enlarged tonsils
M.D.	F	4 yrs.	10	6	tonsillar area	4	300-400	8 x 10	enlarged tonsils and adenoids
T.A.	F	3 yrs.	10	7	face and ant. chest	4	550	10 x 15	sinusitis and peribronchitis
E.W.	F	4 yrs.	14	10	ant. upper chest	4	300	Large	pertussis

* Two cases that are not included in the series are known by correspondence. One, a 14-year-old girl, received X-ray treatments in early childhood to the upper chest for a chronic postpneumonic cough. The other, a 7-year-old girl, was subjected to irradiation to the thymic area in infancy.

The age at the time of irradiation ranged from 2 months to 6 years. The age at the time of histologic diagnosis of tumor varied from approximately 4 to 15 years. The interval from the time of irradiation to that of diagnosis was approximately 3 to 10 years, with the average being 6.9 years.

The total radiation exposure (in air) ranged from 200 to 725 r in 11 of the cases. The sizes of the portals were quite variable, ranging from 3 x 3 to 10 x 15 cm. In the case that was irradiated for pertussis, the size was designated only as large. The portal size in 4 of the cases could not be obtained. The total exposures are comparable to those found by Simpson, Hempelmann, and Fuller,[2] which ranged from 50 to 1500 r, with most being less than 600 r. All of the individuals who subsequently developed tumors of the thyroid received more than 200 r. Six hundred and four (43%) of the 1408 children who were included in their investigation had received under 200 r; none of these had developed any known tumors of the thyroid gland.

The physical development of all of the children described in this report had been normal, and none had a history of any previous thyroid disorder. All of the individuals are living, well, and clinically free of carcinoma except one who still has roentgenographic evidence of pulmonary metastasis.

PATHOLOGY AND EXTENT OF SPREAD

The various histologic types of neoplasms are shown in Table 2. None of the carcinomas in the series had an entirely uniform histologic pattern. In 4 cases, the carcinoma

Table 2

HISTOLOGIC CLASSIFICATION OF CARCINOMAS

Type	No. Cases
Papillary carcinoma	4
Mixed papillary carcinoma	3
Adenocarcinoma	6

was predominantly papillary; in 3, it was an admixture of papillary and alveolar types; and in 6 it was principally alveolar or follicular.

The known extent of the neoplasm in the 13 cases is summarized in Table 3. In only one case was the carcinoma limited to the thyroid gland. Unilateral involvement of the deep jugular lymphatic system was found in 5 cases and bilateral involvement in 6. In one case, 2 pre-tracheal nodes were involved as was the trachea by direct extension from the primary focus in the thyroid. A metastasis to the esophagus was found in 1 patient. Four of the patients had changes in the lungs, demonstrated by chest films, that were compatible with metastases. In 3 of these, the lesions were of a diffuse miliary type involving prin-

Table 3

KNOWN EXTENT OF THE CARCINOMA

Extent	No. Cases
Limited to thyroid gland	1
Unilateral involvement of lymphatics of neck	3
Unilateral involvement of lymphatics of neck and both lungs	2
Involvement of delphian nodes and trachea	1
Bilateral involvement of lymphatics of neck	3
Bilateral involvement of lymphatics of neck and both lungs	2
Bilateral involvement of lymphatics of neck and esophagus	1

cipally the basilar portions of the lungs; and in all 3, the lesions disappeared, as evidenced by roentgenographic examination, after radioactive iodine therapy. The high incidence of metastases in the cases in this series corresponds with that observed by others who studied carcinoma of the thyroid in this age range. In all of the cases, the condition was overt at the time they were first seen.

DISCUSSION

All of the children and adolescents who had carcinoma of the thyroid and who had been treated or seen by the author had been irradiated previously in such a way that the thyroid gland or portions of it had been included in the field of exposure. The observation lends support to the idea that there may be a correlation between irradiation given in infancy and early childhood and the subsequent development of cancer of the thyroid.

During recent years, considerable interest has developed concerning the frequency of carcinoma of the thyroid in children and adolescents. Although it is still a relatively uncommon condition, the number of cases diagnosed is becoming greater. Winship,[3] in his excellent review, found that only 8 cases of carcinoma of the thyroid in individuals under 15 years of age were reported in the literature from 1900 through 1930; 34 cases were recorded from 1931 to 1940; and 50 cases were reported during the period from 1941 to 1950. To these he added 4 of his cases and reported 95 hitherto unpublished cases that he collected by surveying children's hospitals and large approved general hospitals. This brought the total number of cases reported to 191.

On the basis of follow-up information given by Winship[3] for the collected cases and careful study of follow-up data and case histories of the previously published cases, it was possible to ascertain the approximate time of diagnosis of 169 of the 191 cases. As is shown in Figure 1, 18 cases were diagnosed prior to 1930; 30 cases, between 1930 and 1949; and 121 cases during the period from 1940 to 1950. The data show that carcinoma of the thyroid

in children is being diagnosed more frequently. The recent increase in the number of cases is so large that, in the absence of any great improvement in the methods available for diagnosis or in the applicability of diagnostic methods to the whole population during this time, it almost surely represents a true increase in the incidence of carcinoma in the population. It is recognized that many cases have not been recorded and that the statistics have not been corrected for population trends.

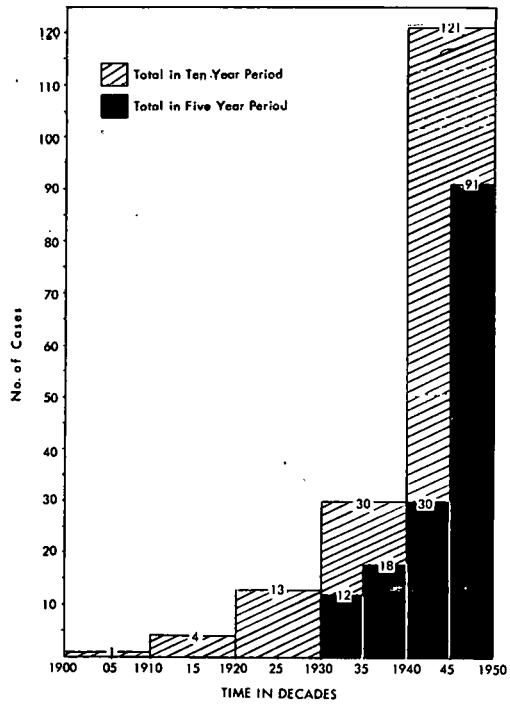

Figure 1. The occurrence of 169 of the 191 cases of carcinoma of the thyroid in children under 15 years of age (Winship).[3] Distribution is based on the approximate time of diagnosis.

The increasing incidence of carcinoma of the thyroid in children and adolescents seems to correlate with the greater use of X radiation for benign conditions about the head, neck, and upper thorax. The use of irradiation for the treatment of enlarged thymus in infancy was first reported by Friedlander[4] in 1907. During the ensuing years there was an increasing number of such cases treated, and X irradiation therapy was extended to the treatment of other benign conditions about the neck. From 1930 to 1945, irradiation in infancy and early childhood was commonplace, not only for enlarged thymus glands,[2] but for enlarged tonsils and adenoids, cervical adenitis, and pulmonary conditions. Although prior X irradiation cannot be incriminated definitely, the association is striking, and the observation suggests strongly that the widespread use of X radiation about the neck in early life may be an important factor in the increasing number of cases of carcinoma of the thyroid diagnosed in late childhood and adolescence. It is hoped that this report will stimulate further investigation of this possibility.

SUMMARY

Fifteen cases of carcinoma of the thyroid in children 15 years of age and younger are reported. All of the cases had been X-irradiated in infancy and early childhood for benign conditions about the head, neck, and thorax. The observation lends strong support to the idea that an association exists between irradiation and the subsequent development of cancer of the thyroid in late childhood and adolescence.

The interval from the time of irradiation to that of diagnosis averaged 6.9 years. The total radiation exposures in air ranged from 200 to 725 r. None of the patients had a history of any previous thyroid disorder. All of the individuals are living and clinically free of carcinoma except one.

The histologic types of cancer of the thyroid and the known extent of spread are presented.

It is shown that during the period from 1900 to 1950 the number of diagnoses of cancer of the thyroid in individuals under 15 years of age increased steadily. This increasing incidence correlates with the increased use of X radiation for the treatment of enlarged thymus, enlarged tonsils and adenoids, cervical adenitis, and benign pulmonary conditions. The correlation suggests that irradiation about the neck in early life may be an etiological factor in the development of carcinoma of the thyroid in late childhood and adolescence.

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THE ROLE OF RADIOACTIVE IODINE IN THE TREATMENT OF THYROTOXICOSIS AND CARCINOMA OF THE THYROID*

By

D. E. Clark and J. H. Rule†

HYPERTHYROIDISM

In 1942, Hertz and Roberts[1] and Hamilton and Lawrence[2] described the response of the first patients with thyrotoxicosis who were treated with radioactive iodine, I^{131}. Since then, many successful remissions of hyperthyroidism have been obtained using this isotope. Although the result of treatment has established a definite place for radioactive iodine in the treatment of selected cases, there is some differences of opinion concerning the criteria for selection of patients. Miller[3] and his group treat all cases of hyperthyroidism with radioiodine regardless of the age of the patient or the type of gland except those associated with pregnancy or lactation. They emphasize, however, that the wholesale treatment of children for hyperthyroidism should not be carried out and they are only administering the isotope to children as part of a long-term study. Chapman, Maloof, Maisterrena, and Martin[4] have concluded that I^{131} should not be used in persons with either a single or multiple toxic nodular goiter because of the high incidence of carcinoma in clinically solitary nodules and because a multinodular goiter may still harbor a carcinoma even though it may diminish in size after radioiodine therapy.

During the past 8 years, we have had the opportunity to institute therapy in over 700 patients with primary or secondary hyperthyroidism, and in this interval we have established fairly definite criteria for the therapeutic application of radioactive iodine. These indications, with a few minor variations, agree with those of many other investigators who are using I^{131} in the treatment of hyperthyroidism.

Criteria for radioiodine therapy: Almost all of the patients that we have treated have met one or more of the following criteria:

1. Uncomplicated hyperthyroidism in patients over 40 years of age.
2. Recurrent or persistent hyperthyroidism after thyroidectomy.
3. Hyperthyroidism with severe cardiovascular disease or some other concurrent disease.
4. Failure to respond properly to antithyroid drugs.
5. Refusal to accept surgery or other therapy.
6. Presence of severe exophthalmos.

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* Based on a paper entitled "Radioactive iodine or surgery in treatment of hyperthyroidism" that appears in the Journal of the American Medical Association, 159:995 (1955).

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The possibility that the radiation from I^{131} may induce carcinoma of the thyroid has been a deterrent to its use in younger patients with uncomplicated thyrotoxicosis. To date, there has been no report of any adverse effects in any of the patients who have received therapeutic doses of I^{131}. Since it will take many years to evaluate any possible long-range harmful effects of internal β radiation on the thyroid or other tissues, we advocate surgery as the treatment of choice in patients under 40 years of age with uncomplicated primary or secondary hyperthyroidism. Older individuals with a large toxic nodular goiter also are better treated with surgery than with radioiodine, providing the surgical risk is not excessive.

Contraindications to I^{131} therapy:

The following conditions are contraindications to therapy:

1. Pregnancy and lactation.
2. Clinically solitary nodule associated with hyperthyroidism.
3. Nontoxic nodular goiter.
4. Nontoxic diffuse or simple goiter.

We believe that the coexistence of hyperthyroidism and pregnancy is an absolute contraindication for radioactive iodine therapy. It has been shown that radioiodine readily crosses the placental barrier and that the fetal thyroid is able to concentrate iodide after the third month. Radioiodine has also been found in the milk of lactating women.

Because of the strong possibility of carcinoma, patients with hyperthyroidism and a clinically solitary nodule should have surgery with or without radioiodine unless the nodule can be proved to be a hyperfunctioning adenoma by suitable techniques.

It should be emphasized that I^{131} will not appreciably alter the size of a nontoxic nodular goiter.

Method of treatment: The diagnosis of thyrotoxicosis is made on the basis of clinical judgment in addition to an evaluation of the results of various laboratory procedures: namely, radioiodine diagnostic studies (conversion ratio and/or uptake), serum protein-bound iodine determination, and one or more determinations of basal metabolic rates. Laboratory study is sometimes foregone in obvious cases of classical hyperthyroidism.

One of the chief problems involved in the treatment of hyperthyroidism with radioactive iodine is the determination of a suitable dose. Many methods have been reported for estimating the minimum effective dose, but to date, none has been entirely reliable. It is extremely difficult to predict accurately the amount of radiation that any given area of thyroid tissue will receive, nor can anyone ascertain how uniformly the thyroid will take up I^{131}. Since most of the methods that have been used to determine dosage do not seem to increase greatly the accuracy of establishing the minimum effective dose and since many of them are quite time-consuming, we have continued to base our dosage determination on the estimated weight of gland, type of the gland, and age of the patient.

It has been estimated that a radiation dose of approximately 10,000 to 12,000 equivalent roentgens per g of thyroid tissue is required to bring about a reduction in thyroid activity. Therefore, a large gland will require much more I^{131} than a small gland. We have

also found that individuals with toxic nodular thyroids require about twice as much I^{131} per estimated g of gland to effect a remission as individuals with a diffuse toxic goiter. Furthermore, older people seem to require more radioiodine to control thyroid activity than younger individuals.

At the present time, 150 μc per estimated g of thyroid tissue is given as an initial dose to patients under 40 years of age with a diffusely enlarged gland, and approximately 250 μc to patients over 40 years of age. From 300 to 350 μc per estimated g of thyroid tissue are given as an initial dose to patients with nodular glands.

Within this dosage range we attempt to obtain a satisfactory remission with one dose. Some individuals prefer to give repeated small doses over a period of time in an attempt to avoid too high an incidence of hypothyroidism.

In hyperthyroid patients who have been pretreated with iodine and/or antithyroid drugs and who are mildly hyperthyroid or euthyroid, therapy is usually deferred for 4 weeks following discontinuance of iodine and 48 hours following discontinuance of the antithyroid drugs.

Almost all of the patients have been treated on an ambulatory basis and have been followed at approximately 8-week intervals until a euthyroid condition developed or the need for further treatment was apparent.

Observations: Therapy has been completed in 628 patients, including 384 that were reported previously,[5] since the summer of 1947. A few patients who had complete remissions discontinued their clinical visits and are not included in the series. A small group of patients offered no opportunity for any follow-up after therapy either because they failed to return or the referring physician was reluctant to forward such information.

One of the big problems with this form of therapy is that many physicians, who do not have a great deal of experience in the care of patients treated with I^{131}, believe that one dose is all that is required to assure a complete remission. If remission is not accomplished by a single treatment, some will condemn the therapy or will allow the patient to continue inadequately treated.

Twenty-five in the group have expired. In all of these patients, a complete remission of the thyrotoxicosis had been accomplished and the cause of death appeared to be unrelated to the isotope therapy.

Of the 628 cases followed carefully, the ratio of females to males was 3.5 to 1. Table 1 shows the therapeutic results in this group. Of the 262 patients with a toxic diffuse goiter, 214 became euthyroid and 48 hypothyroid. Two of the cases had a recurrence 2 and 4 years after a euthyroid state had been established. Two hundred and twenty-nine cases of toxic nodular goiter were treated; 201 became euthyroid and 28 hypothyroid. A recurrence developed in one case 5 years after it had been treated successfully with I^{131}. Post-surgical recurrent thyrotoxicosis was seen in 137 cases, an excellent response was seen in 105, and hypothyroidism followed radioiodine therapy in 32.

In all, 520 or 82.8 per cent of the patients became euthyroid. A total of 108 or 17.2 per cent developed varying degrees of hypometabolism, and the recurrence rate was approxi-

Table 1

GLAND TYPE AND THERAPEUTIC RESULT

Gland type	Therapeutic Result			Total and %
	Euthyroid	Hypothyroid	Post I^{131}* recurrent	
Diffuse	214	48	(2)	262 (41.7%)
Nodular	201	28	(1)	229 (36.5%)
Post-surgical recurrent	105	32	0	137 (21.8%)
Total (%)	520 (82.8%)	108 (17.2%)	(3)	628 (100.0%)

* These cases have all been treated again with I^{131} and are included in the euthyroid groups.

imately 0.5 of 1 per cent. The incidence of hypometabolism may seem high, but in many of these cases the condition is extremely mild; perhaps some might not even be considered to be hypothyroid by less critical standards. Furthermore, some of the patients showed a transitory hypothyroidism that lasted from a few months to a year or more. It is possible that, in time, many of the milder cases of hypometabolism will not require any substitution therapy. A fair number of the patients were purposely rendered hypometabolic in an attempt to improve their cardiac status; if one excludes the latter group and the extremely mild cases, the incidence of hypometabolism drops to 11 per cent.

Table 2 gives the length of the follow-up period and the therapeutic results. None of the patients treated in the latter part of 1954 is included. The highest incidence of hypometabolism occurred among the patients who were treated during 1950 and 1951. This may be explained by the fact that we have recently observed a gradual development of hypometabolism in several patients who were treated during that time. These patients had been euthyroid for several years, which suggests that, in some patients, the biological effect is a gradual one that acts over a period of years.

The number of doses required to effect a remission is shown in Table 3. Only one dose was necessary for 352 or 56.1 per cent of the patients; 2 doses were required for 160 patients or 25.5 per cent; and 3 or more doses for the remainder. There was complete remission with 1 or 2 doses in 81.6 per cent of the 628 patients.

Patients with toxic diffuse goiter received doses ranging from 1.8 to 60 mc, with an average of 11.3 mc. The patients with toxic nodular glands required an average of 20.5 mc to effect a remission, with a range of 3.0 to 140 mc.

The course of exophthalmos following I^{131} therapy is shown in Table 4. There were

Table 2

LENGTH OF FOLLOW-UP TIME AND THERAPEUTIC RESULT

Year treated	Euthyroid	Hypothyroid	Total	Hypothyroid (%)	Recurrence
1947	3	3	6	50.0	
1948	34	3	37	8.1	
1949	75	14	89	15.7	2
1950	105	25	130	19.2	
1951	105	27	132	20.4	1
1952	88	18	106	16.9	
1953	72	14	86	16.3	
1954	38	4	42	9.5	
Total (%)	520 (82.8)	108 (17.2)	628 (100)		3 (0.5)

Table 3

NUMBER OF DOSES OF I^{131} REQUIRED FOR REMISSION

Number doses	Gland Type			Total patients	Remission (%)
	Diffuse	Nodular	Recurrent		
1	164	102	86	352	56.1
2	52	75	33	160	25.5
3 or more	46	52	18	116	18.4
Total	262	229	137	628	100.0

189 patients with exophthalmos of varying degree. An increase in the condition was observed in only 1 patient following therapy. We were unable to evaluate 30 patients adequately. These were being followed elsewhere, and we were unable to determine whether the exophthalmos had improved or remained unchanged. Of the 159 patients who were evaluated satisfactorily, 49 or 30.8 per cent had complete regression; 88 or 55.4 per cent showed varying degrees of improvement; and in 16 or 10.0 per cent it was thought that the degree of exophthalmos remained the same. In five cases, post-therapeutic exophthalmos developed 6 weeks to 17 months after administration of the radioiodine.

It has been our impression that the improvement in exophthalmos with radioiodine therapy is somewhat better than with surgery.

Table 4

COURSE OF EXOPHTHALMOS FOLLOWING I^{131} THERAPY

Exophthalmos	No. of patients	%
Complete regression	49	30.8
Improved	88	55.4
No change	16	10.1
Worse	1	0.6
Developed after therapeutic response	5	3.1
Total evaluated	159	100.0
Inadequately evaluated	30	
Total cases of exophthalmos	189	(30.1% of total pts.)

Untoward effects: Some of the untoward effects of I^{131} therapy are given below:

1. Tenderness of the thyroid gland.

A moderate number of patients reported some tenderness in the region of the thyroid gland following the administration of radioiodine. This usually appeared 1 to 3 days after the administration of I^{131} and continued for several days to a week. Furthermore, some patients complained of a sense of constriction in the neck beginning 5 to 60 weeks after therapy and continuing for a considerable period of time.

2. Transient gastritis.

Some patients complained of symptoms suggestive of gastritis for a few days following therapy.

3. Localized pretibial myxoedema.

Three patients developed localized pretibial myxoedema after they had become euthyroid. They had no other manifestations of hypometabolism.

4. Hypothyroidism.

5. Post-therapeutic exophthalmos.

There has been no radiation sickness and no evidence of bone marrow depression or injury to the parathyroids or kidneys. Menstrual abnormalities have not been observed. Some of the patients subsequently became pregnant and delivered healthy children.

Summary: Radioiodine is an excellent means for treating selected cases of thyrotoxicosis and offers many advantages. There is no mortality, parathyroid destruction, or recurrent nerve damage. Its cost is relatively little compared with that of surgery. No hospitalization is required, virtually no time is lost from work, and the administration is painless and produces an excellent cosmetic result. Furthermore, the recurrence rate is negligible, the incidence of hypometabolism is comparable to that observed following sur-

gery, and the isotope should be effective in all cases of true hyperthyroidism if given in adequate amounts. Once the fear of the possibility of its carcinogenic action in the thyroid has been alleviated, radioactive iodine should largely replace surgical thyroidectomy for Graves' disease unless a better and nondestructive method of therapy is forthcoming.

CARCINOMA

At one time radioiodine was heralded by the lay press as a possible panacea for carcinoma of the thyroid that was not amenable to surgery. Now we know that it has a limited but, we believe, a definite role in the management of such lesions.

In order to obtain a beneficial response from I^{131}, the cancer cell must have an affinity for the isotope. In other words, the cell must have the function of concentrating iodine similar to that of a normal thyroid cell.

In the early days of our program, which is now going into its 8th year, all cases of thyroid carcinoma were treated with I^{131} that could not be satisfactorily cared for by radical surgery. Since not all carcinomas of the thyroid have an avidity for I^{131}, various procedures such as, ablation of thyroid by surgery and/or I^{131}, and the administration of thiourea compounds and thyrotropic hormone were employed in an attempt to stimulate some of the tumors to take up the isotope. As our work progressed, we concluded that certain types of tumors would probably not respond to I^{131}, and its use in such cases was probably not warranted. We also thought that we could ascertain fairly well by careful study of the morphological picture, the types of tumor that might be affected. At the present time, all of our cases are screened on the basis of the pathological type. If a definite decision cannot be made from careful morphological examination, then studies to determine whether the tumor has any avidity for iodine are performed or attempts are made to stimulate the tumor cells to take up the isotope. If no uptake is found, such cases are not treated with I^{131} at this time. In the past, some of these patients were given 35 mc of I^{131} every 2 weeks for a period of 6 months to learn whether some benefit might be observed. The results were very discouraging.

The classification of thyroid cancer that we use is a relatively simple one. There are four main groups:

A. Papillary carcinoma
    1. True
    2. Mixed
B. Alveolar and follicular adenocarcinoma
C. Hürthle cell carcinoma
D. Undifferentiated carcinoma
    1. Small cell
    2. Large cell
    3. Giant cell
    4. Epidermoid

Pathology as it relates to the use of I^{131}: A true papillary carcinoma from a patient

who received 0.5 mc of I^{131} 48 hours prior to surgery is shown in Figure 1. Survey, radioautographs of tissue samples, and direct counting of a known amount of digested tumor tissue failed to reveal any localization. Unfortunately, none of the cases of this type has

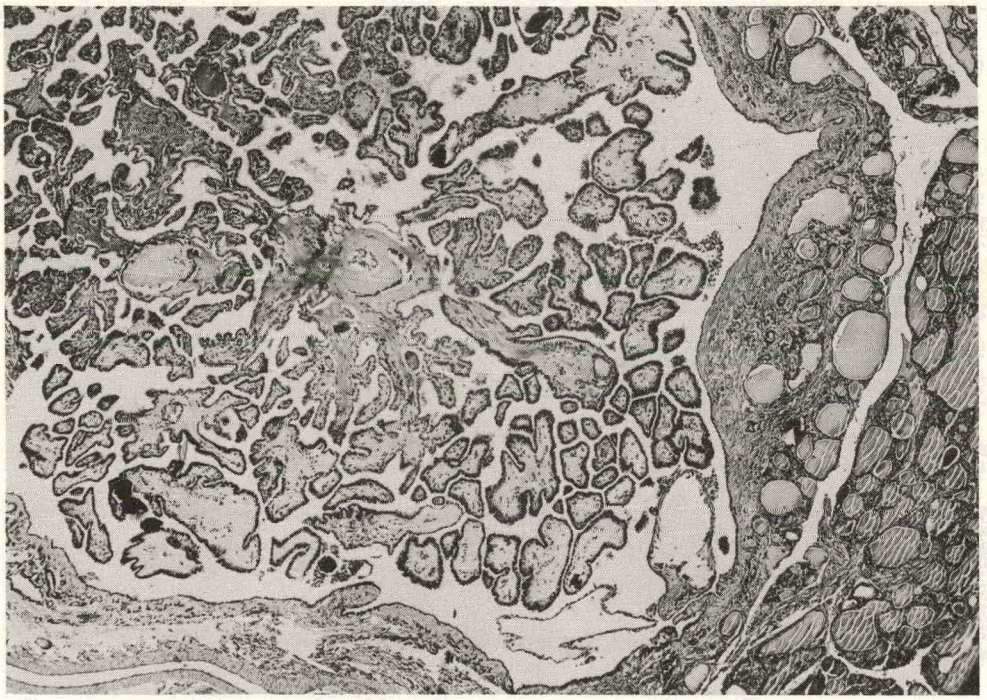

Figure 1. A true papillary carcinoma of the thyroid composed almost entirely of papillary elements. There is no acinar or colloid production. Such a tumor is not functioning.

shown an avidity for I^{131}, nor have we been able to stimulate such a type to take up radioiodine. It is doubtful whether I^{131} is warranted in such cases.

Others have also failed to find evidence of I^{131} concentration in cases with predominantly papillary tumors by radioautographic technique and direct counting of the tissue.

Figure 2 shows a mixed papillary carcinoma. Twenty-five per cent or more of the tumor was made up of acini with colloid formation. This tumor was one that should have been functioning and taking up I^{131}. The specimen was taken from a male, age 41, who had a left radical neck resection in October, 1947. Thirty months later (April, 1950), a recurrence was noted in the lower neck. Forty-eight hours after a tracer dose, the nodule was removed. Direct counting of the tissue and examination of radioautographs revealed a good localization of I^{131} in the tumors. Even though there was no clinical evidence of any residual carcinoma, he was given 35 mc of I^{131} every 2 weeks for a period of 6 months, receiving a total of approximately 450 mc. A fair number of cases with this type of tumor have been treated prophylactically with a similar dosage schedule. He has remained clinically free of any further recurrence since the radioiodine course, a period of approximately 5 years.

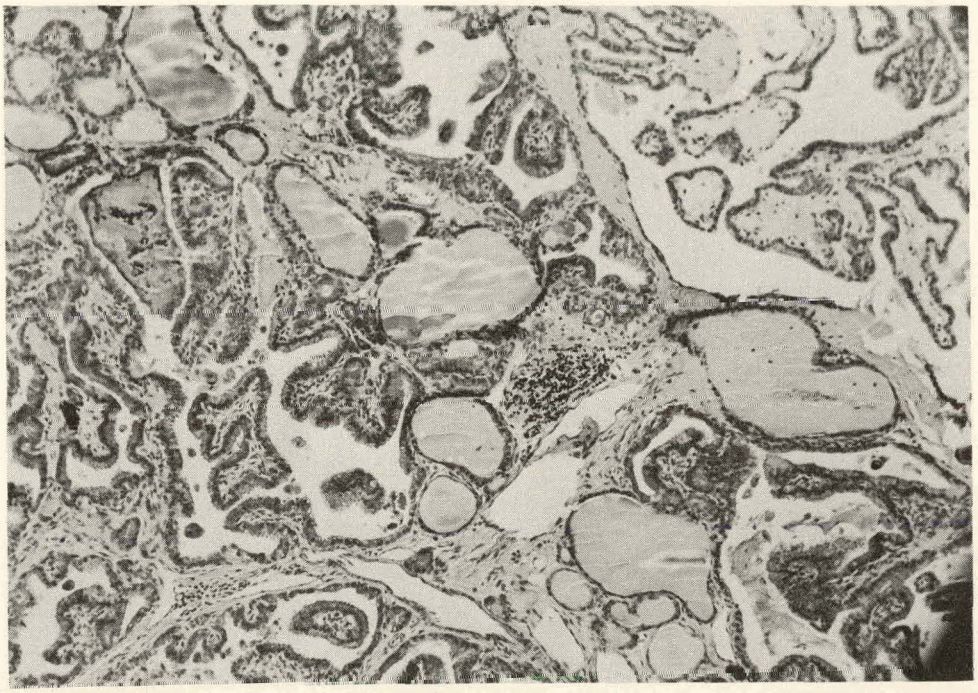

Figure 2. A mixed papillary carcinoma of the thyroid. Twenty-five per cent or more of thus tumor is composed of acini with colloid formation.

The radioautograph in Figure 3 shows that the localization of radioiodine is spotty and corresponds to areas of maximum colloid formation; there is no localization in the papillary areas. When one examines the microscopic sections and corresponding radioautographs, it is evident that some of the glandular areas do not concentrate the isotope. This is one of the principal reasons why we treat carcinomas of the thyroid with multiple moderate doses at regular intervals of time. It is believed that in the tumors that concentrate iodine, the cells have a "phasic function" similar to that of the thyroid. Therefore, by the administration of repeated doses, the cells functioning at the time of one dose would be affected and subsequent doses would affect other cells or follicles as they begin to function.

We realize that there is little chance of affecting the papillary elements in such tomors, but individuals with this type of tumor should probably be given the benefit of I^{131} not only as a therapeutic measure, but as a prophylactic one as well.

An example of an alveolar type of carcinoma of the thyroid is shown in Figure 4. It is a picture of a biopsy specimen taken from a 42-year-old woman, 48 hours after she had received a tracer dose of radioiodine. The tumor was well differentiated with acini and colloid formation. This type of tumor always has an avidity for I^{131}.

Figure 5 is a radioautograph made from the tumor. Direct counts of the tissue also revealed considerable concentration of I^{131}, and an in vivo external survey showed marked localization over the tumor mass in the neck and the mediastinum. This patient had two thyroidectomies, one at the age of 13 and the second at the age of 40. The second thyroid-

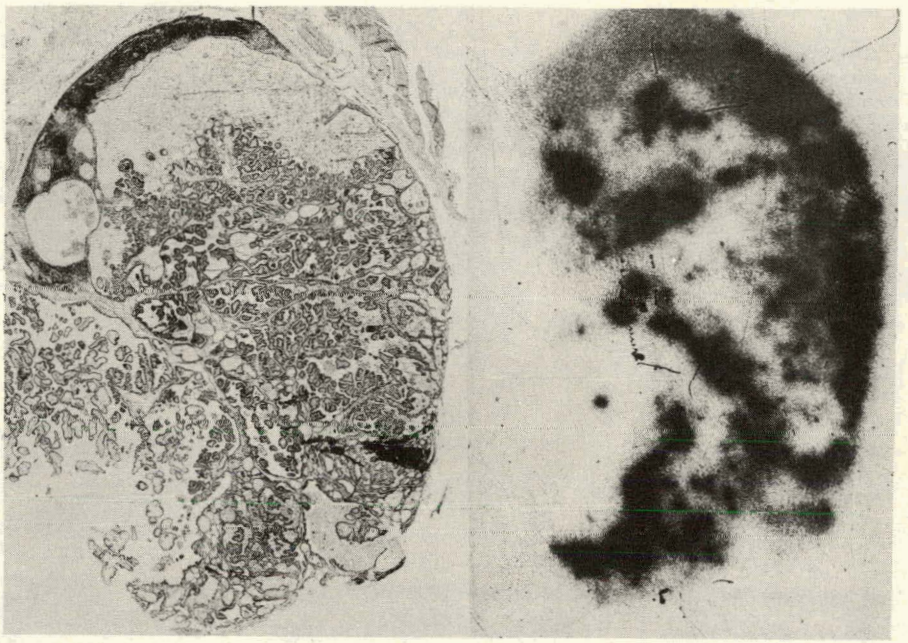

Figure 3. A photomicrograph and corresponding radioautograph of mixed papillary carcinoma of the thyroid. Localization is spotty and corresponds to areas of maximum colloid formation.

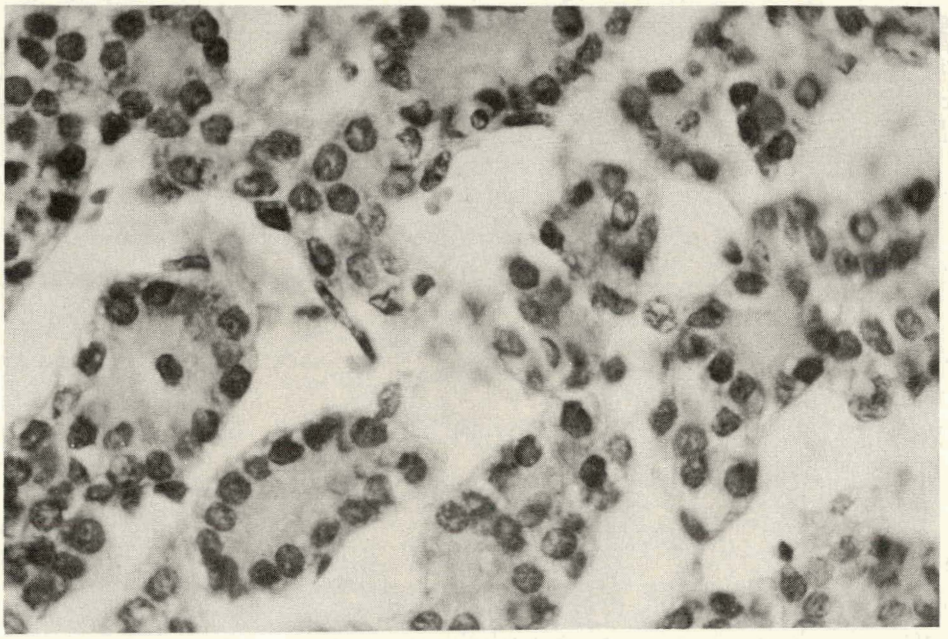

Figure 4. An alveolar carcinoma of the thyroid. The morphological appearance is similar to that of normal thyroid. The acini are well formed, and there is an abundance of colloid.

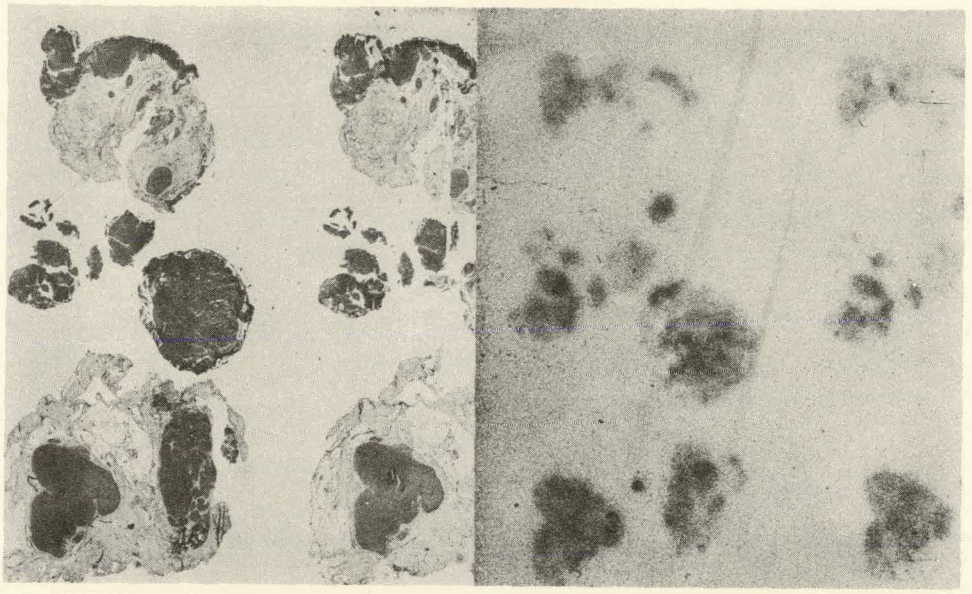

Figure 5. A low power photomicrograph and a radioautograph of the alveolar carcinoma. There is definite localization of radioiodine.

ectomy in January, 1948, revealed carcinoma. We first saw her in December, 1948, at which time she had a large recurrent mass, which was growing rapidly in the lower neck and a mass involving the manubrium of the sternum (Figure 6). A roentgenograph of the chest (Figure 7) revealed large mediastinal masses that were thought to represent metastatic lesions since there was marked localization of the tracer dose over the mediastinum. She was started on I^{131} therapy in January, 1949, receiving approximately 35 mc of I^{131} every 2 weeks for a period of about 2 years. The total dose was approximately 1500 mc. Therapy was discontinued about 4-1/2 years ago. At the conclusion of therapy, the

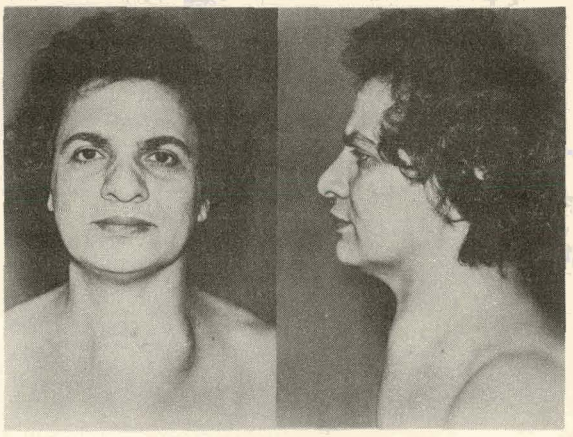

Figure 6. Patient prior to radioiodine therapy. Note large mass in lower anterior neck and over manubrium of sternum.

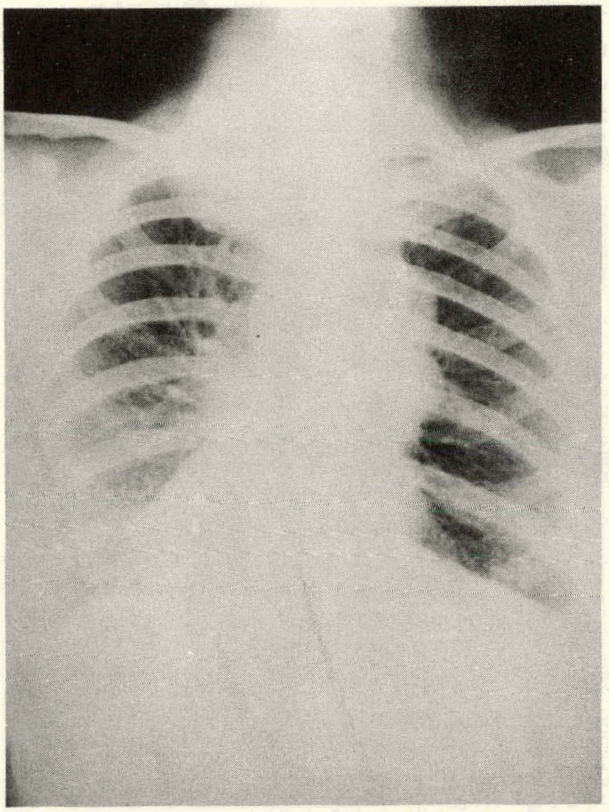

Figure 7. Roentgenograph of chest. Note large mediastinal masses. A large mass is also present in left superior mediastinum deviating the trachea to the right.

masses in her neck and over the manubrium had completely disappeared as judged by external palpation (Figure8); the lesion in the manubrium of the sternum was much smaller and showed marked sclerosis; and the mediastinal masses had completely disappeared as

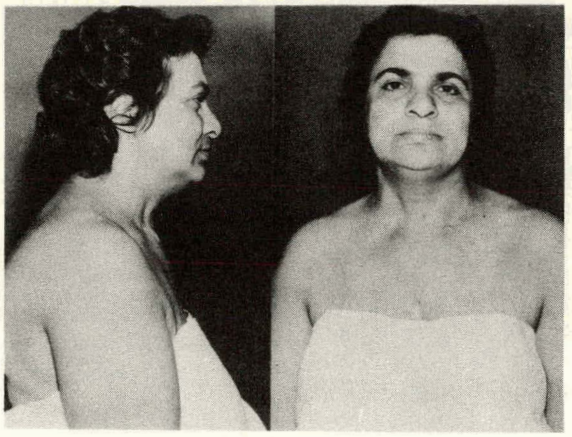

Figure 8. Patient at conclusion of radioiodine treatment. Masses have completely disappeared.

far as could be ascertained by roentgenographs (Figure 9). At the present time she is enjoying excellent health, and the hypometabolism is being controlled with 2 grains of thyroid daily.

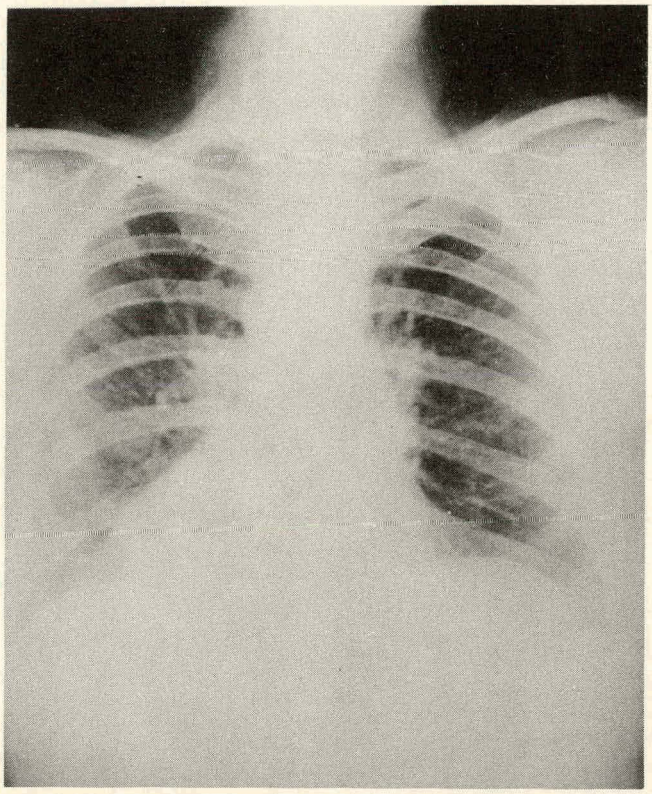

Figure 9. Roentgenograph of chest. Mediastinal masses have disappeared. Trachea is almost in midline.

Figure 10 shows a photomicrograph from another case of an alveolar carcinoma from a young, 22 years of age, who had had several operations including a radical neck resection sometime between 1944 and 1947 (Figure 11).

He was first seen at the University of Chicago Clinics in December, 1947, at which time he complained of headaches, back pain, diplopia, anorexia, nausea, and vomiting. He had lost 11 pounds in weight. Examination of his eye grounds revealed bilateral papilloedema of 3 to 4 diopters. There was no localizing neurological signs. A diagnosis was made of an unlocalized, expanding, intracranial lesion, probably metastatic from the thyroid. He was given a tracer dose of I^{131}, and on survey 24 and 48 hours later, an area of localization was found in the left occipital region (Figure 12).

The patient was treated with multiple doses of I^{131}, receiving a total of 237 mc. His condition improved under therapy and by March, 1948, he felt well. An in vivo survey following a tracer dose of I^{131} at that time failed to reveal any localization. He has remained well for over 7 years and is without any clinical evidence of recurrence at this time.

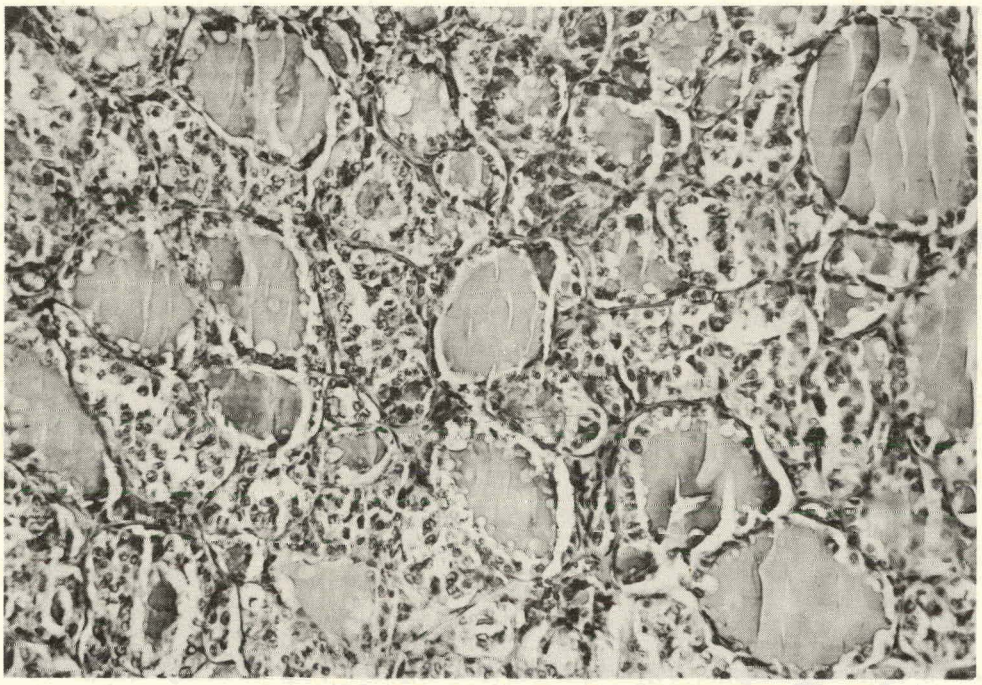

Figure 10. An alveolar carcinoma. Note acini and colloid formation.

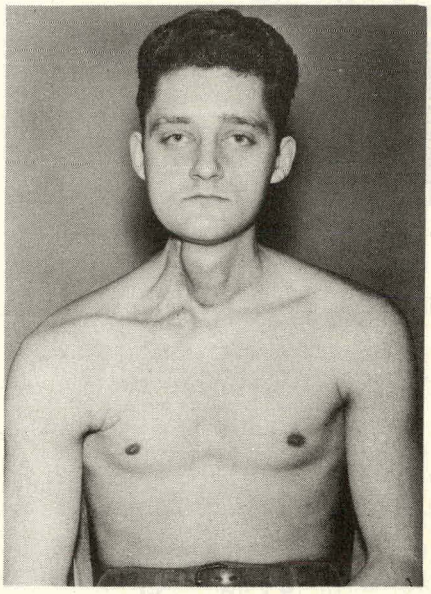

Figure 11. Photograph of patient. Note incision from radical neck resection.

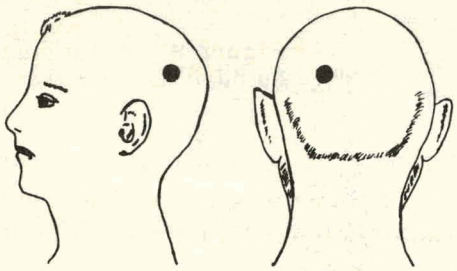

Figure 12. Area of localization of radioiodine in left occipital region.

All of our cases of alveolar and follicular carcinomas that have been treated therapeutically have benefited from isotope therapy, and the patients are still living. One case has recently shown a progression of a metastatic lesion in the spine, and radioiodine treatment was reinstituted.

Since this tumor has a predilection to metastasize to the skeletal system, we feel that all patients with this type should receive I^{131} not only therapeutically but prophylactically following surgery.

A photomicrograph of a section from a Hurthle cell carcinoma is shown in Figure 13. These cells have no resemblance to the cells of the thyroid. There are no acini nor is there any colloid formation. This type of tumor does not function, nor have we been able to stimulate such a tumor to concentrate I^{131}, which has been the general experience of other groups. It is doubtful whether patients with these tumors will benefit from I^{131}.

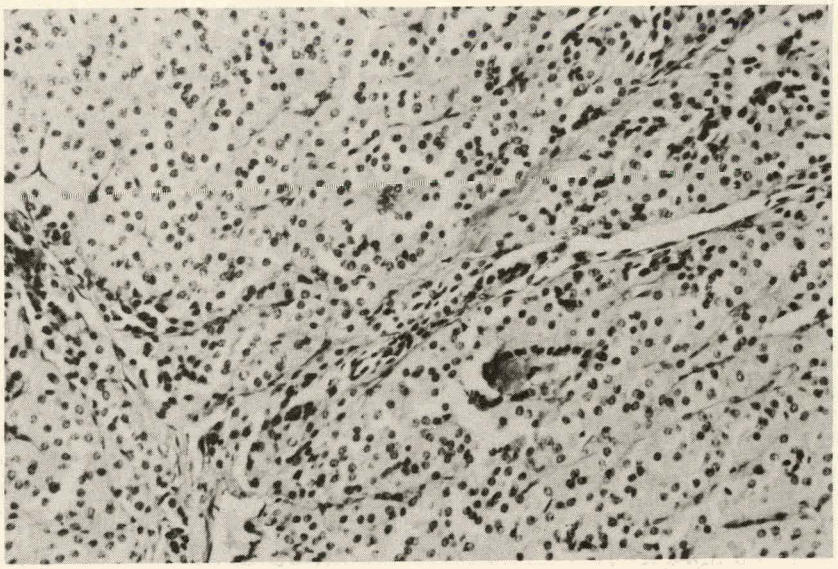

Figure 13. Photomicrograph of a Hürthle cell carcinoma. There is no acinar or colloid formation.

Figure 14 shows a photomicrograph of a section from an undifferentiated carcinoma of the thyroid. No beneficial effect from I^{131} has been observed in individuals with this type of tumor, nor has the clinical course been altered. Such a tumor is not functioning nor have we been able to stimulate such tumors to take up I^{131}. All of the patients who have been treated therapeutically are dead except 2, and, at the present time, such cases are not being given radioiodine.

On the basis of our experience, many patients with mixed papillary carcinoma and alveolar or follicular carcinomas should benefit from I^{131} therapy where surgery has failed. Some instances require the employment of techniques to enhance the avidity. It is also possible that the incidence of recurrence in these types of carcinoma may be mate-

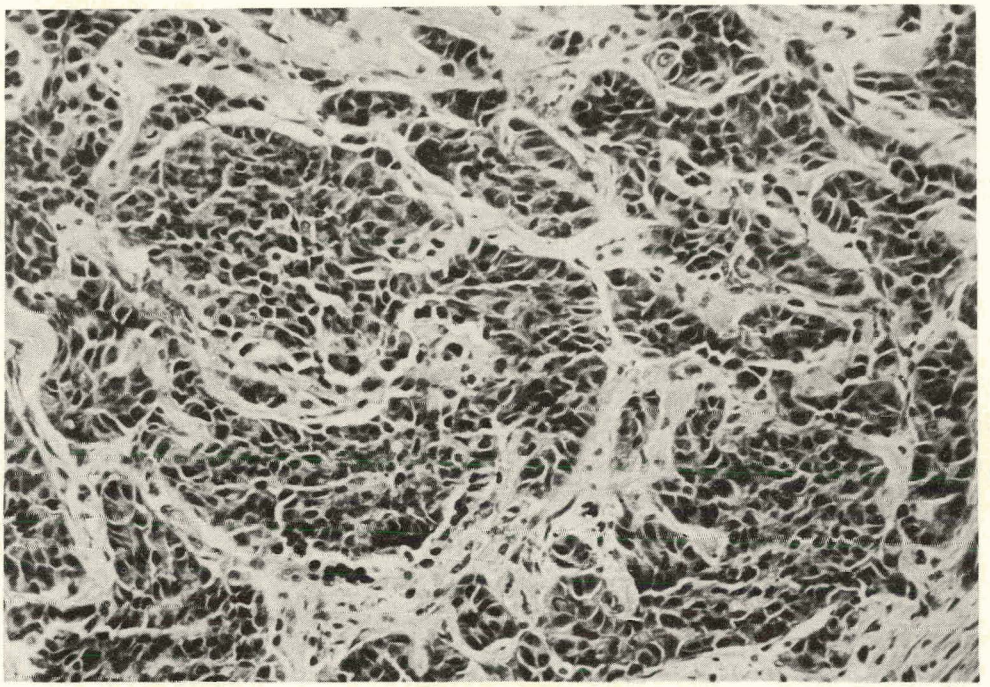

Figure 14. Photomicrograph of an undifferentiated carcinoma of the thyroid. This type of tumor has no avidity for I^{131}.

rially reduced by the administration of a prophylactic course of I^{131} immediately following adequate surgery.

Treatment: There is considerable variation in the methods of treating carcinoma of the thyroid. Some groups ablate the normal thyroid either surgically or with I^{131}, wait for a period of time, then give a course of thyrotropic hormone (25 to 30 mg. I.M. every day for a period of 5 to 6 days) in an attempt to further enhance the uptake of I^{131}, and then administer a large dose of I^{131}. Other groups, after ablation of the thyroid, give thiourea compounds, such as thiouracil (1 to 1.5 g every day) for a long period of time and give massive doses of I^{131} only after it is found by in vivo studies or urinary excretion studies following a tracer dose that a large per cent of the dose is retained. Another group gives moderately large doses at irregular intervals of time. We give moderate doses at regular intervals over a long period of time. Crile and his associates at the Cleveland Clinic are using a similar method.

For an individual with a carcinoma of the thyroid that is thought not to be amenable to surgery and for whom it is decided to consider I^{131} treatment, the following program is usually carried out. Careful blood studies are made as well as necessary roentgenographs. The patient is then given orally a tracer dose of I^{131} which, in most cases, is usually 0.5 to 1 mc. In 48 hours, the patient may be surveyed to determine whether there has been any localization in the metastatic lesions. Also, at this time one of the metastatic lesions, if accessible, or a portion of the primary, if still present, is removed under local or general anesthesia. The specimen that has been removed is divided into three portions.

One portion is weighed and either slowly digested with sodium hydroxide and then counted, or homogenized, and an aliquot of the homogenate is counted in a Texas-well counter. A frozen section is made of the second portion, and the tissue slice is counted and then applied to a nuclear track plate for radioautography. The third portion is used to make permanent microscopic sections. If the tumor is a true papillary carcinoma, Hürthle cell type, or an undifferentiated type and there is no concentration of I^{131}, the patient is not selected for I^{131} therapy. If the tumor is a mixed papillary type or an alveolar type and there is a concentration of I^{131}, the patient is then given 35 mc of the isotope every 2 weeks. The therapy is continued until there is no longer any localization of I^{131} that can be detected by external survey or until it is thought that the patient has obtained maximum clinical benefit. The duration of the therapy is variable—in some individuals, it has been continued for as long as 2 years. The course of the patient during therapy is followed by roentgenographs, tracer studies, and when feasible, biopsies for histopathologic and radioautographic studies. Peripheral blood studies are made at regular intervals in order to evaluate possible radiation effects on hematopoietic function. In many cases, repeated sternal marrow biopsies are performed. Urinalyses are also made at intervals.

Except when very ill, our patients are ambulatory. At the termination of therapy, thyroid extract is administered to control hypothyroidism, and the patients are then seen at periodic intervals for follow-up studies. Thyroid extract is withheld during the course of treatment with the hope that a hypometabolic state will increase the production of thyroid-stimulating hormone by the pituitary, which will enhance the uptake of I^{131} by the tumor. Occasionally, the patients complain so bitterly of the symptoms produced by the hypometabolism that small doses of thyroid extract are given during the latter part of the therapeutic period.

In some patients with an inoperable mixed papillary carcinoma or an alveolar carcinoma, the tumor will <u>not</u> concentrate I^{131}. In such cases, the tumor cells usually can be stimulated to concentrate I^{131} by ablation of the thyroid. At the present time, we are using I^{131} rather than surgery to eliminate the normal thyroid. Thus, such cases are treated with 35 mc of I^{131} every 2 weeks and their clinical course is evaluated in the way described previously. The period of treatment lasts for at least 6 months. If, at that time, there is no clinical evidence that the patient is benefiting from the isotope therapy or we have not been able to demonstrate that ablation of the thyroid by the radioiodine has stimulated the tumor to concentrate iodine, therapy is discontinued. If the patient seems to be responding to the therapy or if there is definite evidence of localization of I^{131} in the tumor, therapy is continued until it is thought that a maximum benefit has been obtained.

Thiourea compounds and thyrotropic hormone may be employed to further enhance the uptake of I^{131} after albation of the normal thyroid gland and are being used quite extensively by others. We feel, at the present time, that the most important aspect in stimulating such tumors to take up I^{131} is ablation of the normal thyroid and hence, we do not use any of these agents.

The rationale for eliminating the normal thyroid is: 1) the normal thyroid has a

greater affinity for iodine than malignant tissue, and its elimination affords the tumor a greater opportunity to concentrate the isotope; and 2) athyroidism may cause the tumor to function and thus concentrate iodine.

Patients who have had recent adequate surgery for a localized tumor of a mixed papillary or an alveolar type are given radioiodine prophylactically. These patients have no clinical evidence of recurrence or distant metastases. They receive 35 mc of I^{131} every 2 weeks for a period of 6 months. It is recognized that this study will be very difficult to evaluate, but it is hoped that, over a long period of time, we will be able to determine whether the incidence of recurrence in such cases has been reduced materially by the prophylactic course of I^{131}.

The best way to treat carcinoma of the thyroid with radioactive iodine is not known as yet. It is gratifying that various methods are being studied thoroughly by different groups. The overall period of observation is still relatively short, but perhaps in the future, some unanimity of opinion will evolve.

Results and comments: The present status of patients treated therapeutically with radioiodine is shown in Table 5. Five cases with true papillary carcinoma have been treated. Four have expired and 1 is living but did not obtain any benefit from the isotope. Seventeen cases with mixed papillary carcinoma have been treated. Of this group, 15 were

Table 5

PRESENT STATUS OF PATIENTS TREATED WITH I^{131} (THERAPEUTIC)

Type	Total no. cases	Number benefited	No. living not benefited	Number expired
Papillary:				
True	5	0	1	4
Mixed	17	15	0	2
Alveolar	11	11	0	0
Hürthle	4	0	1	3
Undifferentiated	16	0	2	14

thought to have been benefited, and 2 have expired. One of the patients who expired also had a carcinoma of the breast, and her death was due to the breast carcinoma. Eleven cases with alveolar carcinoma have been treated and all gave an excellent therapeutic response. None of the 4 cases with Hürthle cell type were benefited, and 3 of these have expired—1 from a cause other than the tumor. Fourteen of the 16 cases with an undifferentiated carcinoma have expired. Two who were treated more recently are living, but were not benefited by a long course of I^{131}.

Table 6 shows the present status of patients treated prophylactically with radioiodine.

Table 6

PRESENT STATUS OF PATIENTS TREATED WITH I^{131} (PROPHYLACTIC)

Type	Total no. cases	No. living without recurrence	No. living with recurrence	Number expired
Papillary:				
True	5	2	3	0
Mixed	14	14	0	0
Alveolar	13	13	0	0
Hürthle	1	0	1	0
Undifferentiated	2	1	0	1

Five cases with true papillary carcinoma have received such treatment; 3 are living with recurrence, 2 without. Fourteen cases of mixed papillary carcinoma have been treated prophylactically. So far as we know, none has developed a recurrence since the I^{131} therapy. A similar course has been observed in the 13 cases with alveolar carcinoma. One of the 2 patients with an undifferentiated carcinoma is living without recurrence. This case had a localized lesion, and it is possible that surgery completely eradicated the tumor.

The time followed since the onset of I^{131} therapy in the therapeutic group is shown in Table 7. Fifteen cases of mixed papillary carcinoma were thought to be benefited by the treatment and are still living; 1 has been followed for 1 year, 3 for 2 years, 5 for 3 years, 2 for 4 years, 2 for 5 years, and 2 for 6 years. Of the 11 cases treated for alveolar carcinoma, 1 has been followed 5 years, 3 for 6 years, 5 for 7 years, and 2 for 8 years.

Table 7

TIME FOLLOWED SINCE ONSET OF I^{131} THERAPY THERAPEUTIC

Type	No. cases	Years							
		1	2	3	4	5	6	7	8
Mixed papillary	15	1	3	5	2	2	2	0	0
Alveolar	11	0	0	0	0	1	3	5	2

Many more years of observation will be required before it can be established how many of these have been "cured." Perhaps none has been cured, but most of them have been benefited and have had added years of comfortable living. Some still have residual tumor, others have no clinical evidence of any remaining tumor.

The time followed since the onset of I^{131} therapy in the prophylactic group is given

in Table 8. Of the 14 with mixed papillary carcinoma, 1 has been followed 1 year, 1 for 3 years, 2 for 4 years, 6 for 5 years, and 4 for 6 years without clinical evidence of recurrence. Of the alveolar group, 3 have been followed 1 year, 2 for 2 years, 2 for 3 years, 1 for 4 years, 3 for 5 years, and 2 for 6 years without clinical evidence of recurrence.

Table 8

TIME FOLLOWED SINCE ONSET I^{131} THERAPY
PROPHYLACTIC

Type	No. cases	Years							
		1	2	3	4	5	6	7	8
Mixed Papillary	14	1	0	1	2	6	4	0	0
Alveolar	13	3	2	2	1	3	2	0	0

The patients in the prophylactic group have all received between 400 and 450 mc over a period of 6 months.

In the therapeutic group, the individuals with alveolar carcinoma have received from 180 to over 1500 mc. Five have received between 1000 and 1500 mc and 5 have received over 1500 mc. The individuals with mixed papillary carcinomas have received from 230 to 2218 mc.

Although a fair number of our cases have been women in the premenopausal period, only 1 case of amenorrhea developed during therapy. This was a woman, age 43, in whom the amenorrhea developed after 450 mc had been given. It is possible the I^{131} was not the cause of the amenorrhea. One girl, 18 years of age, has received over 2000 mc without any disturbance of the menstrual cycle.

Three of the cases have had pregnancies; two post-therapy. One became pregnant while she was undergoing treatment. She had received over 350 mc of I^{131} prior to conception; therapy was discontinued immediately. All bore normal children.

There has not been any clinical evidence of toxic effects on the pituitary, parathyroid, kidney, bladder, or liver in any of the patients. Nor have clinical signs and symptoms of hyperthyroidism appeared during I^{131} therapy. Most of our cases of alveolar carcinoma in the therapeutic group have had very large amounts of I^{131} before hypometabolism became evident. In one case, hypometabolism did not develop until after the patient had received 1400 mc of I^{131}. This suggests that the tumor continues to function and produce thyroxine until it is completely or almost completely destroyed. In individuals with no demonstrable functioning tumor, a hypometabolic state usually develops after 2 or 3 doses.

A fair number of our cases have shown moderate to severe depressions of the peripheral blood elements. Almost all of these have occurred in patients who have received over 500 mc. All have returned to a normal state after the cessation of I^{131} therapy except one who has a persistent leucopenia. Some of our patients have received over 1000 mc without

any demonstrable change in the blood elements. Studies of the bone marrow have shown a marked aplasia in some of the patients who received the larger cumulative doses. No serious complications have developed in any of our patients that could be ascribed to the effects of irradiation on the hematopoietic system.

The changes in the red and white blood cell counts in a patient who received over 1500 mc of I^{131} are shown in Figure 15. There were no demonstrable changes until she had received approximately 1000 mc. ACTH had no effect on the cell counts. The blood values gradually returned to normal levels after I^{131} was discontinued. The bone marrow changes are shown in Figures 16 and 17. In this patient, the bone marrow became quite aplastic after 1000 mc. Most of the marrows that have been studied showed remarkable regeneration after therapy was discontinued.

Conclusions: Carcinoma of the thyroid is still a surgically-treated disease, but we believe that radioactive iodine has a limited but definite value for the treatment of certain patients.

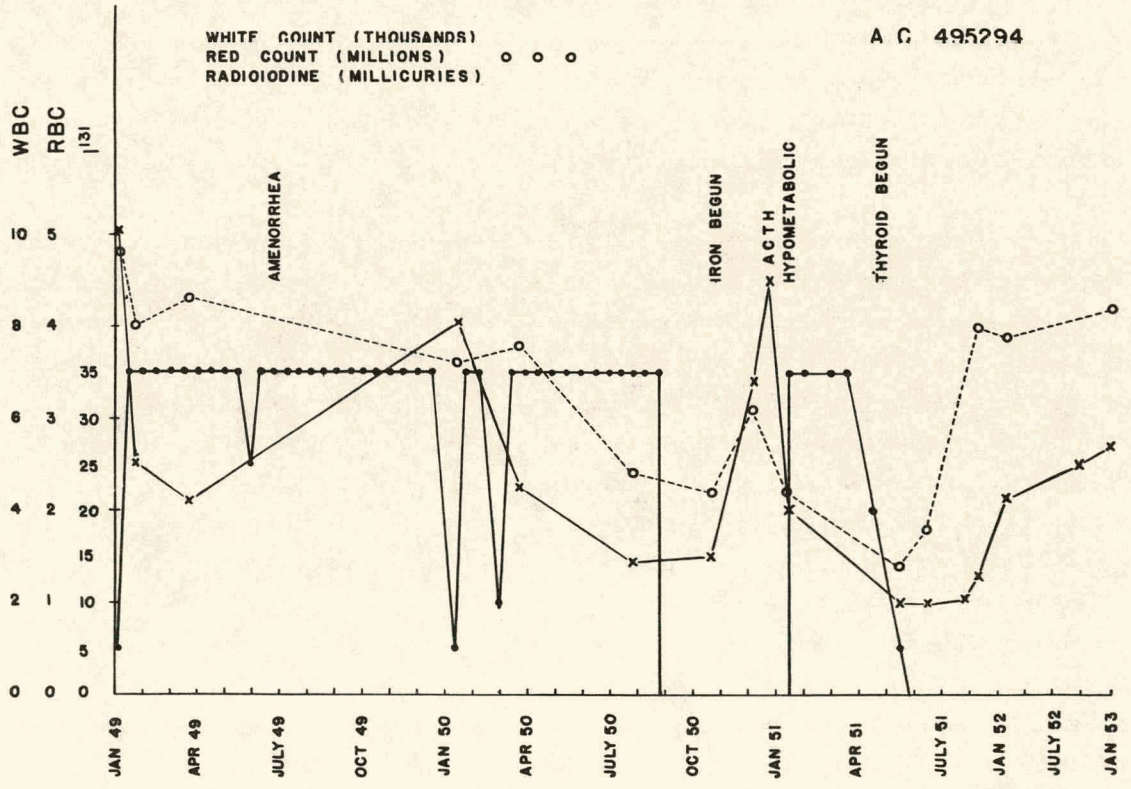

Figure 15. Changes in the red blood and the white blood cell counts in a patient who received over 1500 mc I^{131}.

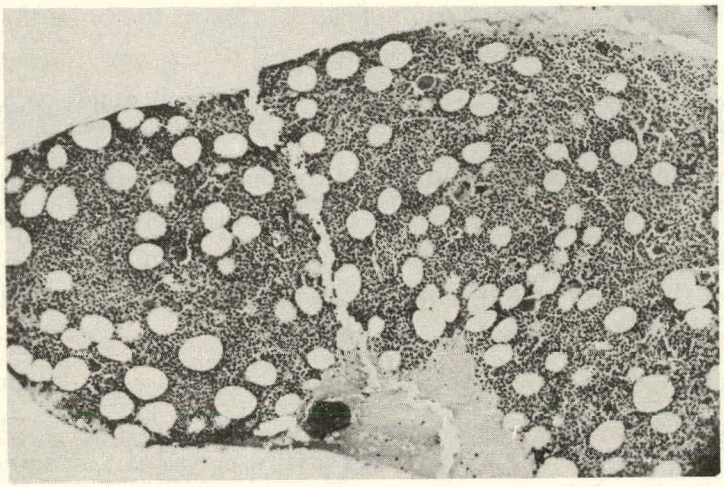

Figure 16. Bone marrow of the patient prior to I^{131} therapy. Note that its appearance is normal.

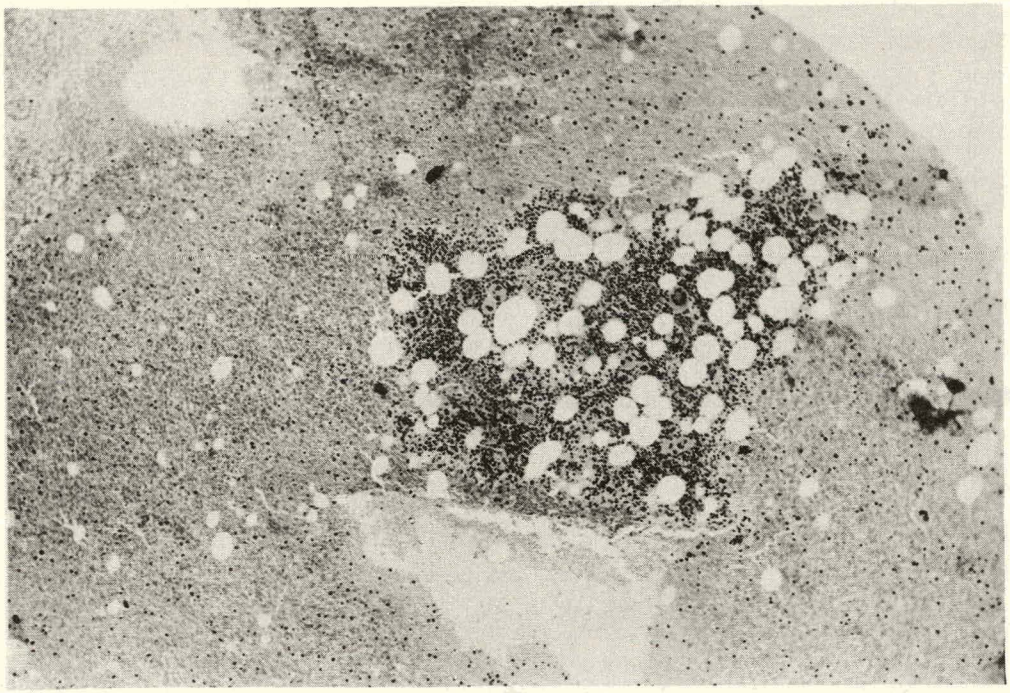

Figure 17. Aplastic bone marrow after 1000 mc of I^{131}.

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HORMONE METABOLISM IN PREGNANCY*†

By

E. J. Plotz‡

The availability of isotopically labeled compounds has provided an ideal tool for the study of the biosynthesis and metabolism of steroid hormones. In his classic experiment, Bloch[1] administered cholesterol labeled with deuterium to a woman in the 8th month of pregnancy. Pregnane-3(α), 20(α)-diol isolated from the urine contained significant concentrations of deuterium, suggesting the probable conversion of cholesterol to progesterone, the principal precursor of pregnanediol. The close co-operation of three research groups, one at the Chicago Lying-in Hospital (M. Edward Davis), one at the Argonne Cancer Research Hospital (G. V. LeRoy and H. Werbin), and another at the Los Alamos National Laboratory (R. G. Gould), has contributed to the understanding of the biogenesis and metabolism of steroid hormones during human pregnancy by the utilization of tagged compounds and thus has led to the results that are discussed in this paper.

METHODS

The experimental procedure, biochemical methods of isolation and identification of compounds, and the methods of radioassay used in this investigation are described in greater detail elsewhere.[2-4] Briefly, a single injection of sodium acetate labeled with C^{14} at the carboxyl group (1-C^{14}-acetate) was administered intravenously to pregnant patients who were scheduled for therapeutic termination of pregnancy for various reasons. A dose of 200 μc 1-C^{14}-acetate was used in all patients. Pregnancy was terminated about 2-1/2 to 3-1/2 hours after injection. In some patients, cholesterol labeled with tritium (T-cholesterol) in doses of 5.5 to 10.0 μc per day was administered orally for a period of 8 to 14 days before surgery. Cholesterol isolated from blood and tissue samples and urinary steroids excreted into the urine were radioassayed for tritium and C^{14} by means of a liquid scintillation counter.[5] In other patients, progesterone labeled with C^{14} at carbon position 4 (C^{14}-progesterone) was administered intramuscularly. Steroids carrying the C^{14} label were isolated from the urine of the patients. The concentration of C^{14} and tritium in a sample (specific activity) was expressed in μc per mM. The tracer doses that were used were approved by the Radioisotope Committee of the University of Chicago Clinics.

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RESULTS AND DISCUSSION

Synthesis of cholesterol in maternal and fetal organs: Cholesterol is regarded as an important precursor of steroid hormones. Therefore, it is important to learn as much as possible about the rate of synthesis in various endocrine organs of the mother and the fetus. Figures 1 and 2 show the specific activity of C^{14}-cholesterol derived from 1-C^{14}-acetate in tissues from two representative cases. The highest specific activity for free C^{14}-cholesterol was found in the corpus luteum of pregnancy, which indicates a very fast rate of incorporation of 1-C^{14}-acetate in cholesterol in this gland. The fetal placenta is able to synthesize cholesterol from acetate early in pregnancy (Figure 1) since the specific

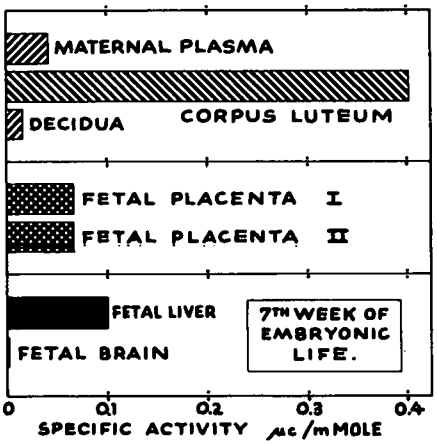

Figure 1. Free C^{14}-cholesterol in maternal and fetal tissues after a single injection of 200 μc 1-C^{14}-acetate.

activity of placental free C^{14}-cholesterol is higher than that of plasma C^{14}-cholesterol. However, the maternal part of the placenta, the decidua, appears not to synthesize cholesterol from acetate. The fetal liver and the fetal adrenals utilize acetate administered to the mother for the synthesis of cholesterol, while cholesterol formation from acetate in other fetal organs (skin, brain, heart, lungs, and aorta) was not demonstrated. As shown in Figure 2, the fetal placenta absorbs maternal cholesterol to a large extent. However, the specific activity of free T-cholesterol found in other fetal organs indicates that a relatively small percentage of cholesterol present was derived from the mother.

Acetate and cholesterol as precursors of progesterone: When T-cholesterol was administered orally to pregnant patients for a period of 8 to 14 days, pregnane-3(α),20 (α)-diol labeled with tritium was isolated from the urine, confirming the results of Bloch,[1] who used the stable isotope deuterium to label the cholesterol that he administered. When 1-C^{14}-acetate was injected into the same patients, who had previously been given T-cholesterol, appreciable radioactivity from C^{14} was found in the urinary pregnane-3(α),20 (α)-diol samples. Pregnane-3(α),20(α)-diol is regarded as the principal metabolite of progesterone. Therefore, C^{14}-progesterone was administered intramuscularly to a patient

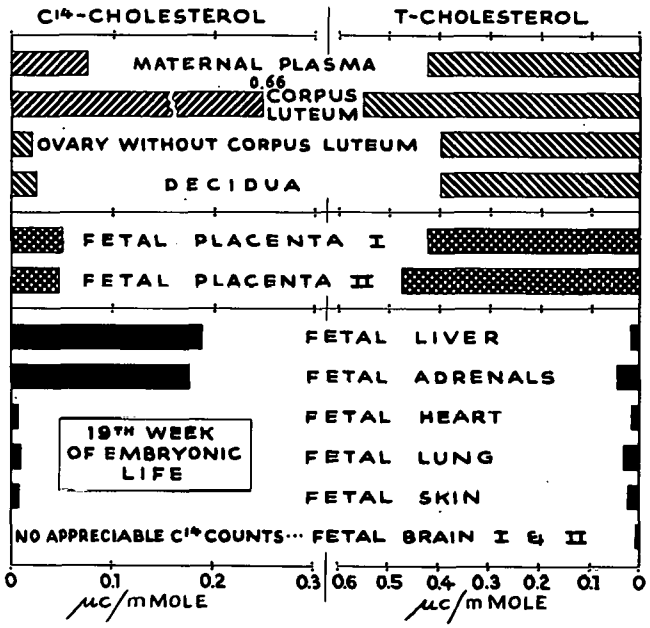

Figure 2. Double label experiment. Free C^{14}-cholesterol and free T-cholesterol in maternal and fetal tissues.

about 2 days before therapeutic interruption of pregnancy was performed. Pregnane-(α), 20(α)-diol isolated from the patient's urine showed an appreciable concentration of C^{14}, which demonstrated the conversion of progesterone to pregnanediol. Radioactivity from C^{14} was also obtained in a fraction of the urinary α-ketonic steroids that very likely represented another metabolite of progesterone, pregnane-3(α)-01,20-one. Figure 3 illustrates the findings concerning synthesis and metabolism of progesterone.

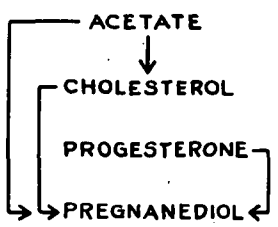

Figure 3. Precursor-product reactions involved in synthesis and metabolism of progesterone in pregnancy.

Figure 4 shows the specific activities of C^{14}-pregnane-3(α),20(α)-diol excreted into the urine of pregnant women following the intravenous injection of 1-C^{14}-acetate shortly before termination of pregnancy. The interval between injection and termination of gestation varied between 2-1/2 and 3-1/2 hours. Therapeutic interruptions of pregnancy were carried out in case Nos. 1, 2, and 3 during the 7th to 12th week of embryonic life. In case

No. 4 an anencephalic fetus was spontaneously delivered during the 34th week of embryonic life. The acetate was administered after labor had begun.

The specific activity of C^{14}-pregnane-3(α),20(α)-diol excreted on the operation day by way of the urine of patients Nos. 1, 2, and 3 was higher than that of C^{14}-pregnanediol excreted by patient No. 4, although the same dose of 200 μc 1-C^{14}-acetate had been given to all four women and the interval between injection and termination of pregnancy was almost the same. This finding indicates that the rate of conversion of C^{14}-acetate to C^{14}-pregnanediol was slower during spontaneous labor as compared with that during the 7th to 12th week of pregnancy.

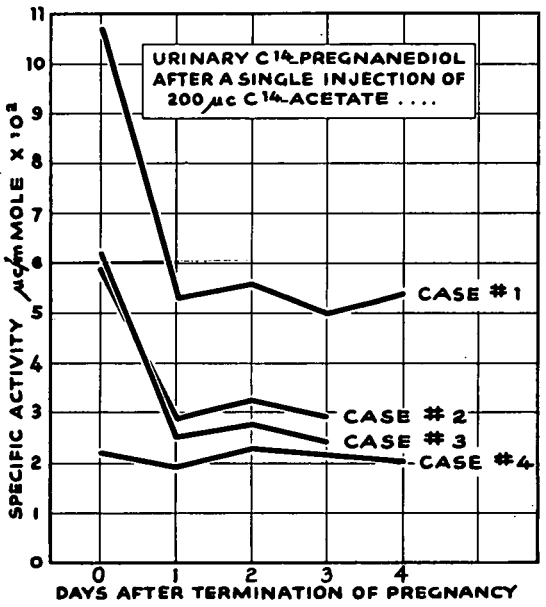

Figure 4. Urinary C^{14}-pregnane-3(α),20(α)-diol after a single injection of 200 μc 1-C^{14}-acetate 2-1/2 to 3-1/2 hours before termination of pregnancy.

The specific activity of C^{14}-pregnane-3(α),20(α)-diol excreted in the urine during the postoperative days remained constant in case No. 4, which indicated that no further synthesis of pregnanediol from acetate and other precursors took place after spontaneous termination of pregnancy. However, the specific activity of C^{14}-pregnanediol decreased significantly to lower values on the first postoperative day in case Nos. 1, 2, and 3 and remained constant during the following days. This decrease in the specific activity suggests that pregnanediol was still synthesized from nonradioactive precursors thus producing a dilution of the concentration of C^{14}-pregnanediol molecules in the urinary pregnanediol sample.

It is rather tempting to conclude from the results of these experiments that the rate of synthesis of progesterone, the principal precursor of pregnanediol, was relatively slow

during spontaneous labor although the absolute amount of urinary pregnanediol was still large at the time of delivery. This conclusion would be correct provided 1) that in all patients no hormonal precursors other than progesterone were converted to pregnanediol to an appreciable extent and 2) that progesterone formed from acetate was metabolized to pregnanediol at the same percentage rate in all instances.

Our findings suggest strongly that pregnanediol was synthesized from nonradioactive precursors (progesterone?) on the first postoperative day in cases Nos. 1, 2, and 3, but not in No. 4. The source of these precursors in case No. 2 may have been part of the corpus luteum that had been removed incompletely. In case No. 3, an hysterotomy and an extirpation of the ovary containing the corpus luteum was performed so that the precursors might possibly have been synthesized in syncitial tissue commonly present in the myometrium after the separation of the placenta. In case No. 1, however, the uterus and the ovary containing the corpus luteum were extirpated. Therefore, another source of precursors of pregnanediol synthesized on the first postoperative day must be taken into account, the most likely being the maternal adrenal cortex.

SUMMARY

Cholesterol is synthesized by the corpus luteum and the fetal part of the placenta early in human pregnancy. There is no evidence of cholesterol synthesis from acetate in the maternal part of the placenta, the decidua. The fetal liver and the fetal adrenals utilize acetate for the formation of cholesterol. Fetal skin and brain do not produce amounts detectable with this method. It appears that a relatively small percentage of fetal cholesterol is derived from maternal cholesterol in early pregnancy, indicating that maternal cholesterol passes the placenta at this stage of pregnancy.

Acetate, cholesterol, and progesterone labeled with C^{14} or tritium serve as precursors of isotopically labeled pregnane-3(α),20(α)-diol excreted into the urine of pregnant patients. The rate of synthesis of pregnanediol appeared to be rather slow during labor as compared with the rate of pregnanediol formation in normal pregnancy during the 7th to 12th week of gestation. This finding may permit the tentative conclusion that progesterone, the principal precursor of pregnanediol, is synthesized at a slower rate during labor than in early pregnancy.

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HEMATOLOGIC RECOVERY FROM RADIATION INJURY*

By

L. O. Jacobson

INTRODUCTION

Evidence from research on radiation injury has accumulated which suggests that a factor(s), present in certain biologic material, appears to have a specific therapeutic value for the treatment of radiation injury. Demonstration of the post-radiation value of this factor(s) is as yet confined to experimental animals and has been shown to apply in mice, guinea pigs, rabbits, rats, and dogs. Thus far, the only effective source of the factor has been found to be hematopoietic tissue or hematopoietic-containing tissue. It is not generally accepted that the effectiveness of shielding or the injection of hematopoietic-containing tissue lies in the production or release of a noncellular substance or group of substances by the shielded or injected tissue. Some investigators maintain that the data thus far reported do not eliminate the possibility that the shielded or injected tissue provides cell precursors that are capable of colonizing the depleted tissues, at least temporarily, holding the irradiated animal in a condition that allows normal regeneration and functional reconstitution to occur. Survival of an irradiated rodent, which has been given a suspension of hematopoietic cells, is correlated roughly with the rapidity of hematopoietic regeneration in the animal but it is not known with any degree of certainty whether systems other than the hematopoietic benefit directly from the shielded or injected tissue. It has been amply demonstrated that above a given dose of total-body X radiation (mice, circa 1500 r), shielding of the spleen or injection of hematopoietic tissue does not prevent death of the animal. In point of fact, Williams and DeLong[1] have shown that deaths occurring after exposures above 1500 r are due to damage to the intestinal tract.

HISTORICAL

In 1949, Jacobson and his co-workers[2] demonstrated that lead shielding of the surgically exteriorized spleen of mice during exposure to a lethal dose of total-body X radiation markedly enhanced survival. The same authors found that the blood-forming tissue under these circumstances, though destroyed by the irradiation, recovered in a phenomenally short time. It was later shown that transplantation of homologous spleens or mouse embryonic tissue or the intraperitoneal or intravenous administration of cell suspensions from these sources given in the postirradiation period likewise had a salutary effect upon survival of the animal and recovery of blood-forming tissue.[3] Lorenz and his associates[4]

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described a similar result with homologous bone marrow suspensions and later reported a comparable effect with heterologous tissue (rat marrow to mouse and guinea pig marrow to mice).[5] The work of these and other investigators has been reviewed comprehensively.[6-10]

THE HUMORAL THEORY

On the basis of a number of admittedly inconclusive experiments, Jacobson postulated the so-called humoral theory. The humoral theory as it has been presented implies that the shielded or injected viable hematopoietic tissue produces a substance or group of substances that are required or utilized by the irradiated cells of the recipient for resumption of functions inhibited by the irradiation. Support of this theory has been based on indirect evidence that has been reviewed critically by Loutit[7] and others.[8,9] The evidence that is the most convincing may be summarized as follows:

1) Homoplastic and heteroplastic regeneration of hematopoietic tissue occurs in spleen-shielded mice or in mice to which transplants or cell suspensions have been given.[3] This type of regeneration in animals recovering from an LD50* of X radiation has been described repeatedly in the literature by a number of authors including Bloom[11] and Block.[12] In other words, it is a normal mechanism of recovery. In animals that have had spleen-shielding or that have been injected with cell suspensions of hematopoietic origin, recovery occurs in a manner that is almost identical with that observed in normal regeneration. It occurs, however, at an earlier period and is more complete.

This fact is worthy of emphasis although the mechanism is not clear. A more or less reproducible pattern of hematopoietic destruction, atrophy, and regeneration occurs in the animals that survive an X-ray LD50. In the mouse, active, sustained, orderly regeneration begins at about 6 days following total-body exposure in the median lethal range. Regeneration of the bone marrow is complete in 12 to 14 days, whereas recovery of lymphopoiesis in the spleen or lymph nodes may be delayed for several weeks longer. If mice are exposed to an LD50 and are spleen-shielded or injected with an adequate amount of a cell suspension of hematopoietic tissue, orderly regeneration begins at 3 days or earlier, and hematopoiesis is normal or hyperplastic in 8 to 9 days. Lymphopoiesis is normal within essentially the same period of time. This is interpreted by the author to be evidence that the shielded tissue or injected cells provide a substance or group of substances that make it possible for the reticular structure of the hematopoietic tissue as well as the free residual cells to begin transformation or proliferation and regrowth. It is not denied that shielded tissue or injected cell suspensions might likewise lead to colonization and multiplication, but this is considered at best to be only a part of the regenerative process.

The minimal number of living cells required in an injection to bring about significant recovery after an LD99 (750 r) of total-body X radiation has been shown to be about

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* The X-ray exposure after which half of the animals die within a 28-day period of observation.

50,000.[13] This is a small number when one recalls that the circulating peripheral blood contains normally between 5 and 10 x 10^6 white cells per cu mm. The source of the cells is important. Normally circulating cells of the order of 5 to 10 x 10^6 have no appreciable effect on the survival of irradiated mice, although this amount from bone marrow, young mouse spleen, or embryonic mouse liver is effective.[13] The relationship between number of cells injected or shielded is not a linear one. After an LD99 of 750 r, survival following the injection of 50,000 cells from the embryonic mouse liver or baby spleen is about 20 per cent. Both survival and the rapidity of hematopoietic regeneration are increased with increasing increments of cells from these sources up to about 10 x 10^6, which gives 75 per cent survival. Above this quantity no appreciable difference in survival or hematopoietic regeneration is noted. The type of cells most important in bringing about recovery under these circumstances has not been determined, if indeed one type is responsible. It is a fair assumption, in view of the failure of normal peripheral mouse blood at 5 or 10 x 10^6 cells per cu mm to favorably influence survival or hematopoietic regeneration, that the more primitive tissue of the hematopoietic system is involved. The homologous tissue thus far studied, e.g., spleen cells, embryonic liver tissue, bone marrow, and bone spicules,[14] all may be considered to contain hematopoietic tissue or potential hematopoietic tissue. A number of publications describes transformation of bone cells to osteoblasts and of osteoblasts to hematopoietic cells.[15]

2) Heterologous tissue transplants or cell suspensions have been reported by Congdon, Lorenz, and co-workers[14,16] and Jacobson[17] to have a significant effect on the survival and hematopoietic regeneration of irradiated recipients. Jacobson first described evidence that suggested the effectiveness of mouse spleen transplants on recovery of the blood-forming tissue of irradiated rabbits.[3] Congdon and Lorenz[14] reported that rat bone marrow and rat bone had a significant effect on survival of irradiated mice and that guinea pig marrow likewise afforded a significant effect on irradiated mice. Jacobson and co-workers corroborated the effectiveness of rat marrow on the survival of irradiated mice. Loutit has been unable to confirm these positive results with heterospecific material.[7] Nevertheless the available data are so convincing that there seems little doubt but that others will eventually confirm these observations.

The interpretation of the positive results observed in mice injected with heterologous tissue is of interest. In general, the findings strongly support the humoral theory. It is difficult to imagine that these injected cells from a foreign species can so quickly take over the functions of normal hematopoietic tissue that death of the irradiated recipient is averted. It is perhaps simpler to imagine the foreign cells providing a factor or factors which allow rapid reconstitution of tissue that is critical for survival. Loutit has called attention to the late deaths (beyond the conventional 28-day period of observation) occurring in irradiated mice that had been given rat bone marrow. Late deaths (up to 100 days) have been observed by Lorenz and Congdon.[16] Loutit has suggested that heterologous material, when injected, may "take" as a graft and that the late death of the animal might be due to the development of isoantibodies in the host that eventually kill the graft and the animal.

On this and other evidence he maintains that proof of the humoral theory is still lacking.

3) Cell-free preparations of hematopoietic tissue have been reported by Cole et al.[6] to increase survival of mice given lethal doses of radiation. These investigators attempted to ascertain the nature of the factor prepared from mouse spleen by the use of a Potter-Elvehjem type of all-glass homogenizer. Their preparation served as a reliable reproducible source of material which invariably gave 100 per cent survival if it was injected soon after a dose of X radiation that ordinarily produces death in all of the mice exposed to it. Using spleen mash or cell suspensions, Jacobson and his associates observed not only increased survival but accelerated weight gain and enhanced hematopoietic regeneration. Cole further found that the effective material resided in the centrifugal residues and sought to determine whether the activity might be associated with the intracellular components. They fractionated the homogenate in sucrose by ultracentrifugation and found that only the fraction containing what they interpreted to be nuclei and nuclear fragments was effective upon assay. No protective effect was obtained with the mitochondria, microsomes, or soluble supernatant fraction. On the basis of enzyme studies, the Cole group postulated that the active principle is a desoxyribonucleoprotein because it is susceptible to the action of desoxyribonuclease and trypsin and is resistant to ribonuclease. They were unable to demonstrate the factor in liver or thymus but found it by their methods in spleen and bone marrow. On the other hand, Jacobson et al.[13] have demonstrated activity in fetal mouse liver, young mouse liver, and, to a lesser extent, in adult mouse liver. Loutit criticized the work of Cole on the grounds that no cell-free solutions of desoxyribonucleoprotein have been used successfully to enhance the survival of irradiated animals. Goldwasser[19] has been unable to obtain data that confirm Cole's interpretation in this latter respect.

In view of the fact that 50,000 spleen cells are all that are required to enhance survival of irradiated mice, less than 1 per cent survival of whole cells from 280 mg of spleen in a preparation made according to Cole's technic would still contain more than 6.4×10^6 living cells. Simmons and Jacobson,[20] in an attempt to test Cole's approach, subjected a leukemic spleen from DBA mice to Cole's technic and found that upon injection of this preparation, 100 per cent of the injected DBA recipients died of leukemia. This implies that treatment of leukemic cells by Cole's technic does not eliminate the required number of whole cells and is therefore not comparable to such treatment of normal spleen or that under the conditions of Simmons' experiments the leukemic agent was indeed present in the cell nuclei fraction, or naked nuclei, or possibly viruses capable of inducing the leukemia were released. The approach of Cole is attractive and offers interesting possibilities for speculation. Like evidence proposed by Jacobson to support the humor-theory, Cole's hypothesis cannot be accepted at this moment as proof that the humoral theory is correct. Were his concept correct, there still remains the task of isolating, identifying, and stabilizing the nucleoprotein responsible since there is, in all probability, a multitude of nucleoproteins in the mammalian nucleus.

RELATIONSHIP OF THE RECOVERY FACTOR TO EXPERIMENTAL LEUKEMIA

Furth,[21] Kaplan,[8] Law and Miller,[22] Lorenz and Congdon,[10] and Simmons and Jacobson[20] have reported a number of interesting observations that may bear some relationship to the so-called recovery factor. Furth[21] first demonstrated that the incidence of lymphoma in susceptible strains of mice was markedly reduced by thymectomy. Kaplan, who has done such outstanding work in this field, has reviewed his own contributions and those of others.[8] He has shown that the high incidence of lymphoma or lymphatic leukemia induced in C57BL mice by exposure to total-body X radiation can be reduced markedly by thymectomy. This observation and that of Furth are especially interesting in view of the fact that thymic cells have been reported by Cole[6] and Goldwasser[23] to be ineffective in reversing the radiation syndrome, whereas spleen or marrow cells will do so regularly. On the other hand, thymectomy prevents the incidence of certain spontaneous as well as radiation-induced lymphomas and leukemia, whereas bone marrow or spleen cells or spleen-shielding greatly reduces the incidence of tumors as demonstrated by Kaplan,[8] Law and Miller,[22] and Lorenz and Congdon.[10] Using DBA leukemia cells, Simmons and Jacobson[20] have shown that acute leukemia induced in CF No. 1 mice weakened by total-body exposure to X radiation and injected with DBA leukemic cells can be prevented entirely by spleen-shielding of the host. Cortisone[24,25] also reduces the incidence of spontaneous and induced lymphoma and leukemia in mice. It is difficult if not impossible at this time to explain adequately in simple terms these various seemingly related findings.

The incidence of leukemia has been found to be increased significantly among the atomic bomb victims in Hiroshima and Nagasaki.[36,37] This information is interesting when considered in the light of the results of laboratory experiments that show the high incidence of leukemia in irradiated mice[10,28] and guinea pigs. One cannot refrain from wondering whether the mechanism of the production of leukemia in the human being and the experimental animal is similar under these special circumstances and from entertaining the thought that the spontaneous and induced leukemias may well be amenable to the influence of the so-called recovery factor.

RELATIONSHIP OF THE RECOVERY FACTOR TO IMMUNE MECHANISMS

It has been demonstrated that the capacity to produce antibodies to an injected particulate antigen is retained in irradiated animals if they are spleen-shielded during the exposure.[29] This capacity has also been observed in irradiated animals injected with spleen cells.[30] The data suggest that the functional reconstitution of cells involved in antibody production is susceptible to the recovery factor. The recovery of the blood-forming tissue and the capacity to produce natural and immune antibodies undoubtedly act synergistically in bringing about survival of irradiated animals that have had the benefit of spleen-shielding or have been injected with hematopoietic cells in the postirradiation period. The restoration or continued production of natural antibodies may well explain the findings of Sim-

mons on leukemia reported above and may, of course, be related to the interesting demonstration by Pillemer and his associates[31] that circulating properdin (euglobulin) is reduced in irradiated animals. Pillemer has also reported that the administration of properdin to animals exposed to radiation in the LD90 range significantly increases survival.

CONCLUDING COMMENT

The evidence available concerning the recovery factor strongly but inconclusively suggests that a humoral agent rather than only the proliferation of the shielded or injected cells of hematopoietic origin is responsible for the recovery of animals subjected to total-body X radiation in the lethal range. It seems appropriate to quote from a 1954 review of the problem by Loutit[7] who states, "It is notable that four years have passed since Jacobson's first report with suggestions about a humoral factor. Many laboratories have been seeking the factor, often no doubt with the aid of the large commercial laboratories who have considerable experience in extraction of active principles from biological tissue. Nevertheless no success has been claimed. If the principle is present in chemical form it is proving a tough customer to isolate." This statement still applies with but one correction —five years have passed instead of four.

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THE EFFECT OF NONCELLULAR AND HETEROLOGOUS PREPARATIONS ON SURVIVAL AFTER LETHAL DOSES OF X RADIATION

By

E. Goldwasser and W. F. White*

Edlund[1] reported recently that the α-octadecyl ether of glycerol (batyl alcohol) had a marked effect over the peanut oil medium in increasing survival of mice irradiated with 750 r. In our tests of synthetic d,l-batyl alcohol, which was administered under the same conditions as those reported by Edlund, we found no increased survival compared with the controls. The discrepancy between the two laboratories at present cannot be explained satisfactorily although it may be attributable either to difference in response by different strains of mice or to difference in the batyl alcohol that was used.

In attempting to pursue further the question of humoral mediation of recovery from radiation injury, a large variety of materials of diverse origin has been tested with irradiated mice. All mice were CF No. 1 strain and were subjected to 750 r under the same radiation conditions indicated previously.[2] All preparations were injected intraperitoneally. Table 1 lists a number of potentially promising materials that were tested because of the well known effects of preparations of fresh homologous spleen, marrow, and embryonic tissue. None of these preparations, under the conditions of the test, demonstrated any capacity to reverse radiation lethality, although in the case of some, early deaths due to toxicity may have obscured such capacity.

Table 1

PREPARATIONS TESTED AND FOUND TO BE WITHOUT EFFECT ON REVERSING RADIATION LETHALITY

Fresh mouse thymus cells
Mouse embryo acetone powder
Fresh bovine spleen cells
Fresh bovine embryonic liver cells
Fresh bovine red marrow
Fresh bovine yellow marrow
90% ethanol extract of beef spleen
Aqueous extract of frozen beef embryo
Lyophilized bovine amniotic fluid

* Armour Laboratories, Chicago, Illinois.

In the absence of any favorable results from these sources of large amounts of materials, we decided to investigate one that seemed to have possibilities. The choice of intestinal and gastric mucosa was based in part upon the results of intestinal shielding experiments[2] and in part upon the well known early effect of X radiation on rat mucosa.

Extracts of hog intestinal mucosa were prepared by squeezing the washed intestine between rollers to extrude the mucosal slime. The mucosa suspensions were then brought to 50 per cent ethanol at -5°C to precipitate most of the protein, and the supernatant fluid was freed of ethanol. The solution of 50 per cent ethanol soluble components was passed through a column of cation exchange resin, and the resin was then eluted with dilute ammonia. This type of eluate was dried by lyophilization and injected into irradiated mice with the results shown in Table 2. Eighty mg of the eluate fraction, which was injected, is equivalent to approximately 10 g (wet weight) of the original mucosal slime. The overall results of the 4 tests are: treated, 37 per cent survivors and controls, 13 per cent survivors.

Table 2

EFFECT OF ELUATE FRACTION ON SURVIVAL OF IRRADIATED MICE

Test no.	Amount injected (mg)	Injections* (no.)	Survival after 30 days
1	80	2	4/10
	controls†		0/10
2	80	3	2/10
	controls		0/10
3	80	3	4/10
	controls		2/10
4	80	2	5/10
	controls		3/10

* Injections were given on 0 and 2, or 0, 2, and 5 days.
† Controls were injected with equal volumes of saline at the same times as treated groups.

Several experiments, the results of which are summarized in Table 3, are indicative that the materials that are responsible for causing the survival of the irradiated mice are probably molecules of relatively small size. All of the preparations were ultrafiltrates of extracts of hog intestinal mucosa.

While it is obvious that the preparations do not have a very marked effect, it appears

Table 3

EFFECT OF DIFFERENT ULTRAFILTRATES ON SURVIVAL OF IRRADIATED MICE

Ultrafiltrate (no.)	Amount injected (mg)	Injections (no.)	Survival after 30 days
1	80	2	3/10
	controls		0/8
1	80	2	1/10
	controls		0/10
2	80	2	3/10
3	80	2	5/10
	controls		1/10
4	80	2	1/8
4	40	2	2/8
	controls		0/8
5	80	2	2/10
6	80	2	0/10
	controls		0/12

likely that ultrafiltrable materials may contain compounds that will reverse the lethal effect of X radiation. A great deal of work is still necessary to separate the active compounds from toxic substances in the extract, if the two effects are not caused by the same materials, and to determine optimum conditions for administration of the test fractions.

While these data cannot yet be considered as conclusive proof, they lend a great deal of credence to the hypothesis of humoral mediation of recovery from radiation injury first proposed by Jacobson.

SUMMARY

Negative results of therapy of X-irradiated mice with a variety of preparations are presented.

Noncellular preparations of hog intestinal mucosa have been shown to have some effect in the reversal of lethality due to X irradiation. There is some evidence that the substances responsible for this effect are of relatively small molecular weight.

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MODIFICATION OF RADIATION INJURY IN THE RABBIT*

By

L. O. Jacobson, E. K. Marks, and E. O. Gaston

INTRODUCTION

Heterologous tissue transplants or cell suspensions of hematopoietic origin have been reported by Lorenz and his associates[1,2] and Jacobson et al.[3] to have a significant effect on the survival and hematopoietic regeneration of irradiated rodents. Jacobson first described evidence that suggested the effectiveness of mouse spleen transplants for hastening the recovery of the blood-forming tissue of irradiated rabbits.[4] Congdon and Lorenz[5] reported that rat bone marrow and, in point of fact, rat bone had a significant effect on the survival of irradiated mice and that guinea pig marrow likewise afforded a significant increase in the survival of irradiated mice. Jacobson and co-workers[3] and more recently Cole et al.[6] corroborated the work of Congdon and Lorenz on the effectiveness of rat marrow on the survival of irradiated mice, but Loutit[7] has been unable to confirm these positive results with heterospecific material.

In the earlier experiments, Jacobson did not observe the effect of mouse hematopoietic tissue transplants on the survival of irradiated rabbits.[4] Hence, the investigation described in this paper was undertaken to determine whether hematopoietic cells from mice enhance the survival of X-irradiated rabbits.

MATERIALS AND METHODS

Swift's snuffle-free, young adult rabbits of both sexes, weighing from 1.5 to 2.5 kg, were used as the recipient animals.

The cells used for postirradiation injection were obtained from tissues of the CF No. 1 mouse embryo or from baby mouse spleens or livers. The donor mice were killed by cervical fracture. The embryo tissue was obtained by making a median incision in the abdominal cavity of the pregnant mouse and removing and plunging the entire embryonic sac into cold Locke's solution. The liver was immediately excised from the embryo and suspended in Locke's solution. A homogeneous cell suspension was produced by drawing the combination into a 2-cc hypodermic syringe and expelling it repeatedly.

Before cell counts were made, the suspension was drawn into a syringe and expelled through a 26-gauge needle. No appreciable cell clumping was found in these preparations. The number of nucleated cells per cu mm was determined by making a dilution with 2 per cent acetic acid in a white blood counting pipette and counting the cells on a standard hema-

* Based on a paper that has been submitted for publication on the Proceedings of the Society for Experimental Biology and Medicine.

cytometer. The cell suspensions were diluted with Locke's solution so that each 10 cc contained the required number of cells for each rabbit. All injections were given within a few minutes after the final dilutions had been made and were administered intravenously into the marginal ear vein of the rabbit with a 24-gauge needle within a few hours (0 to 4) after X irradiation.

Cells from the spleen, liver, and other tissues of the mouse were obtained in similar fashion. The cells obtained from the baby mouse liver or spleen were not contaminated with other mouse tissue cells. A remote possibility, however, exists that the suspensions made from 15- to 16-day embryo liver contained some splenic or intestinal tissue. These latter experiments are being repeated with suspensions that are known to contain no tissue other than liver from the embryo.

The rabbits were exposed to 900 r total-body X radiation* before the administration of the cell suspensions. Irradiated rabbits that were injected with cell suspensions from nonhematopoietic tissue and nonirradiated rabbits that were untreated served as the controls. The experiment designed to study the hematologic recovery of irradiated rabbits following the injection of cell suspensions obtained from various tissues of the mouse is outlined in Table 1. Table 2 gives a detailed outline of the experiments that were devoted

Table 1

OUTLINE OF EXPERIMENT TO STUDY THE HEMATOLOGIC CHANGES IN IRRADIATED RABBITS TREATED WITH CELL SUSPENSIONS OF MOUSE TISSUE

Mouse tissue	Total no. cells ($\times 10^6$)	Rabbits (no.)	X-ray exposure (r)	30-Day survivors (no.)
None	None	3	None	3
1-day liver	250	4	900	2
1- to 4-day spleen	209	2	900	1
16-day embryo liver	240 to 255	6	900	5
None	None	4	900	1

to a study of survival. As indicated in Table 1, hematologic studies were made on a small number of animals. Only one control irradiated animal survived beyong the 14th day. The leucocyte, hematocrit, and reticulocyte values were determined for irradiated rabbits that had been injected with mouse embryo liver or with baby liver or spleen; for irradiated, untreated rabbits; and for rabbits that were normal, nonirradiated, untreated controls (Table 1). Standard hematologic techniques were employed.

X-rays were administered as a horizontal beam to 2 animals at a time. Measurements

* The 30-day X-ray LD50 for these animals in this laboratory is accepted to be 800 r.

Table 2

EFFECT OF CELL SUSPENSIONS OF MOUSE TISSUE ON THE SURVIVAL OF RABBITS EXPOSED TO 900 r TOTAL-BODY X RADIATION

Mouse tissue	Total no. cells (x 10^6)	Rabbits (no.)	Survival			
			30-day		120- to 150-day	
			No.	%	No.	%
Embryo liver	200 to 250	19	11	57.8	10	52.6
1- to 2-day liver	200 to 250	10	6	60	6	60
1- to 4-day spleen	25 to 30	8	6	75	6	75
Heart, kidney, lung, placenta	32	9	1	11	1	11
None	X-ray only	50	5	10	4	10

were made in air with a Victoreen condenser r-meter equipped with a 100-r chamber at the position occupied by the center of the 2 cages. At the midpoint of the total exposure, the animals were reversed from top to bottom and from side to side. The X rays were generated by a 250-kvp Maxitron machine operating at 30 ma. A 1.0-mm Al and 0.5-mm Cu filter were used. The half-value layer in copper of the filtered beam was 1.43 mm. The exposure rate averaged 44 r per minute at a distance of 70 cm.

RESULTS

Survival: As indicated in Table 2, the cells from the mouse embryo or from baby mouse spleen or liver had an appreciable effect on the survival of rabbits that had been exposed previously to 900 r total-body X radiation. Of 8 irradiated rabbits that received from 25 to 30 x 10^6 cells from the spleen of 1- to 4-day-old mice, 6 have survived a period of 150 days, whereas only 1 of 9 has survived 120 days that was injected with cells from nonhematopoietic tissue. All nonsurvivors died within the first 26 days of the observation period, and no deaths have occurred since that time.

Rabbits that received from 200 to 250 x 10^6 cells from the liver of the mouse embryo or from the liver of the 1- to 2-day-old mouse had similar survival. Of 29 thus treated, 17 survived beyond 26 days, and only 1 death has occurred (on day 90) during the remaining 120-day period of observation. Of 50 untreated irradiated controls, only 4 survived (see Table 2).

Hematologic studies: Figures 1 and 2 indicate that the injection of 200 to 240 x 10^6 cells from the mouse embryo or baby liver or spleen had no appreciable effect on the recovery of the leucocyte and hematocrit values of the peripheral blood of the irradiated rabbit. The reticulocyte value of the irradiated cell-injected group at 10 days as compared with the controls (Figure 3) may be significant but should not be considered as evidence of an earlier recovery in these animals unless confirmed by histopathologic findings

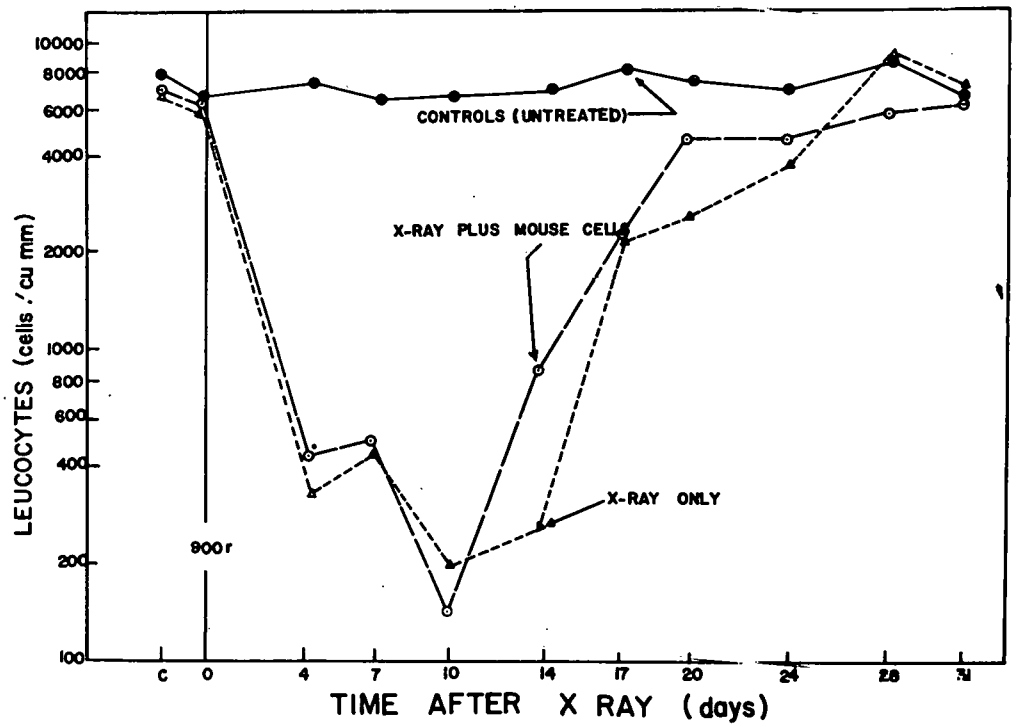

Figure 1. The effect of the injection of mouse cell suspensions on the leucocyte response of the irradiated rabbit.

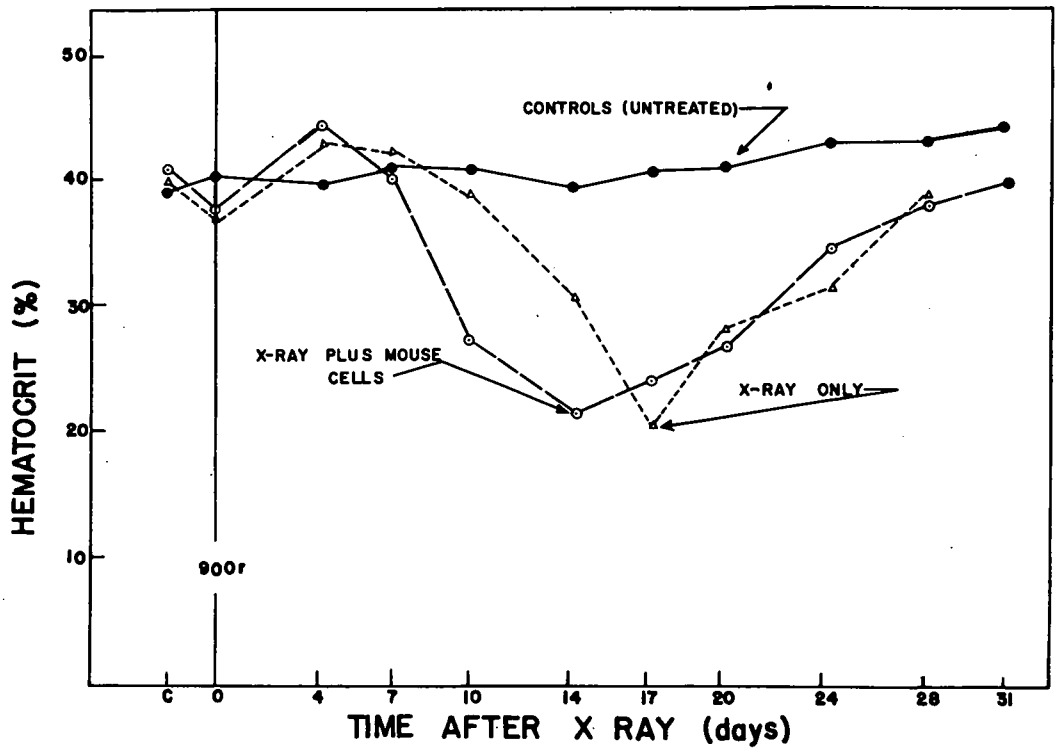

Figure 2. The effect of the injection of mouse cell suspensions on the hematocrit values of the irradiated rabbit.

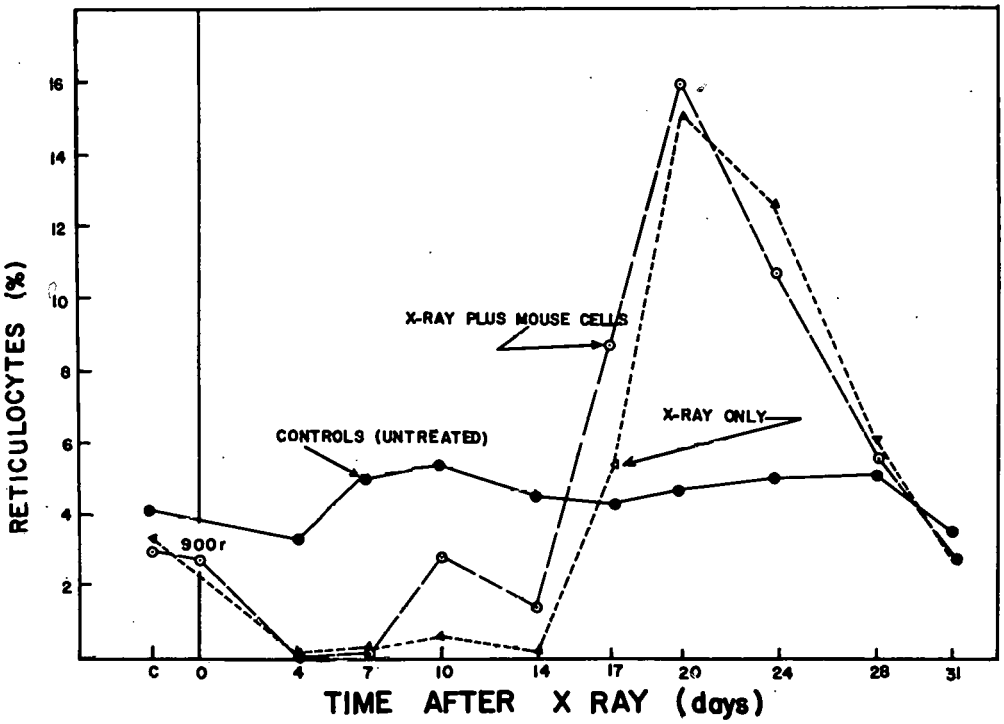

Figure 3. The effect of the injection of mouse cell suspensions on the reticulocyte response of the irradiated rabbit.

or a further hematologic study in which values are obtained at additional periods.

DISCUSSION

The data from this investigation indicate that cells from the embryonic mouse liver or from baby mouse liver or spleen are capable of enhancing the survival of rabbits exposed to 900 r total-body X radiation. These findings supplement those of Lorenz,[1,2] Jacobson and his associates,[3,4] and Cole et al.[6] which show the efficacy of heterologous tissue (rat to mouse, guinea pig to mouse) in prolonging the survival of irradiated mice. Loutit,[7] on the contrary, has not been able to increase the survival of irradiated mice by treating them with heterospecific material. In point of fact, his argument against the benefits of homologous suspensions is based upon the number of late deaths that he has observed in irradiated mice that had been treated with cell suspensions made from animals of the same species but of a different strain. It is thus important to emphasize that, in our study, late deaths did not occur in the surviving group of irradiated rabbits that had been treated with mouse tissue.

Previous studies in this laboratory gave evidence that spleen-shielding had only a slight effect on the survival of X-irradiated rabbits,[8] whereas there was an appreciable effect on the survival of mice.[4] That hematopoietic tissue from the mouse is a more potent source of the recovery factor(s) involved in recovery from radiation injury than hema-

topoietic tissue from the rabbit is a possibility that will require further exploration. Preliminary data in our laboratory suggest that mouse cells from embryonic liver as used in this study are more effective in enhancing the survival of irradiated rabbits than are rabbit cells obtained from rabbit embryonic liver, spleen, or bone.

The fact that hematologic recovery in the rabbit exposed to 900 r and then treated with cells from the mouse was not appreciably different from that of the control rabbits that survived this exposure would seem to indicate that increased survival is not necessarily dependent upon enhanced hematopoietic regeneration. Until histopathologic studies are completed it is perhaps not justified to make such a statement. Evidence of recovery may be present in the bone marrow and elsewhere under the conditions of this experiment which are not manifest in the peripheral blood.

In some respects, the findings of these experiments are similar to those from the spleen-shielding splenectomy experiments that have been reported previously.[4] Survival of spleen-shielded mice (1025 r) that were splenectomized 1 hour after the irradiation spleen-shielding procedure was the same (75 per cent) as that of mice treated similarly with the exception of splenectomy. Hematopoietic regeneration began by 3 days and was complete in the nonsplenectomized spleen-shielded mice in 7 to 9 days, while in mice splenectomized 5 minutes after the spleen-shielding irradiation procedure, regeneration began at 9 days and was only complete at 15 days or longer. These observations suggested that increased survival was not necessarily dependent on early regeneration of the blood-forming tissue. The finding reported in this paper also suggests that early hematologic recovery is not necessary for increased survival.

CONCLUSIONS

Postirradiation injection of mouse embryo cells or baby mouse liver or spleen cells enhances significantly the survival of rabbits exposed previously to 900 r total-body X radiation without producing an appreciable effect upon hematologic recovery as observed in the peripheral blood.

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DIURESIS IN RATS FOLLOWING TOTAL-BODY X IRRADIATION

By

E. I. Pentz and R. J. Hasterlik

The observation that initiated the following experiments was made while extending the studies of Katz and Hasterlik[1] on aminoaciduria in the human being following total-body irradiation. The first observable response elicited from the rat by exposure to low doses of radiation was noted to be an immediate diuresis. Although polyuria in rats has been reported in the literature,* it has not been studied extensively nor have the biological factors involved been elucidated. Since it is a matter of some importance to discern which biological systems are the most radiosensitive, it was decided to explore this response somewhat further.

The physiological factors known to regulate the output of urine are: 1) blood flow through the kidney with the resultant increased or decreased rate of glomerular filtration and 2) the rate of reabsorption of water in the kidney tubules. The mechanisms controlling blood flow through the kidney are rather ill-defined. It appears that "the renal circulation possesses a greater degree of independence in respect to the general circulation than do most other vascular areas. There is little definite information concerning the basis of this autonomy, though the juxta-glomerular apparatus is suspected of playing a dominant role; the controlling mechanism is not impaired by denervation of the kidney."[2] Somewhat more is known about the regulation of water reabsorption in the tubules. General factors such as changes in the protein content of the blood and consequently in its osmotic pressure as well as changes in the rate of flow of urine along the tubule have an obvious influence. Again, nervous stimuli do not appear to have a direct effect on the reabsorption processes.

Known humoral factors influencing water excretion are limited to the antidiuretic hormone elaborated by the posterior pituitary gland.[3] This hormone acts directly on the cells of the renal tubules thereby regulating the reabsorption processes. The mechanisms that stimulate the secretion of the hormone appear to be multiple. Theobald and Verney[4] found that afferent nerve stimulation caused inhibition of water diuresis of the completely denervated kidney. They suggested that this effect was probably induced by pituitary secretion. Changes in the osmotic pressure of the blood caused by the presence or absence of crystalloids are also known to influence the liberation of the posterior pituitary hormone.[5] Since the hormones of the adrenal cortex are important regulators of the blood levels of sodium and potassium, it is not surprising to find the postulate in the literature[6] that the secretion of antidiuretic hormone is dependent upon the integrity of these glands.

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* Prosser, C. L. Radiology, 49:299 (1947).
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Good evidence that there may be as yet unknown antidiuretic substances produced by tissues other than the posterior pituitary has been reported by Mirsky and his collaborators.[7] They found significant increases in the blood level of antidiuretic substance(s) in adrenalectomized rats. These high blood levels fell gradually over a period of time until 30 days after the operation when they were comparable with that of control animals. Rats that had been hypophysectomized previously and that were shown to have blood levels of antidiuretic substance(s) below that of controls upon subsequent adrenalectomy showed very significant increases in blood levels of antidiuretic material(s). Since no pituitary was present, such evidence can only indicate an extra-hypophyseal site for antidiuretic substance(s). The hypothalamus has been suggested, and evidence obtained by other workers[8] supports this.

In addition to the above known and suggested mechanisms for the regulation of water excretion, any of which might be sensitive to low doses of radiation, there was the possibility that the metabolism of the kidney tubule cells might be altered directly.

The following experiments have been carried out with a view to determining which, if any, of the above factors are involved in the observed diuresis in rats subsequent to total-body irradiation.

METHODS AND MATERIALS

All animals used were Sprague-Dawley rats weighing between 125 and 200 g. Except where indicated, they were maintained on Rockland mouse pellets with ad libitum access to drinking water. They were housed at a temperature of $79^{\circ}F \pm 2^{\circ}$. Most of the operated animals were obtained from Hormone Assay Laboratories, Chicago. Where this does not obtain it will be indicated.

Since the urine was being collected for amino acid analyses as well as for measurements of volume, it was necessary to deprive the animals of food during the collection period. This was accomplished in the following manner: For approximately 7 hours during the day the animals were placed in rat metabolism cages* (2 per cage) and during this time they had access to drinking water but not to food. A conditioning period of at least 2 but usually 5 days was always allowed prior to irradiation. Under these conditions, the animals rapidly learn to eat enough during their feeing period to maintain themselves. Even after the first 24 hours, growing animals show no weight loss and thereafter continue to gain.

Urine was collected under toluene in 50-ml graduates placed under the collection tubes of the metabolism cages. Each cage was especially equipped with a removable fine wire (1/8") mesh tray that rested in the collection funnel, about 1 inch below the coarse wire mesh cage bottom. This served to catch the feces thus allowing separation of the urine with satisfactory efficiency.

Irradiation was carried out with a Maxitron 250-kvp X-ray machine operating at 30 ma. The unanesthetized animals were placed in individual sections of a circular aluminum carrier that was perforated uniformly for maximum exposure. The carrier was rotated on a

* Manufactured by the Acme Sheet Metal Works, Chicago.

horizontal plane during irradiation. The target to skin distance was 92 cm. The exposure rate was approximately 30 r per minute.

EXPERIMENTAL RESULTS

A test period of 4 days was selected as a suitable period to indicate the extent of diuresis following irradiation. Control animals were treated in a manner identical with the treated animals except that they were not X-irradiated.

Exp. 1. Sixteen female rats having an average weight of 174 g at the beginning of the experiment were divided into 2 groups of 8 each. They were trained to metabolism cages for 7 days prior to irradiation and their average output of urine per rat per 7-hour-period was found to be 2.7 and 2.9 ml, respectively. Figures for the urine excretion of each group subsequent to irradiation are listed in Table 1.

Table 1

EXPERIMENT 1. DIURESIS IN RESPONSE TO 100 r X RADIATION OUTPUT PER CAGE (2 RATS) PER 7 HOURS

Date	4/6	4/7	4/8	4/9	
0 r 9 a.m.	5 ml	8	7	5	
Group I	4	6	5	3	
Controls	4	5	3	4	
	4	6	4	4	
Ave./rat/7 hrs.	2.1 ml	3.1	2.4	2.0	Ave. 2.4 ml
Ave. wt.	189 g	190	193	193	
100 r 9 a.m.	17 ml	8	8	5	
Group II	14	11	7	9	
Treated	28	14	9	9	
	12	7	5	5	
Ave./rat/7 hrs.	9.0 ml	5.0	3.6	3.5	Ave. 5.3 ml
Ave. wt.	196 g	185	191	193	

Twenty days after the first irradiation the animals of group II were given a second exposure of 100 r, and the diuretic response to this treatment is recorded in Table 2. In this case, the irradiation was done in the late afternoon before the day of the first 7-hour collection period, whereas the first irradiation was carried out on the morning of the first day. The average weights of the animals in the experimental and control groups on the morning of the day of the second irradiation were 232 and 235 g, respectively.

Table 2

EXPERIMENT 1. DIURESIS IN RESPONSE TO A 2nd 100 r EXPOSURE OUTPUT PER CAGE (2 RATS) PER 7 HOURS

Date	4/27	4/28	4/29	5/2	
0 r	7 ml	6	8	8	
Group I	7.5	9	7	8	
Controls	7	8	6	5	
	10	10	10	9.5	
Ave./rat/7 hrs.	4.0	3.8	3.9	3.8	Ave. 3.8 ml
Ave. wt.	234 g	236	237	241	
100 r	6 ml	7	7	5	
Group II	13	10	7	15	
Treated	21	6	10	12	
	16	9	8.5	10	
Ave./rat/7 hrs.	7.0	4.0	4.1	5.2	Ave. 5.1 ml
Ave. wt.	225 g	228	231	240	

Similar volume measurements of urine were made for the next 16 days, and a slight diuresis was found to persist during this period. The averages of the 16 collection periods for the control and irradiated groups were 3.6 and 5.0 ml, respectively.

A second experiment was carried out in the same manner using 12 male rats with an average weight of 168 g. However, the radiation exposure was increased to 250 r. These animals showed a similar diuretic response; the average output per rat on the day of irradiation was 10.3 ml as compared with 3.0 ml for the controls. The average for the 4-day test period was 5.2 ml as compared with 3.3 ml for the controls. Repetition of the irradiation at the same exposure level 14 days later elicited a urine output of 10.4 ml from the exposed animals as against 4.3 ml from the controls for the first 7-hour period. The average for the 4-day test period was 5.5 ml for the irradiated animals and 4.4 ml for the controls. However, in this group of animals, diuresis failed to persist after the 4-day test period. Weight losses following both exposures similar to those observed in experiment 1 were also noted in the second experiment.

Because of the known importance of the posterior pituitary and, less directly, the anterior pituitary in relation to kidney function, a series of experiments has been done in which one or the other or both of these glands have been removed surgically. The results and the relevant data are recorded in Table 3. It is evident that in the case of total hypophysectomy, no diuresis was elicited in response to the first exposure to radiation. Until sufficient data have been collected to allow statistical studies, an average difference in urine volume of 1.1 ml or greater is taken as significant. When this is coupled with an ob-

Table 3

SUMMARY: DIURETIC RESPONSE OF RATS TO TOTAL-BODY IRRADIATION

Type of animal	Group	Ave. beginning body wt. (g)	Ave. urine excretion prior to irradiation (ml)	X-ray Exposure (r)	Post-operative day	Ave. excretion for 4-day test period (ml)	Wt. loss 1st 24 hrs post X ray (g)	Ave. urine vol. prior to 2nd irrad. (ml)	Days after 1st X ray	X-ray Exposure (r)	Ave. excretion for 4-day test period (ml)	Wt. loss 1st 24 hrs post X ray (g)
Normal Females	Irradiated	174	2.9	100		5.3	11	4.2	20	100	5.1	10
	Controls		2.7	0		2.4	0	3.5		0	3.8	0
Normal Males	Irradiated	168	2.8	250		5.2	8	3.0	14	250	5.5	11
	Controls		2.3	0		3.3	0	3.6		0	4.4	0
Totally hypophysectomized females	Irradiated	155	4.6	100	9	3.8	7	4.3	20	100	3.2	7
	Controls		3.7	0		3.7	0	3.5		0	3.4	0
Posterior pituitary removed. Females	Irradiated	153	5.4	100	16	5.1	10	4.6*	22	100	3.7	3
	Controls		5.7	0		5.2	0	5.6*		0	4.9	0
Anterior pituitary removed. Females	Irradiated	128	2.1	100	8	3.0	7	4.1	26	100	3.5	4
	Controls		3.5	0		3.5	0	4.3		0	4.2	0
Adrenalectomized females	Irradiated		5.8†	100		5.5†	5					
	Controls	167	6.3†	0	18	5.3†	0		DISCONTINUED			
Sham Adrenalect.	Irradiated		2.7	100		3.9	4					
Adrenalectomized females	Irradiated		4.4	100		7.2	0					
	Controls	162	3.9	0	11	5.5	0					
Sham adrenalect.	Irradiated		2.4	100		2.7	5					

* Two days only.
† 1 day training period only prior to irradiation.

vious voluminous urine output during the first 7-hour period, there is no difficulty in recognizing a positive diuretic response. A significant decrease in urine volume is somewhat more difficult to decide since to date nothing that approaches complete anuria has been recorded. Therefore, it remains to be determined whether the average drop in urine volume recorded for the irradiated hypophysectomized animals after the second irradiation is significant.

When only the posterior pituitary is removed, no significant change in urine volume after either of two exposures was obtained. This was also true of animals that had only the anterior portion of the gland removed.

Two similar experiments have been conducted on adrenalectomized animals, and in each, an additional group of sham operated animals has been included. These experiments have yielded equivocal results (Table 3). In the first of these experiments, the sham operated* animals gave the expected diuretic response during the first 7-hour collection period. Since the period of adaptation to the metabolism cages had been limited to only 1 day in the 2 adrenalectomized groups, it was felt that the data for these groups were not particularly representative. Owing to an error in manipulation, this experiment was discontinued but is being repeated and the first results are recorded in Table 3. It is evident that a good diuretic response was obtained in the irradiated adrenalectomized animals but not in the sham-operated animals.

It is felt that the equivocal results obtained in sham-operated animals may be related to the number of days that elapse between the time of operation and the first exposure to radiation. Accordingly, the first exposure in some of the hypophysectomized animals may have been carried out too soon. Data from experimentation will have to decide this question.

It is intended also to determine whether a quantitative relationship exists between the dose of radiation the animals receive and the amount of urine that they produce. Body weight losses were apparent in the first 24 hours in every experiment except the last adrenalectomized group. An effort is being made to relate this to food and water balance.

SUMMARY

1) It has been observed that total-body exposure to 100 r produces an immediate diuretic response in normal rats.

2) Some experiments have been conducted on rats that have been totally and partially hypophysectomized, and in all of these groups of animals up to the present, the diuretic response to total-body irradiation has been obliterated.

3 A few adrenalectomized animals and sham-operated controls have been irradiated and in these the results have been equivocal.

* All animals in this experiment were operated by one of us (E.I.P.).

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FURTHER STUDIES ON THE UPTAKE OF RADIOACTIVE SULFUR BY CARTILAGE*

By

C. W. Denko,† D. M. Bergenstal‡ and A. T. Kenyon‡

We have reported that hypophysectomy reduces markedly the fixation of S^{35} by the costal cartilage in the rat.(1) Tibial caps and xiphoid cartilage are much less affected. The activity remaining after hypophysectomy may be attributed to vital processes that continue in cartilage in the absence of hormonal influences. The cartilage must be viable for the incorporation of radiosulfur since this phenomenon does not occur in boiled cartilage nor when $Na_2S^{35}O_4$ is mixed with pure chondroitin sulfate in vitro.(2)

It may be presumed that chondroitin sulfate is a chief repository of accumulated S^{35} although it may not be the sole one. Chondroitin sulfate-containing S^{35} has been isolated from the cartilage of animals that had been given inorganic $S^{35}O_4^{=}$ intraperitoneally.(3,4)

Growth hormone, given daily for 8 days in doses of 100 μg, enhanced the uptake of S^{35} by costal cartilage in the hypophysectomized rat three- to four-fold and also increased the uptake by the tibial cap and xiphoid.(1) Hypernormal levels were often observed. Nevertheless uptake of S^{35} by the cartilage of normal animals was not accelerated. These results are in general accord with the work of others.(5) Since the growth-promoting properties of growth hormone in cartilage have been demonstrated, the enhanced uptake of radioactive sulfur may bear an immediate relationship to this process and may represent the formation of new chondroitin sulfate required by the new cartilage either by enhancing the synthesis or by retarding the degradation of such material.

Steroids of the cortisone family have been found to interfere with various phases of the growth process. A generalized decrease of S^{35} fixation in tissues has been noted in radioautographs of rats that have received cortisone systemically.(6) Similar results were observed in in vitro experiments on rat cartilage.(7) In this report the effect of hydrocortisone on S^{35} uptake is reported.

EXPERIMENTAL

Experimental procedures were similar to those reported previously.(1) Several modifications were made in decreasing by one-half each of the following: the experimental period of treatment to four days, the dosage of radiosulfur to 5 μc, and the carrier barium

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† Work done during tenure of an Arthritis and Rheumatism Foundation Fellowship (1953-55).

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sulfate used in precipitating the radioactive sulfate. These steps did not decrease the sensitivity of the assay procedure. Counting was done to a probable error of 3 to 5 per cent. Correction was made for radiodecay.

Hydrocortisone was injected intraperitoneally as hydrocortisone acetate suspension in saline, 0.5 ml per dose. Essentially no impurity was present. Growth hormone, Armour Lot. No. R491132, was injected in doses of 50 μg in 0.5 ml of saline. S^{35} was also injected in 0.5 ml of saline. The hydrocortisone was given immediately after the growth hormone to the animals that received both hormones.

In Figure 1, each point represents the average of three samples of costal cartilage taken from one animal. Xiphoid cartilage and articular cartilage as the tibial cap gave similar results only less pronounced in magnitude.

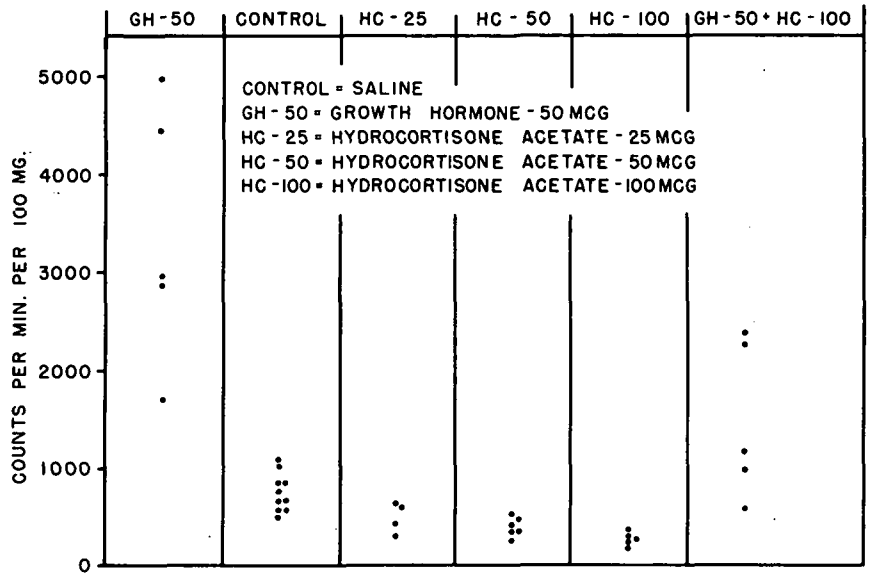

Figure 1. The effect of hydrocortisone, growth hormone, and hydrocortisone with growth hormone on the S^{35} fixation in costal cartilage of hypophysectomized rats.

RESULTS AND DISCUSSION

Hydrocortisone decreased the S^{35} fixation in the costal cartilage of the hypophysectomized rat while growth hormone caused a notable increase (Figure 1). Similar though less marked results were noted in studies on the xiphoid cartilage and articular cartilage as tibial cap. The stimulation of S^{35} uptake is somewhat more striking than is the inhibitory effect of hydrocortisone. Fifty μg of growth hormone produced a three- to four-fold increase in S^{35} binding, while 50 μg of hydrocortisone produced a decrease of about one-half of the control level in this process. An approximation of the proportions of each hormone necessary for counteracting the effects of the other hormone is suggested by the experiment in which the stimulation of 50 μg of growth hormone counteracted the inhibition

of 100 μg of hydrocortisone on the incorporation of S^{35} in cartilage (Figure 1). The number of molecules of each compound must, of course, be known to make comparisons on a molecular basis.

The antianabolic effect of hydrocortisone on weight gain is reflected by the weight loss of 3 g per rat noted during the experimental period of 5 days. Control animals gained 2 g, while growth hormone-treated rats gained 4 g. Rats receiving both hydrocortisone and growth hormone gained approximately the same amount as the growth hormone-treated animals. This process of weight gain or body growth is more complex and difficult to interpret.

The effect of hydrocortisone on the S^{35} binding in cartilage may be due to decreased synthesis of radiosulfate-rich compounds or an increased degradation of this material or to both processes. The effect of growth hormone remains similarly obscure. Growth hormone may increase the synthesis of S^{35}-containing compounds or it may retard their degradation, or both processes may be influenced. The fixation of S^{35} in cartilage may reflect an enzymatic transsulfatation of existing sulfated compounds or an increase in the mass of S^{35}-bearing structures or, again, it may be due to both processes.

Nonetheless, the inhibitory action of hydrocortisone on S^{35} uptake in cartilage remains clear as does the stimulatory action of growth hormone. In addition there is noted the action of hydrocortisone in counteracting the stimulation of growth hormone in S^{35} binding in cartilage while the growth hormone counteracts the inhibitory activity of hydrocortisone with respect to S^{35} incorporation in cartilage.

SUMMARY

1. Hydrocortisone administered to hypophysectomized rats retarded S^{35} fixation in the costal cartilage to one-third to one-half of the level observed in control animals. Similarly though less pronounced results occurred in xiphoid cartilage and articular cartilage studied as the tibial cap.

2. Hydrocortisone inhibited weight gain while growth hormone stimulated weight gain and counteracted the hydrocortisone effect.

3. Hydrocortisone given with growth hormone counteracted the stimulatory action of growth hormone on S^{35} binding in cartilage. Conversely growth hormone counteracted the inhibition of S^{35} incorporation seen with hydrocortisone.

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DEMONSTRATION OF STIMULATION OF ERYTHROPOIESIS BY PLASMA FROM ANEMIC RATS USING Fe^{59}*

By

L. F. Plzak, W. Fried, L. O. Jacobson, and W. F. Bethard

As early as 1906, Carnot and DeFlandre[1] wrote that a humoral factor in the blood plasma of rabbits mediated the production of red cells. Since then, a great deal of evidence has been presented to substantiate the theory of red cell production by means of humoral mediation.[2-6] Such a humoral factor in the plasma of anemic rats, which is capable of stimulating erythropoiesis in normal rats, is described in this paper.

Erythropoietic activity was determined by measuring the rate of appearance of intravenously administered radioiron in the peripheral blood. Fe^{59}, obtained as ferric chloride in 0.08 N HCl, with carrier iron added as $FeCl_3$ was diluted in saline and titrated to pH 6 with saturated sodium citrate. Total iron was determined by the method of Barkan and Walker.[7] Each animal was given 0.8 μc of Fe^{59} at 2 to 5 hours after the last injection of anemic plasma, normal plasma, or physiological saline. Blood samples (0.2 ml) were drawn from the tail vein or by cardiac puncture to determine the erythrocytic concentration of Fe^{59}. The total blood volume was taken to be 5 per cent of the total-body weight. Using this figure, the per cent of iron in the peripheral blood could be calculated.

Fe^{59} is removed from the plasma within 4 to 6 hours after its introduction and it does not exchange with the iron in the red cells[8] nor is it adsorbed on mature erythrocytes.[9] In point of fact, it is incorporated into the maturing erythrocytes soon after it has been injected into the animal.[10] Thus, the radioactivity of the samples (cpm) was a direct indication of the amount of Fe^{59} in the newly-formed erythrocytes, and hence, a direct indication of the rate of erythropoiesis.

Blood volume, determined by the $Na_2Cr^{51}O_4$ labeling method of Gray and Sterling,[11] at the time of sampling was found to be unaltered by previous injections of plasma.

It has been reported[3, 5] that injections of normal plasma do not stimulate erythropoietic activity. To test this and to determine whether normal saline could be substituted for plasma as a control in further experiments, the effects of injections of plasma and saline were compared under the conditions summarized in Table 1. Since no significant difference could be found, saline was injected into the control animals for the experiments described in the following paragraphs.

A series of experiments was performed to determine the amount of anemic plasma that must be injected into normal rats to stimulate erythropoiesis. In this series, the number of injections and the volume of each injection were varied. The results given in Table 2

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* Summary of paper that appears in the Journal of Laboratory and Clinical Medicine, 46:671 (1955).

Table 1

A COMPARISON OF THE EFFECT OF INJECTIONS OF SALINE AND NORMAL RAT PLASMA ON ERYTHROPOIESIS IN RATS

Experiment	1	2	3	4
Number of rats in each group	4	5	7	7
Number and volume of injections	3 x 3 ml	2 x 4.0 ml	4 x 4.6 ml	3 x 4 ml
γ of Fe injected	114	110	38	34
Interval of sample after Fe^{59} injection	22 hr.	19 hr.	18 hr.	18 hr.
Average % uptake of Fe^{59}				
Normal plasma recipients	12.5	13.3	21.2	25
Saline recipients	12.5	14.1	19.5	22.5

Table 2

THE EFFECTS OF NUMBER OF INJECTIONS OF ANEMIC PLASMA ON ERYTHROPOIESIS IN RATS

Experiment	Daily injections of equal volume (No.)	Anemic plasma (Total volume) (ml)	Rats (no.)	Average uptake to % uptake in saline recipients (ratio of %)
1	2	12	4	1.87
1	3	12	4	1.95
1	4	12	3	1.56
2	3	3		1.58
2	1	3	5	1.00

show that the maximum stimulatory effect is obtained with 3 injections of anemic plasma. The data in Figure 1 indicate that 2 ml of plasma administered daily for 3 days effect maximum stimulation with the greatest economy of plasma.

In order to determine the time after injection of Fe^{59} when the rate of uptake of radioiron by erythrocytes in the rate treated with anemic plasma was greatest, single blood samples were withdrawn from the anemic plasma-treated rats at various intervals after the introduction of the radioiron. The curves in Figure 2 show that the greatest difference in Fe^{59} uptake between the anemic-plasma recipients and the saline-treated controls occurs at about 20 hours.

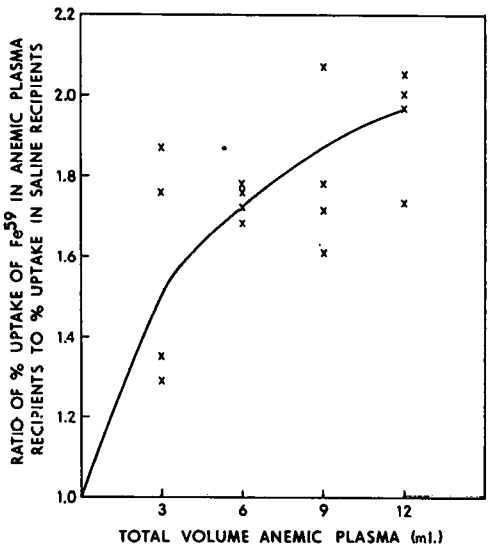

Figure 1. The effect of injections of various volumes of anemic plasma on the rate of uptake of Fe^{59}.

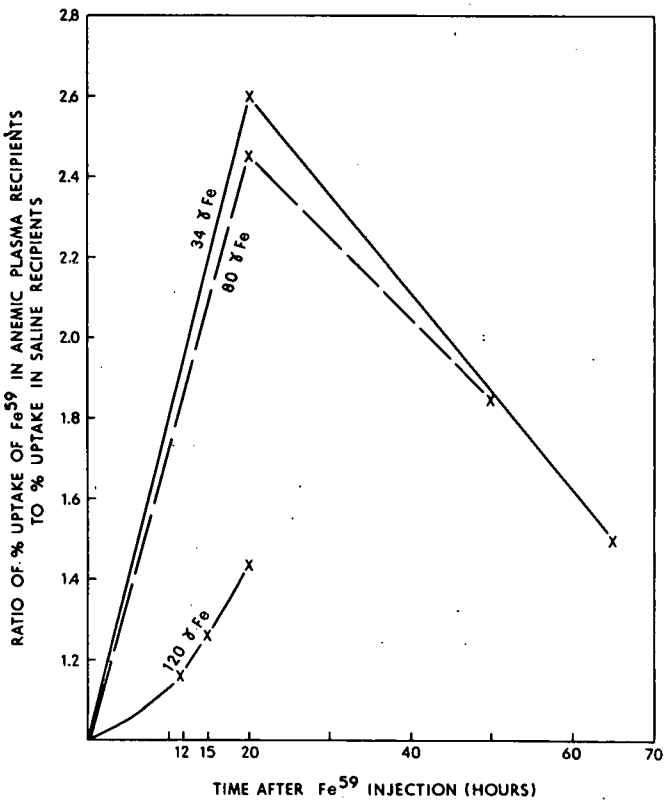

Figure 2. The effect of variations in the amount of carrier iron added to the Fe^{59} injection solution on the rate of uptake of Fe^{59}.

Variations in the amount of carrier iron added to the radioiron were found to influence the per cent uptake of the radioactive form. For this reason some experiments were performed in which carrier iron was not used. Under these circumstances, the sampling period at which time the ratio of Fe^{59} uptake by anemic plasma recipients to that of the saline recipients was found to be 12 hours after the injection of carrier-free Fe^{59}.

No erythropoietic stimulation could be detected if the injection of Fe^{59} was delayed for 24 hours after the last injection of anemic plasma. This is indicated by the slopes of the curves shown in Figure 3 after the 18-hour sample. Therefore, Fe^{59} should be injected within a few hours after the last injection of anemic plasma.

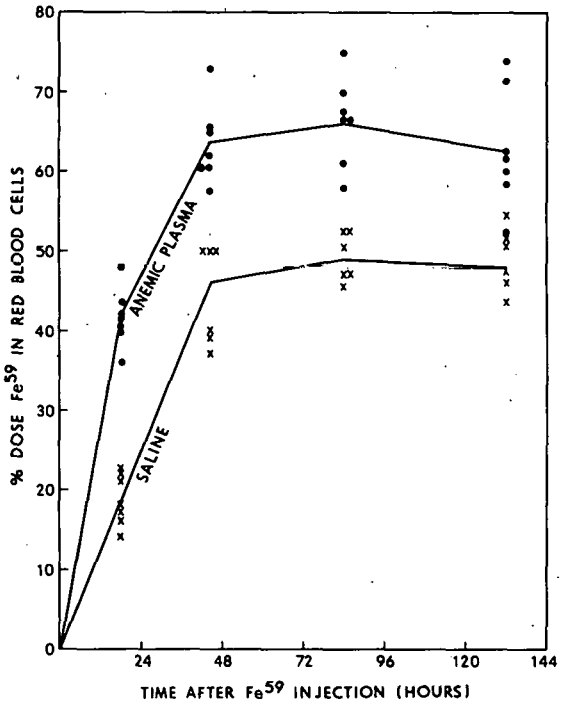

Figure 3. The effect of the length of time between the last plasma injection and Fe^{59} injection on the rate of uptake of Fe^{59}.

ACKNOWLEDGEMENT

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THE ACTION OF ANTI-EHRLICH ASCITES TUMOR ANTIBODY*+

By

M. H. Flax

Many investigations have been directed toward attempts to isolate factors that would act selectively upon malignant cells;[1] but, for the most part, the results have been discouraging. Recent developments, however, in immunological techniques, e.g., improved methods of preparation and purification of highly specific and potent antisera and means of labeling the sera with isotopes,[2-4] have restimulated efforts to find specific antitumor sera that will destroy neoplasms. Thus, a study of some of the antigenic characteristics of one neoplasm, the Ehrlich ascites tumor, was undertaken as well as a determination of the nature of the effect of specific immune sera on the cells of this tumor.

The Ehrlich ascites tumor was chosen because from a cellular viewpoint it is relatively antigenically "pure," i.e., connective tissue contamination is negligible. Furthermore, it is directly accessible to intraperitoneally injected antiserum in vivo. The tumor was maintained by weekly intraperitoneal injections of 0.1 ml of an ascites tumor, approximately 7 days old, into young adult CF No. 1 mice.

The antitumor antisera were produced by repeated injection of Ehrlich ascites cells into rabbits over a 3-week interval. On the day of the first injection of the first week, 5 ml of a 5 per cent suspension of fresh tumor cells was mixed with Freund's adjuvant and injected into multiple intramuscular depots. On the next 2 days the rabbits were injected intravenously, intramuscularly, intraperitoneally, subcutaneously, and intradermally with 1 ml of the tumor suspension in each portal. The multiple portal injections were repeated 3 times each week during the ensuing 2 weeks. Blood was withdrawn from the rabbits by cardiac puncture at the 4th, 5th, and 6th weeks. Control sera from noninjected rabbits were collected at the same time. The sera were lyophilized and stored at $-20^{\circ}C$ over P_2O_5 in a vacuum. Sera from the immunized animals were pooled, and the gamma globulin fraction was separated by alcohol fractionation according to the method of Nichol and Deutsch.[5]

The tumors that were used for cytologic study were fixed in Carnoy's fluid or neutral formalin, dehydrated, embedded in paraffin, and sectioned at 7 microns. Although they were stained with a variety of dye combinations, most of the material described herein was stained with methyl green-pyronin, which permits visual differentiation between the desoxyribonucleic acid of the chromatin and the ribonucleic acid of the nucleoli and cytoplasm.

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* Summary of a paper submitted for publication in Cancer Research.

+ This study was supported in part by institutional grant #3 from the American Cancer Society.

Neotetrazolium was used to determine the activities of the dehydrogenases in the treated cells.[6] It was prepared in a 1 per cent solution in M/10 phosphate-buffered solution at pH 7.4. After the tumor cells had been treated with the various immunological reagents, 0.25-ml aliquots were mixed with 0.75 ml of the neotetrazolium reagent and 0.25 ml of 1 M substrate. The amount of reduction following a 1-hour incubation period at 37°C was estimated visually, or the reduced neotetrazolium was washed from the cells and the amount of the reagent thus obtained was determined in a Coleman spectrophotometer at a wavelength of 540 mμ.

Oxygen consumption of the tumor cells that had been mixed with the various antitumor serum reagents was determined directly by means of the Warburg method.

For some purposes the antitumor gamma globulin was labeled with I^{131}.[3,4] Radioactivity was then measured in a well-type scintillation counter.

A Raytheon 9-kilocycle sonic oscillator was used to rupture the cells, and the various cellular components were obtained by differential centrifugation.

The tumor cells were treated with various combinations of specific antibody and complement prior to their transplantation into CF No. 1 mice. The combinations were as follows: Tube A - 4 mg anti-Ehrlich ascites (AEA) gamma globulin in 1 ml of saline and 2 ml of restored guinea pig complement; Tube B - 4 mg AEA gamma globulin in 1 ml of saline and 2 ml of restored complement that had been heated to 56°C for 30 minutes prior to mixing; Tube C - 4 mg of AEA gamma globulin in 3 ml of saline; Tube D - 2 ml of restored complement; and Tube E - 3 ml of saline.

The tumor cells were taken from a 7-day growth. They were washed 3 times with saline and diluted to twice the original volume. Aliquots of 0.4 ml of the tumor suspension together with 0.6 ml of 1 M dextrose were added to the tubes, which were then shaken in a water bath at 37°C for 2 hours. Five groups of 15 mice each were injected with 0.4 ml from the respective tubes.

Survival of the host mice after injection is shown in Table 1. Only one animal in the AEA-complement-treated group or in the AEA-treated group died in 60 days; all the ani-

Table 1

EFFECT OF TREATMENT OF EA CELLS PRIOR TO TRANSPLANTATION ON HOST SURVIVAL

Pretreatment	Host animals surviving (60 days)
AEA gamma globulin and complement	13/14
AEA gamma globulin and inactivated complement	0/15
AEA gamma globulin	14/15
Complement	0/15
Saline	0/15

mals in the other groups died before this time, most of them by 14 days.

To determine whether complement added to AEA gamma globulin had a greater effect on the tumor in vitro than AEA alone, a double dilution series of both AEA gamma globulin and complement in all combinations was made. It was noted that with certain combinations of AEA and complement the tumor cells appeared quite swollen. The swelling was never observed in preparations of cells that had been treated with only the AEA gamma globulin or complement. A combination of 1.5 mg AEA and 1 ml of complement caused the most marked cytologic changes.

An investigation of whether the gamma globulin-complement (G-C) combination alters the vital stainability of the tumor cells was then undertaken. Azure B made up as a 1 per cent solution in buffered saline at pH 7.0 was used to stain the G-C-treated tumor cells. The nuclei and cytoplasm of these cells stained intensely compared with those of cells treated before staining with AEA gamma globulin alone or AEA gamma globulin with heat-inactivated complement, which remained essentially unstained.

The capacity of G-C treated cells to utilize sucrose or glucose was studied by means of the neotetrazolium method mentioned above. The data in Table 2 reveal that with succinate there was no appreciable difference in the reduction of the reagent by the four groups, whereas with glucose there was reduction by all of the experimental groups except the G-C-treated group. Direct studies using the Warburg respirometer supported the finding that G-C-treated cells were unable to utilize either kind of substrate.

Table 2

EFFECT OF GAMMA GLOBULIN AND COMPLEMENT ON NEOTETRAZOLIUM REDUCTION BY EA (SUBSTRATE VARIED)

Pretreatment (2 hrs, 37°C)	Amount of neotetrazolium reduction	
	Substrates	
	Glucose	Succinate
AEA gamma globulin and complement	0	++++
AEA gamma globulin	++++	++++
Normal rabbit gamma globulin and complement	+++++	+++++
Saline	+++	+++

Several attempts were made to alter the action of tumor cells in vitro with G-C. Three days after EA cells had been introduced into the host mouse, the first IP injections were given. The injection groups consisted of G-C (1.5 mg AEA + 1 ml complement); 1.5 mg AEA; 1.5 mg normal rabbit serum + complement; or 1 ml of saline. The injections were repeated on the next two days. Three animals from each injection group were sacri-

ficed at 12-hr intervals following the initial therapeutic injection to permit observations of cytologic change, and 4 days later, the 3 remaining animals were sacrificed.

The changes that were observed in the cells of the G-C-treated animals occurred gradually in 3 distinct stages. It must be emphasized however, that alterations were not seen in all of the tumor cells of the G-C-treated animals—some remained quite identifiable as tumor cells although the number of normal EA cells in all sections was reduced.

At 12 hours after G-C had been injected into the mouse, there was clumping of the pyronin-staining particulates at the cell membrane in some tumor cells, while others showed a loss of ribonucleoprotein-staining associated with swelling. At 36 hours, the cytoplasmic ribonucleoprotein-staining was lost, and no nucleoli were apparent. In some cells, a methyl-green-staining material could be seen at the periphery of the cellular membrane. At 72 hours, the tumor cells no longer stained with methyl-green pyronin, an indication that the cells probably no longer contained nucleic acid. Numerous polymorphonuclear leucocytes were seen among the nonstainable tumor cells.

To study the effect of G-C on tumor weight and survival of the host mice, tumor-bearing animals were weighed on the 3rd day after transplantation and were then divided into 4 groups that were injected according to the same routine as those used for the cytologic study. Survivors were weighed on the 5th and 8th day after the first therapeutic injection. Weight changes, which were assumed to parallel the amount of tumor present, are given in Table 3.[7,8] After two days of G-C or AEA therapy, there was a marked decrease in

Table 3

EFFECT OF TREATMENT WITH AEA GAMMA GLOBULIN AND COMPLEMENT ON WEIGHT OF TUMOR-BEARING ANIMALS

Treatment	Average wt. increment (over initial animal wt.)		
	Day 3*	Day 5	Day 8
AEA gamma globulin and complement	2.1 g	0.2 g	3.3 g
AEA gamma globulin	2.3	0.5	2.6
Normal rabbit gamma globulin and complement	2.1	2.7	7.0
Saline	1.9	5.8	5.1

* Before treatment was begun.

weight, but by the 8th day, the tumors had apparently begun to grow again, although the tumor mass in these animals was less than in the other treated groups. The significance of this observation is not yet established since the number of survivors was too small to permit a statistical evaluation.

The average length of survival of the various treated groups is given in Table 4. The G-C treated group survived longer than the other three; appreciably longer than the normal rabbit gamma globulin-treated and the AEA-treated groups and twice as long as the saline-treated controls.

Table 4

AVERAGE SURVIVAL TIME OF TUMOR-BEARING MICE AFTER IN VIVO TREATMENT

Treatment	Average survival (days)
AEA gamma globulin and complement	16.5
AEA gamma globulin	10.3
Normal rabbit gamma globulin and complement	11.2
Saline	8.2

The effect of G-C on Krebs ascites tumor was studied to learn whether its action was specific for the Ehrlich ascites tumor. The Krebs cells were treated with G-C, AEA gamma globulin, normal rabbit gamma globulin plus complement, or saline, and their ability to utilize glucose or succinate was studied by means of the neotetrazolium method. The results indicate that the effectiveness of G-C is not entirely limited to the homologous type of tumor.

Another study was made to determine whether G-C acted upon normal tissue as it does upon EA cells. CF No. 1 mouse kidney, liver, and spleen homogenates were subjected to the immunological preparations that have been described. Neotetrazolium tests revealed that the utilization of glucose by the liver and kidney homogenates was inhibited by G-C while that of the spleen was inhibited to a lesser degree (Table 5). Although there was

Table 5

EFFECT OF ANTI-EHRLICH ASCITES GAMMA GLOBULIN AND COMPLEMENT ON NORMAL TISSUES

Pretreatment	Amount of neotetrazolium reduction (ext.$_{540}$)			
	Ehrlich ascites tumor	Liver	Kidney	Spleen
AEA gamma globulin and complement	0.32	0.30	0.30	1.3
AEA gamma globulin	1.58	1.52	0.91	1.72
Complement	1.38	1.26	1.10	2.08
Saline	1.68	1.58	1.22	1.88

considerable variability, the difference between the G-C treated cells and the controls is significant.

The specificity of the active constituent of G-C was investigated by an indirect method. AEA gamma globulin was mixed with variable amounts of lyophilized normal tissues (liver, kidney, and muscle) and with lyophilized EA cells to determine the degree to which the active substance could be adsorbed by these antigens.

The quantity of the active constituent that was adsorbed was indicated by the loss of activity in the corresponding supernatant fluid as tested against living EA cells.

Twenty, 40, 60, or 80 mg of lyophilized antigen were mixed with 1.5 mg AEA gamma globulin and 1 ml of complement, and the resultant mixtures were shaken at room temperature for 2 hours. The tubes were then centrifuged, and the gamma globulin-containing supernatants were decanted. One-half ml of complement was added to each of the solutions to assure adequate levels of complement. Two-tenths ml of EA cells was then added to each solution, and the reaction was allowed to proceed at 37°C for 2 hrs. Alterations in the dehydrogenase activities of the cells were determined by the neotetrazolium method, using glucose as the substrate. The reduced neotetrazolium was extracted from the cells with acetone, and the amounts were determined spectrophotometrically. Extinction under the conditions of this experiment was directly proportional to the total amount of reduced dye (Table 6). The cells treated with G-C that had not first been mixed with any

Table 6

DECREASE OF ACTIVE AEA ANTIBODY BY PRE-ABSORPTION WITH VARIOUS LYOPHILIZED TISSUES

Lyophilized antigen		Amt. of reduced neotetrazolium (ext.$_{540}$)
Ehrlich ascites	20 mg	0.65
	40	1.04
	60	1.05
	80	1.15
Kidney	20	0.19
	40	0.83
	60	1.05
	80	1.25
Liver	20	0.23
	40	0.73
	60	0.76
	80	1.15
Muscle	20	0.24
	40	0.46
	60	0.75
	80	0.79
None		0.09
Untreated EA cells		0.95

antigen were, as expected, almost completely unable to reduce neotetrazolium. The lyophilized EA cells were most active in adsorbing the active constituent. Kidney was next most active in this respect, with liver less so, and muscle tissue the least. These findings support the conclusion that the action of effective antibody constituent is not limited to the immunizing cell type even though it is quantitatively most reactive with it.

AEA gamma globulin was labeled with I^{131} in order to determine the localization of G-C in tumor cells. The tumor cells in 20 ml of ascitic fluid were fractured by sonation. AEA I^{131} was then added, and the mixture was shaken at room temperature for 2 hours. Four fractions were then obtained by differential centrifugation, and the amount of radioactivity in each fraction was then measured directly by means of a well-type scintillation counter. The results are summarized in Table 7. The mitochondrial fraction absorbed from 2 to 4 times as much as the microsome and supernatant fractions and also localized a greater amount of AEA I^{131} per g than the fraction containing the nuclei and cell fragments, which had the highest initial radioactivity.

Table 7

AEA I^{131} UPTAKE BY EHRLICH ASCITES CELL FRACTIONS

Fraction	Exp. no.	% of total AEA I^{131}	% of total AEA I^{131}/g or cc
Nuclei and cell fragments	1	48.3	11.0
	2	41.1	11.5
	Ave.	44.7	11.2
Mitochondria	1	6.0	33.6
	2	6.8	43.1
	Ave.	6.4	38.4
Microsomes	1	5.2	16.2
	2	8.2	14.9
	Ave.	6.2	15.5
Supernatant	1	41.2	39.2
	2	44.3	30.5
	Ave.	42.7	34.8

Thus, it has been demonstrated by a number of differing techniques that anti-Ehrlich ascites tumor gamma globulin with or without complement damages the neoplastic cells.

Cross reactions between the antiserum globulin and normal tissue cells or their components detract from the specificity of these deleterious effects.

Nevertheless when the globulin complement was injected intraperitoneally into mice bearing actively growing ascites tumor, the average survival time relative to untreated controls was approximately doubled.

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THE TIME OF ANTIGEN INJECTION RELATIVE TO WHOLE-BODY X IRRADIATION AND THE DEVELOPMENT OF CIRCULATING ANTIBODY AND THE SPLENIC HISTOLOGICAL REACTION IN THE RAT*

By

F. W. Fitch,† R. W. Wissler, M. La Via, and P. Barker

A marked depression occurs in the concentration of circulating antibodies in rabbits that have been subjected to whole-body X irradiation prior to the injection of antigen whereas there is little or no depression if the antigen is injected prior to exposure.[1-3] Other studies of the rabbit give evidence that the antibody response is relatively radioresistant if antigen is administered before or within a few hours after X irradiation.[4-6] A retardation of the immune response occurred under these conditions but there was no reduction in the peak titer. In the irradiated rat,[7] however, the peak antibody titer is lower, there appears to be a delay in attaining the peak, and there is a delay in the decline of the titer whether the antigen is given before or after X irradiation. These effects are less pronounced in rats that are exposed after the antigen has been injected. Similarly, the findings from studies in this laboratory indicate that there is a pronounced depression in antibody formation in rats that have received total-body irradiation and are then injected with antigen.[8] It thus appears that the immunologic response of the rat is different from that of the rabbit.

A close correlation has been shown between hyperplasia and an increase in the pyronin-staining qualities of the cells of the splenic red pulp in rats that are stimulated antigenically by the intravenous route.[9] This adds to the evidence that cellular proliferation is an important factor in antibody formation.[10-14] Total-body X irradiation of the rat depresses this splenic cellular response without appreciably altering the localization and digestion of labeled antigen by the spleen.[9]

The research outlined in this summary was directed toward a further elucidation of the action of whole-body X irradiation on the antibody-forming mechanism. Observations were made of the concentration of circulating antibodies and the splenic cellular response in the rat given intravenous antigenic stimulation at varying times relative to irradiation.

Young adult male Sprague-Dawley rats, weighing from 215 to 315 g, were used for this study.

Washed sheep cells and formalin-killed Salmonella typhi served as the antigens. Both

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* Summary of a paper that has been accepted for publication in the Journal of Immunology.

† U.S.P.H.S. Research Fellow of the National Microbiological Institute.

were used simultaneously because the typhoid vaccine induces a striking cellular reaction in the spleen and the sheep erythrocytes permit a better quantitative estimation of antibody formation. Methods for antigen preparation and agglutination titration have been described previously.[10,11] The hemolytic content of the sera was measured in 50 per cent units according to the method outlined by Taliaferro and Taliaferro.[12]

X rays were generated by a 250-kvp Maxitron machine operating at 15 ma with 0.25 mm Cu and 1 mm Al added filtration. The exposure rate averaged 39 r per min. at 75 cm. A single exposure of 500 r was delivered to the whole body of rats that were irradiated in groups of 12 in a rotating aluminum cage that was perpendicular to the beam.

Material for histologic study was fixed in Carnoy's alcohol-acetic acid-chloroform mixture or in 10 per cent formal-saline, and the tissues were embedded in paraffin. Sections of Carnoy-fixed material were prepared with the methyl green-pyronin stain[13] and those of the formalin-fixed material were stained with the azure A-eosin B method.

Rats in groups of six were injected with 0.25 per cent sheep cell erythrocytes in typhoid vaccine at 4, 3, 2, or 1 day or a few hours before X irradiation or at 1 or 6 days afterward. Antibody determinations were made on all of the animals at 4, 6, 8, 12, and 18 days after the antigen injection. The animals were sacrificed on the 18th day after injection, and portions of spleen were prepared for histologic study.

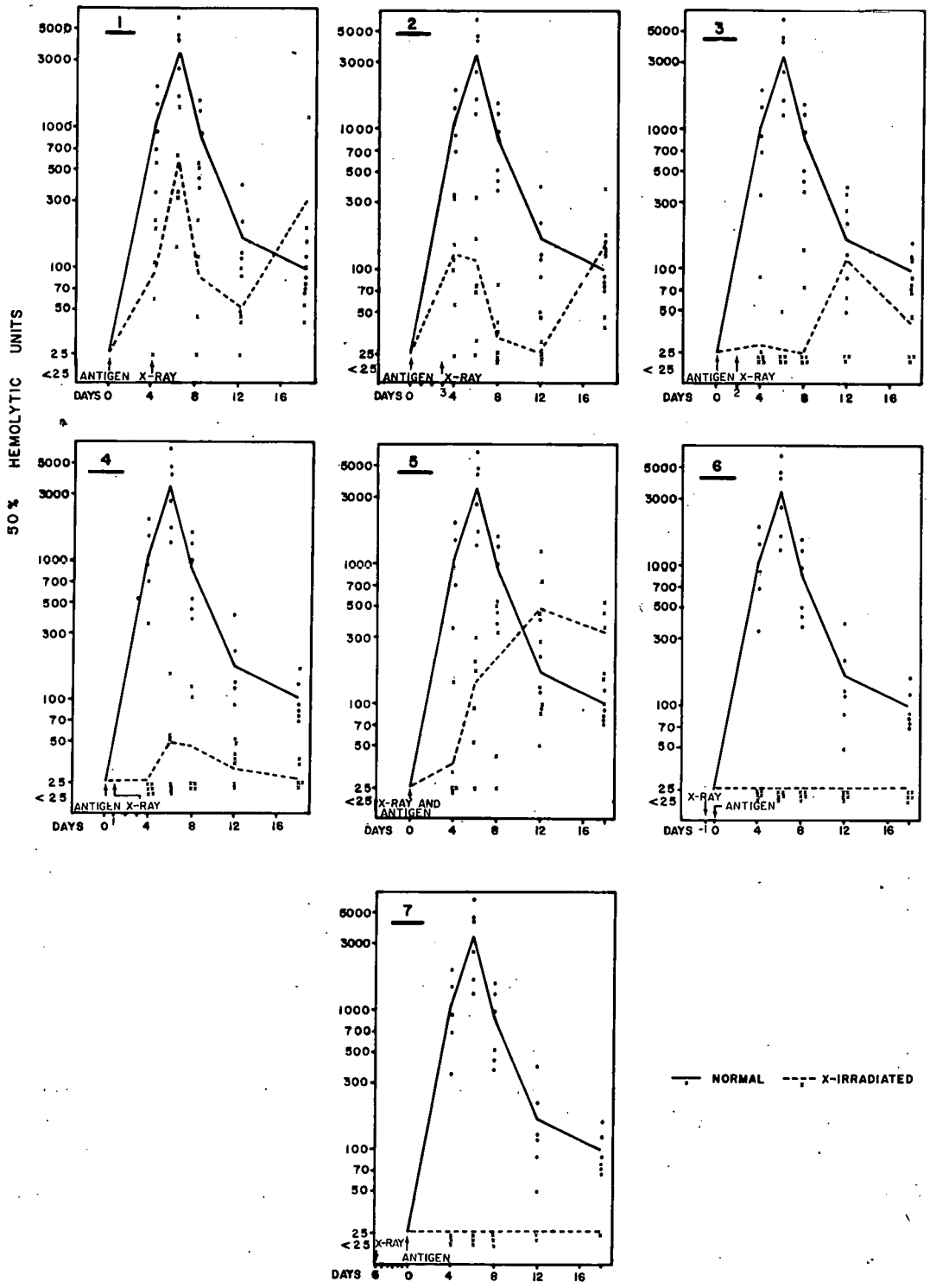
Individual and average antibody levels for each group of rats are given in Figures 1 to 7. All of the irradiated animals had depressed peak antibody titers regardless of when the antigen was given. A secondary peak on the 18th day after injection was observed in animals that had been given the antigen at 4 or 3 days prior to irradiation. A delay in attaining the peak titer with little decrease in total antibody output occurred in rats that had been injected with antigen a few hours previous to exposure to X rays. Complete suppression of circulating antibody was found in animals that had been given the antigen after irradiation.

In another series of experiments, rats in groups of 10 each were injected with antigen at the same intervals in relation to irradiation as those in the preceding series.

Three animals from each group were sacrificed at 4, 6, and 8 days after injection. Blood for antibody determination was drawn at the time of sacrifice when portions of the spleen were also taken for histologic examination.

Although there was considerable individual variation, the average levels of antibodies agreed with those of the previous series.

Since the findings from other investigations[9,14] showed that the peak of splenic hyperplasia and increase in pyronin-staining of the splenic red pulp occurs 2 days before peak circulating antibody concentration, the degree of hyperplasia and increase in pyronin-staining of the splenic red pulp of each animal sacrificed on the 4th day after antigen injection was estimated in arbitrary units from 1 (normal) to 4 (maximum). The degree of hyperplasia and increase in pyronin-staining expressed as average units multiplied by 3 was compared with the average antibody titer on the 6th day after injection. Figure 8 shows the close correlation between the circulating antibody concentration and the degree of splenic cellular proliferation.



Figures 1-7. Individual and average hemolysin levels in normal and X-irradiated rats after a single intravenous injection of antigen at varying times relative to whole-body X irradiation.

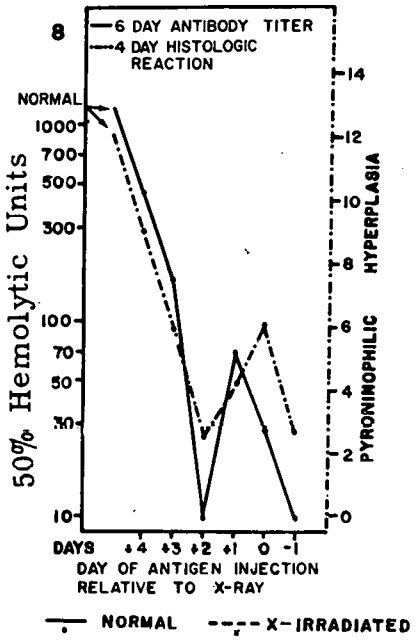


Figure 8. Correlation of antibody response with hyperplasia and increase in pyronin-staining of cells of the splenic red pulp in the rat following a single intravenous antigenic stimulation at varying times relative to whole-body X irradiation.

In the normal rat, there is marked hyperplasia and an increase in pyronin-staining in the cells of the splenic red pulp at 4 days after the injection of antigen. In rats that have been injected with antigen 2 days after exposure to 500 r, the cellular reaction was severely depressed at 4 days after the injection. At 6 days after injection, the circulating antibody concentration was also strikingly depressed in the irradiated rats.

The degree and type of depression of antibody titer and splenic reaction are dependent upon the time that X radiation is delivered in relation to the administration of the antigen. In addition, the extent of hyperplasia and the increase in the pyronin-staining qualities of the cellular elements of the splenic red pulp were found to be correlated with the circulating antibody concentration. The splenic cellular reaction for the most part subsides in nonirradiated rats at 18 days after the injection of antigen, when the concentration of circulating antibodies is declining. At this same time interval, however, the splenic reaction in the animals that had been X-irradiated 4 days after the injection of antigen was still clearly evident. This is correlated with a secondary increase in the concentration of circulating antibodies.

Examination of the histologic sections from nonirradiated rats indicates that as early as 6 hours after antigen injection there are changes in the large immature cells of the red pulp[14] and that proliferation, which is clearly seen at 48 hours, reaches a peak on the 4th day after injection. Most of the proliferating cells disappear after this time. The few that remain appear to differentiate into mature plasma cells.

If then this sequence of cellular change is necessary for antibody formation, any agent that interferes with cell multiplication or cellular protein synthesis will alter the formation of antibodies. The time of interference in the sequence of multiplication, differentiation, and protein synthesis alters the results. The cellular mechanism of antibody formation and the effect of X irradiation upon it are presented diagrammatically in Figure 9. The quantity of antibody formed is shown to be related to the number of splenic red pulp cells

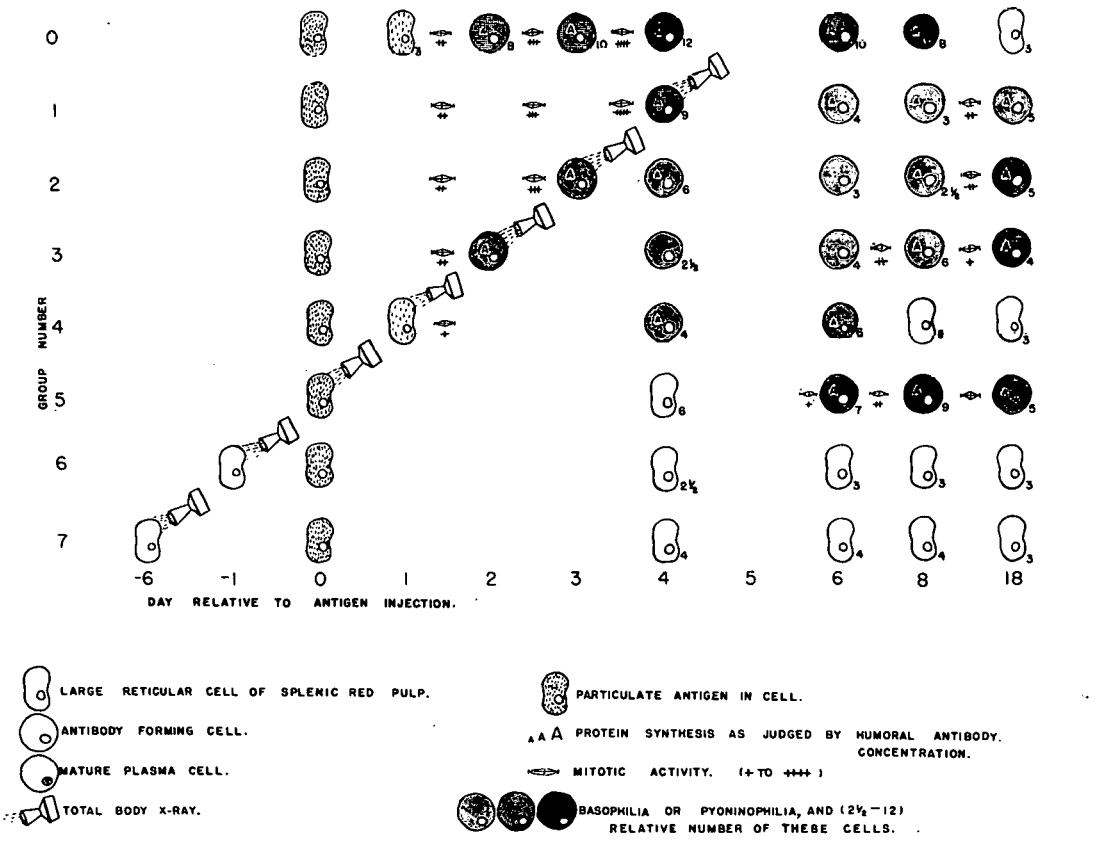


Figure 9. Schematic representation of the cellular mechanism of antibody formation and the effect of X irradiation upon it.

that proliferate in response to antigenic stimulation and to the capacity of these proliferated cells to synthesize protein. It is assumed that irradiation may interfere with either cellular proliferation or protein synthesis and that it has only a slight effect upon antigen fixation by the cell or upon the fate of antigen that is extra- or intracellular. The ineffectiveness of irradiation upon antigen localization has been described previously.(9)

From the investigation, it seems reasonable to state that the radiosensitivity and radioresistance of the immunological phenomena may be explained in terms of the action of the radiation upon the division, maturation, and protein synthesis of cells that either produce antibody or that are precursors to antibody-forming cells.

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ASSAY OF TRITIUM IN URINE USING A LIQUID SCINTILLATION COUNTER*

By

G. T. Okita, J. Spratt, and G. V. LeRoy

A sensitive, rapid, and simple method for monitoring the urine of individuals exposed to tritium has been developed and its description is the basis for this report.

Thirty ml of urine is mixed thoroughly with 1 g of activated charcoal (N.F. IX, Mallinckrodt). This mixture is filtered, and the resulting colorless filtrate is used for radioassay without further treatment. Three samples are then prepared. The first, the test specimen, is made by pipetting 2.0 ml of the decolorized urine, 0.1 ml of distilled water, and 20.0 ml of absolute ethanol into an 85-ml weighing bottle. Twenty-eight ml of reagent grade toluene and 100 mg of scintillation grade p-diphenyloxazole are added after the initial mixing of the urine solution. The second, the background blank, is prepared like the test specimen except that 2.0 ml of water are substituted for the decolorized urine. The third, the internal standard, is also prepared like the first specimen except that 0.1 ml of standard THO† solution replaces 0.1 ml of the distilled water. This last sample is used to determine the counting efficiency (E_s) of the solvent system and counter.

A fast-coincidence, liquid scintillation counter‡ is used to determine the amount of radioactivity that may be present. Samples are first equilibrated at 6°C and then counted long enough to give a standard error of ± 5 per cent.

The following formulae were used to determine the radioactivity in each urine sample (D_u):

$$E_s = \frac{\text{Efficiency of solv.}}{\text{system and counter}} = \frac{(C_i - C_b) - (C_u - C_b)}{\text{dpm of 0.1 ml stand. tritium } H_2O}$$

$$D_u = \text{dpm of 2 ml of urine} = \frac{C_u - C_b}{E_s}$$

The total radioactivity is then expressed as

$$\mu c \text{ per liter of urine} = \frac{D_u \times 500}{2.22 \times 10^6 \text{ dpm}}$$

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* Summary of a paper submitted for publication in Nucleonics.

† Standard THO (tritium oxide) solution assayed by the method of Wilzbach et al.(1) using the vibrating reed electrometer.

‡ Tri-Carb Counter, Model 314, Packard Instrument Co., LaGrange, Ill.

In these, C_u equals the counts per minute (cpm) of the urine sample, C_i equals cpm of the internal standard, C_b equals cpm of the background blank, and dpm equals the disintegrations per minute.

Although a large number of scintillator-solvent systems were studied to determine their efficiency for counting tritiated water, the one finally selected is a modification of the system suggested by Hayes and Gould.[2] The over-all counting efficiency for the solvent system and the counter is approximately 3 per cent.

The effect of color and quenching agents in urine was examined by comparing the counting rate of various urine preparations against an external standard (distilled water used in place of urine filtrate) containing the same amount of radioactivity. As shown in Table 1, the quenching effect of decolorized urine ranged from 0 to 12 per cent, while in the case of unprocessed urine, quenching ranged from 56 to 100 per cent.

Table 1

QUENCHING EFFECT OF VARIOUS SAMPLES

Type of urine sample*	Quenching per cent†
Decolorized urine filtrate	none - 12
Nonprocessed urine (medium color)	56 - 87
Nonprocessed urine (dark color)	78 - 100

* Six different urine samples used for each category.
† Comparison with the external standard.

The reliability of this method of assay was tested by adding varying amounts of standard THO solution to untreated nonlabeled urine. The amount of THO added was never greater than 5 ml per 100 ml of urine. The urine samples were then decolorized and counted in the usual manner. The results are given in Table 2. The recovery of tritium activity for 10 samples treated in duplicate was 96.7 $\pm$ 7.5 per cent standard error of the mean.

The limits of sensitivity of the method are shown in Table 3. For this determination a standard solution of THO was added in decreasing amounts to a series of nonlabeled urine samples, which were then decolorized and counted. The data indicate that urine containing as little as 0.08 μc of THO per liter can be detected by this method. This limit corresponds to a total-body burden of roughly 3 to 4 μc of tritium, which is well below the maximum permissible level of exposure of 1000 μc.[3]

Table 2

RECOVERY OF TRITIUM WATER ACTIVITY ADDED TO URINE

Urine sample	DPM expected	DPM found*	Recovery (%)
1	9,670	8,610	89
2	"	9,280	96
3	"	8,990	93
4	19,350	20,130	104
5	"	17,040	88
6	"	18,390	95
7	38,700	41,050	106
8	"	42,920	111
9	"	37,550	97
10	"	36,420	94
		Mean plus or minus S.D. —	96.7 ± 7.5

* Average of duplicate samples.

Table 3

LIMIT OF SENSITIVITY OF ASSAY METHOD

Urine sample (2 ml)	Sp. act. of urine (μc/liter)	Obs'd net cpm (2 ml urine)
1	0.50	68
2	0.20	27
3	0.10	13
4	0.08	trace*
5	0.05	NSC+
6	0.01	NSC

* Net cpm between one to two S.D. of background counts.
\+ No significant counts—net cpm less than one S.D. of background counts, i.e., less than 6 cpm.

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TRITIUM-LABELING BY MEANS OF THE $Li^6(n,\alpha)T$ REACTION
A PRELIMINARY REPORT

By

H. Krizek, J. Garnett, and W. G. Brown

The introduction of a small amount of tritium, formed by the $Li^6(n,\alpha)T$ reaction, into glucose when mixtures of it with lithium carbonate were irradiated with slow neutrons has been reported.[1] Using a flux of 2×10^9/sec, Wolfgang, Rowland, and Turton obtained glucose having an activity of a few microcuries per gram.

The study of the new technique for tritium-labeling has been initiated in the hope that with larger neutron fluxes, compounds of activity high enough for use in biochemical experiments could be made available. To obtain information about the practicability of the process, several sealed, evacuated quartz tubes containing 0.9- to 1.0-g portions of a mixture of cholesterol and a lithium salt (0.57 to 0.60 mg atom lithium per tube) were irradiated for 113 to 115 hrs in a slow neutron flux of 1×10^{12}/sec in the Argonne National Laboratory research reactor (CP-5). Under these conditions, considerable decomposition occurred, the volume of gaseous products being 45 cc STP when the lithium was dispersed colloidally as lithium myristate. When larger particles (30 μ or 60 μ estimated diameter) of lithium salt (the oxalate was used in these cases) were used, the volume of gas evolved was somewhat smaller, presumably because the highly energetic α particles also formed in the reaction were in part screened out or else underwent a loss in energy inside the lithium oxalate particles. The gas evolved from the irradiated mixtures appeared to be mainly hydrogen. Cholesterol alone irradiated under the same conditions yielded only 3 cc of gas. It may be noted that the volume of the largest sample tube that can be accommodated by a standard irradiation capsule used in the Argonne reactor is less than 100 cc.

The sample for which 30-μ lithium oxalate particles were used was chromatographed on activated alumina and yielded a crude cholesterol fraction representing 35 per cent of the weight of the starting material and had a specific activity of 0.20 ± 0.01 μc per mg. Further purification by conversion to the acetate and one recrystallization gave a product having an activity of 0.145 μc per mg, equivalent to a cholesterol activity of 0.154 μc per mg. This fraction will be subjected to further purification.

To obtain information regarding distribution of the tritium introduced into a molecule by the process, isopropyl benzoate was selected as a model compound for irradiation, since isopropyl benzoate contains three types of C-H bonds; aromatic, primary, and secondary aliphatic. The rate of tritium attack at each of the three types of bonds could be deduced from tritium analyses of suitable derivatives formed from the ester.

Samples of isopropyl benzoate (total weight 3.7 g) containing lithium myristate (total weight 0.6 g) colloidally dispersed were irradiated like the cholesterol. Appreciable de-

composition and discoloration occurred. The irradiated mixture was then purified by three successive distillations, the final activity of the ester being 0.170 μc per mg. The residue (0.6 g) remaining after separation of the isopropyl benzoate and the lithium myristate was dark brown, very viscous, and possessed the highest specific activity (1.010 μc per mg) of any of the products isolated.

The ester was hydrolyzed by the method of Redeman and Lucas,[2] and the alcohol and benzoic acid were isolated. A portion of the alcohol was converted to the 3,5-dinitrobenzoate. The remainder was oxidized to acetone with permanganate in neutral solution by a modification of the procedure given by Shriner and Fuson.[3] According to Kaplan,[4] very little (0.2 per cent) loss of tritium occurs during this procedure. The acetone was precipitated and analyzed as the 2,4-dinitrophenylhydrazone. The benzoic acid, isopropyl 3,5-dinitrobenzoate, and acetone 2,4-dinitrophenylhydrazone were purified to constant activity by recrystallization from suitable solvents, at least four such recrystallizations being required for each compound. From the final activities the ratio calculated for the rate of tritium attack at the three types of bonds was: aromatic C-H : secondary aliphatic C-H : primary aliphatic C-H = 2.68 : 1.32 : 1.00.

All activities, except that of the crude cholesterol, which was assayed by scintillation counting, were determined by means of the zinc fusion technique of Wilzbach, Kaplan, and Brown.[5]

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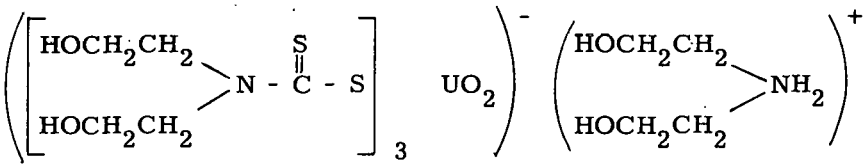
PREPARATION OF ORGANIC URANIUM COMPOUNDS. I. URANYL DITHIOCARBAMATES

By

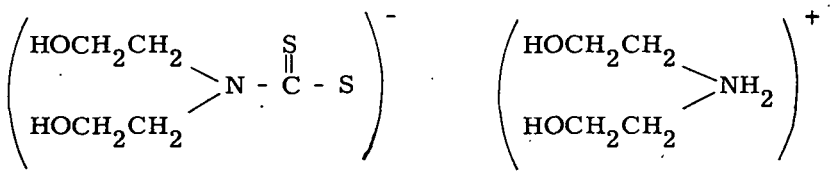
S. Brownstein

The purpose of this work was to obtain water soluble uranium organics in which the uranium is covalently bound.* A series of uranyl dithiocarbamates has been prepared previously.(1) One of these, diethanolamine dithiocarbamate (1)



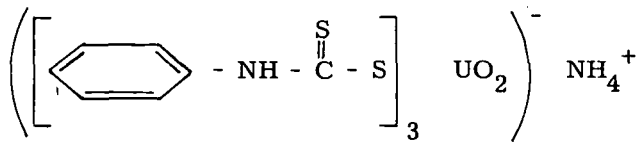
(1)

was reported to be water soluble. This compound was prepared, and found to be excessively toxic to mice, although the parent dithiocarbamate itself (2) was



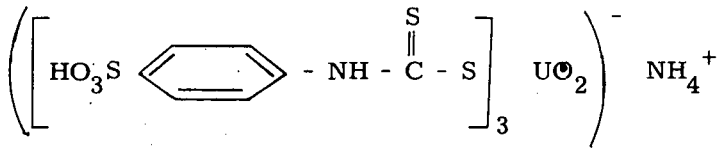
(2)

not toxic.† The uranyl dithiocarbamate of aniline (3) was prepared, but



(3)

was only very slightly soluble in water. An attempt to prepare uranyl sulfanilic dithiocarbamate (4) failed because the uranium tetrachloride preferentially



(4)

* These compounds were parepared at the suggestion of Dr. R. Hasterlik and Dr. W. G. Brown.

† Toxicity studies were done by Dr. R. Hasterlik and Mr. R. Druyan.

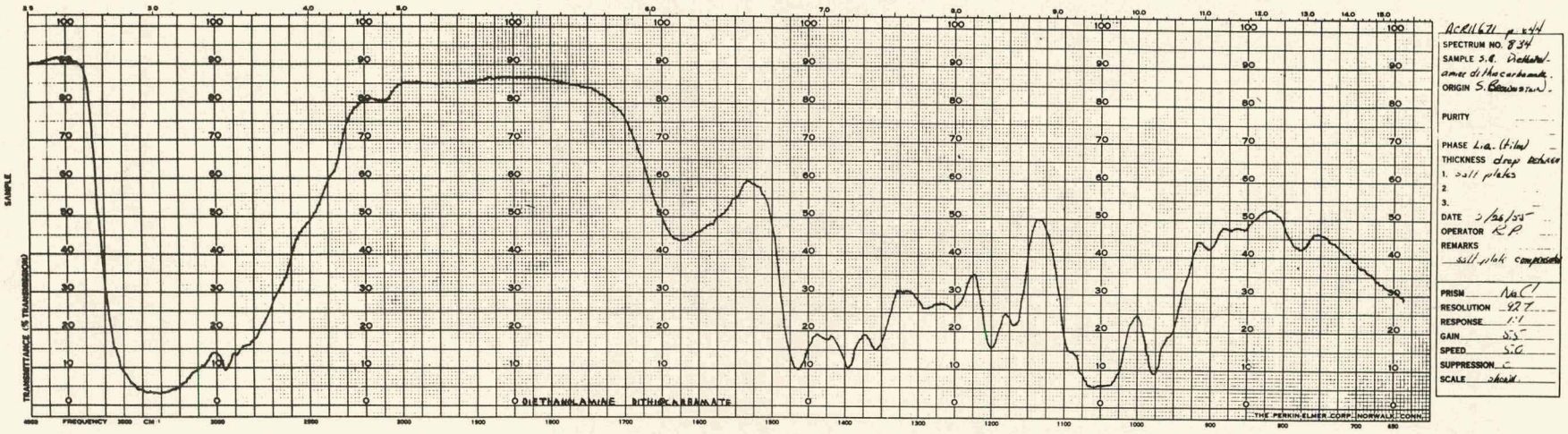


Figure 1. Diethanolamine dithiocarbamate.

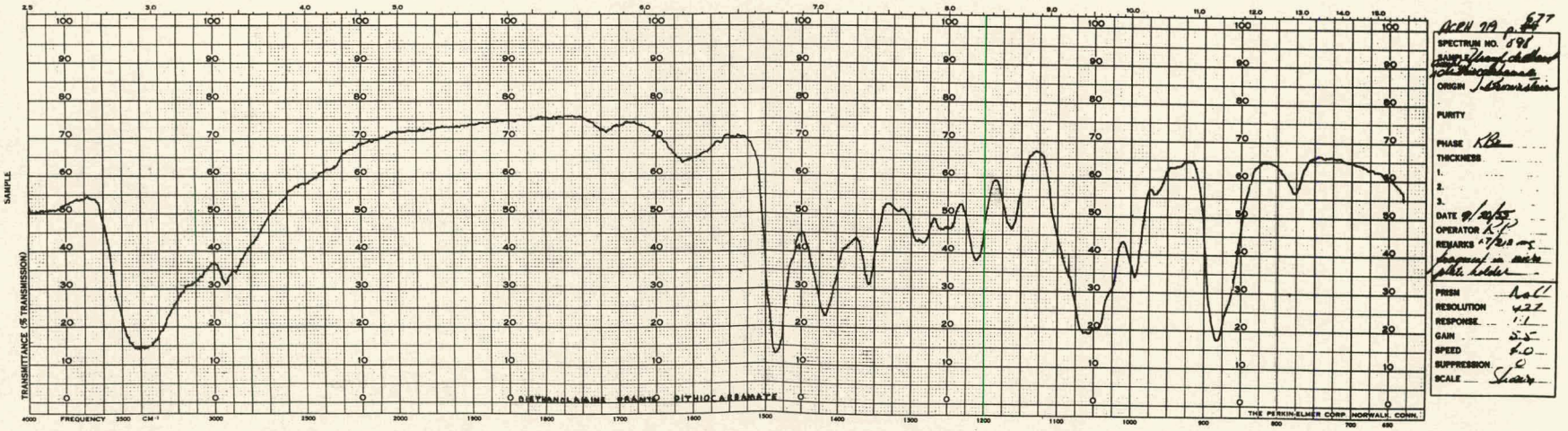


Figure 2. Diethanolamine uranyl dithiocarbamate.

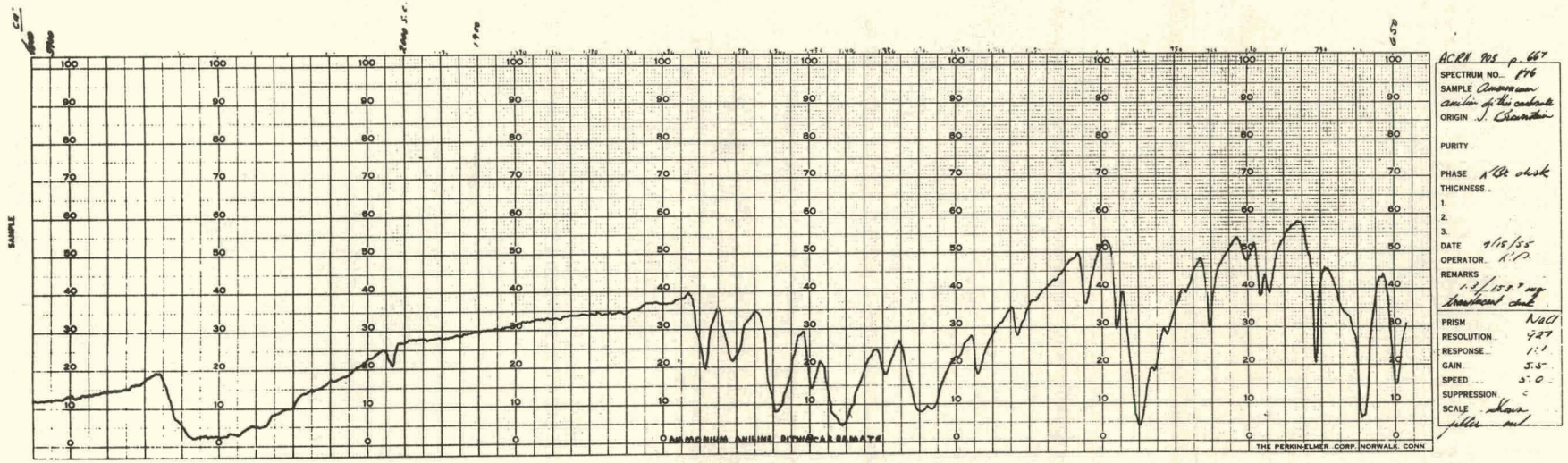


Figure 3. Ammonium aniline dithiocarbamate.

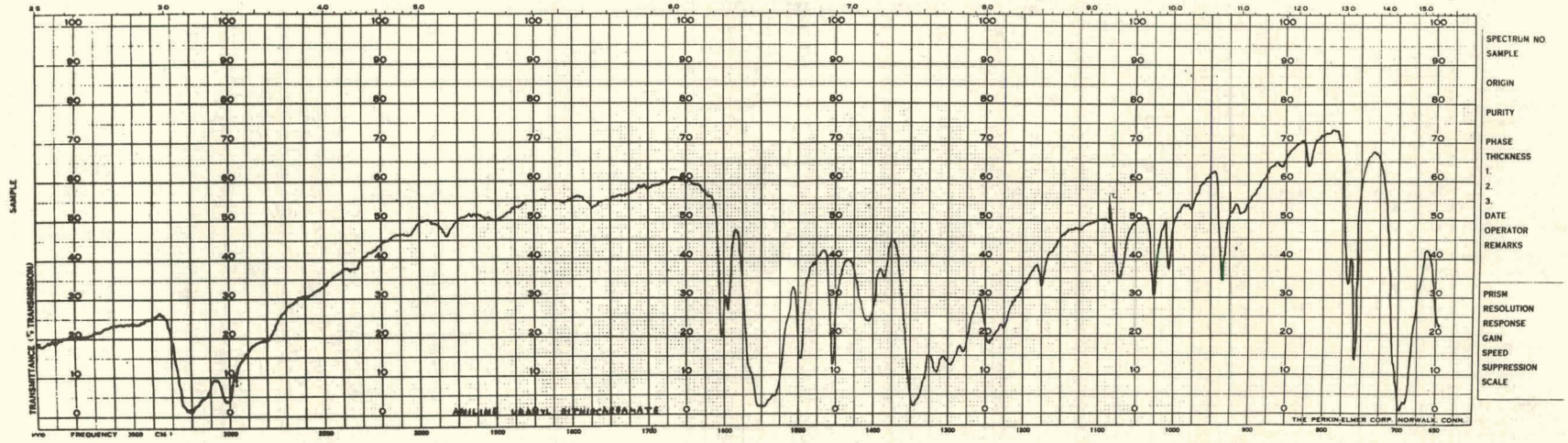


Figure 4. Aniline uranyl dithiocarbamate.

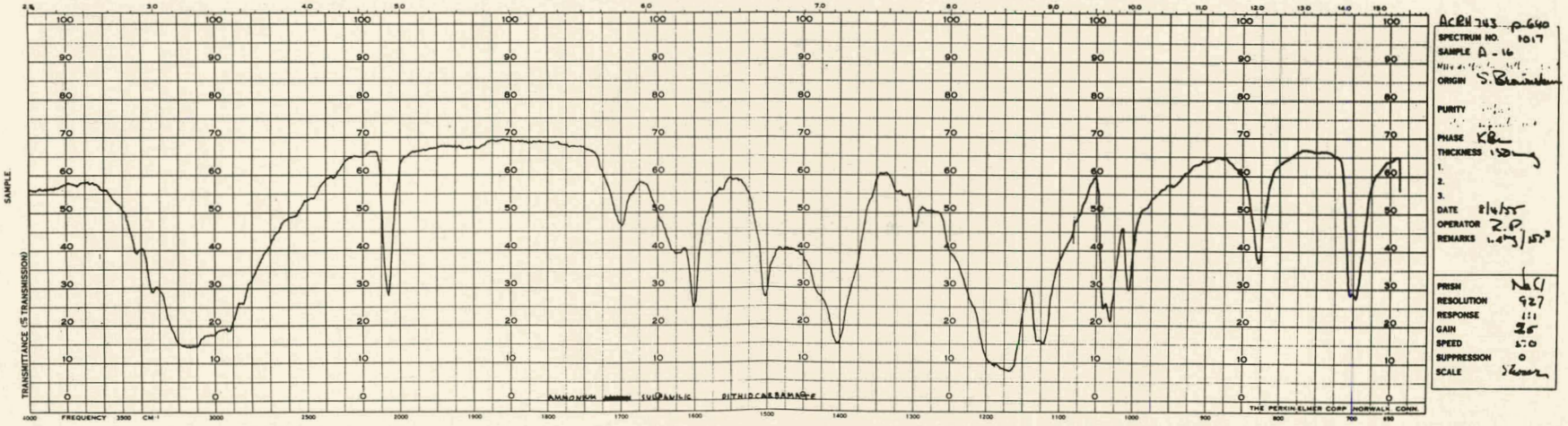


Figure 5. Ammonium sulfanilic dithiocarbamate.

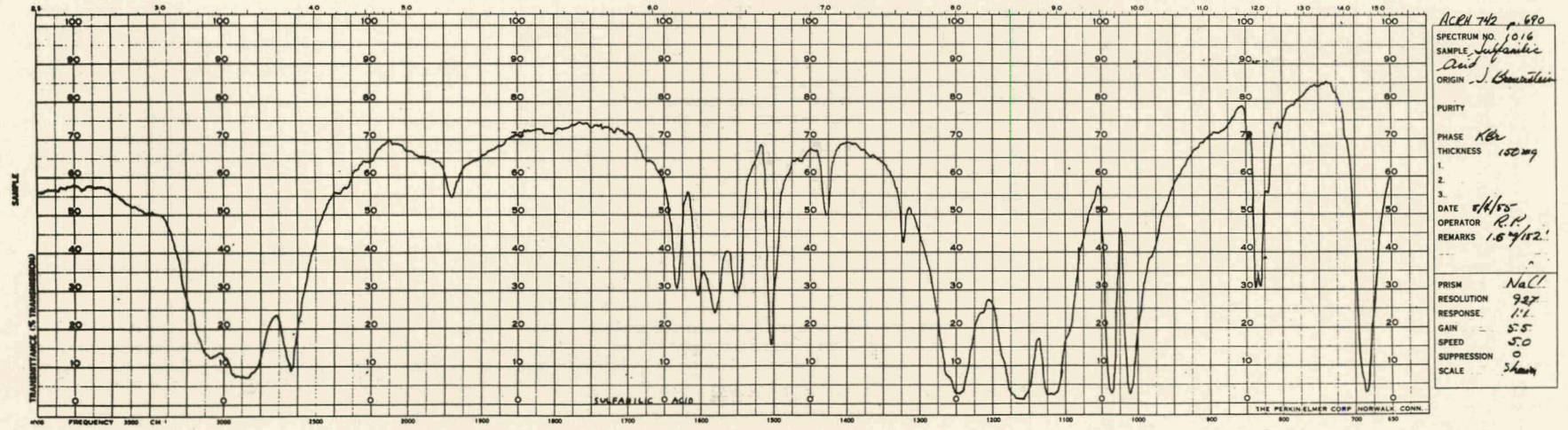


Figure 6. Sulfanilic acid.

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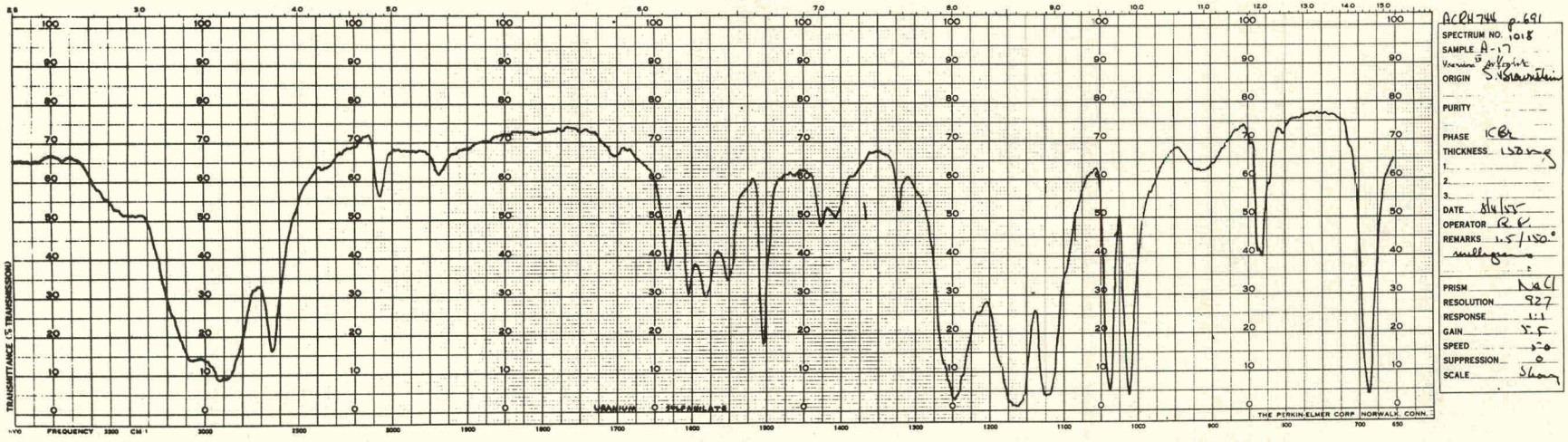

Figure 7. Uranium sulfanilate.

forms a salt with the sulfonic acid grouping. It was noted that the infrared spectra of the uranyl dithiocarbamates are very similar to the spectra of the parent dithiocarbamates. The infra-red spectra are shown in Figures 1-7.

EXPERIMENTAL

Diethanolamine dithiocarbamate and uranyl diethanolamine dithiocarbamate were prepared as mentioned in the literature. The melting point for the uranyl compound was found to be 140-143°C with decomposition. The melting point reported originally was 167-168° with decomposition. The conditions under which the compound crystallizes are variable, and frequently no yield could be obtained. The different melting points are probably due to methanol of crystallization.

Ammonium aniline dithiocarbamate:[2] A cold solution of 11 g of ammonia and 45 g of aniline in 63 cc of ethanol was slowly added with stirring to a cold solution of 50 g of carbon disulfide in 50 cc of ether. Stirring was continued for an hour, and the product was filtered, washed with ether, and dried. A 47 per cent yield was obtained. The M.P. was 81-83°.

Uranyl aniline dithiocarbamate: A solution of 1 g of ammonium aniline dithiocarbamate in 15 cc of methanol was boiled for 1 minute to remove hydrogen sulfide. To this was added a solution of 0.3 g of uranium tetrachloride in 5 cc of methanol. After cooling, oxygen was bubbled into the solution for 2-1/2 hours. The solution turned from a green to a yellow color. When the solution is concentrated under vacuum, yellow crystals of product appear. These were filtered, washed with water, and dried. The M.P. was 145-148°.

Ammonium sulfanilic dithiocarbamate: To a filtered solution of 10 g of sulfanilic acid in 15 cc of concentrated ammonium hydroxide was added 50 cc of absolute alcohol. A solution of 6 g of carbon disulfide in 25 cc of acetone was slowly added to the ethanol solution, and the reaction allowed to continue for 2-1/2 hours. The solution turned dark. It was concentrated under vacuum and cooled. The solid was filtered and dried under vacuum. It does not melt promptly but decomposes gradually.

Uranium sulfanilate: To a solution of 2.4 g of ammonium sulfanilic dithiocarbamate in 25 cc of methanol was added a solution of 0.7 g of uranium tetrachloride in 10 cc of methanol. A white precipitate formed quickly. This was filtered and dried. It is soluble in water, to giving a solution that yields a red color with potassium ferrocyanide solution.

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SEMI-MICRO METHOD FOR ISOLATION AND PURIFICATION OF RADIOACTIVE CHOLESTEROL*

By

J. J. Kabara

The current interest in cholesterol in relation to biosynthesis of steroids has created the need for a semi-micro method for the isolation of radioactive cholesterol in man. A quick and convenient method has been devised that allows the counting of human plasma cholesterol of sufficient radiochemical purity to permit accurate biological interpretation following the administration of a tracer dose (100-200 μc) of C^{14} acetate. By this method, 0.50 to 5.0 mg of cholesterol are isolated as a digitonide and are carefully washed free of adsorbed substances. The resulting filtrate and washings are then used for the isolation of cholesterol esters after hydrolysis in a manner similar to that used for free cholesterol. The digitonin complex is broken up by heating with benzene and alcohol (2:1), and the freed sterol is extracted with toluene. The toluene solution is then divided into two aliquots, one of which is used for a quantitative measurement (Liebermann-Burchard), while the other fraction is assayed for radioactivity by means of the conventional Geiger-Mueller flow counter or the Packard liquid scintillation counter. (The latter counter permitted the counting of both tritiated and C^{14} cholesterol in the same sample.)

The following modification of the Windaus procedure was found applicable in repurifying small amounts (1.0 - 10.0 mg) of assayed cholesterol: Droplets of liquid bromine are added to 1.0 mg of cholesterol dissolved in 1 ml of ether until the solution becomes and remains distinctly orange-colored. The reaction is allowed to continue to completion at room temperature (20 to 30 minutes). The test tube is then immersed in an ice bath. Three ml of glacial acetic acid are added to the mixture. When the solution is thoroughly chilled, water is added drop-wise and very slowly until the first cloudy appearance. This step must be done with great care. The solution is then maintained at room temperature for 1 hour or, preferably, over night in the ice-box. After the precipitate has formed, the solution is centrifuged, and the dibrominated sterol is washed with cold methanol. The cholesterol derivative is then debrominated, using acetic acid and zinc dust as follows: The precipitate is dissolved in 3 ml of acetic acid, and 10 granules of zinc are added to this acid solution. The reaction mixture is heated on a water bath for 30 minutes and then cooled to room temperature. The acetic acid solution is diluted with an equal part of water and extracted with several portions of ether. The ether extract is washed with dilute alkali to remove the acetic acid. The neutral ether solution is evaporated to dryness, and the resi-

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* Summary of a paper presented at the American Society of Biological Chemistry (Fed. Proceedings, Vol. 14, No. 1, March, 1955).

due is extracted with warm toluene solution. By this method, 45 to 55 per cent of the original cholesterol is recovered.

Table 1 is presented to indicate the precision and accuracy of the method.

Table 1

ACCURACY AND PRECISION IN ISOLATING AND COUNTING C^{14}-CHOLESTEROL OF KNOWN SPECIFIC ACTIVITY

Counting method	Mg/sample	Cpm	μc/g exp.	μc/g standard calc.	% of theoretical activity*
Liquid scintillator	0.225	7	0.051	0.054	94
	0.725	31	0.060	0.052	115
	0.725	28	0.055	0.050	110
	0.750	26	0.049	0.053	93
	0.800	24	0.049	0.054	91
	1.325	40	0.046	0.049	94
	1.350	43	0.045	0.050	90
	1.375	50	0.050	0.054	93
Flow counter	0.210	12	0.051	0.054	94
	0.445	25	0.050	0.054	93
	0.320	20	0.056	0.054	104
	0.335	16	0.043	0.054	80

* Standard deviation equals 10 per cent.

The advantages of this procedure are: 1) a rapid and convenient method for the isolation of free and esterified cholesterol of suitable radiochemical purity; 2) a dibromination procedure applicable to semi-micro amounts of sterol; 3) use of a colorimetric instead of a gravimetric method for determining specific activity; and 4) preparation of cholesterol in a form suitable for liquid scintillation or direct counting.

STEROID α-NAPHTHYLURETHANS*

By

H. Werbin, R. Bookchin, and A. Palm†

A brief investigation of the stability of steroid α-naphthylurethans was undertaken to determine their possible usefulness in steroid isolation work. Cholesterol α-naphthylurethan was chosen as the model compound. It is stable at room temperature in concentrated ammonium hydroxide and in ethanol saturated with dry hydrogen chloride gas. The urethan is also stable in refluxing glacial acetic acid and can be recovered unchanged after heating in ethanolamine at 100°.

In refluxing ethanolamine the urethan decomposes to cholesterol. All the steroid α-naphthylurethans that were prepared could be cleaved by refluxing with sodium methoxide in methanol. Cholesterol, β-cholestanol, Δ^5-pregnenolone and dehydroepiandrosterone were obtained in yields of 66, 69, 57, and 38 per cent from their respective urethans. In addition to the free steroid, the α-naphthylurethan of methyl alcohol was isolated by the cleavage. The dissociation of the steroid urethans appears to proceed via a nucleophilic attack of the methoxide ion on the carbonyl carbon of the urethan with the elimination of the o-steroid anion. The chemical characteristics of the derivatives are presented in Tables 1 and 2.

Examination of the absorption bands and extinction coefficients (Table 2) reveals that the N-H band appears to be sensitive to the position of substitution of the α-naphthyl group, while the urethan C=O band remains essentially unaffected, appearing in a range slightly higher than that characteristic of the steroid C=O band.(1) In the urethan of desoxycorticosterone, the bands ascribed to the C=O at carbon 3 and carbon 20 are both displaced from 1660 and 1718 cm^{-1} in the free steroid to 1653 and 1718 cm^{-1} in the urethan.

The C—O stretching mode cannot be assigned unequivocally. It may be identified with the strong bands appearing near 1200 and/or 1250 cm^{-1} in all of the urethans that were studied. The frequencies associated with the α-naphthyl group represent merely some characteristic rather than all the bands ascribed to the aromatic group.(2)

EXPERIMENTAL

α-naphthylurethans 1 to 5 were prepared in the following manner: One-half ml of dry benzene containing 200 mg of the steroid, a slight molar excess of α-naphthyl isocyanate, and 10 μl of anhydrous pyridine were refluxed for 1 hour in a centrifuge tube. The steroid -naphthylurethan was precipitated and washed with a total of 8 ml of ligroin. It was crystallized from the appropriate solvents. One ml of dry ligroin was used instead of 0.5 ml of

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* Based on a paper that appears in the Journal of the American Chemical Society, 77: 4431 (1955).

† Illinois Institute of Technology, Chicago, Illinois.

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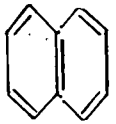
Table 1

STEROID α-NAPHTHYLURETHANS  —NHCOOR

	Derivatives	M.P. (°C*)	Yield (%)	Recrystallization solvent	Formula	Carbon, %		Hydrogen, %	
						(Calcd.)	(Found)	(Calcd.)	(Found)
1	Dehydroepiandrosterone	234-235	97	Benzene-ethyl alc.	$C_{30}H_{35}O_3N$	78.74	78.44	7.71	7.92
2	Etiocholane-17-β-ol-3-one†	229-231	91	Benzene-ethyl alc.	$C_{30}H_{37}O_3N$	78.39	78.06	8.12	8.22
3	Δ^5-Pregnenolone	222-223	95	Acetone	$C_{32}H_{39}O_3N$	79.14	79.17	8.09	8.21
4	Desoxycorticosterone	215-218	84	Acetone	$C_{32}H_{37}O_4N$	76.95	76.94	7.47	7.55
5	Testosterone	258-259	100	Toluene-ethyl alc.	$C_{30}H_{35}O_3N$	78.74	78.55	7.71	7.95
6	β-Cholestanol	156-157	75	Ligroin	$C_{38}H_{55}O_2N$	81.81	81.57	9.94	9.86
7	Cholesterol	169‡	100	Ligroin					

* A Fisher-Johns apparatus was used for the determinations of the melting points, which are uncorrected.
† We are grateful to F. Sondheimer, Syntex Corp., for a gift of this steroid.
‡ V. T. Bickel and H. E. French, J. Am. Chem. Soc., 48:747 (1926), report 160°.

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Table 2

CHARACTERISTIC ABSORPTION BANDS (cm^{-1}) AND EXTINCTION COEFFICIENTS OF STEROID α-NAPHTHYLURETHANS

	Molar extinction coefficient EtOH λ_{max} 290 mμ	N—H (Stretching)	C=O (Stretching)	Naphthyl group				
1*	7950	3432w†	1740vs	775vs		1493vs	1533vs	1597w
2	7200	3275s	1733vs	764vs	793vs	1501vs	1532vs	1597w
3	7100	3407s	1730vs	770vs	789vs	1505vs	1553vs	1584m
4	6250	3306s	1740vs	789s	808s	1504vs	1523vs	1602w
5	6100	3290s	1727vs	768vs	794vs	1502s	1534vs	1612m
6	6600	3456m	1739vs	772vs	793vs	1493vs	1532vs	1562m
7	8600	3487m	1738vs	768s		1491s	1525s	1603m

* Numbers refer to compounds listed in Table 1.
† Estimated intensities are indicated by w = weak, m = medium, s = strong, vs = very strong.

benzene for the preparation of the steroid α-naphthylurethans 6 and 7. The urethans were washed with a total of 4 ml of ligroin.

Cleavage of steroid α-naphthylurethans was accomplished as follows: Thirty mg of the urethan was refluxed at 78°C with 2.5 ml of 3.5 N sodium methoxide for from 30 min. to 1 hour. The steroid was isolated by means of a digitonin precipitation and obtained free in the usual manner by pyridine cleavage of the digitonide. From the nondigitonin precipitable fraction, the α-naphthylurethan of methyl alcohol was isolated and recrystallized twice from ligroin; the melting point ranged from 121 to 122°C, and the mixed melting point with authentic urethan, 122 to 124°C.

A model DU Beckmann spectrophotometer was used for determination of the ultraviolet spectra. The infrared spectra of the steroid derivatives, mulled in nujol, were recorded with a Perkin-Elmer model 21 spectrometer equipped with a sodium chloride prism.

ACKNOWLEDGMENT

We are grateful to Roger A. Pickering for obtaining the infrared data for us.

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INCORPORATION OF ADENOSINE-5'-PHOSPHATE INTO RIBONUCLEIC ACID*

By

E. Goldwasser

Since we have shown that cell-free preparations of pigeon liver that incorporate adenine into ribonucleic acid (RNA)[1] can also convert added adenine into adenosine-5'-phosphate (AMP),[2] it was pertinent to determine whether the mononucleotide was a precursor of the polynucleotide. Previous work indicated that mononucleotides were not as efficient as adenine in RNA formation by intact animals,[3] and that surviving tissue slices did not incorporate 5' nucleotides into RNA.[4] A recent report, however, seems to implicate nucleoside-5'-diphosphates in RNA biosynthesis by extracts of micro-organisms.[5]

AMP labeled with C^{14} in the 4 and 6 positions of the adenine moiety was isolated from the pooled acid-soluble nucleotides derived from the viscera of mice that had been injected with adenine-4,6-C^{14}.† The purified AMP had no detectable contaminant by paper chromatography and assayed 99-100 per cent AMP by enzymic deamination. Its specific activity was 46,000 cpm per μM.

Incubation of pigeon liver homogenate with the test compound was done under the conditions previously described, except that the homogenate was centrifuged for 5 minutes at 500 g and only the supernatant was used.[1] Comparison of AMP and adenine as precursors of RNA yielded the results summarized in Table 1.

Table 1

INCORPORATION OF AMP-4,6-C^{14} INTO RNA

Each flask had 6 ml of a 20 per cent homogenate, incubated for 2 hrs under air at 36°C with labeled precursors. RNA was obtained as a mixture of mononucleotides after alkaline hydrolysis.

Precursor	μ M	% of added cpm in RNA	RNA (cpm/mg)	R.S.A.* x 10^4
Adenine-8-C^{14}	0.8	0.26	1900	3.1
AMP-4,6-C^{14}	2.1†	0.24	107	8.2

* Relative specific activity = $\frac{\text{specific activity RNA adenine}}{\text{specific activity precursor adenine}}$.

† Includes endogenous AMP.

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* To appear as a Letter to the Editor in the Journal of the American Chemical Society.

† The author is indebted to Dr. E. L. Bennett of the University of California Radiation Laboratory for generous gifts of the crude acid-soluble nucleotides and a sample of adenine-4,6-C^{14}.

From these data, AMP may be considered to be at least as effective a precursor of RNA as is adenine in this system.

Evidence that AMP is incorporated into RNA with the ribose-phosphate bond intact was obtained using P^{32}-labeled AMP prepared by Eggleston's method.[6] The resulting AMP, which was chromatographically homogeneous, had a specific activity of 5×10^6 cpm per μM, and assayed 99 to 100 per cent AMP enzymically. The experiment was carried out in the same manner as that described in Table 1, but the alkaline hydrolyzate was separated into its constituent mononucleotides by ion-exchange chromatography.[7] The results are summarized in Table 2.

Table 2

INCORPORATION OF AMP^{32} INTO RNA MONONUCLEOTIDES

Incubations as in Table 1, with 6 μM of adenine-4,6-C^{14} (A) or with 2 μM of AMP^{32} (B).

Isolated nucleotide	Specific activity (cpm/μM)	
	A	B*
Cytidylic acid	26	2780
Adenylic acid	48800	720
Uridylic acid	390	5100
Guanylic acid	1760	3060

* There was one, as yet, unidentified fraction isolated that contained a significant amount of P^{32} in experiment B that was not present in experiment A.

These data demonstrate that the AMP was actually incorporated into the framework of the RNA molecule because the P^{32} was recovered, for the most part, in non-adenine containing nucleotides. This is so because after alkaline hydrolysis the phosphorous which was incorporated into RNA from the 5' nucleotide should be found esterified with the 3' position of the adjacent nucleoside residue. The differences in specific activities indicate strongly that the P^{32} was not incorporated as inorganic phosphate split off the AMP.

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