ACRH-6

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

Semiannual Report to
THE ATOMIC ENERGY COMMISSION

SEPTEMBER 1956

LEON O. JACOBSON, M.D.
Editor

ANTREEN PFAU
Associate Editor

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STUDIES ON THE MECHANISM OF POSTIRRADIATION PROTECTION*

by

E. L. Simmons, L. O. Jacobson, and J. Denko

Experimental data obtained during the past 2 years have stimulated a re-evaluation of the concept of how partial-body shielding and the injection of living cells bring about the recovery of animals that have been subjected to lethal amounts of radiation.

That lead-shielding of the spleen, spleen transplants, and injections of spleen, embryo liver, and bone marrow cell suspensions save the lives of mice following lethal X irradiation is well established by the work of Jacobson *et al.*^(1,2) and Lorenz *et al.*⁽³⁾ Later findings, using these agents, indicate that cells from one species are successful in protecting animals of another related one (e.g., guinea pig or rat marrow to irradiated mice).

Because, at first, it was difficult to believe that cells from one genus could survive in the body of another, the search was directed toward the isolation of a factor(s) in the reticulo-endothelial tissue that hastened the regeneration of undamaged primitive cells already present in the body of the irradiated animal. However, it was demonstrated that the capacity to produce antibodies to an injected particulate antigen is retained in irradiated animals if they are spleen-shielded during irradiation.⁽⁴⁾ Further work by Dixon and Maurer⁽⁵⁾ showed that when irradiated animals are injected with a foreign protein during the refractive immunologic state that follows exposure to radiation, the animal develops a tolerance to the antigen and thereafter will not produce antibodies to it. Hence, it was possible to accept the fact that injected cells can enter an immunologically-altered host to thrive and re-colonize the irradiation-depleted body.

Barnes and Loutit^(6,7) have reported studies indicating that although isologous cell injections confer long-lasting protection, homologously-treated animals die shortly after the initial 30-day period. These findings have been substantiated in our laboratory and by Trentin⁽⁸⁾ and Congdon and Urso.⁽⁹⁾ They postulated that the injected cells protect the hosts until the time that the immunologic response recovers in the irradiated animals. At this time, the foreign cells are destroyed.

We investigated this problem of late death by injecting irradiated CF No. 1 female mice with bone marrow cells from nonirradiated DBA/2 mice. The animals that survived for 24 days or longer were killed when moribund. Histologic preparations of bone marrow were made at the time of death and could be classified as follows: 1) all cells very immature; 2) some immature cells with others showing myeloid differentiation; and 3) cells

* Summary of a paper presented at the Fifth International Conference on Radiobiology, August 15-19, 1956, Stockholm, Sweden. The full text will appear in the Symposium Volume.

showing myeloid and erythroid differentiation. On this basis it might be assumed that one or both series are not maturing or that they are maturing but are being released very rapidly into the peripheral circulation.

All of the specimens of spleen that were examined gave evidence that myelopoiesis and at least minimal erythropoiesis were underway. Although other organs showed a variety of pathologic conditions, no single cause of death could be identified.

Many independent lines of evidence point in the direction of cellular colonization. Lindsley and his co-workers⁽¹⁰⁾ injected bone marrow from rats of one blood type into irradiated rats of another type and found that the donor cell appeared in the peripheral blood and eventually predominated. Nowell *et al.*⁽¹¹⁾ used the alkaline phosphatase test to show that the bone marrow of the mouse, which is typically negative, gives the positive reaction of the rat in mice that were irradiated and treated with bone marrow from the rat.

We have used the sensitivity to lymphatic leukemia to test whether foreign cells may invade the body of a host and function. The experimental subjects were the CF No. 1 mouse, which is insensitive to P-1534 leukemia, and the DBA/2 mouse, which is highly susceptible.

Groups of CF No. 1 female mice were exposed to 750 r X radiation and were then injected intravenously with 5 million DBA/2 bone marrow cells in 0.5 ml of Locke's solution or with 5 million (CF No. 1 x DBA/2)F₁* hybrid bone marrow cells.

One month after X irradiation, the survivors were divided into two groups. The animals in one received an intraperitoneal inoculation of DBA/2 leukemic spleen cells and those in the other, an injection of normal DBA/2 spleen cells. Survival was then compared 30 days after these inoculations (Table 1).

Cells from DBA/2 or (CF No. 1 x DBA/2)F₁ hybrid mice increased the susceptibility of the irradiated CF No. 1 mice to DBA/2 leukemic cells. This suggests that the altered immunological response is due to colonization which confers receptivity to leukemia. The continued sensitivity to leukemia under these circumstances has been observed as late as 68 days after the initial irradiation and injection.

The transfer of sensitivity to leukemia has also been used as a tool to learn how an X-irradiated animal, which has been colonized by foreign cells, responds immunologically to re-X irradiation and subsequent injection of cells from its own strain. CF No. 1 mice were injected with DBA/2 bone marrow cells following irradiation. The survivors were irradiated 28 days later and then injected with cells from their own strain. Twenty-eight days later, the living mice were divided into two groups. The animals in one were then inoculated with DBA/2 leukemic cells while those in the other were given normal DBA/2 spleen cells. Thirty days thereafter, all of the animals given leukemic cells were dead, whereas 86 per cent of the control animals were still alive. It thus appears that the original colonization by the DBA/2 cells had not been destroyed completely or that the immu-

* The injection of leukemic cells into 53 of these hybrids resulted in 100 per cent mortality.

Table 1
THIRTY-DAY MORTALITY IN CF NO. 1 FEMALE MICE
FOLLOWING LEUKEMIC STRESS

Injection material	X-ray exposure (r)	Stress	Mice (no.)	Mortality (%)
CF No. 1 bone marrow	750	Leukemia	23	17.4
DBA/2 bone marrow	750	Leukemia	8	75
DBA/2 bone marrow	750	Normal spleen	19	26.3
Hybrid bone marrow	750	Leukemia	10	100
Hybrid bone marrow	750	Normal spleen	7	14.3
CF No. 1 bone marrow	600	Leukemia	23	0
DBA/2 bone marrow	600	Leukemia	28	78.6
DBA/2 bone marrow	600	Normal spleen	20	30
DBA/2 adult spleen cells	600	Leukemia	17	70.6
DBA/2 adult spleen cells	600	Normal spleen	18	11.1

nologic receptivity that had been conferred by the DBA/2 cells was still in effect.

Ford and his colleagues⁽¹²⁾ have obtained some of the most impressive proof of cellular colonization in irradiated animals. Using a translocated chromosome, they have shown that the defective chromosome can be found in the bone marrow, spleen, and even the thymus of the irradiated host that had been injected with these genetically-tagged cells. Although the bone marrow has not been considered to be a very likely site for antibody production, Askonas and White⁽¹³⁾ have reported that the bone marrow may have antibody-forming properties. In any event, this evidence that cellular colonization spreads to other more likely production sites helps to explain how the immunologic response pattern of the irradiated survivor comes to resemble so completely that of the animal from which the injected cells were obtained.

We do not know whether cellular colonization is involved initially or is maintained by divergent species. In our laboratory, irradiated rabbits injected with mouse cells have lived for more than a year and did not undergo the acute postirradiation failure observed when mice, rats, and guinea pigs were studied.

It is of interest that the mouse cells injected into the irradiated rabbit had no appreciable effect on the hematologic recovery, which is the case when mouse cells are injected into irradiated mice.

In our search for a test to learn whether the rabbit retains its own antibody-forming capacity, use was made of the fact that the rat does not form antibodies (precipitins) to such soluble antigens as bovine serum albumin although the rabbit does. Rabbits were exposed to 900 r and then injected with liver cells from newborn rats. The survivors were

injected with soluble antigen, including a booster dose one month later. When the rabbit serum was tested by the antigen-antibody ring test, a positive reaction typical of the rabbit indicated that antibody had been formed. It would seem then that the rat cells had not taken over the control of antibody sites of production.

Irradiated CF No. 1 mice that were injected with a variety of cells from chicken embryos and young chicks usually died by the 30th day after irradiation. Blood smears from the few survivors taken at intervals during the first year after irradiation revealed no cells of avian origin. It would thus appear that the relationship is perhaps too distant to permit cell growth and colonization.

Additional investigations will be needed to clarify the immunity reactions that have been observed, the possible secretory role of the injected cells, and their transformations into other cell types. Regeneration of lymphoid elements, especially the thymus, does not seem to keep pace with that of the granulocyte series, and may represent a different mechanism. Jaroslow and Taliaferro⁽¹⁴⁾ have demonstrated the restoration of the antibody-forming capacity in the irradiated rabbit when antigen is mixed with cell-free preparations of splenic tissue, yeast autolysate, etc., prior to injection. Their findings suggest that some substance derived from living cells may enter into the reaction. Living cells produce an effect by their work. The challenge that now faces the radiobiologist is to elucidate the functional role of the colonized cell.

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A PRELIMINARY COMPARISON OF THE THERAPEUTIC RESULTS
OBTAINED BY COBALT TELETHERAPY, 2-MEV X RADIATION,
AND 200-250 KV X RADIATION*

by

J. W. J. Carpender and D. J. Lockman

The authors have been provided an unusual opportunity to compare the response of malignant disease to two different supervoltage machines and to correlate the results with those obtained with conventional therapy in the 200- to 250-kv range. This has been made possible by the installation of a 2-Mev Van de Graaff electrostatic generator and a revolving radiocobalt teletherapy device at the Argonne Cancer Research Hospital⁽¹⁾ in conjunction with the Radiology Department of the University of Chicago. The period of observation has been much too short and the number of patients treated far too few to permit conclusions regarding altered survival. However, experience gained in such matters as technique and palliative effects are of interest at this time.

EQUIPMENT

The 2-Mev electrostatic generator is ceiling-mounted with vertical motion and with angular motion in excess of 90 degrees. This range of motion, combined with movements of the treatment couch, the original treatment chair, has been found to be entirely adequate. The rotating chair, which had been supplied with the generator, was dismantled and rebuilt into a couch for convergent beam therapy. Treatment distance has been 100 cm from target to skin, with the exception of a few patients (seminoma) for whom a longer field was desired than that of the 27-cm size obtainable at this distance. The output of 75 r per min at 1 meter (hvl, 12 mm Cu) compares with the output at 50 cm from 250-kv equipment at hvl 2.8 Cu and 70 r per min at hvl 1.3 mm Cu.

The installation was completed and the first patient treated in April, 1953. Since that time, 165 patients have been treated with this unit.

The radiocobalt revolving unit is a relatively specialized instrument. It is designed primarily for moving field therapy in which the source of radiation is carried around the patient who is lying prone or supine with the tumor located at the axis of rotation. It has proved equally useful for fixed field therapy and, in point of fact, has been substituted for the Van de Graaff generator during breakdown periods. The arrangement of angular motion of the source at a fixed radius of rotation usually facilitates the positioning of the patient. The greatest field size is 17 x 17 cm at the source face to axis distance of 81.6 cm.

* Version of a paper invited for publication in the Holthusen birthday issue of Strahlentherapie.

Treatment at any other source to "tumor" distance is, although possible, not practical.

The prone or supine position of the patient is convenient for most applications, much more so than had been anticipated at the time of construction. A plaster or plastic half-shell is of value in immobilizing the patient for treatment of head and neck tumors. If the shells are saved, a stock which meets most patient contours is accumulated. With either moving or fixed field therapy, it is preferable to place the patient in the prone position when treating posteriorly because the skin sparing effect of the high energy radiation is lost if the treatment beam passes through the couch even though low density material is used in the construction.

While the face of the source is only 0.75 cm in diameter, in order to minimize penumbra, the length is 3.0 cm which gives relatively large self absorption. The initial temporary source of 600 curies provided a treatment rate of the order of 10 r per min, making the treatment time uncomfortably long for the patient and severely limiting the number of patients who could be accommodated. The shortcomings have been circumvented by the installation of a 1200-curie source of 105 curies per g. A new source of 2500 curies will be installed shortly. The installation was completed and the first patient treated in April, 1954. Since the device was placed in use, 92 patients have been treated.

TREATMENT POLICY

For obvious reasons, the selection of patients was limited to those who met the following criteria:

- 1) in whom the effects of radiation could be followed on the basis of some established clinical guide;
- 2) in whom the tumor was so sensitive that the dosage could be carried well beyond that which was adequate without encroaching on that which was dangerous;
- 3) in whom only palliation could be expected by conventional methods.

Unlike the skin reaction, the mucosal reaction is not eliminated by supervoltage, and head and neck lesions therefore satisfied quite well the first criterion. Testis seminoma and the lymphoma group of diseases satisfied the second. For the third criterion, there is always a large number of patients in any active radiotherapy department for which palliation is a very significant goal. One exception was made in the case of extremely massive patients with uterine malignancies in whom an adequate dose could never have been achieved with conventional therapy.

Prompted by reports of a lesser relative biological efficiency of higher energy radiation,⁽²⁾ we increased our dosage rate and, initially, our total dosage by a factor of 10 per cent over that with which we were routinely familiar at 250 kv. Most of the patients were treated at a rate of 1000 rads weekly. Subsequently, doses up to 9600 rads were given into the tumor-bearing tissues in a small group of patients with what were thought initially to be excellent results. A few patients have received very rapid treatment at moderately high dosage (e.g., 4000 rads in 2 weeks) with very encouraging results.

RESULTS

It must be emphasized again that this is a preliminary report. The distribution of patients and results are listed in Table 1. Specific lesions of particular interest or larger numbers are shown separately. Successful treatment results have been divided into two groups:

- 1) those with a good primary result and local clinical cure in whom permanent control of the disease is probable;

- 2) those in whom the disease has not been eradicated but who have been able to return to relatively normal life free of disability for a period of time.

Included in the failures are those dying of intercurrent disease during treatment or those who could not complete treatment because of post-operative complications or the early appearance of distant metastases.

In common with the experience of others, ⁽³⁻⁶⁾ we found some advantages in supervoltage as compared to 250-kv radiation. Higher dosage can be given (although we do not agree with advocates of extremely high dose levels) and higher dosage rates can be used with less discomfort to the patient. The skin reaction is either nonexistent or limited to a mild erythema unless, of course, the skin is internal to the surface of the block of radiated material, as when the radiation passes through a bolus or through the top of the treatment couch. Epilation does occur and lasts about as long as with 250 kv. Salivary gland function is repressed in those patients with mouth and upper pharynx lesions where the radiation must pass through this gland. Relative dryness of the mouth may last as long as after 250-kv treatment, but the associated discomfort of the mucosal reaction subsides much more rapidly. The systemic reactions of radiation sickness and bone marrow suppression, as evidenced by changes in the peripheral blood, are considerably less for comparable fields and dose rates. Bone and cartilage necrosis occurred only when the dosage was increased considerably above limits accepted as safe with conventional 250-kv or telerradium therapy. Table 2 lists all dosages above 6500 rads and the effects obtained. It would appear that any advantages derived from doses exceeding 700 rads are gained at a considerable price.

Mouth and pharynx. Reactions on the mucosal surfaces occurred at about the same time-dose level as would have been anticipated at 250 kv, but were more circumscribed in extent and more readily controlled by modifications of the fields. Radiocobalt was thought to show a slight advantage over 2-Mev X radiation in this respect, but the difference was very small and probably due to variations in dosage. The severity of the reaction in those patients treated with excessively high levels was greater than that following 250 kv but was generally well-tolerated.

Results decidedly superior to those of our previous experiences were obtained in cases of extensive lesions of the tonsil. There was local eradication of the disease in 5 of 7 patients. With 250-kv radiation, these lesions would have been considered beyond the possibility of control without the production of severe skin reactions. Decreased bone absorption is undoubtedly an important factor in these patients, reduction of mandibular damage and increased tumor dose being the result. In one patient, there was sequestration of sev-

Table 1

THE DISORDERS AND RESULTS OBTAINED IN PATIENTS TREATED WITH THE 2-MEV ELECTROSTATIC GENERATOR OR THE RADIOCOBALT REVOLVING UNIT

Disease or region	2-Mev X rays				Radiocobalt			
	Number of patients	Good primary result-? cure	Successful palliation	Failure	Number of patients	Good primary result-? cure	Successful palliation	Failure
Vocal cords								
Early	13	12	-	1	1	1	-	
Late	5	2	2	1	2		2	
Head and neck	28	6	15	7*	33	11†	15	7*
Lung	31		17	14‡	12		9	3
Central nervous system	7		6**	1‡‡	2		2	
Lymphoma	18		16	2	3		2	1
Testis								
Seminoma	4	4						
Other	2			2				
Uterus	11	6	3	2	5	5		
Metastases	16		9	7	6		2	4
Others	23		17	6	28	1	20	7
Totals	158	30	85	43	92	18	52	22

* One death from intercurrent disease included in each instance.

† One extensive carcinoma, gingival margin, local cure 4000 rads per 4 weeks, asymptomatic 14 months.

‡ Including 3 cases persistent esophagitis and 5 cases unable to complete therapy.

** One thalamic glioma after 4500 rads per 2 weeks rapidly became asymptomatic and remained so (2 years).

‡‡ Pinealoma.

Table 2

EFFECTS OF DOSAGES ABOVE 6500 RADS IN TISSUES

2-Mev X rays			Radiocobalt		
Dose (rads)	Cancer site	Results	Dose (rads)	Cancer site	Results
7400	Maxillary sinus	recurrence + necrosis	9600	Tonsil	necrosis
7100	Hypopharynx	persistent cancer	9000	Vallecula	recurrence + necrosis
7000	Maxillary sinus	local cure	8600	Epiglottis	local cure, necrosis
7000	Tongue	local cure	8600	Hypopharynx	local cure-fatal necrosis
6700	Mesopharynx	local cure	8300	Tongue	necrosis
6500	Bronchus	severe esophagitis, metastases	8100	Tongue	local cure, well
6500	Hypopharynx	recurrence	8000	Floor mouth	local cure, well
			8000	Esophagus	esophagitis, dysphagia
			8000	Floor mouth	necrosis
			7500	Epiglottis	necrosis + recurrence
			7500	Tongue	necrosis + recurrence
			7100	Esophagus	local cure
			7000	Vocal cord	7-month edema, cured
			7000	Gingiva	persistence
			7000	Bronchus	good palliation
			7000	Tonsil	sequestrum, local cure
			7000	Esophagus	good palliation
			6800	Gingiva	recurrence

eral fragments of bone from the alveolar margin of the mandible after treatment, but this was presumably incidental to pretreatment dental extraction rather than a direct result of the radiation. Before treatment of bone, we have continued the practice of having teeth extracted that are in the zone that is to be irradiated.

Larynx. Treatment of cancer of the vocal cords is entirely satisfactory at 250 kv, yielding, in our series, in the neighborhood of 90 per cent cures (5 yr.), if the disease has not advanced beyond stage 1. Nevertheless, a wet skin reaction is usual and a laryngeal edema not infrequent even with a 6-week course at 900 rads per week. We have used two opposing fields of about 25 cm² each when treating at 250 kv, unmodified by wedging or bolus. The same arrangement was employed at 2 Mev and has been started with the cobalt unit. The dose rate was later increased to 1000 rads per week and the total dose to 6000

rads, midline on the axis of the beam. The development of mucositis closely paralleled that expected from the corresponding 250-kv therapy. No skin reaction developed beyond a minimal dry scaling, and the laryngeal edema was less marked and more transient except in one patient in whom the dose was carried to 7000 rads and in whom the edema persisted for 7 months.

Three patients with extensive lesions of the extrinsic larynx were treated with dosage levels above 7000 rads, and all three developed necrosis of the cartilage.

Lung. Among the patients for whom only palliative therapy could be given, those with bronchial cancer constitute a large enough group to warrant separate consideration. The natural course of the disease from the time of first symptoms to death is brief enough so that a short-term evaluation of results has some meaning. With few exceptions, the tumor is so well-advanced that the fields must include at least the mediastinal lymphatics in addition to the primary lesion. The dosage is readily carried to 6000 rads in 6 weeks or less with 3 or more fixed fields without discomfort to the patient or complications. At this level, there was no improvement in survival, and, because of the frequent occurrence of a persistent esophagitis, over-all palliation was considered inferior. The dosage has therefore been limited to 5000 rads. Although the survival results appear to be no better than may be obtained with 250 kv, the treatment is simpler to plan and administer and is considerably more tolerable for the patient.

Lymphomas. Because of the limited dosage required for the treatment of regional adenopathy in patients suffering from diseases of the lymphoma group, supervoltage is of advantage only in treating certain mediastinal and deep pectoral, intra-abdominal, or localized pelvic masses. Peripheral adenopathy, especially of the neck, is better treated with 250-kv X radiation because the mucosal reaction to the more penetrating radiation produces much discomfort in the esophagus and airways.

SUMMARY AND CONCLUSIONS

1. Minimal skin and systemic reactions to supervoltage radiation enhance patient comfort. Mucosal reactions, although similar in character and development to those from 250-kv radiation, are more localized and better controlled.
2. Carcinocidal dosage, that is otherwise impossible, may be achieved in patients of great body size.
3. The chance of missing the lesion geographically that is inherent in elaborate treatment plans is reduced by the simplification of treatment that is made possible by means of high energy radiations.
4. Tumors that respond to 2-Mev X rays and to radiocobalt γ rays are the ones that respond to conventional radiation.
5. Traditional dosage limits cannot be exceeded greatly without producing damage. On the basis of clinical studies, it is our current impression that to achieve the same effect as with 250-kv radiation, the dose at 2 Mev, as currently measured, should be 10 per cent and the dose for radiocobalt 15 per cent greater.

6. Radiocobalt is ideal from the designer's standpoint as a source for moving field therapy. In clinical use, it is sometimes convenient but never essential provided the ability to use adequate multiple fields is at hand.
7. Shorter treatment courses may perhaps be possible without the sacrifice of patient comfort or reduction in the number of cures.
8. Supervoltage therapy is no more satisfactory than 200- to 250-kv therapy for lesions requiring only moderate dosages and not situated deep in the body.
9. Supervoltage is undesirable in some circumstances because of reactions produced by the higher energy radiation in structures lying beyond the lesion.

Many of the statements given above do not apply to moving field therapy in the supervoltage range.

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THE EXPERIMENTAL PRODUCTION OF ARRHYTHMIAS BY SELECTIVE DESTRUCTION OF THE CARDIAC CONDUCTION SYSTEM*

by

R. W. Harrison,[†] P. V. Moulder,[†] W. E. Adams,[†] and P. V. Harper

INTRODUCTION

At the present time, cardiac surgery is directed primarily toward the treatment or correction of congenital and acquired structural and circulatory defects of the heart. Little attention has been given to the feasibility of surgical correction of rhythm disturbances and defects in the conduction system. This study was undertaken in an attempt to further our knowledge of the anatomy and physiology of the cardiac conduction system. A series of experiments was performed to destroy selectively portions of the conduction system and to observe the resulting effect on cardiac rhythm. The preparations in turn provide experimental material upon which medical and surgical therapy may be developed and evaluated.

The anatomy of the conduction system based on gross dissection and histologic studies has been described in detail by numerous workers.⁽¹⁻⁷⁾ A histochemical technique that facilitates an accurate demonstration and a microscopic delineation of the limits of the conduction system and permits an evaluation of the extent of the lesions produced was used for the investigation that is the subject of this paper.

Observations on the function of the conduction system by early investigators were made frequently on perfused heart preparations or on the heart in situ under acute conditions. These observers utilized techniques of determination of initial negativity,⁽⁸⁻¹²⁾ application,⁽¹³⁾ and acute destruction of areas of the conduction system. The latter was accomplished by cautery,⁽¹⁴⁾ application of sclerosing substances,⁽¹⁵⁻¹⁷⁾ and excision, ligation, or crushing of the tissues.⁽¹⁸⁻²⁶⁾ In the following experiments, well-defined defects in the conduction system were produced by radiation from β -ray point sources. In contrast to earlier studies, these were gradual in production and chronic in nature, the animals being observed during the period of development of the lesion and for long periods of time thereafter.

METHODS

Radiation sources were implanted into 18 mongrel dogs. Prior to surgery, each animal was given 30 mg of morphine sulfate and then anesthetized with positive pressure ether-oxygen. The exact details of the operative technique vary, depending on the portion

* This study was aided by grants from the Damon Runyon Memorial Fund and the Douglas Smith Foundation for Medical Research.

† The Department of Surgery, The University of Chicago.

of the conduction system to be irradiated. Routine right thoracotomy with incision through the fourth interspace was employed throughout.

The sinus node region was exposed by a pericardial incision parallel to the phrenic nerve. Pockets of various material (free pericardial graft, free aortic wall homograft, 0.010-inch polyethylene sheeting) were fashioned to cover generously the area of the right atrium that contained the sino-atrial node. The pockets were sewn to the surface of the atrium on the posterior wall at the cavo-atrial junction and along the sulcus terminalis with 5-0 silk sutures. The radioactive pellets were inserted into the pockets in contact with the atrial wall and were spaced at 5- to 6-mm intervals. Five to 8 pellets were used, depending on the size of the animal.

Hypothermia (24 to 26°C) and complete circulatory interruption with the employment of acetyl choline to render the heart essentially asystolic were utilized to permit a right atriotomy and visualization of the area of the interatrial septum containing the atrio-ventricular node. Four to 5 radioactive pellets were implanted subendocardially, spaced approximately 5 mm apart, in the area between the ostium of the coronary sinus and the septum membranaceum.

Preoperative and serial postoperative standard and augmented unipolar limb lead electrocardiograms were obtained on all animals.

The point source radiation consisted of yttrium oxide pellets containing 0.5 to 1.5 mc of Y^{90} activity. The pellets each weighed 2 to 4 mg and were 0.5 mm in diameter and 1 to 1.5 mm in length. Details of their preparation have been described.⁽²⁷⁾ The radioactive pellets were placed in lead-shielded 17-gauge spinal needles. The tip of the needle was inserted into the atrial tissue to the desired location and the pellet then ejected by means of the obturator of the needle. These sources are β emitters with a half-life of 62 hours. The β rays have a maximum range in tissue of approximately 1 cm. The areas of necrosis produced by these point sources are sharply demarcated and are approximately 8 mm in diameter.

The histochemical technique utilized to demonstrate and study the location of the specialized muscle tissues that make up the cardiac conduction system have been described in detail.⁽²⁸⁾ The technique depends upon incubation with choline esters and demonstration of the byproduct of cholinesterase activity.⁽²⁹⁾ Routine histologic studies were made by means of Gomori's trichrome method.⁽³⁰⁾ Histochemical studies were performed on a series of normal hearts as well as on those in which lesions had been produced to determine the location and extent of the lesions.

RESULTS

The conduction system in the heart of the dog can be characterized enzymatically. The enzyme, which is localized in the myofibrils of the specialized cardiac muscle fibers, was invariably found to be a cholinesterase. The boundaries of the conduction system have a sharp outline, and no transition is seen between the specialized muscle fibers and the cardiac muscle cells.

The enzymatic reaction was most pronounced and strikingly constant around a branch of the right coronary artery, which is located along the axis of the sino-atrial nodal tissue (Figure 1). Homologous fields on consecutive sections show that the reaction develops in delicate fibers woven loosely into a whorl of anastomosing strands, which lie around the artery passing through the crista terminalis. In defining the limits of the sino-atrial node, a positive zone was identified that occupied a portion of the posterior wall of the right atrium and was found to extend inferiorly along the sulcus terminalis to a point near the junction of the inferior vena cava with the right atrium.

Figure 1. Photomicrograph of a section through the sino-atrial node. The results of a positive enzymatic reaction can be seen surrounding the small artery in the crista terminalis. Mag., X 17.

The atrio-ventricular node was found to occupy a position in the lower portion of the interatrial septum between the ostium of the coronary sinus and the septum membranaceum (Figure 2). As this tissue extends forward into the septum membranaceum, the bundle of His is formed, which divides into its right and left branches at the anterior portion of the septum. Another area of positive enzymatic reaction has been identified surrounding the medial portion of the coronary sinus ostium.

Sino-atrial node. Total ablation of the sino-atrial node, as evidenced histochemically by absence of enzymatic activity, results in only minimal permanent rhythm disturbances and electrocardiographic alterations. The changes are as follows: (1) Early in the postoperative period of observation, disturbances in rhythm are quite variable and unpre-

dictable. 2) A nearly constant early finding is the diminution in amplitude or disappearance of the P wave. 3) The P wave has been observed to be reduced markedly in potential within 24 hours after the implantation of the pellets and has been noted to be absent as early as 48 hours postoperatively. 4) Not uncommonly there is a marked reduction in cardiac rate during this early period. 5) In those cases where the P wave remains visible, the P-R interval shows a definite shortening from a control value of 0.09 - 0.12 second to 0.06 - 0.08 second. 6) During the first 2 to 3 weeks following the implantation of the radioactive material, there may be intermittent periods of sino-auricular block or auricular standstill

Figure 2. Photomicrograph of a section through the atrio-ventricular node. The darker area is an indication of the positive enzymatic reaction in the nodal region. Mag., X 18.

with associated beats of "nodal" origin or so-called "escape beats." 7) Frequently, in the early period, there is an absence of the P wave, which is associated with a normal-appearing QRS complex and a non-phasic arrhythmia; in other words, a rhythm more "sinus" than "nodal." 8) In 2 to 3 weeks, a permanent pattern is established with a rate approximating that of the control but with decreased amplitude of the P wave and a shortened P-R interval. Figures 3 and 4 illustrate these electrocardiographic changes.

Atrio-ventricular node. Radioactive Y^{90} pellets implanted in the region of the atrio-ventricular node did not always produce an atrio-ventricular heart block. In the animals in which heart block developed, the disturbance in rhythm was first manifested by a prolongation of the atrio-ventricular conduction time represented by a prolonged P-R interval.

DOG 785

Control Rate 130 PR .12

2 days postop. Rate 120 PR .08

7 days postop. Rate approx. 72 PR 0

LEAD II

9 days postop. Rate approx. 78 PR 0-.08

2 weeks postop. Rate approx. 66 PR .07-.08

1 mo. postop. Rate approx. 132 PR .08

Figure 3. Electrocardiographic tracings following the implantation of five 0.5-mc Y^{90} pellets in the region of the sino-atrial node.

2 days postoperative—Essentially normal EKG except for reduction of PR-interval from 0.12 to 0.08 sec.

7 days—Marked arrhythmia. P waves absent. Intermittent "escape beats."

9 days—Marked arrhythmia. P waves seen only occasionally. QRS complex of normal configuration.

2 weeks—P wave constantly present with P-R interval reduced to 0.07 to 0.08 sec.

1 month—Permanent rhythm established. Note decreased amplitude of P wave and decreased P-R interval.

DOG 669

Control Rate 114 PR .10

3 days postop. Rate 120 PR .08

LEAD II

16 days postop. Rate 84 PR 0-.06-.08

34 days postop. Rate 102 PR .08

Figure 4. Electrocardiographic tracings following the implantation of seven 1-mc Y^{90} pellets in the region of the sino-atrial node.

3 days postoperative—Essentially normal EKG except for reduced amplitude of P wave and reduction of P-R interval from 0.10 to 0.08 sec.

16 days—Frequent "escape beats" with shortening and variability of P-R interval.

1 month—Permanent rhythm established. Note decreased amplitude of P wave and decreased P-R interval.

Often this P-R interval would be variable in duration before becoming constantly prolonged; and later, about 1 week postoperatively, complete heart block would develop with its typical atrio-ventricular dissociation. Figure 5 shows excerpts of serial electrocardiographic tracings of one animal in which a permanent complete heart block was produced. Complete heart block has been observed to be permanent in those cases in which it developed.

Figure 5. Electrocardiographic tracings following the implantation of four 1-mc Y^{90} pellets in the region of the atrio-ventricular node.

- 4 days postoperative—The P-R interval is variable ranging from 0.10 to 0.19 sec. signifying a fluctuating degree of heart block.
- 6 days—A constant first degree heart block with a P-R interval of .40 sec. Due to the prolonged P-R interval the P wave is frequently located within the T wave of the preceding beat.
- 7 days—Complete heart block.
- 1 month—Complete heart block.
- 7 months—Complete heart block.

DISCUSSION

Disturbances in rhythm observed in the "early period" during progressive destruction of the sino-atrial node, e.g., sino-atrial block, auricular standstill, the frequent "escape beats," are similar to the changes one would expect with removal of the "pacemaker" of the heart. Other workers, basing their conclusions on experiments in which the destruction or functional depression of the sino-atrial node has been relatively sudden, believed that the resulting rhythm arose from the coronary sinus or ventricular portion of the atrio-ventricular node. When these observations were made over a longer period of time, the permanent rhythm that was established displayed P-R intervals that were shorter in duration than normal, and they concluded that the permanently established rhythm had its origin in the coronary sinus.^(10,20,31) A small upright P wave and a shortened P-R interval were also obtained when the slow, radiation-induced destruction of the sino-atrial node was

being observed. This is in contrast with the observations of Scherf⁽³²⁾ who depressed the activity of the sino-atrial node with vagal stimulation and increased temperature in the coronary sinus area thereby producing a "coronary sinus rhythm" characterized by deep inverted P waves preceding a normal QRS complex.

The true site of impulse formation in these hearts is not readily apparent. It must be concluded on the basis of the positive character of the P wave that the site of impulse formation in these hearts is located above the coronary sinus and that the direction of spread of the impulse over the right atrium is normal. Nevertheless, the constant decrease in P-R interval suggests the location of the site of origin nearer the atrio-ventricular node than is normal.

It is felt that the slow destructive process used in these experiments simulates changes that occur in most disease processes in which the sino-atrial node is affected. Embolism or sudden thrombosis of the nutrient vessels is, of course, an exception. The difficulty with which sino-atrial block and auricular standstill are produced by gradual ablation of the sino-atrial node corresponds with the arrhythmias in conditions other than those associated with acute drug toxicity.

The inconstancy in our ability to produce heart block by implantation of the pellets in the region of the atrio-ventricular node is very likely attributable to a number of factors. For example, every fiber of the atrio-ventricular system must be affected to produce a conduction disturbance; and as long as any fibers exist, conduction will not be impaired because of auxotonic phenomena. Although it is possible to place the pellets quite accurately, slight variations in the location of the atrio-ventricular node would result in incomplete destruction and, consequently, no impairment of conduction.

In the animals in which atrio-ventricular block develops, the first interference with atrio-ventricular conduction is manifested in the variable prolongation of the P-R intervals. As interference with conduction becomes more complete, the P-R interval becomes constantly prolonged, and with complete destruction of the atrio-ventricular connection, a complete atrio-ventricular heart block develops.

CONCLUSIONS

Beta radiation emanating from small radioactive yttrium-90 oxide pellets provides a means of gradually creating well-localized defects in the conduction system of the heart.

Gradual and complete ablation of the sino-atrial node results in transient rhythm disturbances, e.g., auricular standstill, sino-atrial block, and nodal rhythms; but almost invariably, the permanent rhythm is characterized by an upright P wave of decreased potential and a shortened P-R interval.

The site of origin of the cardiac impulse following ablation of the sino-atrial node is not known.

Permanent complete atrio-ventricular heart block may be produced by application of local β radiation in the region of the atrio-ventricular node.

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THE MECHANISM OF GLUTATHIONE DESTRUCTION AND PROTECTION
IN DRUG-SENSITIVE AND NON-SENSITIVE ERYTHROCYTES.
IN VITRO STUDIES.*

by

E. Beutler, M. Robson, and E. Bittenwieser

An acute hemolytic anemia develops in certain individuals when they are given the anti-malarial drug primaquine or some of the related aniline derivatives, such as sulfanilamide and acetanilid.^(1,2) Although the sensitivity is rare among Caucasians, it is found in about 10 per cent of the American Negroes.⁽³⁾ It has been shown that the sensitivity to the hemolytic action of primaquine is due to an abnormality of the red blood cells of the sensitive subjects.⁽⁴⁾ It has also been demonstrated that the drug sensitivity is not another manifestation of some previously-described disorder.⁽⁵⁾ There is evidence showing that only the older members of a red cell population are affected by the action of the drug,⁽⁶⁾ which suggests that the response may be due to a deficiency in an enzyme system. Measurement of the reduced glutathione (GSH) content of drug-sensitive cells revealed that they were deficient in this compound.⁽⁷⁾ It was found that a fall in the average red cell GSH concentration preceded the major portion of the hemolytic episode, but that as hemolysis progressed, the average red cell GSH again reached pre-treatment levels.⁽⁸⁾ On this basis, it seemed reasonable to assume that GSH might have an important part in the drug-induced hemolysis, and hence, this further study of the destruction of red cell glutathione was undertaken.

The sensitivity of most of the subjects was established by their reaction to 30 mg of primaquine given to them daily at some time prior to this investigation. Samples from Caucasian donors were regarded as non-sensitive and are referred to as being "non-sensitive (Caucasian)." Others, for which it was necessary to establish their sensitivity by in vitro means⁽⁹⁾ are referred to as "sensitive (by test)."

The method for testing the stability of red cell glutathione has been described.⁽⁹⁾ After the GSH content of the blood sample was measured, 1-ml aliquots of the well-oxygenated sample were added to test tubes containing 5 mg of acetylphenylhydrazine. GSH determinations were made after the mixture had been incubated for 2 hrs at 37°C.

Reduced glutathione was determined by a modification⁽⁹⁾ of the method of Grunert and Phillips.⁽¹⁰⁾ A modification of the method of Dohan and Woodward⁽¹¹⁾ was used to establish the total glutathione content (GSSG) of the samples.

Dialyzed destromatized hemoglobin solutions were prepared according to a modification of the method of Drabkin.⁽¹²⁾ Methemoglobin was prepared by suspending the red cells

* Summary of a paper that has been accepted for publication in The Journal of Clinical Investigation.

in 1 per cent sodium nitrite for one-half hour before preparing the Hb solution. Cyanmethemoglobin was prepared by adding 0.1 per cent sodium cyanide to the red cells suspended in 1 per cent sodium nitrite before preparing the Hb solution. Carboxyhemoglobin was formed by passing carbon monoxide through the dialyzed, destomatized hemoglobin solution. "Acetylphenylhydrazine-modified hemoglobin" was formed by incubating 1-ml aliquots of blood with 5 mg of acetylphenylhydrazine before washing with the sodium chloride solutions. Dialysis was continued for 48 hrs. A Beckman Direct Transmittance Recording Spectrophotometer, Model DK, was used to measure the absorption spectra in the range of 325 to 800 $m\mu$.

The substrates and coenzymes that were used were obtained for the most part from various commercial suppliers. The sodium salts were prepared immediately prior to use.

When 1-ml aliquots of the blood from 5 sensitive and 7 non-sensitive individuals were incubated with 5 mg of acetylphenylhydrazine, the GSH content of the red cells of the non-sensitive individuals changed very little whereas that of the sensitive individuals fell markedly (Figure 1).

To determine the fate of the GSH of the sensitive red cells incubated with acetylphenylhydrazine, 1-ml aliquots of blood from a sensitive (by test) subject were incubated with

Figure 1. The results of the GSH stability test carried out on blood samples from 5 sensitive and 7 non-sensitive individuals. One-ml aliquots of blood were incubated with 5 mg acetylphenylhydrazine at 37°C for 2 hours. GSH concentration was measured before and after incubation.

5 mg of acetylphenylhydrazine. The GSH concentration fell rapidly, but the GSSG showed an initial rise. The total glutathione concentration at first fell only slightly which indicated that most of the destroyed GSH had been oxidized to GSSG. After one hr, the GSSG concentration also began to decline. Thus, there was a gradual decline in total glutathione over the 3-hr period.

The degree of oxygenation of the sensitive red cell influences the destruction of its GSH content by acetylphenylhydrazine. Oxygen, carbon dioxide, or carbon monoxide was passed through 3 aliquots of blood from a nonsensitive individual and through 3 aliquots from a sensitive subject. The stability of the GSH content of the nonsensitive cells was not altered appreciably by any of these gases, whereas that of the sensitive cells was markedly influenced. Carbon monoxide and carbon dioxide were found to have a marked protective effect on the GSH level of sensitive cells exposed to acetylphenylhydrazine, a finding which suggested that hemoglobin might be an important factor in mediating the reaction between acetylphenylhydrazine and GSH.

To determine if this were the case, 1 ml of a 307 mg per cent (10 mM) solution of GSH was added to tubes containing 9 cc of varying concentrations of oxyhemoglobin, methemoglobin, cyanmethemoglobin, and carboxyhemoglobin. The GSH content of 1-ml aliquots from the various mixtures was determined before and after incubation with acetylphenylhydrazine at 37°C for 2 hrs. The results of these experiments are shown in Table 1. When GSH and acetylphenylhydrazine are mixed in pure form there is no evidence of destruction. However, the addition of concentrations of oxyhemoglobin to the system results in destruction of GSH. Methemoglobin destroys only a moderate amount of GSH, and the presence of acetylphenylhydrazine has only a slight additional effect. Cyanmethemoglobin and carboxyhemoglobin were found to be relatively inert. The data in Table 1 indicates that the recovery of GSH in the presence of large amounts of some of the hemoglobin compounds was considerably less than 100 per cent.

Oxyhemoglobin modified by acetylphenylhydrazine exhibited the same order of destructiveness toward GSH as did acetylphenylhydrazine in combination with 8 g per cent oxyhemoglobin. The addition of carbon monoxide and of 98 mg per cent sodium cyanide failed to modify the effect.

When blood samples are allowed to stand at room temperature for 24 hours, nonsensitive red cells lose their capacity to protect their GSH against the action of acetylphenylhydrazine. However, this loss of protective capacity is prevented by the addition of excess glucose to the blood.⁽⁹⁾ Further experiments demonstrated that glucose is required for the protection of GSH by nonsensitive cells and that even after incubation for 4 hours without glucose, nonsensitive cells recover their capacity to protect GSH when glucose is made available to them. A number of other substrates were tested for their ability to enable the red cell to protect its GSH. The results are illustrated in Figures 2 and 3. The data demonstrate the capacity of nonsensitive cells to utilize glucose and inosine in protecting their GSH and their incapacity to protect significant quantities of GSH in the presence of lactate, pyruvate, malate, fumarate, and ribose. Sensitive cells only partially protected their GSH

Table 1

THE EFFECT OF SEVERAL HEMOGLOBIN COMPOUNDS ON GSH WITH AND
WITHOUT ACETYLPHENYLHYDRAZINE

Hemoglobin compound	Hemoglobin concentration* Grams per cent	Mg per cent GSH prior to incubation	Mg per cent GSH after 2 hours incubation without acetylphenylhydrazine	Mg per cent GSH after 2 hours incubation with 5 mg acetylphenylhydrazine
Control	0	30.7	31.5	30.9
Oxyhemoglobin	.45	30.1	-	29.7
Oxyhemoglobin	1.8	27.2	-	19.5
Oxyhemoglobin	7.2	25.1	20.3	8.8
Methemoglobin	7.2	26.6	16.9	14.9
Cyanmethemoglobin	7.2	30.3	28.7	25.4
Carboxyhemoglobin	7.2	24.7	22.9	24.5
Acetylphenylhydrazine-modified hemoglobin	6.2	25.8	7.4	-

* Measured as cyanmethemoglobin.

Figure 2. The effect of substrate on the results of the GSH stability test carried out on erythrocytes from a nonsensitive (Caucasian) subject. The red cells were suspended in saline-phosphate buffer, pH 7.0. The final concentration of substrates used were: glucose .02M; glucose .02M with methylene blue .00005 M; inosine .02 M; lactate .04 M; pyruvate .04 M; malate .04 M; fumarate .04 M; and ribose .02 M. Only the glucose and inosine were effective substrates for the protection of GSH. The logarithmic scale emphasizes small differences at low GSH concentrations, a range in which the precision of the method is very limited.

content with glucose and inosine substrates. Methylene blue did not overcome the defect of the sensitive cells.

Further studies were made of the enzymatic basis of the protection of red cell GSH in a hemolyzed system because of the impermeability of these cells to phosphorylated sugars⁽¹³⁾ and to the enzymes and co-enzymes involved in their metabolism. The results of experiments in which hemolysates from both nonsensitive (Caucasian) and sensitive donors were used are shown in Table 2. No consistent difference could be found between the GSH stability of hemolysates of sensitive and nonsensitive cells. Both types of hemolysates protected their glutathione when glucose-1-phosphate, glucose-6-phosphate, 6-phosphogluco-

Figure 3. The effect of substrate on the results of the GSH stability test carried out on erythrocytes from a sensitive subject. The red cells were suspended in saline-phosphate buffer, pH 7.0. The final concentration of substrates used were: glucose .02 M; glucose .02 M with methylene blue .00005 M; inosine .02 M; lactate .04 M; pyruvate .04 M; malate .04 M; fumarate .04 M; and ribose .04 M. Only the glucose and inosine were effective substrates for the protection of GSH. The greater degree of protection of GSH observed than in the GSH stability test carried out on whole blood is attributed to differences in the properties of the suspended medium. The logarithmic scale emphasizes small differences at low GSH concentrations, a range in which the precision of the method is very limited.

nate, fructose-6-phosphate, inosine, fructose diphosphate, ribose-5-phosphate, ATP, DPNH, TPNH, and DPN were used as substrates. In addition, there was no consistent difference between the protection afforded by sensitive and nonsensitive cells under these experimental conditions. Irregularities in the results are probably explained by the impure nature of the substrates used.

On the basis of the data obtained from the experiments outlined above and the findings of others, it appears to the authors that the fall in the GSH concentration of sensitive cells

Table 2

GSH STABILITY IN HEMOLYSATES

		Conditions: Each hemolysate preparation contains the following final concentrations: packed cell hemolysate 25 per cent; nicotinamide .02 M; <u>added</u> GSH 50 mg. per cent.			
Experiment#		1	2	3	4
Donor		Sensitive (by test)	Non-sensitive (Caucasian)	Non-sensitive (Caucasian)	Sensitive
GSH concentration of hemolysate preparation before incubation		53.4 mg %	55.8 mg %	57.4 mg %	64.0 mg %
Final substrate concentration (100% purity and yield assumed except for TPNH and DPNH).		Per cent of GSH recovered after 4 hrs. incubation of 1-ml aliquots of hemolysate preparation with 5 mg acetylphenylhydrazine.			
Substrate or coenzymes					
None (control)	0	34	41	24	33
Glucose	.008 M	36	51	26	30
Glucose-1-phosphate	.008 M	-	-	103	90
Glucose-6-phosphate	.008 M	110	105	96	91
Glucose-6-phosphate	.004 M	-	-	71	-
6-phosphogluconate	.008 M	111	106	100	-
Fructose-6-phosphate	.008 M	108	56	57	22
Fructose-1,6-diphosphate	.008 M	63	80	71	63
Inosine	.025 M	96	103	-	-
Inosine	.008 M	-	-	91	85
Ribose-5-phosphate	.008 M	-	104	52	65
Ribose	.008 M	-	-	23	30
DPNH	.0015 M	-	103	-	-
DPNH	.0020 M	-	-	72	83
DPN	.0026 M	-	-	-	50
TPNH	.0007 M	-	48	-	70
TPNH	.0020 M	-	-	68	-
ATP	.009 M	-	-	90	58

may be explained as follows: upon incubation with acetylphenylhydrazine, the oxyhemoglobin of red cells is altered to a product that oxidizes GSH. In cells from normal individuals, the GSSG formed is reduced rapidly to GSH through the action of TPN which is reduced when glucose-6-phosphate and phosphogluconate are oxidized. In sensitive cells, a defect in glucose-6-phosphate oxidation limits TPN reduction,⁽¹⁴⁾ both directly and indirectly by restricting the amount of phosphogluconate available for further oxidation. The GSSG formed is therefore not reduced effectively and some of it is changed to hydrogen sulfide.⁽¹⁵⁾

Additional studies are necessary to elucidate the role of GSH in the economy of the red cell.

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PROTEINS IN MULTIPLE MYELOMA. VI. CRYOGLOBULINS.*

by

F. W. Putnam and A. Miyake

Cryoglobulins are rare serum globulins that have in common the unique property of precipitating, crystallizing, or gelling upon cooling.⁽¹⁾ The presence of these proteins is a nonspecific indication of a disease process. They are detectable only in sera from patients with multiple myeloma or cryoglobulinemia or related diseases that exhibit syndromes of vascular and renal involvement, anemia, and hyperproteinemia. In patients with cryoglobulinemia, the physical properties of the cryoglobulins are such that chilling the extremities results in temperature sensitivity, high blood viscosity, purpura, thromboses, strain on the circulatory system, and possibly death induced by the intravascular deposition of the abnormal protein.⁽¹⁻³⁾

A comparison of the physical properties of a few of the cryoglobulins that have been isolated reveals that there is no uniformity.⁽³⁻¹⁰⁾ However, such a comparison is based upon a variety of reports, each dealing with a single protein studied under a particular set of conditions. Thus, in order to establish the relationship of cryoglobulins to normal plasma components and to each other, eight highly purified specimens were studied by physicochemical methods and by N-terminal amino acid analyses. All of the cryoglobulins differed from normal serum proteins in two or more of the following properties: crystal form, solubility, isoelectric point, electrophoretic mobility, sedimentation constant, ultracentrifugal or electrophoretic homogeneity, and N-terminal amino acid residues. The same properties served to differentiate all but two of the cryoglobulins from each other. Since it is unlikely that at least seven different cryoglobulins occur naturally in normal serum, we believe that the cryoglobulins that we have investigated are unnatural proteins which are individually specific.⁽³⁾

The purified globulins or sera containing them were obtained from several sources.[†]

* Summary of a manuscript that was invited for publication in the Archives for the Linderström - Lang Festschrift.

† Sera containing cryoglobulins (designated Th, Ag, etc.) were kindly supplied by the following physicians: Dr. Charles B. Higgins, Ben May Laboratory for Cancer Research, University of Chicago (Th); Dr. Steven A. Schwartz, Hektoen Institute for Medical Research, Cook County Hospital, Chicago, Illinois (Ag and Mi); Dr. Elliott F. Osserman, Dept. of Medicine, College of Physicians and Surgeons, Columbia University, New York City (Se); and Dr. F. W. Gunz, Pathology Department, Christchurch Hospital, Christchurch, New Zealand (Gu). Dr. Jan Waldenström, University of Lund, Lund, Sweden, supplied a cryoglobulinemia serum not fully reported on herein (Me). Purified cryoglobulins previously described in the literature^(2,9) were obtained in an amount of about 100 mg each from Dr. David Barr and Miss Ella Russ, Dept. of Medicine, Cornell University Medical College (R and I), and from Dr. A. S. McFarlane, National Institute for Medical Research, Mill Hill, London, England (WK). A diagnosis of

The serum concentration of the cryoglobulins ranged from 3 to 12.7 g per cent. The normal globulins were diminished both relatively and absolutely. In one case (WK), the cryoglobulin comprised 98 per cent of the total globulin. The variability in solubility made it impossible to use any uniform procedure for purifying the cryoglobulin. In general, the purification was accomplished by a modification of the method of Lerner and Greenberg.⁽⁴⁾ Two cryoglobulins (R and I) were purified by cold ethanol fractionation, another (WK) by salt fractionation.

Electrophoresis was carried out at 1°C with the Pearson or Model H Spinco apparatus. * Area analyses and mobility calculations were performed as described previously.⁽⁶⁾ Mobilities corrected to 0° are given for the descending boundary and are expressed in units of $10^{-5} \text{ cm}^{-2} \text{ sec}^{-1} \text{ volt}^{-1}$ designated . Univalent buffers of 0.1 ionic strength were employed.⁽¹¹⁾ Acetate buffer at pH 4.65 was used for electrophoresis of some globulins that were insoluble at 1° in Veronal buffer at pH 8.6 and/or 4.7 are given in Table 1, which also gives the isoelectric points for some. Representative electrophoretic diagrams of

Table 1
PHYSICAL CONSTANTS OF HUMAN PLASMA CRYOGLOBULINS*

Protein	Crystal form	pI	Mobility at 1°C		S _{20,W}
			pH 8.6	pH 4.7	
			$10^{-5} \text{ cm}^{-2} \text{ sec}^{-1} \text{ volt}^{-1}$		S
Normal γ_2		7.2	-1.3	3.0	6.6
Th		7.5	-1.1	3.0	6.6
Ag		6.4	-1.1		6.6
Mi	Needles		-1.3		6.7
R	Hexagons		Insoluble	3.0	7.6 [†] , 11
Gu			Insoluble	3.3	6.0
Se			-0.8		6.4
WK		5.5			7.18 [†] , 28
I			Insoluble	3.3	7.6 [†] , 11

* Mobilities and sedimentation constants were determined in 0.1 ionic strength Veronal buffer pH 8.6 or 0.1 ionic strength acetate buffer, pH 4.7. S_{20,W}, given in Svedberg units (S) is extrapolated to infinite dilution except for ultracentrifugally heterogeneous proteins. pI signifies the isoelectric point.

† Major component. All values so indicated are data of other authors obtained from analysis with turbine driven ultracentrifuges instead of the Spinco apparatus.

multiple myeloma was given in all cases except WK which resembled Waldenström's "macroglobulinemia"⁽¹⁰⁾ and Gu for which a diagnosis has not been established although the complaint has persisted for some years.

* Some of the ultracentrifugal and electrophoretic analyses were done by Miss Gloria Rasch.

purified cryoglobulins are shown in Figure 1.

Analyses of sedimentation velocity were accomplished by means of a Spinco analytical centrifuge. All of the sedimentation constants are corrected to the water basis at 20° (S_{20}^W) and are expressed in Svedberg units (Table 1). The sedimentation constants of proteins in the range of $S_{20}^W = 6.6$ S are corrected to infinite dilution by a relationship derived from the slope of the sedimentation constant plotted against the protein concentration curve determined previously at room temperature for cryoglobulin Th and for normal gamma globulin.⁽⁶⁾

Figure 1. Electrophoretic diagrams of purified cryoglobulins. Ag, Th, and Se migrated at pH 8.6 at a current of 16 ma for 273, 157, and 223 minutes, respectively. Gu was analyzed at pH 4.7 with photographs taken after 152 minutes migration at the same current. Note the sharpness of the boundaries except for Gu, which was photographed after diffusion overnight. Although the mobility is low, homogeneity is indicated by the sharpness of the boundaries after electrophoresis for up to four hours.

The amino terminal residues of the proteins were identified and estimated by a series of procedures based upon the fluorodinitrobenzene (FDNB) method of Sanger.⁽¹²⁾ In the early part of this investigation, the DNP-amino acids were separated and tentatively identified by chromatography at pH 6.5 on buffered silica gel. Later, the chromatographic separation was begun on buffered celite and completed on buffered silica gel. Solvent systems that contained various mixtures of methyl ethyl ketone and chloroform (5-95, 15-85, and 30-70 v/v) were used for the silica gel columns. Ethyl acetate was used for the celite. The quantity of DNP-amino acid was estimated from the light absorption measured in 1 per cent NaHCO_3 at 350 $m\mu$ in a Beckman spectrophotometer.⁽¹²⁾ Values cited in the literature for

the recovery of the various DNP-amino acids were assumed to apply in the calculation of the molar ratios, and the protein content of the DNP-cryoglobulins was taken as 75 per cent.⁽¹³⁾ The tertiary amyl alcohol paper chromatography system of Blackburn and Lowther⁽¹⁴⁾ and the toluene and decaline-glacial acetic systems of Biserte and Osteux⁽¹⁵⁾ were used to confirm the identification of the terminal groups. Further confirmation was obtained by subjecting the DNP derivatives to cleavage by hydrolysis in sealed tubes with barium hydroxide at 105° and then identifying the free amino acids by means of paper chromatography.⁽¹⁶⁾

No evidence was found for the cleavage of the N-terminal amino acids of either normal or abnormal serum globulins during the reaction with FDNB although this has been reported.⁽¹⁷⁾ The data obtained from analysis of eight cryoglobulins and normal gamma globulin are given in Table 2.

Table 2

N-TERMINAL GROUPS AND IMMUNOLOGICAL CLASSIFICATION OF
HUMAN PLASMA CRYOGLOBULINS*

Protein	Immunological group	N-terminal groups		Serine	Unidentified
		Aspartic acid	Glutamic acid		
		<u>Moles per 160,000 g</u>			
Normal γ_2	I	<u>1.12</u>	<u>1.75</u>	0.11	0.29
Th	I	<u>1.80</u>	0.16		
Ag	I	<u>2.04</u>			
Mi		<u>2.06</u>	0.16		
R		<u>2.00</u>	<u>2.64</u>		
Gu	I	<u>2.29</u>	<u>2.21</u>		
Se	I		<u>2.65</u>		
I		0.15	<u>2.77</u>		
WK		0.12	<u>3.10</u>		

* The major end groups are underlined.

The agar diffusion method of Ouchterlony was used by Dr. Leonhard Korngold* to perform the immunological analyses for us. His findings indicate that myeloma globulins can be classified according to antigenic structure into three major groups, all of which cross react with an antiserum against normal gamma globulin. The nature of the cross reaction, however, suggests that the myeloma globulins lacked antigenic determinants present in the gamma globulins of the human serum Fraction II. The specimens from our laboratory were

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all classified as group I (Table 2) although there was little correlation with electrophoretic mobility at pH 8.6. Precipitin lines caused by γ_2 formed a faint spur at the site of fusion with the precipitin lines caused by cryoglobulins Ag and Th. The spur formation may be attributed to the fact that the cryoglobulins are antigenically more deficient than γ_2 globulin.

The crystallizability of the cryoglobulins may be taken as a qualitative index of their homogeneity. This is borne out by the electrophoretic and ultracentrifugal diagrams shown in Figures 1 and 2. Comparable peaks were observed in the mobility region of the cryoglobulin in the sera that were subjected to electrophoretic analysis. Some of these patterns indicated an almost total absence of other globulins. Hence, by means of a purification procedure based on the characteristic property of gelation in the cold it was possible to prepare highly purified cryoglobulins for physicochemical study and end group analysis.

Figure 2. Sedimentation diagrams of purified cryoglobulins. All photographs taken at 16-minute intervals at a rotor speed of 59,780 r.p.m.

On the basis of the physical constants (Table 1), the cryoglobulins that we studied cannot be placed in a single group or even into several related groups. The isoelectric points varied from pH 5.5 to 7.5 in the samples for which complete pH mobility curves were obtained. However, all of the cryoglobulins appeared to migrate in the general range of gamma globulin, either at pH 4.7 or 8.6 or at both pH's when two analyses were made. The single sharp migration boundary that was obtained upon electrophoresis (Figure 1) was evidence that these proteins had greater electrical homogeneity than normal human γ_2 globulin. Five of the cryoglobulins sedimented with a single sharp boundary with no indication of heavier components (Figure 2). In two, a small proportion of the S_{9-11} component that is characteristic of normal gamma globulins was seen. One protein was a macroglobulin with a sharp major peak having an S_{20} of 18 S. The diversity of these findings is in agreement with those cited in the literature. (1-10)

Normal human gamma globulin has been reported to contain both N-terminal aspartic and glutamic acids.⁽¹⁸⁻²⁰⁾ Evidence has also been given for the existence of myeloma globulins that have only N-terminal aspartic acid.⁽¹⁸⁾ It has been suggested⁽¹⁸⁾ that normal human gamma globulin consists of at least two components; one having only N-terminal aspartic acid (an N-aspartyl globulin), and the other, only N-terminal glutamic acid (an N-glutamyl globulin). Since this suggestion, five types of myeloma globulins have been described:⁽²⁰⁾ i.e., those that contain 1) only N-terminal aspartic acid (5 specimens); 2) only N-terminal glutamic acid (5); 3) both N-terminal aspartic and glutamic acids (3); 4) only N-terminal leucine or a related amino acid (2); and 5) a heterogeneous group of proteins (6). The cryoglobulins that are listed in Table 2 fit the first three categories. Three N-aspartyl globulins have about two moles of N-terminal aspartic acid and are nearly devoid of N-terminal glutamic acid. Two cryoglobulins have both types of end-groups in about the ratio of normal globulin fractions but in twice the normal amount. Three cryoglobulins have 3 N-terminal glutamyl groups and are almost devoid of N-terminal aspartic acid. On this basis, the cryoglobulins can be differentiated from the normal pooled human globulins. The cryoglobulins that were studied lacked the minor quantities of N-terminal serine and unidentified groups that are characteristic of the heterogeneous normal gamma globulin. Therefore, both in amino end-groups and in physical properties, the cryoglobulins are more homogeneous than the customary ethanol fractions of gamma globulin from normal pooled human sera.

The data indicate that all of the specimens, with the possible exception of the macroglobulin WK, are closely related to normal human gamma globulin. It may be that the differences in mobility are attributable to the carbohydrate content as is the case with certain myeloma globulins.⁽²¹⁾ In addition, a difference in end-groups owing to number, amidation, or type might lead to a chance difference of two or three groups per mole, which is sufficient to produce appreciable mobility changes near the isoelectric point. None of these factors, however, adequately explains the origin of the insolubility in the cold, the unique property of these proteins.

It is possible that at least seven cryoglobulins occur normally in trace amounts in human serum, each available to be elaborated singly in the disease at the expense of all other globulins. However, to us it seems more likely that each cryoglobulin is an unnatural protein, the product of an abnormal cell. This hypothesis should be given particular consideration in view of the fact that the selfsame plasmacytoma that produces cryoglobulins is involved in the formation of Bence-Jones proteins. An understanding of the profuse and selective synthesis of abnormal globulins may help to elucidate the problem of normal serum globulin formation.

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EVALUATION OF TRITIUM CHOLESTEROL AS A TRACER IN MAN^{*†}

by

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This paper describes an investigation that was undertaken to evaluate the use of cholesterol-t as a tracer in man. A 60-year-old male patient with chronic rheumatoid arthritis was injected intravenously with a mixture of 33.8 μ c cholesterol-t and 4.24 μ c 4-C¹⁴-cholesterol dissolved in sterile human plasma with propylene glycol. Plasma free cholesterol and five urinary steroids were isolated and in each, the ratio of the specific activities of tritium to C¹⁴ was determined by means of a liquid scintillation counter.

The findings demonstrated that exogenous cholesterol is a precursor of three urinary adrenal steroid metabolites, pregnan-3- α -17- α -triol-11,20-dione (THE), pregnan-3- α -11- β -17- α -21-tetrol-20-one (THF), and etiocholan-3- α -ol-11,17-dione (11-ketoetiocholanolone) in vivo. The data also showed that cholesterol-t is sufficiently stable to be used as a tracer in man for catabolic studies involving that sterol.

MATERIALS AND METHODS

All melting points were taken on a Fisher-Johns apparatus.

Tracers: Cholesterol-t was prepared by an exchange reaction with THO.^(1,2) Labile tritium was removed by refluxing the cholesterol for 2 hours with 5 per cent potassium hydroxide in methanol.⁽³⁾ After 2 crystallizations from 80 per cent alcohol, the cholesterol-t had a melting point of 146-147^o, and a specific activity of 7.3 μ c/mg. The 4-C¹⁴-cholesterol, m.p. 147-148^o, s.a. 5.0 μ c/mg, was obtained from Radioactive Products Inc., Detroit, Michigan.

Distribution of tritium in cholesterol: Cholesterol-t was diluted with "cold" cholesterol and converted to cholesteryl chloride-t by the method of Blecher and Gurin.⁽⁴⁾ The steroid melted at 97^oC after purification by chromatography on aluminum oxide⁽⁵⁾ and crystallization from acetone-alcohol. Three g were pyrolyzed by the procedure of Bloch and Rittenberg.⁽⁵⁾ After pyrolysis, the mixture of isooctane and isooctene was distilled off. The residue was chromatographed on aluminum oxide, and the C₁₉H₃₀ hydrocarbon was eluted from the column and counted.

C₁₉H₃₀. Calculated, C 88.4, H 11.6; found, C 88.4, H 11.3.

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‡ The Los Alamos Scientific Laboratory, The University of California.

One g of cholesterol-t was subjected to the Oppenauer oxidation as described by Jones *et al.*⁽⁶⁾ After chromatography and crystallization from acetone, the Δ^4 -cholestenone-t melted at 87.5-88°. The steroid was refluxed with aqueous methanolic potassium hydroxide under nitrogen for 2 hours, and was found to have the same melting point after re-isolation.

Twelve g of cholesterol-t were converted to methyl-3 β -acetoxy- Δ^5 -cholenate-t by the procedure of Fukushima and Gallagher.⁽¹⁾ After repeated crystallization from acetone-methanol, the steroid melted at 147.2-158.6° (146.5-157.5° reported).

Isolation of plasma free cholesterol: The method for the isolation and radioassay of plasma cholesterol has been described.⁽⁷⁾

Hydrolysis and extraction of the urine: Twenty-four-hour urine samples were adjusted to pH 6.5, and 75 units/ml of β -glucuronidase (Sigma) was added. Following incubation for 48 hours at 37° and the addition of 90 g/liter of anhydrous sodium sulphate, the urine was extracted continuously with ether for 2 days, and a crude neutral steroid extract was prepared according to the method of Baggett *et al.*⁽⁸⁾

Isolation and identification of urinary steroids: The paper chromatographic methods of Burton *et al.*⁽⁹⁾ and Savard⁽¹⁰⁾ were used to separate the steroids. Good separations of THE from THF were obtained when the Whatman #1 paper was impregnated with a solution containing 2 parts methanol to 1 part propylene glycol prior to application of the crude steroid mixture. A flow sheet for the separation of the steroids is shown in Figure 1. The THE and THF were assayed by the method of Chen *et al.*,⁽¹¹⁾ and the 17-ketosteroids were determined by the procedure described by Callow *et al.*,⁽¹²⁾ using stabilized alcoholic potassium hydroxide.⁽¹³⁾

The C₁₉ steroids were identified by paper chromatography in two solvent systems (ligroin-propylene glycol and ligroin-phenylcellosolve). An aliquot of each eluted steroid was converted to the acetate and was identified by paper chromatography (ligroin-formamide). Initial identification was assumed if the unknown, the authentic steroid, and the mixture had the same R_f values in all the paper chromatographic systems that were studied. At this point, carrier steroid was added, and after several crystallizations, if the specific activity remained constant, identification was considered complete, provided that the melting point of the steroid agreed with that recorded in the literature. In the case of etiocholan-3- α -ol-17-one (etiocholanolone), the infrared spectrum of a sample containing 36 per cent urinary steroid was identical with the spectrum of authentic etiocholan-3- α -ol-17-one. 11-Ketioetiocholanolone was isolated and counted as the acetate.

The procedure for the isolation and identification of THE has been described.⁽¹⁴⁾ Part of a pooled sample of THF was converted to the diacetate and was crystallized from methanol without the addition of carrier steroid. Identification of the THF was carried out in the same manner as that described for THE. The ultraviolet spectrum of the THF diacetate after 2 hours in concentrated sulphuric acid was identical with that of the spectrum of pure THF diacetate. Carrier was added to the remainder of the pooled THF diacetate, and the mixture was crystallized from methanol-ligroin (1:1). The infrared spectrum of the crys-

Crude neutral steroid extract from urine hydrolyzed with β -glucuronidase placed on 15-cm. wide sheet of paper for 7 days in Zaffaroni system

talline material (70 per cent urinary steroid, 30 per cent carrier) coincided with the spectrum of authentic THF diacetate.

Radioassay: The steroid was dissolved in 50 ml of toluene containing 100 mg of 2,5-diphenyloxazole in a tall form-weighing bottle.⁽¹⁵⁾ The solution was cooled to -20°C and counted in a Packard liquid scintillation counter. The C¹⁴ was assayed by screening out the tritium pulses by a proper choice of discriminator settings. Then, after resetting the discriminators, both the carbon-14 and tritium counts were registered simultaneously. The following equation was employed to calculate the C¹⁴ and tritium disintegrations per minute:

$$\text{DPM } C^{14} = \frac{\text{cpm } C^{14}(\text{B})}{C^{14} \text{ efficiency (B)}}$$

$$\text{DPM T} = \frac{\text{cpm (T + C}^{14}\text{) (A) - DPM C}^{14} \times \text{efficiency C}^{14}\text{ (A)}}{\text{T efficiency (A)}}$$

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Table 1

COUNTING ERRORS FOR TRITIUM AND CARBON-14 MIXTURES

Mixture prepared*	DPM T	792	Ratio T/C ¹⁴ = 3.26		
	DPM C ¹⁴	342			
Found					
T (dpm)	% Error	C ¹⁴ (dpm)	% Error	Ratio T/C ¹⁴	% Error
736	7.1	244	0.4	2.77	15.0
831	4.9	266	9.5	3.09	5.2
903	14.0	266	9.5	3.39	4.0
996	25.7	269	10.7	4.08	25.2
Av.	Mean	Av.	Mean	Av.	Mean
886	12.9	261	7.5	3.31	12.4

Mixture prepared*	DPM T	396	Ratio T/C ¹⁴ = 3.27		
	DPM C ¹⁴	121			
Found					
T (dpm)	% Error	C ¹⁴ (dpm)	% Error	Ratio T/C ¹⁴	% Error
327	13.4	130	7.4	2.50	23.5
423	9.3	131	8.3	2.97	9.2
460	16.2	136	12.4	3.40	4.0
571	44.1	146	20.7	4.39	34.2
Av.	Mean	Av.	Mean	Av.	Mean
448	21.8	136	12.2	3.31	17.7

* The same amounts of tritium and carbon-14 in four weighing bottles.

sufficiently long so that the probable error was no greater than 5 per cent.

After counting, the steroid was reisolated chromatographically on aluminum oxide or silica gel. The scintillator was eluted with benzene, and the steroid was eluted by increasing the polarity of the benzene with alcohol.

Five mg of THE diacetate had no quenching effect on the carbon or tritium that was counted. It was assumed that the other steroids behaved similarly.

RESULTS AND DISCUSSION

The distribution of tritium in the cholesterol-t as determined by several degradative procedures is given in Figure 2. The distribution of the tritium between the side chain and the tetracyclic rings is similar to that found by Fukushima and Gallagher⁽¹⁾ for deuterated cholesterol. Thus, 47.2 per cent of the tritium was located on carbon atoms 24, 25, 26, and 27. (The deuterated steroid had 52 per cent of the total radioactivity at these positions.)⁽¹⁾ There was no tritium in the hydroxyl group because the cholesteryl chloride-t had the same specific activity as the cholesterol-t from which it was prepared. Positions 2, 3, 4, and 6 had 37.5 per cent of the tritium. (The deuterated cholesterol had 40 per cent and 3 per cent of the deuterium on carbon atoms 6 and 3, respectively.)⁽⁴⁾ Since the C₁₉H₃₀-t

hydrocarbon and the methyl-3- β - Δ^5 -cholesterol-t had the same specific activity within the experimental error (Figure 2), it was concluded that tritium was not stably bound at carbon atoms 20, 21, 22, and 23. About 16.2 per cent of the tritium in the rings was located at undetermined positions.

Figure 2. Distribution of tritium in cholesterol-t.

The ratios of the tritium to C^{14} specific activities of the plasma free cholesterol that was isolated during the 10-day period following administration of the tracers are given in Table 2. The T/C^{14} ratio of the mixture of cholesterol that was administered was 8.0. The average value of the ratio in the samples of blood cholesterol that were isolated was 7.0 ± 1.0 . The 13 per cent discrepancy is probably not significant in view of the mean error of 12-17 per cent in determining the ratios of T/C^{14} . These data indicate that cholesterol-t is stable in the blood for at least 10 days. The C^{14} and tritium specific activities of the three C_{19} and two C_{21} urinary steroids that were isolated in this study are presented in Table 3. The ratios of the T/C^{14} specific activities are shown in the last column. The mean value for all the ratios was 4.0 ± 0.9 .

By multiplying the percentage of tritium found in the rings of cholesterol-t, 53.2 per cent, by the mean value of the T/C^{14} ratio of the plasma free cholesterol, one obtains 3.8, the value expected for the T/C^{14} ratio of the urinary steroids. If 8.0, the value of the ratio of specific activities of the administered cholesterol, is used, then the expected ratio for

the urinary steroids is 4.3. These calculations are based on the following assumptions: 1) that the conversion of cholesterol-t to the urinary steroids involves no loss of tritium from the rings; 2) that the tritium from positions 24, 25, 26, and 27 on the side chain is not converted to urinary steroids to any appreciable extent; and 3) that the tetracyclic rings of 4-C¹⁴-cholesterol are not degraded to smaller fragments, thereby releasing C¹⁴ for possible steroid synthesis.

Table 2
STABILITY OF CHOLESTEROL-T IN VIVO

Time after administration of tracers (days)	Plasma free cholesterol		Ratio of specific activities T/C ¹⁴
	$\mu\text{CT/mM}$	$\mu\text{C}^{14}/\text{mM}$	
0.17	0.44	0.65	6.7
0.33	0.24	0.031	7.7
2	0.30	0.034	8.8
3	0.19	0.029	6.6
4	0.30	0.053	5.7
6	0.26	0.039	6.8
8	0.20	0.025	8.0
10	0.12	0.017	6.8
	0.12	0.019	6.3

The difference between the calculated values for the T/C¹⁴ ratio and that actually found in the urinary steroids was about 7.5 per cent.

The results of adrenal perfusion⁽¹⁶⁾ and adrenal homogenate^(17,18) experiments show that cholesterol can serve as a precursor of cortisol. The data in Table 3 demonstrate that this conversion can take place in vivo since cortisol has been isolated from adrenal vein blood^(19,20) and THE, THF, and 11-ketoetiocholanolone are metabolites of this steroid.⁽²¹⁾

Ungar and Dorfman⁽²²⁾ isolated C¹⁴ androsterone and etiocholanolone from the urine of a female patient with an adrenal cortical carcinoma who had been fed 3-C¹⁴-cholesterol. The isolation of labeled androsterone and etiocholanolone in the present study suggests that under normal physiologic conditions, cholesterol is also a precursor for the 11-desoxy-C₁₉-steroids.

There is now ample evidence from tracer studies that adrenal steroids are derived from cholesterol, and from acetate. In the case of the latter precursor, it is not known whether adrenal steroids are synthesized directly from acetate or come from cholesterol produced from this precursor in the adrenal, the liver, or both.

Table 3

SPECIFIC ACTIVITIES OF CRYSTALLINE STEROIDS ISOLATED FROM THE
URINE BY CARRIER TECHNIQUE

Day urine collected	Amount isolated from urine (mg)	Carrier added (mg)	Steroid m.p. °C	Counted (mg)	Calculated by isotopic dilution		Ratio of specific activities
					mcT/mM	μcC ¹⁴ /mM	T/C ¹⁴
<u>Androstan-3-α-ol-17-one</u>							
1	1.75	17.59	186	10.77	0.11	0.21	5.2
5	1.24	10.44	185	8.30	0.0089	0.035	2.5
5 + 6	2.40	7.91	186.5	1.82	0.10	0.029	3.5
7 + 8	1.50	10.14	186-187	6.38	0.072	0.022	3.4
<u>Etiocholan-3-α-ol-17-one</u>							
1 + 2	3.18	8.60	151-152	8.05	0.078	0.020	4.0
4	1.90	5.00	151-152	4.04	0.10	0.027	3.6
5 + 6	4.92	8.72	150-151	5.25	0.12	0.025	5.3
<u>Etiocholan-3-α-ol-11,17-dione Acetate</u>							
1 + 8	1.67	9.91	161-164	6.20	0.084	0.028	3.0
<u>Pregnan-3-α-17-α-21-triol-11,20-dione Diacetate</u>							
2	3.46	10.09	232-233.5	5.01	0.087	0.017	5.2
4	2.39	7.89	234	7.60	0.062	0.024	3.9
5 + 6	4.60	4.87	232.5	3.73	0.099	0.027	3.6
<u>Pregnan-3-α-11-β-17-α-21-tetrol-20-one Diacetate</u>							
2 + 6	1.1	-	209-217	1.1	0.11	0.021	5.2
	7.64	3.75	213-222	2.69	0.12	0.024	4.9

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FACTORS INFLUENCING DIURESIS IN RATS FOLLOWING TOTAL-BODY X IRRADIATION*

by

E. I. Pentz and R. J. Hasterlik

Polyuria^(1,2) and significant decreases in the serum content of antidiuretic substances⁽³⁾ have been reported to occur in rats following total-body X irradiation. It has been suggested that the secretion of the antidiuretic hormone of the posterior pituitary is dependent upon the integrity of the anterior pituitary and the adrenal cortex.⁽⁴⁾ Mirsky and his co-workers⁽⁵⁾ have obtained good evidence that the hypothalamus is the site of origin of an extra-hypophyseal antidiuretic substance, and their findings have been supported by those of others.⁽⁸⁾

The purpose of the investigation described in this paper was to determine which endocrine mechanisms are involved in the polyuria that is observed in rats during 24 to 48 hours after total-body X irradiation.

For 5 or 7 hours during the day, for 4 days or more prior to irradiation, Sprague-Dawley rats were fasted in metabolism cages (2 per cage), but given adequate drinking water. Some groups of rats consisted of intact animals while other groups consisted of animals from which various tissues had been removed. Urine was collected in 50-ml graduate cylinders placed under the collection tube of the cage. The rats were given Rockland Mouse pellets and water ad libitum when they were not in the metabolism cages.

Unanesthetized animals were placed in individual sections of a slowly rotating aluminum carrier for exposure to X radiation, which was provided by a 250-kvp Maxitron X-ray machine, operating at 30 ma at a rate of 30 r per min. The total exposure was 400 r. The target to skin distance was 92 cm. Groups of normal and sham-operated animals were randomized on the basis of body weight and their diuretic response to X radiation was calculated (Table 1). The control volume for all of the experiments was obtained by averaging the urine output of the last 4 pre-irradiation days.[†] The first collection period was the 5 or 7 hours immediately following irradiation, the second on the following day. In each experiment, the volumes of the two collection periods were averaged and the value of the control volume was subtracted from this amount to obtain the "average difference."

The data in Table 1 reveal the type of quantitative changes occurring in urine volume following exposure to X irradiation. In these experiments, the primary concern was to

* Summary of a paper presented at the International Congress of Physiology, Brussels, Belgium, August 23 - 26, 1956, and accepted for publication in The American Journal of Physiology..

† For one group (Table 1) a 3 - day collection was used.

Table 1

DIURESIS IN RATS IN RESPONSE TO 400 r X RADIATION

Surgical Treatment	Animals (no.)	Body wt. (g)	Base line volume (ml/rat)	Exposure (r)	1st collection period (ml/rat)	2nd collection period (ml/rat)	Average Difference
Sham Adrenalectomized ^{1,2}	8	273	2.4	400	4.5*	4.5	2.1
None ²	6	240	2.6	0	2.2*	2.0	-0.5
None ²	6	279	2.7	400	3.8	6.6	2.5
Sham Hypophysectomized ³	10	194	2.7	400	6.3	6.3	3.6
Sham Hypophysectomized ³	6	191	2.6	400	5.5	9.5	4.9
Sham Hypophysectomized ³	6	182	2.7	0	2.5	3.3	0.2

* P = 0.01

1. These animals had been X-irradiated 38 and 55 days previously with doses of 100 r. They were sham-operated 67 days prior to the above irradiation.
2. The period of training to the metabolism cages in these groups was only 3 days.
3. 20 days subsequent to operation.

Table 2

URINE OUTPUT BY RATS IN RESPONSE TO 400 r X RADIATION FOLLOWING
EXTIRPATION OF THE ANTERIOR OR POSTERIOR PITUITARY

Surgical Treatment	Animals (no.)	Body wt. (g)	Post-operative day	Base line volume (ml/rat)	Exposure (r)	1st collection period (ml/rat)	2nd collection period (ml/rat)	Average Difference	P**
Anterior pituitary removed ¹	8	134	76	3.2	400	3.4	4.0	0.5	0.05
Anterior pituitary removed	6	148	76	4.4	0	2.7	5.2	0.6	
Posterior pituitary removed ²	10	250	82	6.1	400	9.7	6.2	1.8	0.001
Posterior pituitary removed	10	253	82	7.9	0	6.4	7.2	-1.1	
Posterior pituitary removed	6	175	12	3.5	400	5.8	5.2	2.0	0.01
Posterior pituitary removed	8	177	12	4.4	0	2.7	5.0	0.6	
Posterior pituitary removed,* Adrenals removed	8	173	12 7	4.2	400	4.5	4.7	0.4	0.1
Posterior pituitary removed,* Adrenals removed	8	166	12 7	5.0	0	2.5	4.9	-1.3	

* 5-hour urine collection period.

** First collection period relevant to the base line volumes.

1. Animals previously exposed to 100 r on 42 and 88 days prior to above irradiation.

2. Animals previously exposed to 100 r on 44 and 66 days prior to above irradiation.

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Table 3

URINE OUTPUT BY RATS IN RESPONSE TO 400 r X RADIATION FOLLOWING
EXTIRPATION OF THE PITUITARY OR ADRENAL GLANDS

Surgical Treatment	Animals (no.)	Body wt. (g)	Post-operative day	Base line volume (ml/rat)	Exposure (r)	1st collection period (ml/rat)	2nd collection period (ml/rat)	Average Difference
Hypophysectomized ¹	8	177	113	4.8	400	5.0	3.4	-0.6
Hypophysectomized	6	177	113	5.6	0	5.0	5.8	-0.2
Hypophysectomized	12	139	20	5.9	400	4.3	4.0	-1.7
Adrenalectomized ²	10	170	12	4.8	400	4.3	3.2	-1.1
Adrenalectomized ²	8	174	12	3.7	0	3.2	4.1	-0.1
Sham Adrenalectomized ²	10	182	12	2.5	400	9.1*	8.2	6.1
Sham Adrenalectomized ²	10	184	12	2.8	0	2.2*	2.9	0.5

* P = 0.001.

1. Animals previously exposed to 100 r on 84 and 104 days prior to above irradiation.

2. Adrenalectomized by one of us (E.I.P.).

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Table 4

DIURESIS IN RATS IN RESPONSE TO 400 r X RADIATION FOLLOWING
EXTIRPATION OF VARIOUS ORGANS AND TISSUES

Surgical Treatment	Animals (no.)	Body wt. (g)	Post-operative day	Base line volume (ml/rat)	Exposure (r)	1st collection period (ml/rat)	2nd collection period (ml/rat)	Average Difference	P*
Sham splenectomized	8	194	24	3.3	0	2.5	2.8	-0.6	0.02
None	6	170	-	2.5	400	4.6	8.5	4.0	
Adrenal medulla removed	10	176	12	1.9	400	4.3	3.6	1.9	0.01
Thyroid removed	12	155	12	2.5	400	4.8	4.1	1.9	0.01
Sham splenectomized	6	227	24	3.5	400	9.0	10.0	6.4	
Spleen removed	8	170	8	2.8	400	8.1	12.0	7.3	
Ovaries removed	10	170	8	2.1	400	7.8	6.2	4.9	

* First collection period relevant to the base line volume.

identify a significant increase in urine volume occurring in either of the first two collection periods or in both. For calculation of P the data of only the first collection period has been used. Where diuresis has persisted through the second collection period the significance of P will, of course, be considerably increased.

The anterior pituitary and the adrenal cortex were found to be of prime importance in this diuretic response (Table 3). Removal of the anterior lobe of the pituitary or of the adrenal glands was sufficient to completely inhibit polyuria. In addition, the reaction was found to be independent of the presence of the posterior pituitary because animals having only this structure removed (Table 2) were diuretic following exposure to 400 r. The effect of removal of some other tissues on diuresis following X irradiation is shown in Table 4.

The experimental findings suggest that the release of antidiuretic substances from tissues other than the posterior pituitary is under the control of the anterior pituitary and the adrenal cortex and that immediate changes in water balance are specifically related to this control.

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THE CATABOLISM OF UREA BY THE TOTAL-BODY IRRADIATED RAT*

by

J. H. Rust,[†] W. J. Visek,[‡] and L. J. Roth[‡]

In a previous communication⁽¹⁾ data on the metabolism of glucose, alanine pyruvate, and carbon dioxide by the irradiated rat were presented. It was shown that there is a reduced ability of such animals to convert glucose to urea. In the irradiated rat, alanine and carbon dioxide were anabolized^{**} to urea as in the controls, but glucose, alanine, and carbon dioxide were all more actively anabolized to glycogen. The development of this information created an interest in the retrograde movement of the urea carbon to plasma carbon dioxide and to liver glycogen.

METHOD

Sprague-Dawley female rats, weighing 70-90 g, were placed in individual metabolism cages so designed as to collect the expired carbon dioxide in bubbling towers filled with 5N NaOH and also to collect separately urine and feces.⁽²⁾ Before being placed in the cages, the six randomly selected control rats were administered intraperitoneally C¹⁴-labeled urea with an activity of 3.77×10^6 DPM per 3.0 mg dose. A similar group of animals was irradiated 48 hours prior to the metabolic test. In a previous trial run, it had been determined that the metabolic disturbance was well manifest at that time. Exposure was made with the cobalt-60 rotational therapy unit of the Argonne Cancer Research Hospital of The University of Chicago. The animals were held in a lucite box and permitted to run free. The target distance was 49 cm, and the rate of exposure approximately 20 r/minute in air for 50 minutes. The total dose was 1000 r.

One rat each was sacrificed at 1, 2, 3, 4, 5, and 7 hours. The urine, expired air, and livers were assayed. From the urine, the urea was determined by the Conway microdiffusion method,⁽³⁾ and the glycogen was extracted from the liver by the hot KOH and alcohol method.⁽⁴⁾ Total carbon and total radioactivity were determined by means of a modified

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** In this communication, anabolism is used where there is a higher net energy in the final or end product and catabolism where there is a lower net energy in the final or end product.

Van Slyke apparatus using liquid nitrogen traps. Stable carbon dioxide was measured manometrically, and radioactivity by a vibrating reed electrometer. This entire procedure is described elsewhere.⁽²⁾

The accumulated carbon-14 and total carbon from the serially sacrificed rats was in effect a biological integration which represented the specific activity (accumulated carbon-14/accumulated total carbon) in the expired carbon dioxide and urea. The glycogen values represent its state at the time of sacrifice and not an accumulated or integrated value. For reasons adequately discussed by another investigator,⁽⁵⁾ it has been assumed that the expired carbon dioxide is representative of the plasma carbon dioxide (and those closely related forms which are in equilibrium with it) and that the urea voided is representative of the plasma urea from which it was derived.

RESULTS

The results of the trials are given in Tables 1, 2, and 3. The pool size, turnover time, and turnover rate given in Table 2 were determined by the method described by Weinman et al.⁽⁶⁾

Table 1
THE CATABOLISM OF UREA TO PLASMA CARBON DIOXIDE*

Hours after injection	Control			Irradiated		
	Urea	CO ₂	% Contribution	Urea	CO ₂	% Contribution
1	9466 (4.21)	102 (0.66)	1.07	5971 (2.25)	28 (0.07)	0.469
2	42428 (55.24)	107 (1.51)	0.25	134634 (81.28)	25 (0.25)	0.019
3	54393 (61.86)	82 (2.07)	0.15	108657 (88.48)	24 (0.33)	0.022
4	51635 (51.09)	72 (1.95)	0.14	90048 (---)	23 (0.40)	0.026
5	48878 (79.25)	81 (1.67)	0.08	123658 (64.04)	21 (0.88)	0.017
7	45055 (67.06)	33 (1.67)	0.07	101863 (78.26)	19 (0.91)	0.019

* Values are given in accumulated specific activity (DPM/mg of carbon), accumulated percentage of the dose administered (in parentheses) appearing in the plasma carbon dioxide and urea and the percentage contribution (accumulated specific activity of the CO₂ divided by the accumulated specific activity of the urea carbon x 100). All data have been adjusted to a 10⁶ DPM/dose and to a standard 100-g rat.

DISCUSSIONS AND CONCLUSIONS

It is noted first that the percentage of the dose appearing in the urine as urea is slightly but generally higher in the irradiated rats than in the controls. Assuming that the administered urea is quickly and uniformly mixed with the plasma urea pool, this must mean that there is a slightly larger proportion of the plasma urea pool eliminated in the irradiated animal. It is also seen (Table 2) that there is more than a twofold increase in the plasma urea pool in the treated rats. So in spite of a moderate prolongation of turnover time, there is an absolute increase in urea elimination, which is good evidence that even with a supralethal dose of 1000 r of total-body radiation, and at the critical 48-hour period following exposure, the rat kidney is able to clear urea effectively.

Table 2

THE CALCULATED PLASMA UREA POOL SIZE, TURNOVER
TIME, AND TURNOVER RATE DERIVED FROM THE
PLASMA UREA SPECIFIC ACTIVITY DATA
IN TABLE 1

	Pool size (mg of carbon)	Turnover time (hrs)	Turnover time (mg/hr)
Control	11.64	7.63	1.52
Irradiated	22.40	9.65	2.32

Utilizing the isotope dilution concept, one can estimate the portion of the plasma urea carbon that finds its way to the carbon dioxide of the plasma and thence to the expired air. This may reach, at an equilibrium time of 3 to 4 hours, 0.14-0.15 per cent in the control rats (Table 2). In the irradiated group, however, at the same equilibrium time, these values are from 0.022-0.026 per cent. This represents a considerable reduction in contribution to the plasma CO_2 pool by the urea following irradiation. Two explanations are suggested. First, there may be an increased cell barrier to plasma urea. Second, there may be a decrease in the ability of the catabolic mechanism to function under these circumstances. The first suggestion implies that the urea does not reach a site in the cell where there is active catabolism of urea, and the second that there is a defective enzyme or enzyme system which is reached by the urea but by which it is inadequately degraded.

Turning to the data from the glycogen analysis (Table 3) for further information, it is seen that the percentage of the dose finding its way from the urea into liver glycogen is reduced by irradiation. The specific activity of the liver glycogen is also less but there is some question as to the usefulness of the values. There is not an absolute mixing of the isotopic carbon in the glycogen since it is utilized on a "last in first out" basis.

With the glycogen data it is possible to make further speculations with regard to lo-

cation of the defect in the liver cell of the irradiated animal. As mentioned before in a previous communication,⁽¹⁾ the remarkable enhancement in the anabolism of the carbon from glucose, alanine, and carbon dioxide in the irradiated rat was pointed out. This at times was more than 10-fold. In this present study, the specific activity of the expired CO₂ is reduced in the irradiated rats to about 1/3 of the control values, while the percentage of the dose appearing in the liver as glycogen is reduced to about 1/30 of the control value. This then is clearly a 10-fold reduction in glycogen synthesis. It would seem then, that even if urea passed the cell barrier as mentioned above and was catabolized at the greatly reduced rate now noted, the net conversion of urea to glycogen should be a near balance in the treated and control animals. But instead, there is such a marked reduction in labeled glycogen that it is concluded that the urea never passed the cell barrier and, therefore, was not in a site of active urea catabolism. It is believed then that total-body irradiation at the supralethal level of 1000 r has by 48 hours increased a cell barrier to urea. Inasmuch as urea is anabolized by the liver cells and the same mechanism must also catabolize urea, the cell in question undoubtedly is the hepatic cell.

Table 3

THE SPECIFIC ACTIVITY (DPM/MG OF CARBON) AND THE PERCENTAGE OF THE DOSE OF CARBON, GIVEN AS UREA, APPEARING IN THE TOTAL LIVER GLYCOGEN*

Hours after injection	Control specific activity*	% of dose	Irradiated specific activity*	% of dose
1	45	0.64	3	0.016
2	59	1.10	4	0.030
3	74	0.79	4	0.023
4	32	0.29	5	0.010
5	83	1.12	3	0.020
7	108	0.63	4	0.018

* Specific activity values have been adjusted to 10⁶ DPM per dose and to a standard 100-g rat.

SUMMARY

Evidence is presented that the urea plasma pool is increased by total-body irradiation in the rat and that there is a larger total movement of urea from the animal.

There is a reduction in the amount of urea catabolized to carbon dioxide. There is also a striking reduction in the glycogen synthesized from urea in the total-body irradiated rat. These observations would seem to be related to an increased barrier of the liver cell to plasma urea following total-body irradiation.

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CARBON DIOXIDE FIXATION AND UREA SYNTHESIS IN THE RAT^{*†}

by

J. H. Rust,[†] W. J. Visek,^{**} and L. J. Roth^{**}

Previously published reports either make no mention of or leave the impression that the carbon of urea is derived from a general CO₂ pool. In a study concerned with some aspects of the intermediary metabolism in the irradiated rat, we deemed it essential to establish the part played, in glycogen and urea synthesis, by the CO₂ fixation process in the metabolism of glucose and alanine. Our findings were revealing with respect to urea formation and are recorded in this communication.

METHOD

Twenty-four young Sprague-Dawley female rats, weighing from 50 to 88 g, were used in the study. Animals were selected randomly into groups of 6 each. The rats in these groups, i.e., A, B, C, and D, were injected intraperitoneally with C¹⁴-labeled sodium bicarbonate, uniformly labeled glucose—two trials—and alanine labeled in the 2-C position. After injection, they were placed in metabolism cages⁽¹⁾ designed to permit the separation and collection of excreta and to permit collection of expired CO₂ in towers of NaOH. Collections of urine and expired air were made at 1, 2, 3, 4, 5, and 7 hours after injection. At the time of collection, the animal was sacrificed, and its liver and muscle glycogen contents were determined. Thus, the value at each sacrifice time represents the production of one animal integrated biologically over that period of time. Some variations were introduced that probably would not have been present if sequential collections could have been made from one or more animals. Only one set of values was discarded—the 4th hour of the alanine series. An adaptation of the method of Dean and Dixon⁽²⁾ for the determination of an extraneous datum was used in this exclusion.

The radioactive materials, with the exception of glucose, were obtained from Nuclear Instrument and Chemical Corporation, Chicago, Illinois. The glucose was extracted by Dr. L. B. Achor from colchicum corms grown by Dr. E. S. Mika of The University of Chicago,

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Department of Pharmacology Biosynthetic Program.* The disintegrations per minute (DPM) per dose administered were: sodium bicarbonate 2,825,683; glucose, 330,374; and alanine, 402,759. In the calculations that are presented, all data are adjusted to a standard dose of 1,000,000 DPM per 100-g rat. Carrier was added as follows: glucose 2.85 mg; alanine 4.66 mg; and sodium bicarbonate, 2.19 mg.

Stable and radioactive carbon were measured as CO_2 by a modified Van Slyke wet combustion method.^{†(3)} The C^{14}O_2 was placed in an ionization chamber.⁽⁴⁾ The change in the chamber was then measured with a vibrating reed electrometer,[‡] and the DPM were calculated by the rate of drift method.⁽⁵⁾ Urea was determined by the Conway urease microdiffusion method.⁽⁶⁾ Carbon dioxide was trapped in NaOH, and the CO_2 was released, as was that collected from the expired air, from the collecting medium with an excess of phosphoric acid. C^{12} and C^{14} analyses were calibrated respectively with a National Bureau of Standards benzoic acid C^{14} sodium bicarbonate reference standard.

RESULTS

The data in Table 1 are expressed as per cent of the dose administered that appeared in the expired air or urea. These data are also tabulated as specific activity of carbon derived from the expired air and from urea.

DISCUSSION

There are two basic assumptions that we have made: 1) the CO_2 of the expired air is representative of the CO_2 of the plasma pool** and has the same specific activity;⁽⁷⁾ and similarly, 2) the urea carbon in the voided urine has the same specific activity as that in the body pool of urea from which it was derived. In both cases, these entities are readily and quickly diffusible across the lung alveoli and kidney tubule walls and represent an end product of metabolism.

If the CO_2 of the plasma pool is the sole precursor for urea, the specific activity of the carbon of urea should eventually equal the specific activity of the carbon of the CO_2 of expired air. In our study, the carbon fixed as urea from labeled bicarbonate reached an asymptotic level of only about 25 per cent of the carbon in the expired air after 7 hours (Table 1, column 5). Delluva and Wilson⁽⁸⁾ gave radioactive sodium bicarbonate hourly for 18 hours to a rat and found that the ratio was 0.53 at the end of that time. MacKenzie and du Vigneaud⁽⁹⁾ fed a rat L-methionine containing C^{14} in the methyl group and found the ratio at 24 and 48 hours to be 1.0. In both of these studies, there was essentially a continu-

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† A method devised by one of us (L. J. R.) to trap CO_2 with liquid nitrogen for manometric and radiometric measurements. To be published.

‡ Applied Physics Corporation, Pasadena, California, Model No. 30.

** Plasma carbon dioxide as used in this paper includes the dissolved CO_2 , H_2CO_3 , HCO_3^- , and CO_3^{2-} as they exist in equilibrium.

Table 1

PER CENT OF DOSE OF LABELED CARBON IN EXPIRED AIR AND
UREA AND ITS SPECIFIC ACTIVITY

Hours after injection and animal number		Per cent of C-14 expired (1)	Specific activity of carbon expired (2)	Per cent of C-14 in urea (3)	Specific activity of C in urea* (4)	Specific activity ratio† (5)
<u>Group</u>		<u>Carbonate Fixation</u>				
A	1	73.87	7028	0.34	333	.023
	2	74.75	4035	0.61	404	.101
	3	83.54	3600	0.73	503	.140
	4	84.42	1930	0.85	455	.236
	5	86.53	1575	0.89	384	.244
	7	84.24	1184	0.93	273	.231
		<u>Glucose Metabolism (Trial I)</u>				
B	1	5.30	888	0.32	1926	2.169
	2	23.10	1550	1.31	5318	3.431
	3	31.65	1206	1.97	5735	4.755
	4	33.65	1127	1.20	4455	3.943
	5	35.75	975	1.77	4365	4.480
	7	32.45	614	0.57	1865	3.037
		<u>Glucose Metabolism (Trial II)</u>				
C	1	12.57	1430	0.68	2302	1.610
	2	21.95	1123	3.72	4097	3.648
	3	23.52	979	2.74	4692	4.793
	4	24.00	720	7.90	4318	5.997
	5	27.00	614	6.90	5872	9.563
	7	21.50	403	4.23	4327	10.737
		<u>Alanine Metabolism</u>				
D	1	4.71	426	0.71	1020	2.394
	2	17.62	957	3.17	3820	3.992
	3	20.95	792	2.46	2920	3.687
	5	25.90	484	1.83	2368	4.892
	7	28.18	404	2.22	2302	5.698

* Accumulated radioactivity in DPM/accumulated mg of carbon.

† Specific activity of carbon in urea/specific activity of expired carbon dioxide, i.e., column 4/column 2.

ous or extended administration of the labeled carbon as carbonate or as a metabolite. It is quite probable that because of this, more than just the CO_2 of the plasma was labeled. This could account for the higher ratio values they obtained.

Our data show a greater specific activity in the urea than in the plasma CO_2 after administration of labeled glucose and alanine. Even if labeled plasma CO_2 had been totally incorporated into urea, because of the greater specific activity of the urea, one would still have to conclude that the CO_2 of the plasma pool is not the sole source of carbon for urea production. Undoubtedly, there is another and more significant pathway for CO_2 fixation as urea. The relationship suggests that a large per cent of the urea carbon is derived from

a source that is not in ready equilibrium with the plasma CO_2 pool. One explanation is that the pool or source of CO_2 in urea is relatively independent of the pool of plasma CO_2 because of a different point of origin. It seems reasonable to suggest that the liver cells form most of the CO_2 for the local synthesis of urea and that other sources form the bulk of the plasma pool CO_2 under normal circumstances. There is also the possibility that there is direct transcarbonation or a "hand-off" mechanism that moves carbon directly to the urea cycle without recourse to the plasma CO_2 pool. The nature of the mechanism cannot be characterized by this study. The specific activity ratios of the glucose and alanine trials (groups B, C, and D) seem to be approximately the reciprocal of the carbon fixation trial (group A). The significance of this is not apparent at this time.

SUMMARY

Carbon fixation as urea can be determined by the ratio of the specific activities of urine urea and the CO_2 of expired air. At 7 hours after injection of the radiotracer, this is approximately 25 per cent. After the injection of uniformly labeled carbon-14 glucose and alanine (labeled in 2-C position), the specific activity of urea is higher than that of expired CO_2 . This indicated that urea was formed within the liver cells from plasma carbon dioxide and by some other unknown but more effective mechanism than carbon dioxide production and fixation as urea.

ACKNOWLEDGMENT

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THE ACUTE EFFECTS OF BETA₃-THIENYLALANINE IN THE ADULT MALE ALBINO RAT^{*†}

by

R. W. Wissler, L. F. Frazier, K. H. Soules, P. Barker,
and E. C. Bristow III

The acute and reversible effects of β_3 -thienylalanine, a specific inhibitor for phenylalanine, upon the growth of young rats and the protein repletion of protein-deficient adult rats have been summarized.^(1,2) The purpose of this investigation was to study the acute effects of a phenylalanine antagonist in the normal adult rat. The experiments permitted evaluation of the influence of β_3 -thienylalanine upon body weight, nitrogen balance, the synthesis of specific antibody globulin, and neoplastic growth within a few days after the antagonist had been introduced into the diet.

β_3 -thienyl-DL-alanine was synthesized by a modification⁽³⁾ of the method reported by Dittmer.⁽⁴⁾

A maintenance amino acid ratio was given to the rats by gavage twice daily to insure control of other dietary ingredients fed with the inhibitor. This diet included a mixture of pure amino acids present at maintenance level (MAA - Table 1). The level at which the mixture was given was calculated on the basis of the average weight of the rats in each experiment, 3.07 g of MAA/kg/rat/day being necessary to maintain nitrogen balance and body weight.

The MAA diets used for all of the experiments were essentially the same. The amount of corn oil was varied to meet the caloric requirements of the average rat in each experimental group. In the control protein-free ration, dextrin replaced the amino acid mixture. The dry diet was blended with enough water to permit its passage through the stomach tube.

The amounts of β_3 -thienylalanine and phenylalanine added to the diets varied within an experiment—and among experiments (Tables 2 and 3). When phenylalanine was added in excess, the amount given was a multiple of that given daily in the MAA diet and replaced an equal weight of dextrin. The amount of β_3 -thienylalanine ranged from 25 mg/rat/day to 100 mg/rat/day.

A protein hydrolysate aminosol[†] was utilized as a nitrogen source during the preliminary tube-feeding period because of its solubility and assortment of essential and nonessential amino acids. The amount of aminosol was comparable to the quantities of amino

* Summary of a paper that appears in the Archives of Pathology, 62:62, 1956.

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‡ Donated by Abbott Laboratories, North Chicago, Illinois.

Table 1

COMPOSITION OF BASIC DIET AND OF MAINTENANCE AMINO ACID
MIXTURE USED FOR TUBE-FEEDING EXPERIMENTS IN ADULT
PREVIOUSLY WELL-NOURISHED RATS

Tube-Fed Maintenance Ration*		Water-Soluble Vitamins	
Ingredient	G/100 ml.	Ingredient	Mg/100 ml
Dextrin	20.63	Nicotinic acid	2.12
Corn oil	5.63	Ca pantothenate	1.06
MAA	3.43	Pyridoxine HCl	0.53
Cellulose	2.63	Riboflavin	1.06
Salt mix	2.11	Thiamine HCl	0.53
Vitamin mix	0.53		
50% Choline	0.32		
Percomorph liver oil	0.16		
Water	67.7		

Maintenance Amino Acid (MAA) Mixture	
Amino Acid	Mg/kg/rat/day
DL - Alanine	288.7
L - Arginine HCL	255.7
DL - Aspartic acid	324.7
L - Cystine	18.3
L - Glutamic acid	1474.1
Aminoacetic acid	26.0
L - Histidine HCl, HOH	38.3
DL - Isoleucine	330.0
L - Leucine	103.3
L - Lysine HCl, HOH	80.0
DL - Methionine	93.3
DL - Phenylalanine	76.7
DL - Threonine	136.7
DL - Tryptophan	28.3
L - Tyrosine [†]	76.8
DL - Valine	256.7
Total	3607.7

* Composition for Experiment 3. Basic composition varied slightly in each experiment, depending on the weight of the rats being used.

† In the first experiment, 329.3 Mg of tyrosine and 1221.7 mg of glutamic acid were used. The tyrosine was reduced to the tabulated value in all subsequent experiments to decrease the possibility of dietary tyrosine reducing the effectiveness of the β_3 -thienylalanine. Glutamic acid was increased by a comparable amount.

acids given subsequently.

The total amount of diet given per feeding varied, depending on the study; e.g., 15 ml in the first and fifth experiments and 20 ml in the others. Three experiments were performed to demonstrate whether the effects of β_3 -thienylalanine on nitrogen balance and antibody formation were consistently reproducible. For a preliminary period of 8 days, well-nourished Sprague-Dawley adult male rats of comparable weight were given increasing amounts of a liquid ration in which the amino acids were supplied by the protein hydrolysate (Table 1). Then the experimental rations were started after the rats had been on the various diets for 2 days. Nitrogen balance determinations were initiated and continued for the subsequent 4-day period. Kjeldahl nitrogen determinations in triplicate were made on aliquots from the urine, feces, and diets, and from these, the 4-day nitrogen intake, total 4-day nitrogen excretion, and nitrogen balance (Table 3) were calculated.

Table 2
AVERAGE RAT WEIGHTS AND THE DIETARY VARIABLES IN
EACH EXPERIMENT

	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5	Exp. 6
No. rats used	35	35	25	12	10	21
Av. rat wt., g	350	380	550	450	300	300
MAA/rat/day, g	1.14	1.39	1.98	1.98	1.98	1.06
Cal./rat/day	50.6	73.7	89.5	89.5	89.5	60.8
1 X phenylalanine*/rat/day/g	0.025	0.030	0.042	0.042	0.042	0.025

* Since the molecular weights of phenylalanine and β_3 -thienylalanine are similar, the quantities of the inhibitor (1 X to 4 X) incorporated in each experimental ration represent multiples of the phenylalanine in that ration.

For determinations of antibody-forming capacity, the rats were injected via the tail vein with 1 ml of a 0.25% suspension of washed sheep erythrocytes in 0.86% saline after they had been maintained on the diet for 2 days. One ml of blood was withdrawn from each rat on the 4th and 6th post-injection days and hemolysin determinations were performed using the serum.

Tissues for histologic examination were taken from the animals at the time of killing in three experiments. Samples of liver, spleen, thymus, mesenteric lymph node, bone marrow, kidney, and adrenal were prepared for study. Samples of tumor and small intestine were also taken from the rats that were being used to determine the effects of the antagonist on neoplasms.

For the tumor study, on the second day of the preliminary diet, the rats in two groups were injected with 0.3 ml of a 1:2 sterile saline homogenate of Jensen sarcoma into the anterior thigh muscles. The tumors were measured daily with calipers to obtain a three-

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Table 3

AVERAGE BODY WEIGHT, NITROGEN BALANCE, AND SERUM PROTEIN
DATA, WITH STANDARD ERRORS*
EXPERIMENTS 1, 2, AND 3

Diet group	8-day wt. change, g			4-day N-balance, mg			8-day serum protein, g/100 Cc†
	Exp. 1	Exp. 2	Exp. 3	Exp. 1	Exp. 2	Exp. 3	Exp. 1
20% Hydrolysate	+ 5(±1)	+10(±3)	-	+144(±41)	+676(±90)	-	6.9 (±0.05)
MAA	- 8(±2)	- 5(±2)	+ 7(±1)	0(±16)	0(± 6)	0(±21)	6.5 (±0.1)
MAA + 1X TA	-16(±2)	-12(±2)	-	- 65(±15)	- 43(±38)	-	6.2 (±0.09)
MAA + 1X TA + 4XPA	-12(±2)	-	-	- 85(± 6)	-	-	6.4 (±0.04)
MAA + 1X TA + 6XPA	-	-15(±5)	-	-	- 67(±31)	-	-
MAA + 3X TA	-	-	-12(±4)	-	-	-411(±33)	-
MAA + 4X TA	-41(±1)	-	-	-504(±10)	-	-	5.1 (±0.2)
MAA + 3X TA + 6XPA	-	-	-21(±3)	-	-	-476(±55)	-
MAA + 6XPA	-	-	+12(±4)	-	-	- 1(±17)	-
MAA without PA	-	-15(±3)	-	-	- 49(±24)	-	-
Protein-free basal	-21(±2)	-12(±1)	-	-148(±15)	-234(±11)	-	6.1 (±0.1)
Fasted	-81(±4)	-82(±3)	-	-491(±57)	-431(±14)	-	6.1 (±0.1)

* These data are the averages of five rats in each diet group. Each group of five rats was comparable to the other groups used in that experiment in age, weight, and previous dietary history. All groups within any given experiment were tested simultaneously, and no animals were used for more than one experiment.

† Serum protein concentration determined on blood obtained at killing, eight days after the diets were started.

dimensional picture of tumor growth.

Results: The addition of variable quantities of β_3 -thienylalanine to the maintenance diet fed to previously well-nourished rats caused an acute but variable depression in body weight and nitrogen balance. In addition, weight loss and nitrogen imbalance increased in proportion to the amount of β_3 -thienylalanine added to the diet. These effects could not be reversed by the addition of phenylalanine to the ration, which is in contrast with the results obtained using protein-depleted rats.

An acute depression in antibody formation followed the single intravenous injection of antigen given two days after the inhibitor had been added to the diet. This effect was reversible by the addition of more phenylalanine to the amino acid ration. A similar effect was not obtained by a period of fasting or by feeding rations that were free of amino acid nitrogen or phenylalanine.

There was a depression of the pyroninophilic cellular proliferation in the red pulp of the spleens of rats that had been given β_3 -thienylalanine during the antibody-forming period. In the spleen of the rat, this proliferation is usually associated with antibody formation in response to a particulate intravenously-injected antigen. There was very little histologic evidence of damage to any of the normally rapidly-dividing rat cells.

With β -3-DL-thienylalanine there was marked retardation in tumor growth (Figure 1) accompanied by a reduction in mitosis (Figure 2) and by characteristic neoplastic nuclear changes in rats with a well-established Jensen sarcoma. Tumor necrosis was also clearly evident in these treated animals. All of these effects could be reversed by adding phenylalanine to the diet. A study of the mechanism of the selective action of β_3 -thienylalanine upon tumor growth and its potential as a synergist with other chemotherapeutic agents is in progress.

Figure 1 (Experiment 5). The mean volumes of Jensen sarcomas in two groups of five rats each receiving identical amino acid rations except for the presence or absence of β_3 -thienylalanine in the diet. The eight-day volumes are calculated from measurements of the tumor after removal at autopsy. The ante-mortem values are calculated from three-dimensional measurements in situ.

Figure 2. A section of a Jensen sarcoma from a rat which received 80 mg of B_3 -thienylalanine per day added to the MAA ration for eight days. Note the almost complete absence of mitotic figures, the increase in chromatin in the nuclei, and the less prominent nucleoli. Hematoxylin and eosin; Mag. X 1135.

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PARTIAL DEGRADATION OF THE BENZENE RING OF ESTRADIOL-17*

by

H. Werbin

This note describes the synthesis of 2,4-dinitroestradiol-17 β and its partial degradation to tribromonitromethane (representing both carbon atoms 2 and 4 of the benzene ring) by a similar application of the bromopicrin split. The 2,4-dinitroestradiol-17 β was characterized by its ultraviolet and infrared absorption spectra. The steroid had absorption bands at 741 cm⁻¹, 759 cm⁻¹, and 904 cm⁻¹ which had been found previously to be characteristic of dinitrophenolic pentasubstituted compounds.⁽¹⁾ The ultra-violet spectrum of the dinitrosteroid showed absorption maxima in absolute ethanol at 277 m μ (ϵ 6655), 352-354 m μ (ϵ 3340), and a plateau of 424-426 m μ (ϵ 910). These values are in excellent agreement with those recorded for similar pentasubstituted aromatic compounds.⁽¹⁾ The 2,4-dinitroestradiol-17 β was also characterized by conversion to a colorless 3,17-diacetate which was purified by filtration in ether over charcoal. The diacetate was hydrolyzed readily to a yellow 17-monoacetate by elution from a column of aluminum oxide. The bromopicrin resulting from the cleavage of the benzene ring of the dinitrosteroid was identified by infrared analysis.

EXPERIMENTAL⁺

2,4-Dinitroestradiol-17 β . One hundred mg of estradiol-17 β was dissolved in 3 ml of glacial acetic acid, and 60 μ l of concentrated nitric acid was added slowly at room temperature with good mixing. After standing for 24 hours, the solution was poured into 50 ml of water and was extracted several times with ether. The ether solution was concentrated to dryness in vacuo. The yellow residue was treated with 5 ml of 5 per cent methanolic potassium hydroxide for 2 hours to hydrolyze any ester that may have formed, after which the solution was acidified and centrifuged. The precipitate was washed several times with water and was crystallized from aqueous ethanol, m.p. 240-250^o (dec.).

Anal. Calculated for C₁₈H₂₂N₂O₆: C, 59.66; H, 6.12; N, 7.73. Found: C, 60.12; H, 6.50; N, 7.35.

2,4-Dinitroestradiol-3,17 β -diacetate. Twenty mg of 2,4-dinitroestradiol-17 β was mixed with 100 μ l of acetic anhydride and 50 μ l of dry pyridine. After standing at room temperature overnight, the mixture was evaporated to dryness with a stream of nitrogen.

* Summary of a note that has been accepted for publication in the Journal of Organic Chemistry.

⁺ Melting points were taken on a Fisher-Johns melting point block and are uncorrected.

The residue was taken up in ether, 70 mg of activated charcoal was added, and the solution was filtered. Evaporation of the ether left a colorless solid that was crystallized from aqueous methanol, m.p. 161.5-164.5°.

Anal. Calculated for $C_{22}H_{26}N_2O_8$: N, 6.28. Found: N, 6.30.

2,4,-Dinitroestradiol-17 β -acetate. The diacetate was taken up in benzene and was placed on a column of 5 g of aluminum oxide (Merck acid-washed). Benzene containing 0.5 per cent methanol caused elution of a yellow solid that was crystallized from aqueous methanol, m.p. 197.5-198.5°.

Anal. Calculated for $C_{20}H_{24}N_2O_7$: N, 6.93. Found: N, 6.97.

Bromopicrin split. The procedures for the bromopicrin split and the isolation of tribromonitromethane have been described.⁽¹⁾ Method B was employed. From 48 mg of 2,4-dinitroestradiol-17 β there was obtained 30.5 mg of tribromonitromethane. The infrared spectrum of this material was compared with the spectrum of a sample of tribromonitromethane from Eastman Kodak that was vacuum distilled. The spectra were identical except for a few additional weak bands in the bromopicrin from the cleavage of the dinitrosteroid, indicating the presence of a minor component.⁽²⁾ After evaporation of the bromopicrin on a watchglass at room temperature, a few micrograms of colorless crystals remained, subliming at 123-127°, KBr λ_{max} 1475, 1442, 1323, 1118, 1058, 881, 642 cm^{-1} . These bands accounted for the weak bands in the bromopicrin from the degradation of the dinitrosteroid. The small quantity precluded identification.

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PARTIAL DEGRADATION OF THE BENZENE RING OF ESTRONE.
ISOLATION OF CARBON ATOMS 2 AND 4*

by

H. Werbin and C. Holoway

INTRODUCTION

The biosynthesis of the estrogens from carbon-14-labeled acetate has been accomplished under both in vivo and in vitro conditions.⁽¹⁻⁵⁾ Determination of the distribution of the radioactivity in the labeled estrogens may facilitate elucidation of the mechanisms involved in the biosynthetic pathway from acetate. Although the cleavage of the D ring of the estrogens has been reported,^(6,7) the degradation of the benzene ring does not appear to have been accomplished. This paper describes the use of the bromopicrin split[†] of nitroestrones[‡] to isolate carbon atoms 2 and 4 of the benzene ring as bromopicrin (tribromonitromethane). The 2-, 4-, and 2,4-dinitroestrones and 2,4-dinitro-17-desoxoestrone were synthesized for this purpose. A comparison of the melting points of the nitroestrones with the same steroids reported in the literature⁽¹¹⁻¹³⁾ indicates that previous mixtures rather than the pure steroids have been isolated previously. On the basis of the spectroscopic data reported in this communication, it appears that the previous assignment of the 2- and 4- nitroestrones should be reversed.

EXPERIMENTAL

All melting points were taken on a Fisher-Johns melting point block.

2- and 4-Nitroestrones. The nitrating procedure described by Niederl and Vogel⁽¹¹⁾ was employed. One hundred mg of estrone was dissolved in 3 ml of boiling acetic acid. When the temperature of the solution reached 45°, 47 μ l of concentrated nitric acid was added slowly with mixing, and the yellow solution was allowed to stand overnight at room temperature. The yellow crystals of 4-nitroestrone were filtered, washed with acetic acid, and dried. The steroid, after crystallization from aqueous acetic acid, decomposed between 270 and 280° C. Other workers^(11,13) have reported a value of 258° for this steroid.**

* Version of a paper that has been accepted for publication in the Journal of Biological Chemistry.

† This phrase refers to the well-known degradation of nitro and polynitrophenols by heating with hypohalite.^(8,9) Rafelson *et al.*⁽¹⁰⁾ used this reaction to degrade a quinoline derivative.

‡ The term estrone is used for the sake of simplicity. The basic structure referred to is $\Delta^{1,3,5(10)}$ estratrien-3-ol-17-one.

** The 2-nitro and 4-nitro group assignments are the reverse of those made by Niederl and Vogel⁽¹¹⁾ and Hillmann-Elies *et al.*⁽¹³⁾ The spectroscopic data presented in this paper support the new assignments.

$C_{18}H_{21}NO_4$. Calculated: C, 68.55; H, 6.71; N, 4.44. Found: C, 68.49; H, 6.86; N, 4.41. Treatment of 4-nitroestrone with acetic anhydride and pyridine at room temperature yielded the acetate, which melted at $139.5-140^\circ$ after crystallization from aqueous methanol. The acetate was chromatographed on a column of aluminum oxide (Merck, acid washed) and was eluted with benzene. After two crystallizations from aqueous methanol, the colorless steroid melted at $163-165.5^\circ$. Hillmann-Elies *et al.*⁽¹³⁾ report 166° .

$C_{20}H_{23}NO_5$. Calculated: N, 3.92. Found: N, 4.00. Refluxing the 4-nitroestrone in absolute alcohol containing methyl iodide and sodium resulted in the formation of 4-nitroestrone methyl ether, m.p. $254.5-257.5^\circ$ (decomposition). Niederl and Vogel found 150° .

$C_{19}H_{23}NO_4$. Calculated: N, 4.25. Found: N, 4.22.

The filtrate from the 4-nitroestrone was poured into 30 ml of water, and the yellow precipitate was filtered and dried. A benzene solution of the steroid mixture was placed on a column of 20 g of Merck aluminum oxide. Elution was carried out with benzene and benzene containing 1 per cent methylene chloride. Evaporation of the solvents left 2-nitroestrone, which was crystallized from 80 per cent alcohol, m.p. $183.5-184^\circ$, reported 155° .^{*(13)}

$C_{18}H_{21}NO_4$. Calculated: C, 68.55; H, 6.71; N, 4.44. Found: C, 68.41; H, 6.63; N, 4.52. 2-Nitroestrone acetate, m.p. $174.5-176.5^\circ$ (literature 146°),⁽¹³⁾ was prepared as described above for the 4-nitro derivative.

$C_{20}H_{23}NO_5$. Calculated: N, 3.92. Found: N, 3.98.

The rest of the material in the column of aluminum oxide was eluted with methanol. Infrared examination of the mixture indicated the presence of 4-nitroestrone and 2,4-dinitroestrone. It seems likely that the steroids precipitated by the other workers^(11,13) by pouring all of the nitrated estrone mixture into water were the 2-, 4-, and 2,4-dinitroestrones. Their crystallization procedures do not seem to have yielded pure mononitroestrones. This may account for the discrepancies in melting points between the nitroestrones and acetates isolated in this study and those reported previously.

2,4-Dinitroestrone. One hundred μ l of concentrated nitric acid was added with stirring to 10 ml of glacial acetic acid at 45° , containing 200 mg of estrone. The solution, after standing overnight at room temperature, was poured into 200 ml of water, and the mixture was extracted exhaustively with ether. It was then dried with anhydrous sodium sulphate and concentrated in vacuo to dryness. After two crystallizations from 80 per cent alcohol, the residue yielded 2,4-dinitroestrone, m.p. $185-187^\circ$ (literature 275°).⁽¹³⁾

$C_{18}H_{20}N_2O_6$. Calculated: C, 59.99; H, 5.59; N, 7.78. Found: C, 60.08; H, 5.64; N, 7.65. After chromatography on aluminum oxide and crystallization, the dinitroestrone had the same melting point. The acetate melted at $185.5-186^\circ$.

$C_{20}H_{22}N_2O_7$. Calculated: N, 6.96. Found: N, 7.01.

* See footnote (**) on page 69.

2,4-Dinitro-17-desoxoestrone. 17-Desoxoestrone, m.p. 131-133.5°, was prepared by a Wolff-Kishner reduction of estrone. Ninety-three mg was dinitrated as described above. The crude dinitroesteroid was taken up in a low boiling petroleum ether-benzene mixture (3:1) and placed on a 30 g column of Merck aluminum oxide. The column was developed with 400 ml of 3:1 petroleum ether-benzene and 200 ml of 1:1 petroleum ether-benzene. Elution of the desired steroid was accomplished with 600 ml of benzene and 500 ml of benzene containing 0.3 per cent methanol. Evaporation of the solvents left an oil that could be crystallized from a 20:1 methanol-water mixture. 2,4-Dinitro-17-desoxoestrone, m.p. 100-103°, was obtained. Further crystallization gave a product that melted at 103.5-104°.

$C_{18}H_{22}N_2O_5$. Calculated: C, 62.41; H, 6.40; N, 8.09. Found: C, 62.46; H, 6.59; N, 8.14.

Conversion of 4-nitroestrone to 2,4-dinitroestrone. 4-Nitroestrone dissolved in glacial acetic acid was mixed with one equivalent of concentrated nitric acid at 45°. After standing overnight at room temperature, the dinitrosteroid was isolated as described above for 2,4-dinitroestrone. Following crystallization from 80 per cent alcohol, the compound was found to be identical with 2,4-dinitroestrone on the basis of mixed melting point and infrared spectrum.

Preparation of nitro-3,4-dimethylxlenols. The 2-nitro-, 6-nitro-, and 2,6-dinitro-3,4-dimethylxlenols were prepared by the procedures described by Holler *et al.*⁽¹⁴⁾ The melting points were in agreement with those that have been reported.

7-Nitro- and 5,7-dinitro-6-hydroxytetralin. A mixture of 5- and 6-hydroxytetralins in glacial acetic acid was nitrated with one equivalent of concentrated nitric acid at room temperature. The resultant mixture of nitro compounds was chromatographed on a column of aluminum oxide. The material eluted with 30 per cent benzene in petroleum ether gave 7-nitro-6-hydroxytetralin, m.p. 88-88.5°, when crystallized from alcohol (literature 88°).⁽¹⁵⁾ Elution with 75 per cent benzene in petroleum ether resulted in an oil that could not be crystallized. Elution with benzene and evaporation of the solvent left 5,7-dinitro-6-hydroxytetralin. After crystallization from alcohol, the m.p. was 140-142° (literature 142°).⁽¹⁵⁾

Bromopicrin split of the nitrosteroids. To 0.3 mM of dinitro-steroid or 0.6 mM of mononitrosteroid (small crystals) and 600 mg of calcium oxide in a 50-ml round bottom flask was added 3 ml of distilled water. The flask was heated in a boiling water bath to permit formation of the calcium salt and was then placed in an ice-salt mixture at -5°. Over a 5- to 10- minute period, 250 μ l of bromine was added with stirring. Five ml of water was added, the contents of the flask were mixed thoroughly, and a downward condenser was attached. The outlet was inserted in the mouth of a chilled 5-ml centrifuge tube. The flask was heated gently with a free flame until distillation of the water and bromopicrin occurred. For the cleavage of 2,4-dinitro-17-desoxoestrone, it was necessary to steam-distill, or, alternatively, the contents of the flask were brought to a boil and the bromopicrin was carried over with a gentle stream of nitrogen.⁽⁹⁾ The aqueous layer was siphoned off after centrifugation, leaving a small droplet of bromopicrin on the bottom of the centrifuge

tube. The bromopicrin was transferred with 300 μ l of methylene chloride to a small test tube. The methylene chloride was dried over sodium sulphate, filtered through a small sintered funnel and evaporated at 65° in another previously weighed test tube. The open tube was re-weighed after standing one hour at room temperature. This method of isolation when applied to 6.7 to 77.3 mg of pure bromopicrin resulted in a loss of 3.6 ± 1.4 mg. A sample of bromopicrin isolated in this manner was subjected to infrared analysis, and the absorption spectrum agreed with that of pure bromopicrin. ^{*(6)}

Isolation of carbon dioxide resulting from bromopicrin split of nitrosteroids. Freshly burned calcium oxide and carbon dioxide-free distilled water must be used to determine quantitatively the carbon dioxide formed during the degradation of the nitrosteroids. A 50-ml, 3-necked flask was used instead of a 1-necked flask so that there could be simultaneous acidification and bubbling of nitrogen through the mixture after the bromopicrin was distilled off. The existing gasses were passed through molten phenol, and the carbon dioxide was trapped in a barium hydroxide-potassium hydroxide solution. The precipitated barium carbonate was filtered under nitrogen through a previously weighed sintered glass funnel fitted with an asbestos mat. The blanks were of the order of 1 to 2 mg. The carbon dioxide from known amounts of sodium carbonate could be determined with an accuracy of ± 3 per cent when the above procedure was employed.

The infrared data were obtained using a Perkin-Elmer Model 21 spectrometer and potassium bromide disks. A Beckmann Model DU spectrophotometer was employed for measurements of ultraviolet absorption.

RESULTS AND DISCUSSION

The bromopicrin split of the nitrosteroids may be useful in following the biosynthetic pathways of estrogens only if the positions of the nitro groups are established. The data in Tables 1 and 2 support the tentative assignments given in this paper and are at variance with assignments made previously. ^(11,13) Table 1 shows that in the 280-m μ region, 2-nitro-3, 4-dimethylxlenol has a lower absorption maximum and extinction coefficient than the 6-nitro derivative. This may be attributed to steric inhibition of the 2-nitro group resonance with the benzene ring. ⁽¹⁷⁾ The same explanation was offered by Holler *et al.* ⁽¹⁴⁾ to explain the data in the 400-m μ region for the sodium salts of these compounds (Table 2). These phenomena also seem to apply to the 2- and 4-nitroestrones. A Fisher-Hirschfolder model of 4-nitroestrone reveals that the methylene group adjacent to the nitro group prevents full coplanarity of the latter with the benzene ring. The agreement in absorption maxima and extinction coefficients shown in Table 1 for 2-nitroestrone, 2,4-dinitroestrone, and their corresponding model compounds is very close, while the absorption spectra of 4-nitroestrone and its model compound do not coincide. However, it was converted to 2,4-dinitroestrone, identical in every respect with the same steroid prepared by the dinitration of estrone.

* A few weak bands indicated a trace quantity of some other component.

Table 1

ULTRAVIOLET ABSORPTION MAXIMA AND MOLAR EXTINCTION
COEFFICIENTS OF NITROESTRONES AND MODEL COMPOUNDS

Compound	Max. m μ	ϵ	Max. m μ	ϵ	Max. m μ	ϵ
2-Nitroestrone	293-294	8220	364-366	3710		
7-Nitro-6-hydroxy- tetralin	293	8170	367-369	3655		
6-Nitro-3,4-dimethyl- xylenol*	282-284	9285	356-358	4585		
4-Nitroestrone	278	1885				
2-Nitro-3,4-dimethyl- xylenol*	278	5310	360	2780	244	3560
2,4-Dinitroestrone	276	6890	353	3405	430	1050
2,4-Dinitro-17- desoxoestrone	278-279	6760	353-354	3575		
5,7-Dinitro-6- hydroxytetralin	275-276	6495	354-356	3270	433-437	1310
2,6-Dinitro-3,4- dimethylxylenol	274	6795	350	3605	424	1535

* Hexane solution. All the others were in absolute alcohol. The data on the xylenols is taken from the work of Holler *et al.*⁽¹⁴⁾

Table 2

ABSORPTION SPECTRA OF SODIUM SALTS OF MONONITRO
ESTRONES AND XYLENOLS

Compound	Max. m μ	ϵ
2-Nitroestrone	436-437	4635
4-Nitroestrone	426-431	1031
6-Nitro-3,4-dimethylxylenol*	432	5380
2-Nitro-3,4-dimethylxylenol*	418-420	780

* Data taken from Holler *et al.*⁽¹⁴⁾

Compounds of similar structure frequently exhibit close agreement in their infrared absorption bands because, for example, of the presence of such groups as nitro and aromatic C-H. There should be closer agreement in the absorption spectra (fingerprint region) of each nitrosteroid and its model compounds than between the various nitrosteroids. The data in Figure 1 indicate that this is the case and offer further evidence for the nitro

Figure 1. Infrared spectra of nitrosteroids and model compounds in the 700-1000 cm^{-1} region. The lengths of the lines represent relative intensities.

group assignments made on the basis of the ultraviolet absorption data in Tables 1 and 2. The four dinitro compounds show strong absorption bands at 744-745 cm^{-1} , 763-766 cm^{-1} . 2-Nitroestrone and its model compounds have absorption bands at 760-762 cm^{-1} , 865-866 cm^{-1} , and 886-889 cm^{-1} . The 865 cm^{-1} band falls in the region of the aromatic C-H defor-

mation vibration expected in a 1, 2, 4, 5 tetrasubstituted aromatic compound.* 4-Nitroestrone and 2-nitro-3,4-dimethylxlenol show bands, respectively, at 1029 and 1027 cm^{-1} that are absent from all the other compounds studied. Bands at 824 cm^{-1} in the former and at 812 cm^{-1} in the latter may be caused by aromatic C-H deformation vibrations.*

The amounts of bromopicrin and carbon dioxide isolated from the partial degradation of the nitrosteroids is shown in Table 3. Since both the 2- and 4-nitroestrones yield

Table 3
BROMOPICRIN SPLIT OF NITROESTRONES

Steroid	mMole	Bromopicrin isolated mMole	Carbon dioxide isolated mMole
2-Nitroestrone	0.635	0.074	0.35
	0.635	0.060	0.43
4-Nitroestrone	0.635	0.032	0.25
	0.635	0.057	0.46
2,4-Dinitroestrone	0.280	0.041	0.42
	0.302	0.16	-
	0.305	0.12	0.53
	0.305	0.22	0.51
2,4-Dinitro-17-desoxoestrone	0.289	0.055	0.34
	0.289	0.064	-

bromopicrin, it may be assumed that the bromopicrin resulting from the cleavage of both dinitrosteroids comes from the 2 and 4 carbon atoms. Carbon dioxide was isolated from the bromopicrin split of every nitrosteroid. In the base of the dinitrosteroids, part of the carbon dioxide comes undoubtedly from carbon atom 3. However, more carbon dioxide was isolated than can be accounted for from this position alone. The degradation of cyclopentanone with calcium hypobromite gave rise to carbon dioxide. Some of the carbon dioxide in the case of 2,4-dinitroestrone may result from the degradation of ring D. It will be necessary to determine the nature of the degradation products before the origin of the carbon dioxide can be known with certainty.

* In penta and 1,2,4,5 tetrasubstituted aromatic compounds, the C-H deformation vibration has been reported to occur between 876 and 859 cm^{-1} , while in the 1,2,3,4 tetrasubstituted derivatives, the absorption is found around 829 and 800 cm^{-1} (18,19). Polynitro aromatic compounds appear to be exceptions to these correlations. (20)

Hillmann-Elies *et al.* (13) found a band of moderate intensity at 864 cm^{-1} in the spectra of their mononitroestrone acetate, m.p. 166°. Thus, they assigned the nitro group to the 2 position in this steroid. This band was not present in the spectra of either the 2-nitroestrone acetate or the 4-nitroestrone acetate prepared in this study. The use of a single absorption band (864 cm^{-1}) appears to be insufficient evidence upon which to base a nitro group assignment.

SUMMARY

2-Nitro-, 4-nitro-, and 2,4-dinitroestrone and 2,4-dinitro-17-desoxoestrone were synthesized. A comparison of the spectroscopic data from these steroids with those obtained from model compounds makes it possible to assign tentatively positions to the nitro groups on the benzene ring.

All the nitrosteroids were partially degraded to bromopicrin and carbon dioxide when subjected to the bromopicrin split. The origin of the carbon dioxide has not been established. The bromopicrin from the 2-nitro- and 4-nitroestrones comes respectively from carbon atoms 2 and 4.

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IODINATION OF SERUM PROTEINS AND ITS INVOLVEMENT IN THYROID CHEMISTRY

by

B. Rabinovitch

Thyroxine is now known to appear in the circulating blood, not as the free molecule but as a protein-thyroxine complex. The nature of this complex has been somewhat elucidated recently⁽¹⁾ and is known to involve a specific protein whose properties are similar to those of the α_2 -glycoprotein of Schmid and the M2 mucoprotein of Winzler.⁽²⁾ It is also known that serum albumin is capable of binding thyroxine, albeit to a much lesser extent than the specific protein. In point of fact, the albumin-thyroxine complex appears only when the level of thyroxine in serum is high enough to saturate the specific protein.⁽³⁾

In the treatment of hyperthyroidism, the administering of iodine as potassium iodide over a period of time leads to a remission of the condition, although a higher level of protein-bound iodine (PBI) is found concomitantly. Moreover the application of an iodine-iodide solution (Lugol's solution) instead of an iodide solution leads to the same results in the patient but decidedly more quickly and with a concomitantly higher level of PBI. These facts suggest that the PBI is not calorogenic but most probably arises from the presence of iodinated proteins. These iodinated proteins may be a by-product from the direct action of the iodine in remitting hyperthyroidism or they may play a primary role in the remission. If the latter is true, then this role may be one of direct participation in thyroid-pituitary balance or may simply be one of interference with the normal action of thyroxine in this mechanism.

The purpose of the present work is to determine whether the iodination of serum proteins interferes with normal thyroid chemistry. In this paper, we are concerned with the action of iodinating media on serum proteins in vivo and in vitro.

IODINATION IN VIVO

Each of three guinea pigs of approximately equal weight was given a single intraperitoneal injection of a solution of potassium iodide, elemental iodine, and iodide-iodine. Each of the injected solutions was labeled with I^{131} , and the last-mentioned system was simply a mixture of the first two. The elemental iodine was produced in solution by reacting a known volume of potassium iodate solution with a known volume of potassium iodide solution in the presence of a known concentration of hydrochloric acid, adding 10 ml of propylene glycol and making up to 25 ml with water. These three reagents were internally standardized against the potassium iodide solution. The composition of the three systems is given in Table 1.

Immediately after injection of these iodine solutions, 0.5-ml aliquots of blood were

withdrawn into heparinised syringes, by cardiac puncture, at intervals over a period of some four hours. The plasma was separated by centrifugation after adding 2 ml of a normal saline solution, and the total supernatant liquid was counted for radioactivity in a well-type scintillation counter. The plasma proteins were separated by addition of 5 ml of a 10% trichloroacetic acid solution (TCA), centrifugation, thorough washing with 2 ml 10% TCA, and final centrifugation. The sediment was counted as above.

Table 1
COMPOSITION OF THE IODINE INJECTION SOLUTIONS

Guinea Pig	Body Wt. (g)	Soln Volumes (ml)			Final pH	Activity present (mc)
		KI	KIO ₃	HCL		
B3	540	2.35	0	0	7	0.40
B1	465	1.65	3.0	2.8	5.2	0.22
B2	475	4.	3.0	2.8	5.2	0.62

Concentration of reagents: KI 0.25M, KIO₃ 0.025M, HCl 0.15N

The results of these experiments are shown in Figure 1. Two salient features of these curves appear. While iodine appears in the plasma at a much reduced rate when injected as elemental iodine as compared with the other systems, nevertheless its iodinating properties are much better. The iodinating properties of the iodine-iodide system seem to be at a lower level but probably more enduring. Secondly, a cooperative effect seems to exist between elemental iodine and the iodide ion, allowing for a faster absorption of iodine from the peritoneal cavity into the blood stream. The iodide, though appearing quite rapidly in the blood stream, has little effect during this interval of time on the protein bound iodine.

IODINATION OF SERUM IN VITRO

Fourteen ml of blood were drawn from the hearts of 3 guinea pigs and pooled. The serum was obtained by allowing the blood to clot over a 16-hour period at 6° C. Some hemolysis seemed to take place. Aliquots of this serum were iodinated to different extents using I¹³¹-labeled iodine dissolved in iodide solution. The iodinating solutions used were of the same composition as that used in the in vivo experiment but diluted appropriately so as to incorporate 1, 2, 3, 4, 6, 8, and 10 iodine atoms per mole of protein. This dilution was calculated on the basis of the protein concentration in guinea pig serum being 5% and the average molecular weight being 10⁵; furthermore that all the elemental iodine present be consumed in substitutive iodination (none in oxidation), and that the serum be diluted by a factor of 2 by the iodinating solution.

These serum samples were then electrophoresed on paper strips, using the Spinco

Model R Paper Electrophoresis Apparatus, together with an uniodinated sample of the same concentration. Barbital buffer of pH 8.6 was used, 0.020-ml quantities of serum were applied to the strips, and a current of 15 ma was passed through 8 strips for 6 hours. At the end of this time, the strips were dried in an oven at 120 to 130° C for 30

Figure 1. Appearance of radioiodine in the plasma and the course of iodination of plasma proteins after intraperitoneal injections in guinea pigs.

minutes and upon withdrawal, some of the strips, especially those with the more heavily iodinated serum on them, appeared somewhat discolored. In order to correct for this discoloration, a trace of the density of the color was taken, using the Spinco Analytrol, before dyeing. The results, given in Figure 2, show clearly that the discoloration appears as x

bands on the strips, being absent both in the uniodinated serum and in the iodinated strip 1 (the least iodinated serum), incipient in strip 2, highly developed as a major peak and a minor peak in strips 3 and 4, and highly developed as a major peak in strips 5, 6, and 7, with the minor peak diminishing in height until disappearance in strip 7. The major peak, moreover, appears always at the same place on the strip, being adjacent to the γ globulin and displaced toward the cathode. These bands cannot be attributed to the small amount of hemoglobin present, and must therefore be due either to some unreacted elemental iodine, fixed to the paper in some way, or to a serum fraction appearing only on iodination.

On dyeing the strips with bromphenol blue, it became clear that these colored bands did not pick up any dye and were therefore most likely not due to an iodinated protein. Unfortunately, the specific activity of the iodinating solutions and the extent of iodination did not allow for a scanning of the strips for location of the radioactivity.

A similar set of experiments was carried out using the serum from the same pooled blood but using iodinating solutions having an activity at least 20 times ($240 \mu\text{C}/10 \text{ ml}$) that used in the first series. The extents of iodination, calculated on the same assumptions stated above, were 3.1, 6.2, 8.8, and 12.6 atoms per mole of protein. The conditions for electrophoresis were changed from the first series only in that 8 strips were electrophoresed for 16 hours at 5 ma. After drying the strips, no colored bands could be detected by eye as in the first series, and this was confirmed when, after dyeing and analyzing with the Analytrol, no peaks could be seen on the cathodic side of the γ globulin.

This would suggest that the former colored bands were due to unreacted elemental iodine which disappeared during the slower rate of electrophoresis and longer contact time with the unseparated proteins. However, this point remains in some doubt so long as no explanation can be given for the appearance of more than a simple colored band on strips 3, 4, and 5, and none for their slow migration toward the cathode.

The radioactivity on the two most highly iodinated serums was sufficiently high to allow the corresponding strips to be scanned with great precision with a Tracerlab Geiger-Muller tube ($1.9 \text{ mg}/\text{cm}^2$) attached directly to an Atomic Instruments Company Model 1030A scaler. The strips were fastened to the recording chart of a Bristol Dynamaster Model PH560 recorder, set to travel at a chart speed of 12 inches per hour, and the chart was passed under a lead shield in front of the G-M tube, the window being a slit in the shield approximately $1.3 \times 0.2 \text{ cms}$. The strips could be lined up very accurately with the window and were moved by turning on the chart movement switch for exactly 10 seconds at a time. In 206 such intervals, the strip moved 6.875 inches, which gives a movement of 0.0334 inches per 10 seconds, agreeing closely with 12 inches per hour. With each movement of the strip, a count of 1 minute was taken. Figure 3 shows the results of this scanning and the variation in cpm due to changes in the background. Figure 4 shows, for the two strips under consideration, the superimposition of this radioactivity scan on the Analytrol scan of color density after dyeing the same strip. It must be emphasized that the strips were lined up in the analyzer with great accuracy simply by removing the photocell, exposing the slit and lining it up with a pencil line on the strip. Thus the superimposition in Figure

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Figure 2. Electrophoretic patterns of guinea pig serum iodinated in vitro to different extents. The appearance and location of an unidentified band.

Figure 2 (continued)

Figure 2 (continued)

Figure 3. Radioactivity scan of two electrophoretically separated iodinated serums.

5 was done with great confidence.

It can be seen that the peaks ascribed to the γ globulin and to the α_1 globulin coincide perfectly on the two scans, whereas the albumin peak on the density trace only partially coincides with the large peak on the radioactivity trace, the latter being displaced toward the cathode. The same picture is presented by both strips. Radioactivity in the lesser iodinated serums was again, unfortunately, too low for usefulness.

Figure 4. Comparison of optical density scan of dyed strips with radioactivity scan.

Figure 5. Showing the relative uptake of iodine along two strips and the positions of the serum proteins on the strips.

DISCUSSION

It appears certain that iodination of serum proteins in vivo occurs when a strong iodinating solution is injected intraperitoneally into the guinea pig. The appearance of radioactivity in the protein in the case of the iodide injection cannot arise out of the synthesis of thyroxine⁽⁴⁾ in the short intervals of time involved here, and therefore probably is due

to the oxidation of the iodide ion to elemental iodine in the thyroid gland⁽⁵⁾ and subsequent iodination of the proteins available there. The appearance of such iodinated products in serum have been recognized under certain conditions of trauma.⁽⁶⁾ The failure of the activity in the protein to increase after 45 minutes must be due to the inhibition of iodination by the excess iodide ion accumulating in the thyroid gland.⁽⁷⁾

That elemental iodine should be the most successful iodinating agent is not surprising inasmuch as the presence of iodide ion will inhibit the rate of iodination by elemental iodine due to the formation of the tri-iodide complex ion I_3^- .⁽⁸⁾ However, the radioactivity appearing in the plasma of the iodine/iodide injection is much higher than could be expected from the same amounts of elemental iodine and of iodide ion given in the other two cases. This would suggest that the tri-iodide ion has the special property of being absorbed more rapidly from the peritoneal cavity than the other species. In spite of the reduced rate of iodination, the higher iodine level present in the blood in the iodine/iodide case compensates for this. Maximum iodination appears to be reached in about 2 hours in the case of elemental iodine, but with iodine/iodide a considerably longer period must be expected. If the sustained presence of iodoprotein in the serum is important in the remission of hyperthyroidism, this might explain both the more rapid and more complete action of Lugol's solution in clinical treatment.

Iodination in vitro of guinea pig serum was allowed to proceed for some 16 hours at 6° C, before electrophoresing. Hughes and Straessle⁽⁹⁾ have shown that even after 170 hours, two-thirds of the elemental iodine in an iodine/iodide solution remains unreacted with human serum albumin at pH 7.13. However, even so, some 30 atoms of iodine per mole of albumin are incorporated in the molecule. At a pH close to 6, similar behavior might be expected and this would account for the possible presence of some free iodine in the first series of electrophoresis experiments, and also for its absence in the second series.

The displacement of the maximum uptake of iodine from the albumin peak can be demonstrated clearly by plotting the quotient cpm/optical density against distance along the strip. Figure 5 represents such a plot for the two strips counted and shows unambiguously that there must be a serum component, different from albumin or any of the major constituents of serum, which takes up iodine five times more strongly than most of the serum proteins. It appears that the γ globulins also have a somewhat elevated uptake, perhaps twice the normal level.

It is known that the α_2 -glycoprotein normally found in human serum migrates electrophoretically very close to the albumin,⁽²⁾ having an isoelectric point of about 4.0 compared to the albumin of 4.64. Moreover, the thyroxine-specific protein complex has a mobility at pH 8.6 intermediate between the α and the α_2 globulins.⁽¹⁰⁾ It is not impossible that the iodination of such a specific protein might so alter its electrophoretic mobility so as to shift its location on paper electrophoresis from the anodic side of the albumin peak, where the α_2 -glycoprotein would be, to the cathodic side, but not so far as the α_1 and α_2 globulins where the thyroxine-specific protein complex lies. It is therefore suggested that the spe-

cific protein involved in thyroxine transport is much more readily affected by iodination than is the albumin. That this interferes with its capacity to carry thyroxine awaits further direct experiments.

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IODINATION OF HUMAN SERUM ALBUMIN AND THE UPTAKE OF THYROXINE

by

R. B. Bartosiewicz and B. Rabinovitch

The fact that thyroxine is bound very strongly to a specific protein in serum is well established.⁽¹⁾ It is also known that serum albumin binds thyroxine, although less strongly than the specific protein.⁽²⁾ Very little quantitative work has been done by way of elucidating the nature of these complexes. Lein⁽³⁾ has used the method of the spectrum shift of thyroxine bound to bovine serum albumin, and while the details of his experiments have not been published, he appears to have obtained significant quantitative results. Larson and Albright⁽⁴⁾ have used the method of displacement of bound thyroxine from the thyroxine-specific protein complex by thyroxine analogues and have discussed the relative importance of functional groups and spatial arrangements on the binding capacities.

In this paper we have investigated the quantitative effects of iodination of human serum albumin on its capacity to bind thyroxine.

MATERIALS USED

The human serum albumin (HSA) used was a 25 per cent solution of normal albumin, salt-free, obtained from the Cutter Laboratories. The solution was 0.02 M in sodium caprylate and 0.02 M in sodium acetyl tryptophanate, which act as stabilizers. Their presence was considered unimportant in this study. The thyroxine used was sodium L-thyroxinate obtained from the Nutritional Biochemical Corporation; the I^{131} -labeled thyroxine was obtained from Abbott Laboratories and was chromatographically pure.

METHODS

The method suggested by Klotz⁽⁵⁾ was used in which the shift in an absorption peak of thyroxine after adsorption by the protein is observed. A 1-cm path-length quartz cuvette was used in conjunction with a Beckman spectrophotometer, model DU. Thyroxine shows a convenient peak in its ultra violet spectrum at 330 $m\mu$, where the absorption by the protein is minimal. Systems of protein-thyroxine-buffer were made up, so that various concentrations of protein were found in a fixed concentration of thyroxine. The buffer was a 0.075 phosphate system at pH 11.2. The optical densities (OD) of these systems were measured at 331 $m\mu$ against a blank of exact duplicate systems without thyroxine. The OD of unbound thyroxine was taken as that found in the system containing no protein, and the OD of bound thyroxine as the asymptote reached in the presence of very high protein concentrations.

Since iodinated proteins have an increasingly high absorption at 331 $m\mu$ as the iodine

content of the protein increases, the above-mentioned method becomes useless for highly iodinated proteins. The method of equilibrium dialysis was therefore used for these latter systems; no correction for the Donnan membrane equilibrium was considered necessary since maximum protein concentrations were of the order of 1 per cent and in a buffered system. Dialysis sacks and dialysate were shaken at room temperature for periods ranging from 24 hours to 3 days, depending upon the protein concentration. The equilibrium concentration of thyroxine was determined either by measuring the OD of the dialysate at 331 $m\mu$, or by using labeled thyroxine and counting aliquot samples of the dialysate.

Iodination of the serum albumin was carried out according to Hughes and Straessle,⁽⁶⁾ using a carbonate-bicarbonate buffer at pH 10.0 (unless otherwise stated) with subsequent dialysis at 5° C to remove excess electrolyte.

RESULTS

In Figure 1 is shown the change in OD of a given concentration of thyroxine in the presence of increasing amounts of protein, in the one case uniodinated at pH 11.2 and in the other slightly iodinated (1.66 atoms/mole) at pH 11.12. The displacement of these curves on the OD axis arises out of the different concentrations of thyroxine used, 1.10×10^{-4} M and 1.17×10^{-4} M, respectively. Figure 2 gives these results in terms of moles of thyroxine bound per mole of albumin (r) against equilibrium concentration of unbound thyroxine (A). Included on this figure are the data obtained with a fairly heavily iodinated protein (18.7 atoms/mole) using the same method as with the others. However, the data could not be extended down to low values of A , i.e., high iodoprotein concentrations, because of the high OD at 331 $m\mu$ of the protein alone.

Because the effects of iodination are by no means clear from these data and inasmuch as any effect is therefore likely to be small, the following experiment was devised: From the same HSA solution, aliquots containing 0.5 g of protein were withdrawn and made up to 25 ml in a volumetric flask, first with a calculated volume of iodinating solution at pH 9.4 (carbonate-bicarbonate buffer) and then with phosphate buffer at the same pH. The solutions were left overnight at 6° C and tested for the presence of elemental iodine with a starch solution. None was found on any of the solutions. The calculated iodine contents of the protein samples so prepared were 0, 2.0, 4.0, 6.0, 10.0, 20.0, 30.0, and 40.0 atoms per mole. One-ml volumes of these systems were then placed in dialysis sacks together with 14.0 ml of a 1.00×10^{-4} M labeled thyroxine solution in phosphate buffer at pH 9.4 and were allowed to reach equilibrium against 10 ml of the same thyroxine solution. These systems were shaken for 48 hours at room temperature. Each system was made up in duplicate, and the equilibrium concentration of thyroxine determined by a count of 1 ml of the dialysate. The results of this experiment are shown in Figure 3, where it appears that, as expected, the effect of iodination is clear, albeit small.

A similar series was carried out at pH 7.05 in which the equilibrium was approached from both sides by running duplicate systems in one of which the thyroxine was included in the dialysis sack, the other of which had the same quantity of thyroxine solution placed out-

Figure 1. Change in optical density of fixed concentration of thyroxine in presence of various amounts of protein.

A - Uniodinated HSA

B - Slightly iodinated HSA

Figure 2. Binding of thyroxine by HSA.

Figure 3. Change in uptake of thyroxine with degree of iodination of HSA at pH 9.4.

side the sack. The results of this experiment appear in Figure 4. The equilibrium concentration was taken as the mean of the two dialysates after 4 hours. Owing to the much reduced solubility of thyroxine at pH 7.05, much lower equilibrium concentrations are involved in these experiments than in the previous ones, so that much smaller quantities of protein are involved. Moreover, the absorption of thyroxine by the dialysis sacking becomes under these conditions an important quantity, and all results are corrected for this loss of thyroxine. The iodinations of the protein were carried out as in the previous series at pH 9.4.

DISCUSSION OF RESULTS

The results obtained with the uniodinated HSA agree well with those of Lein, using bovine serum albumin, only insofar as the kind of binding is concerned. In order to demonstrate this, the smoothed data of Figure 1A are plotted in Figure 5A according to Klotz's equation;⁽⁷⁾

$$\frac{1}{r} = \frac{K}{n} \frac{1}{A} + \frac{1}{n} \quad (1)$$

Figure 4. Change in uptake of thyroxine with degree of iodination of HSA at pH 7.05.

and in Figure 5B according to Scatchard's equation;⁽⁸⁾

$$\frac{r}{A} = nk - rk \quad (2)$$

where r and A have the meaning already assigned them above, n is the maximum number of bound molecules or ions and k and $1/K$ have the significance of association constants.

It is clear that according to these data, a total of 8 thyroxine molecules may be bound per HSA molecule. At the lowest concentrations, just one molecule is bound with a k value of 9.9×10^4 liters $\cdot$ mole⁻¹, and above a thyroxine concentration of about 3×10^{-5} M, a second series of sites is engaged, characterized by a k value of 3.2×10^3 liters $\cdot$ mole⁻¹. Lein gives the following results: 4 sites per molecule are occupied first with a k value of 7.9×10^4 liters $\cdot$ mole⁻¹, and above a thyroxine concentration of about 5×10^{-5} M, 8 more sites are occupied, having a k value of 1×10^3 liters $\cdot$ mole⁻¹.

The effect of iodination is seen more clearly in Figures 3 and 4 than in Figure 2. This

Figure 5. Plot of smoothed data for uniodinated HSA according to: A - Klotz's Equation; B - Scatchard's Equation.

is due simply to the inaccuracies inherent in the spectral shift method, especially at low protein concentrations (high free thyroxine concentrations). At pH 9.4, iodination of the albumin inhibits the uptake of thyroxine up to an iodine content of between 10 and 20 atoms/mole, and above this value there is a slight promotion of uptake. There are 18 tyrosine residues per molecule of HSA,⁽⁹⁾ and it is known that these are attacked primarily in iodination.⁽¹⁰⁾ It might appear, then, that if the tyrosine residues are involved in thyroxine binding, iodination at these sites would affect the disposition of thyroxine there. In tyrosine, the -OH group begins to be titrated at about a pH of 11.0, while in diiodotyrosine, the pK of the phenolic group falls to about 8.5.⁽¹¹⁾ Thus it would appear that as iodination occurs at a fixed pH of 9.4, more and more tyrosine -OH groups lose a proton and become negatively charged. This would certainly hinder the uptake of the thyroxinate ion. The promotion at higher levels of iodination is probably a result of secondary effects due to denaturation.

At the lower pH of 7.05, however, uptake of thyroxine is promoted at all levels of iodination that were examined, but it is well to remember that at this pH the tyrosine -OH groups remain uncharged no matter what the iodine content of the albumin is. If the binding of thyroxine is promoted by iodination but inhibited by the presence of a negative charge on the tyrosine residue, this would explain the results observed.

This picture is partially substantiated by comparison of the values of the quotient r/A for uniodinated albumin at the three experimental pH's. From equation 2 it is apparent that as r/A increases, either the binding capacity (n) increases or the free energy of binding increases (function of k); this assumes that over the range of free thyroxine concentrations encountered, r/A is independent of r (see Figure 2). This comparison is made in Table 1:

Table 1

pH	Free Thyroxine conc. (A)	r/A
11.2	up to 2.5×10^{-4} M	1.8×10^4
9.4	ca. 4×10^{-5} M	1.1×10^5
7.05	ca. 4.8×10^{-7} M	3.0×10^5

It is more likely that r/A is here a reflection of the binding strength rather than binding capacity, since it cannot be expected that the latter can increase 17 fold over this pH range. Thus we can say that thyroxine is more strongly bound the lower the pH in the region of 7 to 11, and this would be a natural consequence of charge effects.

CONCLUSIONS

We may say that the effect of iodination on the capacity of HSA to bind thyroxine is made up of two factors: the steric effect of the iodine atoms, probably at the tyrosine residues, and the effect of the iodine atoms on the pK of the tyrosine phenolic groups. At pH's lying below the pK of diiodotyrosyl -OH groups, iodination has a promoting effect caused by the steric factor. At pH's above the pK of tyrosyl -OH groups, iodination has little or no effect because of the presence of the negative charge on the tyrosine residues. At pH's lying between these two pK values, the effect of iodination is one of inhibition due to the successive loss of protons from the tyrosyl -OH groups with increasing levels of iodination.

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TRITIATION OF DEUTERATED COMPOUNDS*

by

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PART I. RECOIL TRITIATION OF DEUTERATED COMPOUNDS

A method for synthesizing organic compounds labeled with tritium has been reported recently by Wolfgang, Rowland, and Turton⁽¹⁾ who find that tritium atoms, which are produced in the $\text{Li}(n, \alpha) \text{T}$ reaction, are capable of replacing hydrogen atoms in organic compounds with a high efficiency. In the irradiation of a powdered mixture of glucose and lithium carbonate, approximately 10 per cent of the total tritons produced were located in the non-labile positions of the glucose. From the results of degradation studies with the glucose, the authors indicated that labeling is nonuniform within the molecule.⁽²⁾ Further experiments⁽²⁾ by the same authors have included the labeling of crystalline galactose whereby activities approximating $100 \mu\text{c}$ per g were obtained before radiation damage became excessive. In an independent check of this technique, Wolfgang and coworkers⁽¹⁾ have reported the results obtained by Kaplan at Argonne National Laboratory from the irradiation of a mixture of benzoic acid and lithium sulphate. Kaplan found that 25 per cent of all tritons produced were in the ring hydrogens of labeled benzoic acid, a specific activity of $96 \mu\text{c}$ per g being obtained with little radiation decomposition.

A review article covering the results obtained from the tritium-recoil labeling of 22 different organic compounds has been written.⁽³⁾ The authors claim that the procedure is generally applicable to low-level synthetic work, although the technique cannot produce the very high specific activities obtainable by chemical synthesis or by exchange reactions with materials containing large percentages of radioactive atoms.

Pertinent to the mechanism of the reaction, the distribution of tritium in isopropyl benzoate has been studied so that a comparison can be made of the replacement of aromatic hydrogen and of the two different types of hydrogen in the isopropyl group. The nature of any observed deviations from random distribution can be correlated with the type of processes that are occurring. The same system was used for the study of the occurrence of isotope effects during tritiation, comparative distribution experiments being performed with 2-propyl-1,1,1,3,3,3- d_6 (isopropyl- d_6) benzoate and 2-propyl benzoate- d_5 (isopropyl benzoate- d_5). Another aspect of the problem, important to the mechanism of hydrogen-tritium substitution and to its practical applications in the labeling of biologically-important materials, is the question of stereochemical configuration of the tritium atoms when substitution occurs on an asymmetric carbon atom. The high yield of tritiated

* Abstract of a dissertation that has been accepted by the Department of Chemistry, The University of Chicago, as one of the requirements for the Ph.D. degree.

glucose reported by Wolfgang, Rowland, and Turton⁽¹⁾ suggests a tendency towards retention of configuration. Rowland and Malson⁽⁴⁾ confirm this by their results with the tritiation of 1-alanine. They found that 16 per cent of the tritons stopped in crystalline 1-alanine were retained in nonlabile positions in dl-alanine. This activity was distributed between 1-alanine and d-alanine in a ratio of more than 7 to 1, indicating a strong preference for the formation of the same optical isomer as that irradiated.

In this paper, tritium substitution on an asymmetric carbon atom has been studied in the case of 2-octyl phthalate using carrier techniques to detect the formation of the enantiomorph.

All samples were irradiated in the Isotope Trays of the CP-5 Reactor at a flux of 1×10^{12} neutrons per cm^2 per sec for periods of 118 to 153 hrs. For this procedure, solid organic materials were mixed with lithium oxalate and sealed in evacuated quartz ampoules, while liquid samples (isopropyl benzoate and deuterated derivatives) were irradiated in the form of lithium myristate gels.

Tritium distribution studies were performed on the recovered esters by the following degradation procedure:

The results of the experiments are summarized in Tables 1 and 2.

A general feature of the results (see Table 1) is that the largest proportion (30 to 42 per cent) of the total tritium activity produced in each irradiation is in the gas phase; the total activity in the recovered ester is inversely proportional to the activity in the gas, e.g., isopropyl benzoate- d_5 possesses the lowest activity (10 per cent) of the recovered esters and the highest gaseous activity (42 per cent). This substantiates the theory that the activity in the gas arises from secondary reactions in the irradiated material.

The observed tritium distribution in the various esters, together with the estimated errors, are listed in Table 2. The hydrogen in the mixed H-D species serves as an internal standard and permits an evaluation of H-D isotope effects not too dependent upon the unavoidable variations in radiation dosage received by different samples.

From the data in Table 2, it is observed that in the comparison of the aryl and primary alkyl hydrogen atoms of isopropyl benzoate, the former are replaced more effectively by tritium by a factor of 2.66 (this factor is based upon the average of the five aryl hydrogens, the distribution of tritium in the benzoate group not being determined). Higher efficiencies have also been observed in the tritiation of aromatic nuclei as compared with aliphatic systems.⁽⁵⁾

Table 1
RESULTS OF THE ISOPROPYL BENZOATE IRRADIATIONS

	Isopropyl benzoate	Isopropyl-d ₆ benzoate	Isopropyl benzoate-d ₅
Mixture irradiated	3.6900g ester 0.595g lithium myristate	3.0530g ester 0.529g lithium myristate	3.256g ester 0.514g lithium myristate
Length of irradiation hrs.	118	128	153
Flux* n/sq cm/sec	1×10^{12}	1×10^{12}	1×10^{12}
Calculated T activity	2223 μ curies	2110 μ curies	2450 μ curies
Recovered ester specific activity	0.188 μ c/mg	0.187 μ c/mg	0.217 μ c/mg
Lithium myristate specific activity	0.20 μ c/mg	0.159 μ c/mg	0.181 μ c/mg
Polymer [†] specific activity	1.096 μ c/mg	0.549 μ c/mg	0.562 μ c/mg
Recovered ester total activity	377.5 μ c (16.8%) [‡]	402.0 μ c (19.0%) [‡]	249.6 μ c (10.2%) [‡]
Polymer [†] total activity	657.5 μ c (29.3%) [‡]	467.0 μ c (22.1%) [‡]	562.0 μ c (22.9%) [‡]
Gas: activity	750 μ c (33.4%) [‡]	625.0 μ c (29.6%) [‡]	1036 μ c (42.3%) [‡]
Volume S.T.P.	137.2 cc	100.7 cc	138.5 cc
Pressure	237.9 cm	210.8 cm	311.4 cm

* Flux is accurate to $\pm 10\%$.

[†] Non-distillable oil remaining after recovery of lithium myristate and isopropyl benzoate from the mixture after irradiation.

[‡] Percentage figures based on calculated T activity.

The difference in tritium incorporation (factor 1.28, see Table 2) between the secondary and primary hydrogen atoms of the isopropyl group is almost within experimental error. This is in contrast with the very considerable departure from uniform distribution observed in glucose and galactose by Rowland, Turton, and Wolfgang.⁽²⁾ At present, there is no basis for associating the relative degree of tritium substitution on an aliphatic CH group with the number of hydrogen atoms bound to the carbon atom, i.e., according to whether it is primary, secondary, etc.

The isotope effect, referring to the incorporation of tritium by replacement of H as compared with the replacement of D, is of small magnitude for tritiation at either an aryl position or in a methyl group (observed H/D ratios 1.16 and 1.41, respectively) but is significantly different from unity in the latter case. Thus, regardless of mechanism, it can be stated that a process resulting in tritiation of a methyl group involves a weakening, if not complete rupture, of a C-H bond in the rate-determining step. To a much smaller degree, if at all, C-H bond weakening or rupture is not involved as a rate-determining factor in aromatic tritiation.

From the results of the octyl phthalate irradiations (Table 3), there is a tendency

Table 2

TRITIUM DISTRIBUTION IN THE ESTERS

Component	Isopropyl benzoate activity ($\mu\text{c}/\text{mmole}$)	Isopropyl- d_6 benzoate activity ($\mu\text{c}/\text{mmole}$)	Isopropyl benzoate- d_5 activity ($\mu\text{c}/\text{mmole}$)
Benzoic acid	18.83	20.59	18.94
Acetone-2,4- dinitrophenylhydrazone	8.50	6.55	9.89
Isopropyl-3,5- dinitrobenzoate	10.32		
Activity per CH (μc): Aryl	2.66 ± 0.05	3.78	2.30
primary aliphatic	1.00 ± 0.05	1.00	1.00
secondary aliphatic	1.28*		
Ratio of activity per CH (or C-D): aryl/primary aliphatic	2.66	3.78	2.30
Ratio of activity: primary aliphatic CH/ primary aliphatic C-D	$\frac{1.41}{1.00}$		
Ratio of activity: aromatic CH/ aromatic C-D	$\frac{1.16}{1.00}$		

* This figure has been derived by difference whereas other data have been computed from direct measurements.

towards retention of configuration when substitution of recoil tritium occurs on an asymmetric carbon atom. From the irradiation of 1(-)-2-octyl phthalate, the recovered 1(-)-2-octanol possessed an activity per C-H position of $11.3 \mu\text{c}$ (based upon the average of the 17 available C-H positions in 2-octanol) and was almost three times greater than the total activity ($4.72 \mu\text{c}$) found in the carrier enantiomorph. This activity in the d(+) form must also be corrected for the possible presence of optical isomer since resolution was only accurate to 1 per cent, so that $4.72 \mu\text{c}$ is an upper limit to the total carrier activity. From the irradiation of d(+)-2-octyl phthalate, the data for the recovered d(+)-2-octanol (average activity per CH, $6.7 \mu\text{c}$ as compared with total carrier activity of $4.82 \mu\text{c}$) substantiate the conclusion that there is a strong preference for the formation of the same optical isomer as that irradiated.

The possible mechanisms for the incorporation of recoil tritons by organic molecules have been discussed by Rowland, Turton, and Wolfgang⁽²⁾ principally in terms of three possible types of reactions involving neutral tritium atoms: (1) hot atom reaction with capture of T and simultaneous elimination of H; (2) fragmentation of target material into radicals by low energetic tritium atoms and recombination; and (3) tritium atoms, after reach-

Table 3

d(+) AND 1(-) OCTYL PHTHALATE IRRADIATIONS*

Mixture irradiated	d(+)-2-Octyl Phthalate 3.5314g d(+) form 0.112g lithium oxalate*			1(-)-2-Octyl Phthalate 3.709g 1(-) form 0.118g lithium oxalate†		
Gas:Activity μc	664.7			702.1		
Volume cc. S.T.P.	68.4			84.4		
Carrier added	Irradiated mixture* 3.35g 1(-)-2-Octyl phthalate 0.80g			Irradiated mixture† 3.50g d(+)-2-Octyl phthalate 2.90g		
	Spec. activity $\mu\text{c}/\text{mg}$	Wt. of material (g)	Total activity (μc)	Spec. activity $\mu\text{c}/\text{mg}$	Wt. of material (g)	Total activity (μc)
Phthalic acid	0.0664	1.200	79.5	0.0861	2.550	219.6
Polymer†	0.420	0.320	134.3	0.490	0.470	230.3
Recovered d(+)-2-octanol	0.118	0.940	114.5 (average 6.7 μc per C-H)	0.00504	0.937	4.72‡
Recovered 1(-)-2-octanol	0.0129	0.375	4.82‡	0.139	1.380	192.0 (average 11.3 μc per C-H)

* Length of irradiation was 120 hrs for each mixture. The flux was approximately 1×10^{12} neutrons per cm^2 per sec.. Both mixtures were irradiated at the same time, so that any change in flux does not appreciably affect comparative results.

† Non-distillable oil remaining after recovery of 2-octanol, phthalic acid and lithium oxalate from the mixture after irradiation.

‡ The activity in these compounds was still dropping to a lower value. These figures must also be corrected for the possible presence of optical isomer since resolution was only accurate to one per cent.

ing thermal energy, eventually combining with free radicals. From their observations on the labeling of the hexoses, mechanism (2) is consistent with the high gross entry of tritium. To account for the peculiar distribution of tritium in the products, it is further assumed that shielding effects may be important and that, in the interval between fragmentation and recombination, varying amounts of radical isomerization may occur.

From the results of the present work, the following additional comments can be made:

1. The H-D isotope effect in tritiation on an aliphatic carbon atom is consistent with mechanism (2).
2. In aliphatic systems, hydrogen abstraction by tritium atoms is a predominant reaction.
3. The virtual absence of an H-D isotope effect in the tritiation of an aromatic nucleus points toward a mechanism involving capture of tritium with subsequent elimination of H (or D).
4. The higher efficiency of tritiation on an aromatic nucleus, despite the known greater susceptibility of aliphatic structures to free radical attack, probably reflects the disappearance of a substantial fraction of the aliphatic radicals by paths other than combination with T atoms. The appearance of polymeric by-products containing tritium in amounts greater than that found in the recovered parent substance is a measure of the importance of such side reactions.
5. The retention of asymmetry in the tritiation of the asymmetric carbon of 2-octyl acid phthalate is an observation of some practical significance in connection with the labeling of natural products. It is to be noted that this situation, as well as the hexoses and 1-alanine studied by Rowland and co-workers, involved the irradiation of crystalline solids.

PART II. DEUTERIUM EXCHANGE REACTIONS OF SUBSTITUTED BENZENES

The study of these exchange reactions originated from the need to find an improved method for synthesizing benzoic acid-d₅. The most suitable procedures reported in the literature involve the preparation of hexadeuterobenzene⁽⁶⁾ followed by bromination and a grignard reaction to yield pentadeuterobenzoic acid. Leitch⁽⁶⁾ has prepared hexadeuterobenzene by the exchange of benzene with heavy water in the presence of active platinum at 130° C.

In this research, the same procedure⁽⁶⁾ has been applied to the deuteration of substituted aromatic systems such as benzoic acid, sodium benzoate, bromobenzene, isopropyl benzoate, nitrobenzene, p-nitrobenzoic acid, and trimesic acid (benzene-1,3,5-tricarboxylic acid) in an attempt to study the influence of substituent upon the ease of exchange. The rate at which sodium benzoate exchanges has been investigated, and the possibility of observing orientation effects during deuterium incorporation into bromobenzene and sodium benzoate has been examined.

The results of the exchange reactions with the aromatic compounds are shown in Table 4.

In marked contrast to the ease with which sodium benzoate and benzoic acid exchange, the remaining benzene derivatives show less tendency to incorporate deuterium. Trimesic acid and p-nitrobenzoic acid do not exhibit exchange; nitrobenzene and bromobenzene exchange to a small degree (3 to 10 per cent), whereas isopropyl benzoate undergoes theoretical deuterium equilibration in the ring hydrogens only, almost negligible exchange occurring in the isopropyl group.

Table 4
DEUTERIUM EXCHANGE OF SUBSTITUTED AROMATIC COMPOUNDS

COMPOUND	Wt. (g)	D ₂ O (g)	Platinum oxide (g)	Time of reaction at 130-5°C (hrs)	Theoretical α^*	Found α^*	% Deuteration completion
Sodium benzoate	0.130	0.489	0.012	24	0.915	0.910	99.5
Benzoic acid	0.100	0.402	0.012	12	0.891	0.731	82.0
Benzoic acid + K ₂ CO ₃ (0.007g)	0.112	0.370	0.012	12	0.870	0.728	83.7
Isopropyl benzoate	1.500	1.00	0.150	72	0.476	0.475	99.8
Bromobenzene	1.495	2.20	0.120	24	0.822	-	≈ 10
Nitrobenzene	0.550	2.20	0.050	24	0.908	-	≈ 3
p-Nitrobenzoic acid	0.500	2.20	0.120	24	0.936	0	0
Trimesic acid	0.500	2.20	0.120	24	0.939	0	0
Trimesic acid + K ₂ CO ₃ (0.025g)	0.500	1.98	0.120	24	0.933	0	0

$$* \alpha = \frac{\text{m.e.D.}}{\text{m.c.}(D+H)}$$

The evidence presented by the infrared spectra A and B, Figure 1, indicates that random distribution of deuterium is obtained with sodium benzoate at complete equilibration. The deuterobenzoic acid in spectrum A was prepared by equilibrating sodium benzoate with water of known deuterium content (calculated to give 25 atom per cent excess D in benzoic acid), whereas spectrum B represents a deuterobenzoic acid (30 atom per cent excess of D) synthesized from deuterobenzene. The two spectra are very similar in the positions of all absorption bands.

The data obtained in a series of rate experiments with sodium benzoate are reported in Table 5. The slow initial rate, or apparent induction period, is followed by a rapid up-

Figure 1. A. Deuterobenzoic acid $\alpha = 0.256$ from D_2O exchange of sodium benzoate.
B. Deuterobenzoic acid $\alpha = 0.301$ from deuterobenzene.

Table 5

DEUTERIUM EXCHANGE RATE WITH SODIUM BENZOATE

Sample	Wt. of sodium benzoate (g)	D_2O (g)	Platinum oxide (g)	Time at 130-5°C (hrs)	Theoretical α^* in benzoic acid	Found α^* in benzoic acid	Per cent reaction completion
A	0.130	0.485	0.012	1	0.761	0.00120	0.15
B	0.130	0.491	0.012	3	0.762	0.0574	7.5
C	0.130	0.497	0.012	6	0.764	0.608	79.6
D	0.130	0.489	0.012	12	0.762	0.732	96.0

$$* \alpha = \frac{m.e.D.}{m.e.(D+H)}$$

take of deuterium and eventually complete equilibration with respect to all nuclear positions.

Infrared spectra of the benzoic acid samples resulting from the above-mentioned experiments, together with the reference spectra of o-, m-, and p-monodeuterated benzoic

acids are reproduced in Figures 2 and 3. The pertinent C-D absorption frequencies for samples A and B as well as the corresponding reference bands from the o-, m-, and p-monodeuterated benzoic acids are summarized in Table 6.

Our interpretation of the data is that deuteration occurs least rapidly at the meta-positions and probably less rapidly at the para- than at the ortho-positions. This orientation effect was opposite that observed in the deuteration of bromobenzene. From the infrared spectra of deuterated bromobenzene, it was noticed that deuteration occurs almost exclusively in the meta- and para- positions (para- appears to be twofold more reactive).

There are several theories^(8,9) to account for the observed orientation effects in exchange reactions involving substituted benzenes in the presence of active metal catalysts. Lauer and Errede⁽⁸⁾ found, in deuterating aniline with heavy water and sodium deteroxide in the presence of Raney nickel, that the ortho hydrogens exchanged more readily than the meta and para. The authors assumed that the aniline was anchored to the catalyst through the electron pair on the nitrogen and that the ortho hydrogen touches the reactive sites on the catalyst more often than do the meta and para hydrogens.

Tiers⁽⁹⁾ has reported that in each of the ortho-, meta-, and para-monodeuterotoluenes, the deuterium will be redistributed throughout the ring and the alpha hydrogens of the methyl group, under the influence of Raney nickel catalyst. The ortho species in toluene exhibits a relatively low reactivity in redistribution experiments ($k_o/k_m = 0.1$) where as meta and para undergo redistribution at the same rate ($k_m/k_p = 1.0$). Tiers⁽⁹⁾ attributes this low ortho effect to the steric factor of the methyl group rather than to any differences in activation energy of the species involved.

On the basis of the Lauer and Errede theory,⁽⁸⁾ the orientation effect observed in the initial exchange of sodium benzoate (Table 5) can be explained by assuming that the benzoate ion is absorbed at the catalyst surface through its carboxylic acid group. The ortho positions are then in close proximity to the catalyst, and possibility of exchange in these positions is strong. This conclusion is supported by the trimesic acid results (Table 4) where it is presumed that the acid is very strongly adsorbed on the catalyst surface through its three carboxylic acid groups and the ortho hydrogens are not free to touch an active site to undergo exchange.

The low ortho reactivity that was observed during the initial exchange of bromobenzene is consistent with the results from the toluene experiments⁽⁹⁾ and can be attributed to the relatively large steric requirements of the heterogeneous catalytic surface.

The poor reactivity of nitrobenzene and p-nitrobenzoic acid towards catalytic exchange can be attributed to reduction of the nitro group by the residual deuterium gas available at the catalyst surface, thus lowering the efficiency of the catalyst towards the promotion of further exchange.

The reason for the absence of exchange in the aliphatic hydrogens of isopropyl benzoate as compared with the ease of deuteration of the methyl hydrogens in toluene may be attributed to different types of mechanisms for the exchange of each compound. Tiers⁽⁹⁾ has suggested the formation of a dissociative intermediate complex involving the catalyst

E

F

G

Figure 2. A. Nondeuterated benzoic acid.
 B. Deuterobenzoic acid 0.15% complete reaction (Sample A, Table 5).
 C. Deuterobenzoic acid 7.5% complete reaction (Sample B, Table 5).
 D. Deuterobenzoic acid 45% deuterated for comparison.
 E. Deuterobenzoic acid 79.6% complete reaction (Sample C, Table 5).
 F. Deuterobenzoic acid 96.0% complete reaction (Sample D, Table 5).
 G. Deuterobenzoic acid completely deuterated.

Figure 3. A. Nondeuterated benzoic acid.
 B. Ortho mono deuterated benzoic acid.
 C. Meta mono deuterated benzoic acid.
 D. Para mono deuterated benzoic acid.

Table 6*

INTERPRETATION OF IR SPECTRA OF SAMPLES (A) AND (B) IN TABLE 5

Sample	Intensities	Observed in reference spectra		Observed absorption frequency in cm^{-1}	Interpretation
	Ortho-D-benzoic	Meta-D-benzoic	Para-D-benzoic		
A	180		55	779 (shoulder)	ortho and para
B	120		90	693 (shoulder)	ortho and para
		116		770 (shoulder)	meta
	180		55	779 (strong)	ortho and para
		72	150	878 (strong)	meta and para
				1140 (shoulder)	ortho

* All spectra were recorded from KBr discs on a P.E. double-beam, double-pass instrument at a concentration of 1.9mg/150mg KBr. The reference bands for ortho, meta, and para species in KBr agreed with the values in solution reported by Tiers.⁽⁷⁾ The intensities listed are also from the paper by Tiers.⁽⁷⁾

and benzyl radicals to account for the toluene results. However, any theory for the isopropyl benzoate exchange presumably does not involve the dissociation of the hydrogens on the isopropyl group in the formation of the transition state.

SUMMARY

1. The mechanism of tritium labeling of organic compounds by recoil tritons generated in the Li (n, α) T reaction has been studied using isopropyl benzoate, isopropyl benzoate- d_5 and isopropyl- d_6 benzoate as model systems.

2. In tritium distribution experiments with labeled isopropyl benzoate, a high efficiency of tritium incorporation on the aromatic nucleus has been observed; the ratio of the average tritium incorporation in aryl; primary; secondary C-H positions being 2.66:1.00:1.28.

3. The isotope effect, referring to the incorporation of tritium by replacement of hydrogen as compared with replacement of deuterium, is of small magnitude for tritiation at either an aryl position or in a methyl group (observed H/D ratios 1.16 and 1.41, respectively) from the data with labeled isopropyl benzoate- d_5 and isopropyl- d_6 benzoate.

4. The t-labeling of optically active compounds has been studied using d(+) and l(-)-2-octyl phthalates and in each case the results suggest that there is a strong preference for the formation of the same optical isomer as that irradiated.

5. The data available for aliphatic systems is consistent with a mechanism in which hydrogen abstraction by tritium atoms is the predominant reaction whereas evidence for aromatic tritium incorporation indicates that a mechanism involving capture of tritium with subsequent elimination of H (or D) will be more plausible.

6. An exchange reaction using heavy water and catalyzed by active platinum has been applied to the deuteration of substituted benzenes such as benzoic acid, bromobenzene, isopropyl benzoate, aniline, nitrobenzene and trimesic acid.

7. Exchange with sodium benzoate involves an apparent induction period, followed by rapid deuteration and eventually complete equilibration with respect to all nuclear positions.

8. Differences in deuteration rate have been observed for the various compounds and these differences have been correlated with the type of substituent which is present in the compound undergoing exchange.

9. An initial orientation effect occurs in the deuteration of both sodium benzoate and bromobenzene and this has been attributed to the influence of the substituent upon the ease of formation of the transition state during the exchange reaction.

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I-II + Nd

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