rapidly disappear from the bloodstream of rats and may be fixed at specific tissue sites. If so, absorbed PA not only cannot react with subsequently injected LF to produce the lethal effect but also undergoes some change that inhibits the lethality of toxic mixtures. The exact nature of this change or changes is unknown but probably involves subtle physicochemical changes rather than extensive splitting of the molecule.

The observation that lethality of toxic mixtures can be inhibited by large doses of PA suggest that both are fixed at the same tissue site and that toxic mixtures are fixed through their PA component. The toxin continuously released during anthrax infection might well be antagonized by repeated injections of PA to occupy all the available tissue sites. Since it is generally recognized from the data of Smith *et al*(6) that toxemia is the primary cause of death in anthrax infected animals, these observations may be useful in exploring a rational therapy in anthrax.

Summary. Evidence is presented to show that protective antigen undergoes subtle alterations that affect its biological activity. For one hour following intravenous injection of protective antigen (PA) into the rat, subsequent inoculation of lethal factor leads to death of the rat as expected. Two hours after injection of protective antigen, injection of lethal factor does not kill the rat although challenge with whole toxin produces the classical lethal response. At this time, PA is no longer demonstrable in the bloodstream by immunological methods. In contrast, 4 hours following an injection of protective antigen, rats not only do not die when injected with lethal factor but also are protected from the lethal effect of whole toxin. Some preparations of protective antigen can detoxify *in vitro* whole toxin of known potency. These facts are discussed in relation to the clearance from the bloodstream of injected whole toxin or its components and a possible role for PA in anthrax therapy is suggested.

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Plasma Protein Thyroid Hormone Complexes: Separation by Continuous Flow Paper Electrophoresis. (28657)

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The transport of the thyroid hormones thyroxine and triiodothyronine by plasma proteins has received much attention in the last few years(1). It has been shown that their transport is accomplished by several plasma proteins, but there seems to be no general agreement as to which protein plays the main physiological role. The differences in results obtained by the use of various experimental methods are manifest(2). The considerable amount of work performed using single- and 2-dimensional gel and paper electrophoretic techniques, although very useful in identification of thyroid hormone-plasma protein complexes does not allow a determination of the chemical composition of these complexes (3,4). We are reporting our attempt to isolate these complexes by means of continuous flow electrophoretic fractionation. This method enables us to separate the protein-bound thy-

roxine complexes in amounts large enough to facilitate further identification and chemical characterization.

Materials and methods. Blood samples from 6 patients receiving doses varying from 2 to 6 mc of I-131 for treatment of hyperthyroidism were collected by venipuncture 24 and 48 hours after the dose and allowed to clot. The serum removed after centrifugation was treated with an anionic exchange resin (Amberlite IRA-400 in its chloride phase) to remove the inorganic I-131. The nature of the iodinated compound found in the circulation of patients receiving therapeutic doses of radioiodine for treatment of hyperthyroidism has been established by Robbins *et al* (5) to be essentially thyroxine. As a check we also performed descending paper chromatographic analyses of the butanol extractable portion of sera obtained from 2 of the patients using butanol-acetic acid-water (4:1:5) as solvent on Whatman No. 1 paper. The activity appeared to be located in one spot corresponding to that of commercial radioiodinated thyroxine in control runs.* In three additional experiments the serum from each of 2 subjects showing abnormalities in their plasma proteins and from a normal individual was incubated *in vitro* with commercial thyroxine for 30 minutes with constant shaking at 37°C. Each serum was treated as explained above to remove unbound thyroxine or inorganic I-131.

Serum proteins were separated using the Spinco C.P. Continuous Flow Paper Electrophoresis Cell enclosed in a special refrigerator at a temperature of 5°C† Pre-cut upper and lower curtains and wicks from Schleicher and Schuell No. 470 filter paper were used. Barbital buffer of pH 8.6 and ionic strength 0.02 was used as the electrolyte. A continuous and steady flow of buffer was maintained with the wick syphons and the overflow tube in the electrolyte reservoir set at a level of 10 cm and 5.5 cm respectively. The current from a Spinco constant power supply was adjusted to 60 milliamp which required 710 volts. The curtain was allowed to come to equilibrium by applying the current for 30 minutes prior to sample fractionation.

Before sample separation the serum was dialyzed against the electrolyte solution overnight in the refrigerator. The serum samples were applied at a rate of 1.5 ml per hour to the top of the lower curtain using a tab 4 inches from the negative side of the cell. The sample was then carried downward by the flow of the buffer and collected in a series of 32 tubes. The volume recovered in each tube was measured and a 0.5 ml aliquot of each tube was qualitatively analyzed for protein with 10% trichloroacetic acid. The radioactivity present in each of the fractions was measured using a well type scintillation counter in conjunction with a scaler and radiation analyzer.‡

The protein fractions were then identified by paper electrophoresis employing filter paper strips (3 × 30.6 cm Whatman 3 mm) as stabilizer and barbital buffer (pH 8.6 and ionic strength 0.05) as electrolyte. A Spinco Duostat power supply was used to apply 240 volts for 4 hours. The strips were dried at 120-130°C for 30 minutes and stained 30 minutes with bromphenol blue in methanol.§ Aliquots of the whole and dialyzed sera were run simultaneously so as to identify each fraction by its mobility. The lipoprotein fractions were likewise identified by staining a duplicate set of strips for 16 hours with a saturated solution of oil red O in 60% ethanol(6). Total protein determinations were performed on all protein fractions by the standard biuret technique as described by Reinhold(7).

Total beta lipoprotein determinations were performed on all protein fractions by the Beta-L-Test using the "Immunocrit Method."‖ Total cholesterol was performed on the

* Commercial preparations supplied by Abbott Laboratories, North Chicago, Ill.

† Spinco Division of Beckman Instruments, Inc., Belmont, Calif. Operating Instructions: Spinco Model CP, Continuous Flow Paper Electrophoresis Cell.

‡ Nuclear Chicago Corp.: Model DS-5 Scintillation Well Counter, Model 181A Royal Scaler and Model 1810 Radiation Analyzer.

§ Spinco Division of Beckman Instruments, 1957. Paper electrophoresis system. Operating instructions. Revised serum protein analysis. Technical Bulletin No. 6027A.

‖ Beta-L-Test and Immunocrit are trademarks of Hyland Laboratories, Los Angeles, Calif.

TABLE I. Percent Radioactivity in I-131 Labelled Protein-Thyroxine Complex After Curtain Flow Electrophoresis Fractionation of Human Sera.

Exp No.	Gamma globulin	Beta globulin	Alpha-2 globulin	Alpha-1 globulin + albumin	Albumin
1	1.0	3.8	52.2	35.6	7.4
* 2	0	3.8	64.7	27.3	2.9
† 3	6.8	12.7	17.8	16.0	30.8
4	1.4	4.2	74.6	14.2	4.3
5	.2	3.0	52.7	33.8	8.9
6	.5	2.8	75.4	15.4	5.7
7	.3	3.4	61.9	29.7	3.7
* 8	0	.8	88.0	8.1	2.9
* 9	.1	18.1	56.8	22.4	2.3

* *In vitro* experiments, subjects not hyperthyroid.

† Remaining radioactivity (to complete 100%) migrated ahead of albumin.

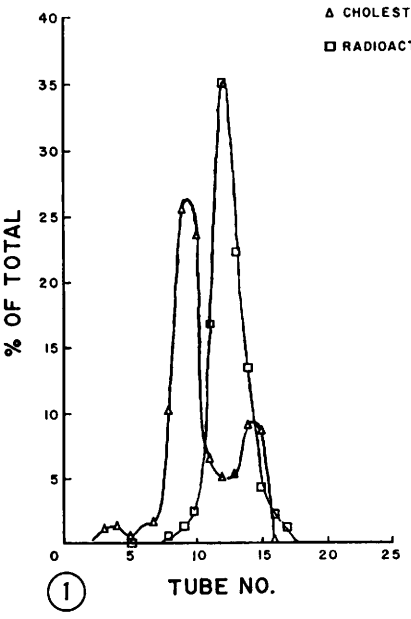
fractions recovered by the microtechnique of Zak(8).

Results and discussion. Our results are summarized in Table I. Curtain flow electrophoresis confirms the previous findings that almost all the protein bound radioactivity in patients receiving therapeutic doses of I-131 is found as thyroid hormone-protein complex with proteins having the mobility of alpha globulins and albumin(1). Very small amounts were found to be bound by the gamma globulins. In 5 *in vivo* experiments, the radioactivity bound to gamma globulins was less than 1.5% of total protein bound radioactivity. Identical results were obtained in 3 *in vitro* experiments. The sample from one subject showing a marked alteration in his plasma proteins contained 6.8% of the total radioactivity as thyroid hormone gamma globulin complex (Exp. 3). The beta globulins were found to be capable of forming thyroid hormone protein complexes to a larger extent than the gamma globulins. In 5 *in vivo* and 2 *in vitro* experiments the per cent of total radioactivity found as thyroxine-beta globulin complex ranged from 2.2 to 4.2%. In an additional *in vivo* experiment, 12.7% of the radioactivity was present as thyroxine beta globulin complex. This same subject had a considerable amount of organic-bound radioactivity as thyroid hormone-gamma globulin complex. An additional *in vitro* experiment (Exp. 9) performed on the serum of a pateint with cirrhosis of the liver showed the presence of an unusual amount of organically bound radioactivity (18.1% of total radioactivity) as thyroxine-beta globulin complex. This was the only case in which the fraction having the highest beta lipoprotein contents, as determined by the Immunocrit Method, contained appreciable radioactivity. In all other cases the radioactivity present in the fraction containing the bulk of the beta lipoproteins was less than 2%.

In 5 *in vivo* and 3 *in vitro* experiments the largest percent of the radioactivity was found to exist as an alpha globulin-thyroid hormone complex. The individual fraction containing the highest percent of the total radioactivity was, with only one exception, always the fraction containing alpha-2 globulin. Once again, the exception was the subject whose gamma and beta globulin showed a higher than usual affinity for thyroid hormone. In his case, the highest percent of organic bound radioactivity was found in a fraction showing the mobility of an albumin.

Protein and cholesterol analysis of the individual fractions obtained after curtain flow electrophoresis indicated that the bulk of the radioactive thyroxine is bound by proteins migrating between the beta and alpha lipoproteins. Although the alpha globulin fractions containing the bulk of the radioactivity also contain cholesterol, in none of the experiments did either of the peaks of cholesterol coincide with the peak of the radioactivity (Figure 1).

Based on cholesterol contents, our data indicate that the plasma protein having the highest affinity for thyroid hormone is lipid poor, confirming the previous findings of Tata (9). This illustrates that under the experimental conditions used, the lipid-rich proteins (beta and alpha lipoproteins) are not responsible for the binding of the bulk of thyroxine. Such results contrast with previous work in which the techniques of immunoelectrophoresis and radioautography have been used(2, 10). No detailed analysis of the carbohydrate contents of these fractions was attempted, but tests for reducing sugars performed using the Folin-Malmros(11) micromethod after partial acid hydrolysis gave the highest values in the fractions showing the



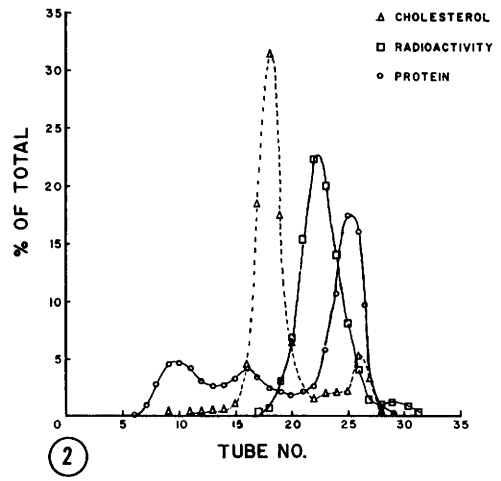


FIG. 1. Distribution of cholesterol and radioactivity in different fractions after continuous flow fractionation (Exp. 1).

FIG. 2. Distribution of protein, cholesterol and radioactivity in different fractions after continuous flow fractionation (Exp. 2).

highest affinity for radiothyroxine.

Fig. 2 illustrates the relationship between the protein, cholesterol and radioactivity contents of the fractions obtained.¶ This indicates that the existence of a protein-thyroid hormone complex does not depend on the abundance of a particular protein but on the number of specific sites that a particular protein may have for binding the hormone and on thermodynamic factors influencing the strength of the complex so formed, *i.e.*, the magnitude of its association constant.

¶ The highest radioactivity levels appear in fractions having very low protein content.

Summary. Nine serum protein fractionation experiments using the continuous flow paper electrophoresis technique were performed. Six experiments were performed using the serum collected from individual patients 24 and 48 hours after receiving therapeutic doses of I-131 and 3 using the serum of euthyroid individuals which was incubated *in vitro* for ½ hour at 37°C with I-131 labelled thyroxine prior to fractionation. Protein fractions obtained were identified by conventional paper electrophoresis and analyzed for radioactivity, total protein and cholesterol, the latter being taken as an index of lipid contents. Similar results were obtained for both *in vivo* and *in vitro* experiments. Beta globulins were found to be capable of forming thyroid hormone protein complexes to a larger extent than gamma globulins, but the largest amount of radioactive hormone was found as an alpha-2 globulin-thyroid hormone complex. A patient showing a marked abnormality in his plasma proteins showed the highest percent of radioactive hormone as protein thyroid hormone complex with the mobility of albumin. The highest percent of the radioactivity was found in fractions having a low protein concentration, indicating that the existence of a protein thyroid hormone complex does not depend on the abundance of a particular protein but on the number of specific binding sites that a particular protein may have and on thermodynamic factors influencing the association constant of the complex so formed. It was also found that the fractions having the highest cholesterol level and hence higher lipid contents contained little radioactivity, indicating that the bulk of thyroid hormone binding is accomplished by a lipid poor protein.

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Effects of Microbial Flora on Cardiac Output and Other Elements of Blood Circulation.* (28658)

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It has been observed that the microbial flora imparts elements of mild ("physiological") inflammation to the wall of the intestinal canal which are portrayed by an increment in weight, hydration and reticulo-endothelial cell population of the tissue(2). These are accompanied by elevated levels of histamine, though observations on this issue are not always consistent(3,4). It has also been observed that the germfree‡ gut appears paler in the living animal(5).

Observations of this nature drew attention to effects which the microbial flora might exert on elements of blood circulation and it was decided to explore the possible effects more systematically. As an initial step, it was endeavored to obtain expressions on composition, volume, pressure and flow of blood in germfree and conventional control rats.

Methods. 1. *Animal material.* The rats used were of Wistar origin. The germfree animals represented the 12th and 13th generation offspring of the original cesarean born ancestors. The conventional controls were genetically closely related to the germfree stock. All were males, normally born and suckled. Age at term was 3-4½ months. The diet was steam sterilized, fortified practical type L-462(6). The germfree rats were housed in the steel isolators, the conventional controls in the open colony room. In all details of rearing, routine procedures were followed(7).

One day before sacrifice the germfree rats were transferred into plastic isolators(8). All animals were starved overnight but were given free access to water. At term they were anesthetized inside the isolator (35 ± 5 mg steam sterilized pentobarbital sodium/kg body weight i.p.), aiming at identical depth of narcosis. Thus, the needed exposure and cannulation of blood vessels was started at the time of disappearance of the corneal reflex and pain response to tail-pinching. A uniform time schedule was maintained in

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‡ At this writing the germfree animal may be defined as a host reared in absence of demonstrable living bacterial mycological, protozoan and macroparasitic associates, according to the test procedures routinely employed(1). The possible presence of pleuropneumonia (PPLO) like organisms, rickettsiae and virus has been investigated, but so far results are negative.