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MEASUREMENT OF FREE THYROXINE CONCENTRATION IN HUMAN SERUM *

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In the present investigation the existence of "free" thyroxine in human serum was repeatedly verified by dialysis through cellophane, with paper chromatographic identification of thyroxine in the dialysate. Methods were developed for measurement of the minute amount of thyroxine present in the dialysate that was considered to represent the unbound or diffusible fraction of the hormone in serum.

Similar values were obtained whether a tracer amount of labeled thyroxine was added to serum, or serum containing endogenously labeled hormone after I^{131} therapy was employed.

MATERIALS AND METHODS

Materials

I^{131}-labeled L-thyroxine was obtained from E. R. Squibb and Co. or from Abbott Laboratories. Shipments were tested for purity by descending paper chromatography as previously described (1, 2), and were discarded if found to be less than 95 per cent pure. Nonradioactive L-thyroxine was purchased from Mann Research Co., New York City.

All blood sera not used the day they were drawn were stored frozen. Repeated freezing and thawing had no effect upon the results. Large pools of sera were supplied by the Medical Chemistry Laboratory and run as standards with each set of dialysis experiments.

Clinical material

Thyrotoxicosis. Sixty hyperthyroid patients were available from the Thyroid Clinic and the Department of Radiological Research of the Presbyterian Hospital. Blood samples were obtained from 35 patients 1 to 13 days after I^{131} therapy. The remaining 25 patients did not receive radioactive iodine therapy before study of their blood sera. The diagnoses were verified by thyroidal uptake of I^{131}, serum protein-bound iodine (PBI) determination (done by Bio-Science Laboratories, Los Angeles 25, Calif.), and in some instances by determination of basal metabolism and triiodothyronine resin uptake (3) as well.

Normal. Blood sera were obtained from 29 healthy subjects either after normal physical examinations at the Personnel Clinic of the New York State Psychiatric Institute or upon return visits to the Vanderbilt Clinic of the Presbyterian Hospital.

Hypothyroidism. Twenty hypothyroid subjects were available from the Thyroid Clinic of the Presbyterian Hospital, the diagnoses being verified by thyroidal uptake of I^{131}, serum PBI determination and, in some instances, by other parameters. Three had hypothyroidism following thyroidectomy; three were hypopituitary patients. The remaining 14 represented spontaneous idiopathic myxedema or hypothyroidism.

Pregnancy. Blood sera were obtained from 28 women, beyond the first trimester of normal pregnancy, in the Obstetrical Clinic of the Presbyterian Hospital.

Euthyroid "sick" group. Blood samples were obtained from a group of 12 patients with various illnesses but with no significant thyroid disorder. The illnesses included hepatic cirrhosis, pulmonary emphysema with CO_2 retention, congestive heart failure, metastatic carcinoma, peptic ulcer, and progressive muscular dystrophy.

Methods

The general procedure employed was similar to that described previously (1). The dialysis membranes were specially cleaned by soaking them in 0.1 M nitric acid for 16 to 24 hours, and then for 2 to 3 days in 0.01 M nitric acid, followed by storage in deionized water at 4°C. Prior to use the dialysis bags were washed repeatedly with deionized water and dried for 6 to 16 hours. A marble placed within the bag before addition of serum insured optimal mixing. Dialysis was carried out, as previously described (1), in 50-ml plastic (cellulose nitrate) centrifuge tubes, which obviated adsorption of thyroxine from dilute solution by glassware. After the addition of 5 ml of serum inside the bag, an equal quantity of isotonic phosphate buffer was added to the outside. In a few instances, when necessary to conserve serum, 3- to 4-ml quantities of serum and buffer were employed.

In addition to the potassium phosphate buffer, ionic strength 0.15 (isotonic) at pH 7.4, Tris-sodium chloride buffer (0.05 M Tris–0.1 M NaCl) was also used on a

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few occasions, and gave results indistinguishable from those obtained with phosphate buffer.

The dialysis was carried out for 16 hours at 37°C in a water-bath shaker. No significant increase in thyroxine radioactivity in the dialysate occurred after 16 hours of equilibration; hence 16 hours was selected as an adequate interval before bacterial contamination became a problem. In control tubes with no protein inside the bag, radioactivity was essentially the same in both compartments with 16 hours of shaking at 37°C. As in the equilibrium dialysis studies with serum albumin solutions, the recoveries of radioactivity ranged from 95 to 105 per cent, indicating the absence of appreciable binding by dialysis bags or the walls of the plastic tubes.

The fraction of radioactivity in the dialysate was obtained by counting aliquots of the solutions from the inside (serum) compartment and the outside (buffer) compartment. The radioactivity in the dialysate consisted predominantly of iodide, which presented the technical problem of identification and measurement of a very small amount of thyroxine in the presence of a relatively larger quantity of radioactive iodide. In contrast to previously reported equilibrium dialysis studies, which employed very dilute albumin solutions with relatively much greater amounts of thyroxine (1, 2), the present work with undiluted sera yielded a much higher fraction bound—very close to 100 per cent. Therefore the thyroxine was always present with a larger amount of iodide in the dialysate.

The identification of the minute amounts of thyroxine in the dialysate was undertaken by two methods: paper chromatography, and fractionation with a column of the cation exchange resin, Dowex 50W–X8. In many instances, because of the preponderance of iodide, paper chromatography was not sensitive enough to detect the presence of thyroxine with certainty. However, in the few cases of thyrotoxicosis in which the thyroxine concentration happened to be sufficient, the thyroxine peak was clearly evident, and its area could be determined accurately by planimetry. The identification of the thyroxine peak as such was verified by two-dimensional paper chromatography by the method of Werner, Row and Radichevich (4).

The single-dimensional paper chromatography of dialysate radioactivity was undertaken after concentration to dryness in a rotating ("flash") evaporator. The dried residue was extracted three times with 0.2-ml portions of redistilled *n*-butanol, and applied directly to duplicate strips of Whatman 3MM filter paper which had been prepared previously with appropriate iodide, thyroxine, and triiodothyronine carriers at the origin. The residue was then extracted rapidly with three 0.2-ml deionized water washings which were also added to the filter paper strips for chromatography. Subsequent water washings and surveys of the evaporation flask revealed no appreciable residual radioactivity, and the procedure recovered 95 to 100 per cent of the total dialysate radioactivity. Descending paper chromatography in a single dimension gave satisfactory separation of iodide and iodothyronines with 56-cm strips and a butanol: dioxane: ammonia solvent system (4:1:5, vol/vol). Intervals of 22 to 24 hours usually provided satisfactory migration and resolution of iodide, thyroxine, and triiodothyronine carriers. The strips were dried and then scanned with a Nuclear-Chicago Actigraph IIB, model C-100 B. After scanning, the position of the nonradioactive carriers was obtained by the color development upon spraying with palladium chloride for iodide, and 4-aminoantipyrene for thyroxine and triiodothyronine (5). It should be mentioned that the buffer salts extracted with the water washings tended to reduce the Rf values and the speed of migration of all of the compounds. This aqueous extraction step was essential in order to approach complete recovery of dialysate radioactivity, but had to be carried out with care and rapidity to avoid overloading the paper strips with excessive inorganic salts from the dried residue, which cause failure of adequate migration and consequently inadequate separation of the peaks.

Because of the difficulties of achieving satisfactory quantitative determination of the amount of thyroxine when it was less than 20 per cent of the dialysate radioactivity, the use of Dowex 50W–X8 columns was undertaken to fractionate the radioactivity of the whole dialysates. Resin column fractionation proved satisfactory for routine application to all sera examined. Dowex 50W–X8, 200–400 mesh, ionic form H^+, was the resin employed for the separation of thyroxine from the larger amounts of radioactive iodide. This cationic exchange resin was obtained from the manufacturer, Dow Chemical Co., Midland, Mich., or from their distributor, J. T. Baker Chemical Co., Phillipsburg, N. J. Dowex lot 20642 was used for most of the determinations in this report, but no difference was apparent in other lots tested. Fifty- to 80-g quantities of the dry resin were washed once with 200 ml deionized water which was decanted, and the resin was stored in water at 4°C. By means of a wide-bore pipet, the resin was added to 1-cm diameter glass tubes to a height of approximately 3 cm, the column of resin being supported by glass wool. After washing with 10 ml sodium acetate buffer, pH 4.4, the resin column was ready for use.

Three ml of the serum dialysate was acidified to approximately pH 6.4 by the addition of 0.1 ml 1 N hydrochloric acid. The acidified dialysate was poured onto the resin column and the effluent collected in a screw-cap tube for radioactive assay. This initial tube, labeled "1," contained a variable amount of iodide radioactivity. A series of solutions was poured through the column to elute first the predominant I^{131}-iodide radioactivity and finally all of the thyroxine radioactivity. Three-ml portions of the following solutions were added to the column and the effluents collected in counting tubes, as follows: 2) acetate buffer, pH 4.4; 3) sodium bromide, 3 N; 4) sodium bromide, 3 N; 5) acetate buffer, pH 4.4; 6) ammonium hydroxide, 4 N; 7) ammonium hydroxide, 4 N; 8) acetic acid, pH 1.4; 9) acetic acid, pH 1.4; 10) ammonium hydroxide, 4 N.

Finally, the resin remaining was aspirated and added to a final counting tube, labeled "11." The iodide radio-

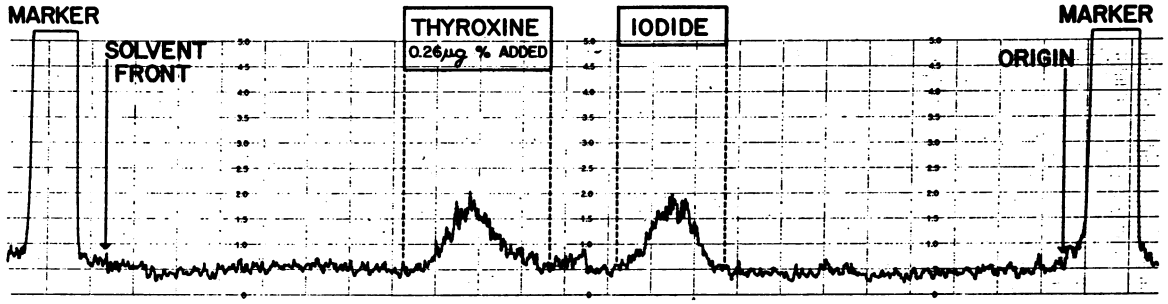

FIG. 1. DIALYSIS OF THYROXINE ADDED TO SERUM. Radioactive scan of chromatographic strip of dialysate.

activity was found in tubes 1 through 5 (mainly in 1, 2, and 3). The thyroxine radioactivity was recovered in tubes 6 through 10, most of it being found in 6 and 7. The radioactivity in tubes 5, 10, and 11 was negligible in all acceptable column fractionations. The recovery ranged from 85 to 105 per cent and was within the 90 to 100 per cent range in most of the procedures. Extensive separations of I^{131}-iodide from "cold" thyroxine were carried out, and "cold" iodide from I^{131}-thyroxine in varying proportions, with agreement within 5 per cent of the known amounts of radioactivity added to buffer solutions. Similarly satisfactory results were obtained with cold serum dialysates to which known mixtures of thyroxine and iodide were added. Paper chromatography of I^{131}-thyroxine revealed no appreciable alteration that could be attributed to the column fractionation procedure.

The tubes were counted in a Packard Auto-Gamma well-type scintillation counter. The relatively small amount of thyroxine radioactivity, often 5 per cent or less of the total in the dialysate, made it impossible to obtain enough radioactivity in endogenously labeled sera after diagnostic tracer doses of I^{131}; consequently, endogenously labeled sera were obtained after I^{131} therapy. When labeled thyroxine was added to cold sera, it was advantageous to employ as much radioactivity as possible, consistent with the objective of using true "tracer" amounts. The quantities of I^{131}-labeled thyroxine added to sera were such that the increment in thyroxine iodine concentration approximated 0.2 μg per 100 ml, a physiologically insignificant change.

In the early stages of the series two or more procedures were carried out on each serum. As the study proceeded it was considered unnecessary to make more than a single free thyroxine determination. With the dialysis of each group of six or seven sera a pool of sera from the Medical Chemistry Laboratory was included to serve as a standard. These pools invariably gave values close to the normal free thyroxine of 0.11 per cent. The reproducibility of the procedure may be judged from the findings on 13 consecutive determinations on the pool run most frequently, which yielded a mean of 0.11 per cent ± standard deviation of 0.011.

RESULTS AND COMMENTS

1. Identification of free thyroxine in serum. Radioactive scans of paper chromatographic strips of serum dialysates are illustrated in Figures 1 and 2 representing, respectively, dialysis after addition of thyroxine to serum, and serum obtained after radioactive iodine therapy. In the two strips illustrated, no attempt was made to determine the proportion of thyroxine in the radioactivity of the dialysate but rather to indicate its presence. In these studies the dialysates were extracted with *n*-butanol which was then concentrated by rotary evaporation for descending paper chromatography. The procedure employed tended to extract more thyroxine than iodide, and therefore could not be employed for quantitative determinations of free

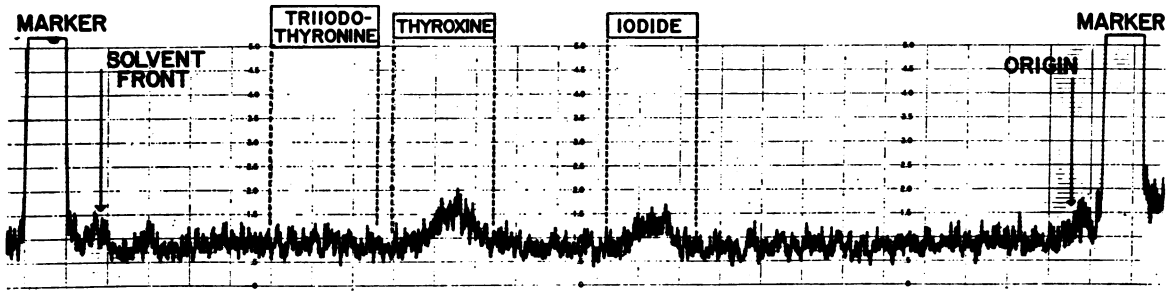

FIG. 2. DIALYSIS OF SERUM AFTER RADIOACTIVE IODINE THERAPY. Radioactive scan of chromatographic strip of dialysate.

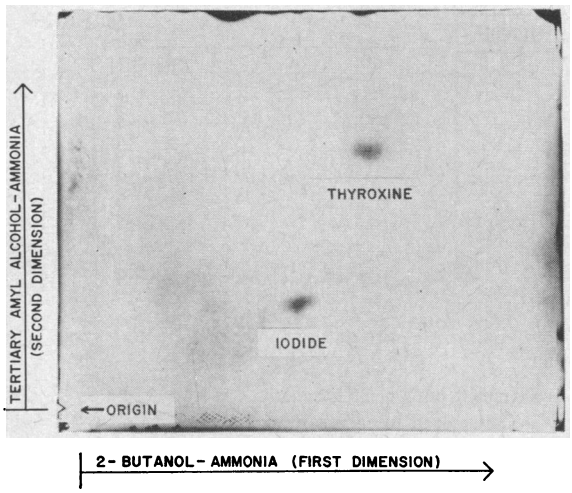

FIG. 3. DIALYSIS OF SERUM AFTER RADIOACTIVE IODINE THERAPY. Radioautograph of two-dimensional paper chromatogram of dialysate. The positions of the carrier compounds corresponded precisely with those of the radioactive spots indicated on the radioautograph.

thyroxine, in contrast to the butanol and water extraction described in *Methods*.

Two-dimensional chromatography was undertaken on the dialysate of serum obtained 5 days after I^{131} therapy, in order to verify that the radioactive peak in question was indeed thyroxine. The radioautograph in Figure 3 reveals darkening due to radioactivity which corresponded precisely to the position of the carrier thyroxine. In no case did a chromatogram of a serum dialysate reveal a peak suggestive of triiodothyronine.

2. Measurement of free thyroxine. With the resin fractionation method described, normal sera contained 0.11 per cent of their total thyroxine in the unbound state, as indicated by dialyzability. The free thyroxine concentration was higher in thyrotoxic than in euthyroid subjects. This fact made it possible to compare the results obtained by the resin fractionation method with paper chromatographic separation of the whole dialysate recovered by rotary evaporation (cf *Methods*). A scan of such a chromatographic strip is seen in Figure 4, and the values of duplicate strips are compared with the results of resin fractionations on different days in Table I. The final column represents a correction factor of 0.8 employed to correct for volume change produced by osmosis of 0.5 ml caused by the undiluted serum, as well as the relatively insignificant factor (above 0.95) due to the Gibbs-Donnan membrane equilibrium. The water shift was evaluated by biuret determinations of total protein of the serum before and after dialysis. The Gibbs-Donnan effect was assessed by chloride determinations on the fluid inside and outside the bag at the end of dialysis. In most instances, in endogenously as well as exogenously labeled sera, the thyroxine in the dialysate radioactivity was below 10 per cent, precluding accurate planimetry of flat thyroxine peaks.

Some studies were undertaken with diluted sera, which yielded appreciably higher free thyroxine values with 1:10 dilutions. In the equilibrium dialysis studies of the interaction of albumin and thyroxine, reported separately (1), a twofold variation in protein concentration had no appreciable effect upon the binding constants. The implications of the equilibria between thyroxine and several binding proteins at different dilutions, however, have not been studied in the present work. Such considerations may have relevance to

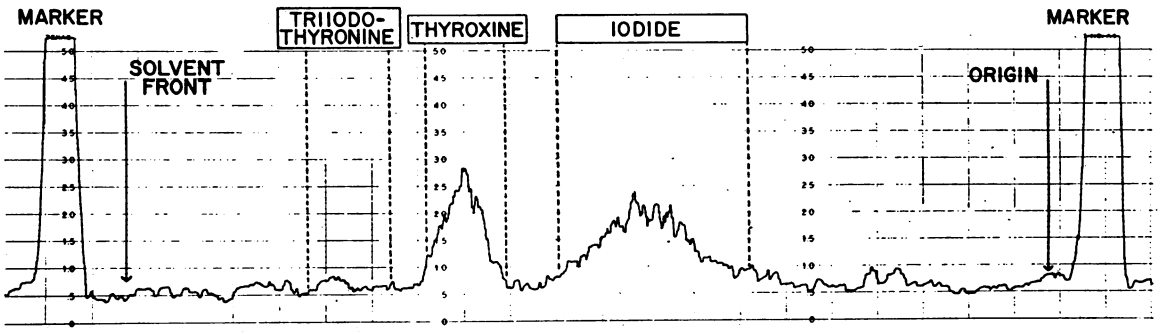

FIG. 4. DIALYSIS OF SERUM AFTER RADIOACTIVE IODINE THERAPY. Radioactive scan of chromatographic strip of dialysate. In contrast to the previous figures the above chromatograph was carried out after concentration and essentially complete recovery of dialysate radioactivity.

TABLE I

Per cent thyroxine in dialysate radioactivity as determined by two methods

Procedure	% Dialysate radioactivity ($\frac{100\% \times \text{cpm outside}}{\text{cpm inside}}$)	% Thyroxine in dialysate radioactivity	Uncorrected % free thyroxine*	Corrected % free thyroxine
5/16/61	1.0	35.8	0.358	0.29
5/19/61	1.0	36.8	0.368	0.29
Chromat. strip A		37.2		
Chromat. strip B		38.0		

* The "uncorrected % free thyroxine" was obtained by the product of "% dialysate radioactivity" and "% thyroxine in dialysate radioactivity." Theoretically, however, the most precise value should be given by the following equation: uncorrected % free thyroxine = (thyroxine cpm in dialysate)/(total cpm in bag − iodide cpm in dialysate). Iodide is treated as though it were distributed equally between the compartments. Dialysis of serum with added I^{131}-iodide resulted in approximately 10% greater radioactivity within the bag, a discrepancy too small to require correction. In actual practice, the two methods of computation have given almost indistinguishable values.

the problem of free thyroxine concentration in interstitial fluid and its availability at the cell membrane. Despite the relatively less exacting technical requirements entailed in measuring a larger amount of thyroxine in dialysates of diluted sera, it was considered preferable to work under conditions approximating circulating blood serum—undiluted serum at 37°C, pH 7.4, with isotonic phosphate buffer. The free thyroxine values obtained with Tris-sodium chloride buffer were indistinguishable from studies run with phosphate buffer.

The values were not affected by the presence or absence of EDTA, since all studies were run with the radioactivity initially on the protein side of the bag. In previous studies with human serum albumin (1, 2) EDTA was necessary to achieve proper equilibrium when thyroxine was added outside the bag, but was not necessary when the tracer was added to the protein solution. In studies with serum in the bag, tracer added to the outside in the presence of EDTA resulted in variable but always higher values than those reported in the present work. This indicated that equilibrium was more closely approached when the tracer was added to the inside.

3. The effect of addition of increasing amounts of carrier thyroxine to serum. Since previous electrophoretic studies have included sera "loaded" with successive increments of thyroxine to determine the binding capacity of the various carriers (6–8), a similar procedure was undertaken in the present dialysis experiments. A serum pool with normal (0.12 per cent) free thyroxine concentration was enriched with graded increments, resulting in progressively greater free thyroxine values (Table II). The thyroxine additions (which may be expressed as PBI additions if multiplied by the factor 0.655) represent increments above the original normal PBI greatly in excess of that seen clinically. Nevertheless, the free thyroxine percentages, in spite of the vast "loading," are not in excess of those observed in thyrotoxicosis. The results above are typical of four such experiments. It will be noted that increments of 100 μg per 100 ml or greater resulted in relatively slight increases in the per cent free thyroxine (a relative plateau) in contrast to the steep rise with lesser additions. Further investigation is needed to explain satisfactorily this observation as well as the fact that thyrotoxic sera may show higher free thyroxine percentages than do euthyroid sera enriched with very large thyroxine increments.

4. Endogenous and exogenous thyroxine. As a further examination of the technique, several sera obtained after I^{131} therapy were stored frozen for several months until the residual radioactivity had fallen to three or four times background. They were then studied again by addition of la-

TABLE II

Effect of "loading" serum on per cent free thyroxine

Added thyroxine	Free thyroxine
μg/100 ml	%
None	0.12
20	0.14
50	0.20
100	0.24
200	0.24
500	0.27
1,000	0.32

TABLE III

Comparison of free thyroxine values in endogenously labeled serum versus "cool" serum with added tracer

Patient	Endog. labeled	"Cool" serum with added I^{131}-thyrox.
	%	%
C.W.	0.24	0.22
S.B.	0.22	0.21
M.W.	0.26	0.25

beled thyroxine. Representative free thyroxine values by the two techniques are given in Table III; the two sets of values show no appreciable difference.

Of the 60 hyperthyroid patients studied, 35 had studies of endogenously labeled sera. With the exception of two euthyroid subjects who received I^{131} in therapeutic doses, all other studies in this report were carried out by the addition of tracer thyroxine to sera. Two or more sera were examined after I^{131} therapy in 29 patients, with no significant difference in free thyroxine observed 1 to 13 days after treatment.

5. Free thyroxine concentration in health and disease. The mean per cent free thyroxine in thyrotoxicosis was twice the normal mean, as illustrated in Figure 5. However, the free thyroxine iodine concentration (the product of the per

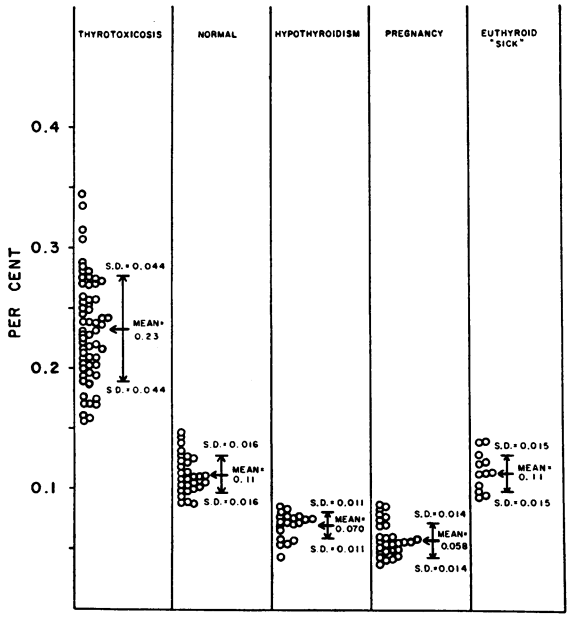

FIG. 5. FREE THYROXINE IN SERUM AS PER CENT OF TOTAL THYROXINE.

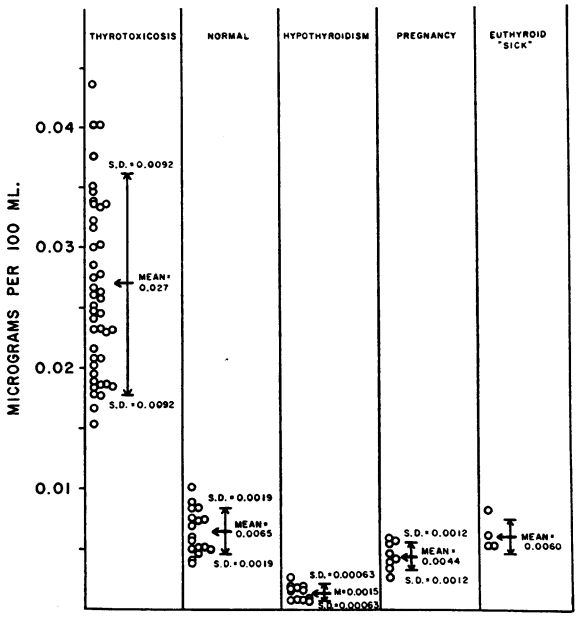

FIG. 6. FREE THYROXINE IODINE IN SERUM.

cent free thyroxine and the PBI value) revealed that the mean in thyrotoxicosis was more than four times greater than the normal mean (Figure 6).

The mean per cent free thyroxine in both the hypothyroid and pregnant groups was slightly more than half that of the normal mean. Expressed as free thyroxine iodine, the mean value in hypothyroidism was less than one-fourth that of the normal mean. In contrast, the absolute values computed for pregnancy revealed considerable overlapping with the normal range; the mean values for free thyroxine iodine were, however, significantly different ($p < 0.05$). This finding is in agreement with that of Robbins and Nelson (9), who calculated a small but significant diminution in the free thyroxine concentration in pregnancy, based upon free thyroxine values computed from electrophoretic data with the aid of certain assumptions. The two groups with thyroid disease had highly significant differences ($p \cong$ or < 0.001)

TABLE IV

Effect of treatment on per cent free thyroxine

Patient	Untreated	Euthyroid after I^{131}
	%	%
M.J.	0.22	0.10
A.S.	0.22	0.11

TABLE V

Free thyroxine in normal and pathological sera *

Sera	Free thyroxine	Free thyroxine iodine	Free thyroxine concentration
	%	*μg/100 ml*	*M*
Thyrotoxicosis	0.23 ±0.044	0.027 ±0.0092	5.3×10^{-10}
Normal	0.11 ±0.016	0.0065 ±0.0019	1.3×10^{-10}
Hypothyroidism	0.070 ±0.011	0.0015 ±0.00063	0.29×10^{-10}
Pregnancy	0.058 ±0.014	0.0044 ±0.0012	0.87×10^{-10}
Euthyroid "sick"	0.11 ±0.015	0.0060 ±0.0014	1.2×10^{-10}

* Mean values ± standard deviations are given in the first two columns.

from the normal group both with respect to per cent of free thyroxine and the computed free thyroxine iodine in micrograms per 100 ml.

The euthyroid "sick" group revealed values virtually identical with those of the healthy volunteers. Fortuitously, blood sera were obtained from two euthyroid subjects who had received appreciable doses of I^{131}. The first was an elderly hypertensive woman with montoxic nodular goiter and severe angina pectoris, and the second was a man with cervical lymph node metastases given I^{131} at another institution for supposed thyroid carcinoma. The free thyroxine values of 0.13 and 0.12 per cent were well within the normal range, based upon studies in which tracer was added to serum.

The effects of therapy of thyrotoxicosis are evident in the data on per cent free thyroxine in Table IV. The per cent free thyroxine became normal after therapy and attainment of the euthyroid state (cf Figure 5).

The values of Figures 5 and 6 are presented with calculated mean molar concentrations in Table V.

DISCUSSION

The concept that thyroxine rather than triiodothyronine is the major calorigenically active hormone that enters the tissue cells is consistent with the distribution studies of Ford, Corey and Gross (10), which present evidence that intravenously injected thyroxine may penetrate the tissues without conversion to triiodothyronine.

The present evidence is compatible with the possibility that unbound thyroxine is the form of the hormone that diffuses across cell membranes and exerts physiological effects. The alternative hypothesis that a protein-bound moiety is the active principle has not been disproved, but it appears to be distinctly less plausible when the present findings are viewed in relation to earlier work. Previous reviews (6–8) have pointed out the existence of clinical states in which significant variations in binding capacity of thyroxine-binding globulin (TBG), with appropriate alterations in serum PBI concentration, have been associated with euthyroid status. Thus, pregnancy and estrogen administration are characterized by high TBG-binding capacity and PBI's, while androgen administration and the nephrotic syndrome have led to definite, if less consistent, diminutions. The euthyroid clinical status usually observed in these circumstances probably depends upon essentially normal free thyroxine concentration despite variations in the amount of protein-bound hormone (6–8). Moreover, Beierwaltes and Robbins (11) reported the existence of congenitally elevated serum TBG and PBI associated with euthyroid status; there was presumably normal free thyroxine concentration as judged by the indirect evidence of erythrocyte uptake of triiodothyronine and by radiothyroxine turnover studies. Subsequently, Beierwaltes, Carr and Hunter reported similar findings in other members of this family (12). The converse situation of low serum PBI and low TBG but presumably normal free thyroxine has been reported by Tanaka and Starr (13) and studied in detail by Ingbar and Freinkel (7, 14).

Christensen has reported a method of dialysis intended to measure the relative free thyroxine values of sera in arbitrary units. This method is based upon the *rate* of passage of radiothyroxine through a dialysis membrane separating samples of the same serum, with the tracer added to one side in a concentration of 1 μg per 100 ml serum (15, 16). The results were expressed as the percentage of total thyroxine radioactivity passing through the membrane in 24 hours, and the curves illustrated a linear rate of passage. It should be emphasized that Christensen did not attempt to measure free thyroxine directly but gave, rather, a figure related to a rate constant in a specific apparatus. This rate for free radiothyroxine transfer as measured by Christensen should be proportional to the per cent free thyroxine as determined in the present study; the findings of the two methods are in general agreement.

The mean free thyroxine content of normal sera was 0.11 per cent of the total in euthyroid healthy

subjects. Taking a normal butanol-extractable iodine of 5.1 μg per 100 ml, corresponding to a normal thyroxine concentration of 7.8 μg per 100 ml, the normal *total* serum thyroxine concentration is 10^{-7} moles per L. Therefore, the *free* thyroxine concentration would be 1.1×10^{-10} M. In the present series of normal sera the free thyroxine iodine values, calculated in each case from the plasma PBI, gave a mean normal free thyroxine concentration of 1.3×10^{-10} M. These values are approximately twice the results computed with the aid of some assumptions by Robbins and Rall (6, 8) and by this laboratory (1).

The value of 0.6×10^{-10} M for free thyroxine was calculated from the binding constants for the interaction of thyroxine and human serum albumin (1) with the assumption that 15 per cent of the serum thyroxine is bound by albumin, which was observed on electrophoresis at pH 8.6 by Ingbar and Freinkel, using Tris-maleate buffer (7), and by Robbins and Rall, who used ammonium carbonate at pH 8.4 with the reverse-flow technique (17). The binding constants (1) are presumably quite accurate for the interaction under the conditions described for dialysis of dilute albumin solutions, principally 0.1 g per 100 ml, or approximately one-fiftieth of the actual concentration in serum. The extrapolation of the results to undiluted sera, with its many small molecules (18), the partial removal of fatty acids bound to albumin, and the use of data from electrophoresis at pH 8.6 may account for the discrepancy.

The addition of successively increasing "loading" amounts of thyroxine to serum pools has given free thyroxine values rising to a flatter slope in the 0.2 to 0.3 per cent range even with increments as great as 500 to 1,000 μg of thyroxine per 100 ml. Since the free thyroxine observed in thyrotoxicosis is commonly 0.2 to 0.3 per cent, the possibility must be entertained that hyperthyroid sera are not precisely equivalent to normal sera with an increased total amount of thyroxine. This is also suggested by observations with the triiodothyronine resin uptake (3) in which normal sera or pools had to be enriched to a thyroxine concentration of more than 100 μg per 100 ml to duplicate the higher range of resin uptake values occasionally observed in thyrotoxicosis. A frequent but not invariable alteration in the serum proteins in thyrotoxicosis is suggested by the electrophoretic data of Ingbar and co-workers (7, 19, 20), indicating diminished thyroxine binding by the prealbumin fraction. The present sera have not been studied by electrophoresis.

In thyroid disease the deviations from the normal range in the calculated absolute values for free thyroxine iodine were quite pronounced and could conceivably explain the differences in removal rate reported several years ago (21, 22). In the discussions of these papers at that time, the variations from normal were considered primarily in relation to possible differences in tissue metabolism, although mention was made of possible differences in serum protein binding. While the present data are compatible with the concept that the free thyroxine concentration may be an important determinant of the daily removal rate of hormone, the earlier hypotheses regarding possible rate-limiting processes in the tissues are by no means excluded. The slow radiothyroxine turnover curves of myxedematous patients were changed only by relatively prolonged replacement therapy; the biological half-time of labeled thyroxine turnover was not altered by intravenous injections of thyroxine doses as great as 2 mg, which abruptly raised the PBI concentration as much as 26 μg per 100 ml. In subsequent "loading" experiments with intravenous doses as great as 4 mg of thyroxine, however, Ingbar and Freinkel (7) demonstrated increased hepatic radioactivity on external counting, as well as an abrupt and transitory fall in the plasma thyroxine radioactivity.

The euthyroid "sick" group in the present study revealed no significant deviations from the normal range. It is of interest that Ingbar, Freinkel, Richards and Dowling (7, 19, 20) have observed diminished prealbumin binding capacity in subjects with a variety of illnesses. In the absence of electrophoretic determinations in the present group it is not possible to state whether this represents a significant disagreement. Some of Ingbar's patients had normal prealbumin levels; moreover, his patients may well have been more seriously ill than those of the present group. It is of interest that the Ingbar group (7, 19, 20) had reported an increased erythrocyte uptake of thyroxine and triiodothyronine from sera of patients with nonthyroidal illnesses in which the prealbumin was found to be diminished. This increased uptake

was accentuated by prior enrichment of the sera with thyroxine to thyrotoxic levels.

The findings of the present paper are compatible with the assumption that the physiological activity of thyroxine depends upon the concentration of unbound hormone. The serum proteins, including the carriers of thyroxine, evidently exist in low concentrations in extravascular fluids including interstitial fluid, lymph, cerebrospinal fluid, and so forth (6–8). It is considered likely that the unbound hormone diffuses across the cell membrane where it may be in equilibrium with binding sites within the cell (23).

SUMMARY

1. The existence of "free" thyroxine in human serum was repeatedly verified by dialysis through cellophane, with chromatographic identification of thyroxine in the dialysate. The identity of thyroxine was confirmed by two-dimensional paper chromatography.

2. Measurement of the minute amounts of thyroxine in the dialysate was undertaken by paper chromatography, and by fractionation with a column of the cation exchange resin, Dowex 50W–X8.

3. Evaporation of the dialysate, followed by both butanol and water extraction of the dried residue, made it possible to recover more than 95 per cent of the radioactivity. Descending paper chromatography, strip scanning, and planimetric measurement of the areas under the thyroxine and iodide peaks yielded values agreeing to within 5 per cent of the values obtained by the resin columns.

4. The mean free thyroxine content was 0.11 per cent of the total thyroxine of normal serum, corresponding to a concentration of 1.3×10^{-10} M.

5. The same values were obtained whether I^{131}-labeled thyroxine was added to serum, or serum containing endogenously labeled hormone after I^{131} therapy was employed.

6. The observed free thyroxine values, expressed as per cent of the total thyroxine content of the sera, were as follows (mean ± standard deviation):

thyrotoxicosis	0.23 ± 0.044
euthyroidism	0.11 ± 0.016
hypothyroidism	0.070 ± 0.011
pregnancy	0.058 ± 0.014

7. The product of the per cent free thyroxine and the protein-bound iodine value gave a result for free thyroxine iodine concentration that showed marked deviation from normal in thyrotoxicosis and hypothyroidism.

8. The findings are compatible with the concept that physiological activity of thyroxine depends upon the concentration of unbound hormone.

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