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Dialyzable Thyroid Hormone–binding Material in Human Serum*

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ABSTRACT: Human serum was dialyzed through cellophane against four times its volume of water, and the freeze-dried dialysate was extracted with 0.3% methanolic ammonia. The extract contained a material which interacted with added L-thyroxine on paper chromatography. In aqueous solution, in the presence of the major electrolytes of serum, it decreased the rate coefficient of escape of L-thyroxine and 3′,3,5-triiodo-L-thyronine through cellophane. Thyroxine and triiodothyronine were quantitated with the ceric sulfate–arsenious acid fading test for iodine.

The method, adapted for the present work, had 95% precision limits of ±0.055 ng iodine in the range of 0.1–2 ng iodine. The small-molecular-weight material interacting with thyroxine and triiodothyronine might conceivably be of importance as a regulator for the concentrations of thyroid hormones not bound to protein.

Models used for describing the behavior of thyroxine in blood include as an essential feature the equilibrium for thyroxine between free and bound states. The association constants for the thyroxine complexes with thyroxine-binding globulin, thyroxine-binding prealbumin and albumin, accepted for the physiological situations, are those obtained from dialysis equilibria and electrophoretic data. No allowance is made for the possible presence of a dialyzable substance that interacts with thyroxine. By reducing the chemical activity, such a substance would increase the concentration of thyroxine not bound to the three proteins. Furthermore, the possibility exists that this substance, by combining with thyroxine, could change the biological reactivity of the latter. The present work is concerned with evidence for a dialyzable material in human serum which binds, or interacts with, thyroxine and with triiodothyronine.

Materials and Methods

Sera from healthy men, 20–50 years old, were used fresh or after storage in the deep-freeze. L-Thyroxine and 3′,3,5-triiodo-L-thyronine were supplied by Sigma Chemical Co., St. Louis, Mo., or donated by Warner-Lambert (Morris Plains, N.J.). Analytical Reagent grade anhydrous methanol was supplied by Mallinckrodt. NH_4OH (Fisher) was purified by distillation with 3 volumes CH_3OH. From this distillate, CH_3OH-NH_4OH solutions I and II were prepared by diluting with 7 or with 24 volumes CH_3OH. The chemicals used in the determination of iodine were the same as described previously (Hoch *et al.*, 1964) except for $KClO_3$, which was recystallized four instead of three times from hot water. Reagent solutions: (1) 73% $HClO_4$: approximately constant-boiling, sold as "double vacuum distilled from Vycor, 70% $HClO_4$ and shipped in Vycor ampuls" by G. Frederick Smith Chemical Co., Columbus, Ohio. (2) $HClO_3$ reagent: 22.5 ml 73% $HClO_4$ was added slowly to a hot solution of 29 g $KClO_4$ in 58 ml H_2O, and after cooling the supernatant was separated and stored at −14° before use. (3) CrO_3 solution: the aqueous solution, approximately 0.6%, was adjusted with water so that when 0.050 ml was added to 1.35 ml H_2O the absorbancy was 0.30 at 430 mμ. (4) Sodium arsenite reagent: stock solution, 19 g As_2O_3, 7.8 g NaOH, and 2.0 g NaCl diluted with H_2O to 500 ml; for use, diluted further 1:10 with H_2O and stored at room temperature. (5) Ceric sulfate reagent: 3.32 g $Ce(SO_4)_2$, anhydrous, purified (Fisher), was suspended in 4.8 ml 10 N H_2SO_4, diluted to 100 ml with H_2O, and stored several days to let the undissolved and the freshly forming precipitate settle. It was accurately standardized so

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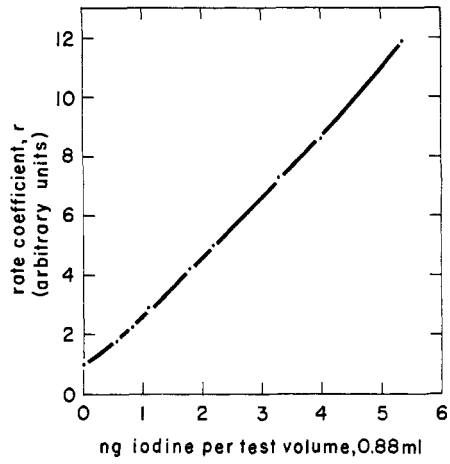

FIGURE 1: Calibration curve for iodine determination.

that the volume used, when diluted with 2.00 ml 0.5 N H_2SO_4, gave an absorbancy of 0.668 at 317 mμ.

Determination of Iodine. The previous technique (Hoch *et al.*, 1964) of preparing the samples and of measuring the catalytic effect of iodine on the ceric sulfate–arsenious acid reaction (Sandell and Kolthoff, 1937) has been modified for the present purposes. The samples were digested in weighed, thin-walled Pyrex test tubes of 20 mm outside diameter, cut to a length of 110 mm. In succession were added 0.50 ml $HClO_3$ reagent, 0.050 ml CrO_3 solution, and 0.15 ml 73% $HClO_4$. The tubes were placed in a beaker filled with glass beads (3 mm diameter) which was immersed in a sand bath, heated from below by a hot plate and from above by heat lamps. The temperature of the glass beads at the level of the digestion mixture rose from about 120° to 150 or 160° during digestion lasting 45–55 minutes. The digestion was considered completed when the hot solution became nonwetting. The tube was weighed and the volume of the contents was calculated by dividing the weight of the contents by 1.71, the specific gravity of constant-boiling $HClO_4$. To the digest were added, in succession, 0.10 ml 10 N H_2SO_4, after 10–20 minutes 2.0 ml sodium arsenite reagent as rapidly as possible, by blowing the arsenite solution from a wide-tipped graduated 10-ml pipet toward the bottom of the tube, and then constant-boiling $HClO_4$ in amounts calculated to make the total $HClO_4$ in the tube equal to 0.60 ml. The tube was capped with Parafilm, heated to 50 or 60°, and left at room temperature overnight. Aliquots of 0.88 ml were used for the ceric sulfate fading test which was followed at 317 mμ (Sanz *et al.*, 1956). The volume of ceric sulfate reagent for 0.88 ml test solution was 3.60 μl, added with a marked 5-μl pipet, the tip of which had been reground to a more acute angle. The ceric sulfate pipetted against the wall of the cuvet does not come in contact with the test solution until the cuvet is tipped, and then the mixing is rapid. Before, the 0.065 ml ceric sulfate reagent floated on the test solution until the cuvet was tipped about 10 seconds later. Apparently reactions taking place during this time contributed to the variability of results; a Beckman DB spectrophotometer with a tungsten lamp and a Beckman potentiometric recorder were used in conjunction with a Sola constant-voltage transformer. The recorder paper supplied by the manufacturer is divided into quarter inches, each division corresponding to 15 seconds for the recorder speed used. For convenience, the unit of time was taken as 15 seconds. The reciprocal of the time, s, required for the absorbancy to decrease from 0.93 to 0.50 was used as a measure of the ceric fading-rate coefficient. Reading accuracy for the time was better than 0.1 unit (1.5 seconds). The absorbancy of 0.93 occurred at 25° after about 5 minutes for the reagent blank and after about 1.5 minutes for a sample containing 2.8 ng iodine. The temperature of the cuvet holder was regulated by water circulating from a 12-liter container held at room temperature. After every test the temperature of the solution in the cuvet was measured to the nearest 0.05° with a micro thermistor probe of a Tele-Thermometer.[1] The values for the reciprocal time were corrected to refer to 25.0° by the empirical equation

$$r = \frac{1}{s} + \left(\frac{0.05777}{s} + 0.08134\right)(25.0 - t) \qquad (1)$$

where r is a temperature-corrected reading proportional to the "average" rate coefficient in the not-quite-first-order (Hoch *et al.*, 1964) fading reaction, s is the time in 15-second units, and t is the temperature in degrees C. The coefficients 0.05777 and 0.08134 were obtained by the method of least squares from calibration experiments at three iodine levels and over the temperature range 20–28°. Equation (1) reflects the complexity of the temperature dependence of the fading reaction. Temperature probably affects the catalytic reaction and the interference by chromic ion in different ways. With the precautions described, the precision of replicates done in succession is, in terms of the 95% confidence range for a single test, ±0.055 ng iodine (n = 48, degrees of freedom = 24) in the range of 0.1–2 ng iodine (average 1.0 ng) and ±0.078 ng iodine (n = 96, degrees of freedom = 52) in the range of 2–5 ng iodine (average 3.4 ng) per test volume of 0.88 ml—an improvement by a factor of 3, respectively, 2 over our previous figure of ±0.00015 μg (Hoch *et al.*, 1964). The reason the precision is still below the unusual one, reported by Sanz *et al.* (1956), of better than ±1% for 1 ng iodine (in 0.25 ml test solution) should be sought partly in the interference by chromic ion which we did not wish to omit from the reaction mixture and partly in the lack of stability of the spectrophotometer, probably caused by fluctuations in the power supply. Figure 1 shows the calibration curve for r, used as a measure for iodine concentration.

Dialysis. The apparatus[2] was similar to that described

[1] Manufactured by Yellow Springs Instrument Co., Inc., Yellow Springs, Ohio.

[2] Dialystat, manufactured by National Instrument Laboratories, Inc., Rockville, Md.

previously (Hoch and Williams, 1958). Membranes were cut from Visking tubing (19 mm inflated diameter) and tied with type B, size O surgical linen (Ethicon) onto a piece of glass tubing with 22 mm inside diameter (exposed membrane area 3.8 cm^2) or with 12 mm inside diameter (exposed membrane area 1.5 cm^2). Absence of leaks was ascertained by measuring the flow rate of 0.01 N NaCl solution or of H_2O under 30–40 cm liquid head. After storage in CH_3OH, the membrane was agitated 30 minutes in 0.01 N NaOH–0.05 N NaCl, and then 30 minutes in the salt solution to be used in the quantitative experiments; or, first in 0.001 N NaOH–0.01 N NaCl and then in H_2O for the preparative dialysis. The membrane was adjusted to a horizontal position and the glass tube slightly off the axis of rotation. Snapping movement through 90° provided efficient stirring inside and outside.

Chromatography. Strips of 30-mm-wide filter papers, CS and S 2043A, supplied by Beckman for electrophoresis, were cut in half lengthwise, soaked in several changes of CH_3OH-NH_4OH solution II for at least 1 week, and then air dried immediately before use. Two halves belonging together were used side by side for equal aliquots of the same sample, and one strip was stained to serve as pilot for sectioning the other, unstained strip. The samples were applied dissolved in CH_3OH-NH_4OH solution II under a stream of air at room temperature. The solvent system of Mandl and Block (1959), consisting of 2-butanol–3% (w/v) NH_3 (3:1 v/v) was used in descending chromatography at 19–21°. Chromatography was terminated when the solvent had passed 140–160 mm past the point of application; the solvent front was marked with scissors, and the strips were dried horizontally in air drawn off by a large fan. The pilot strips were sprayed with a solution prepared fresh before use from 2 parts arsenite (19 g As_2O_3 in 10 ml H_2O containing 7.8 g NaOH, diluted to 500 ml) and 1 part ceric sulfate (10 g $Ce(SO_4)_2$ in 100 ml 10% [v/v] H_2SO_4) (modified from Mandl and Block, 1959). The patterns were counterstained by spraying both sides of the strip lightly with 0.03% aqueous methylene blue.

Preparation of Serum Dialysate. Aliquots of 0.5 ml serum were dialyzed against 2 ml H_2O in a 50-ml beaker for 1 hour at 20–21° (membrane area 3.8 cm^2). The pooled dialysates from 3 ml serum were freeze-dried in a desiccator over concentrated H_2SO_4 and pellets of NaOH, and stored in the refrigerator.

Quantitative Analysis of Chromatograms. The freeze-dried dialysate was extracted with 0.15 (or 0.3) ml CH_3OH-NH_4OH solution II; 0.02-ml aliquots of the extract, corresponding to 0.4 (or 0.2) ml serum, were spotted on two neighboring strips. To an 0.02-ml aliquot contained at the drawn-out tip of a graduated 1-ml pipet was added 2 μl of thyroxine solution (containing 26 ng, = 0.026 μg, thyroxine) from a lambda pipet by emptying it toward the tip of the 1-ml pipet. The fluid, drawn inside the pipet by capillary action, was mixed with the extract by back and forward movement of the solution, effected through manipulation of thin-walled rubber tubing attached at the top of the 1-ml pipet. The extract with the added thyroxine was then spotted directly on the paper. The neighboring strip was prepared in the same manner. A third pair of strips received 2 μl of the thyroxine solution pipetted directly on the paper, and one strip without sample was used as a blank. The unstained strips were cut into sections at positions of R_F values determined from the stained pilot strips. Each section was placed in a digestion tube and left to soak in 4 ml CH_3OH-NH_4OH solution I for 2 days. Finally, the filter paper was removed with a glass rod, the solution evaporated to dryness in a vacuum oven, and the residue used for iodine determination.

Isolation of Chromatographic Fraction Containing Binding Factor. Freeze-dried dialysate from 3 ml serum was extracted with 0.2 ml CH_3OH-NH_4OH solution II, and the extract was spotted on seven filter paper strips (15 mm wide) and chromatographed for 6 hours. Six unstained strips were cut at R_F values 0.36 and 0.67, as determined from the stained seventh strip. The combined paper sections were extracted with 8 ml CH_3OH-NH_4OH solution I; after removal of the paper with a glass rod, an aliquot of $^1/_{11}$ was used for iodine determination and the extract was evaporated to dryness in a vacuum oven below 28°. The material eluted from the sections above R_F = 0.36 was designated fraction I and that from the sections between R_F = 0.36 and R_F = 0.67, which contained the binding factor, fraction II.

Analytical Dialysis. The solutions to be placed inside were prepared as follows: (3 drops) CH_3OH-NH_4 solution II was added to each of the tubes containing the dry chromatographic fractions and to an empty tube. For each dialysis experiment, 15 μl thyroxine solution (38.7 μg thyroxine/ml) was added with the tip of the pipet immersed in the liquid, and the solution was taken to dryness at room temperature; 0.25 ml salt solution was added and the test tube was agitated for several minutes, during which time 59–88% of the thyroxine went into solution. The salt solution was prepared immediately before use by addition of 174 mg $NaHCO_3$ to 50 ml of a solution containing 6.09 g NaCl, 0.254 g KCl, 0.060 g K_2SO_4, and 0.059 g Na_2HPO_4 (Na 146, K 316, Cl 107, SO_4 0.34, and HPO_4 4.5 meq) per liter. Dialysis was carried out in a temperature-controlled room with the outside fluid at 20.5° ± 0.3°.

Results

In Figure 2 are shown diagrams of the stained chromatograms from an experiment with thyroxine and a similar one with 3′,3,5-triiodo-L-thyronine. They demonstrate one salient feature: the very intense spot, caused by the strong catalytic action of what appeared to be a combination of thyroxine and a compound of the dialysate extract. 3′,3,5-Triiodo-L-thyronine showed a similar effect. The material of the extract which remained close to the point of application and reduced ceric sulfate was not involved in thyroxine binding.

The ranges of R_F values observed in fourteen experiments were, for the interacting material X from serum, 0.50–0.60; for thyroxine, 0.56–0.73; and for 3′,3,5-triiodo-L-thyronine, 0.75–0.88. The R_F values were lowest

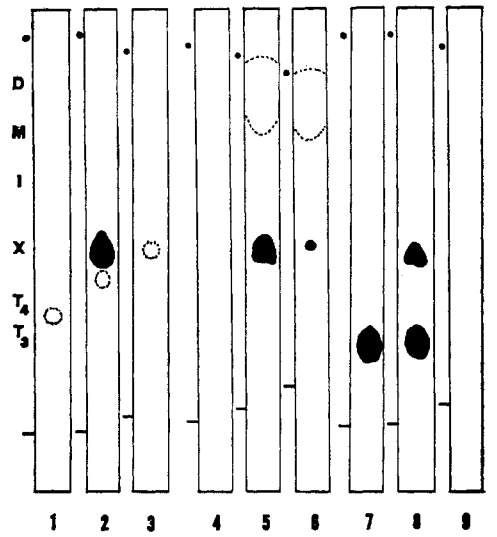

FIGURE 2: Paper chromatograms of thyroxine and 3′,3,5-triiodo-L-thyronine in the presence and absence of serum dialysate extract. Solvent: 2-butanol–3% (w/v) NH_3 (3:1 v/v). (1) 0.025 μg thyroxine (Warner-Lambert). R_F = 0.69. (2) 0.025 μg thyroxine (Warner-Lambert) and dialysate extract from 0.1 ml serum M. R_F = 0.55. (3) Dialysate extract from 0.1 ml serum M. R_F = 0.58. (4) 0.026 μg thyroxine (Sigma). Spot too weak for location. (5) 0.026 μg thyroxine (Sigma) and dialysate extract from 0.4 ml serum D. R_F = 0.54. (6) Dialysate extract from 0.4 ml serum D. R_F = 0.55. (7) 0.6 μg 3′,3,5-triiodo-L-thyronine (Warner-Lambert). R_F = 0.80. (8) 0.6 μg 3′,3,5-triiodo-L-thyronine (Warner-Lambert) and dialysate extract from 0.1 ml serum M. R_F = 0.56 and 0.78. (9) Dialysate extract from 0.1 ml serum M. Spot too weak for location. The symbols on the left indicate the approximate positions, not necessarily the presence, of the compounds diiodotyrosine (D), monoiodotyrosine (M), iodide (I), thyroxine (T_4), and 3′,3,5-triiodo-L-thyronine (T_3).

with fresh solvent and rose as the solvent remained exposed in the trough of the chromatographic chamber. Stale solvent was preferred because of the better separation of material X and thyroxine. The ratio of the displacement for material X over the displacement for thyroxine fell from 0.91 for relatively fresh to 0.82 for stale solvent.

It should be kept in mind that substances not containing iodine, e.g., cysteine, reduce ceric sulfate and are not distinguishable by the simpl espraying technique. Further, as is well known, the intensity of the spot is no measure of the amount of iodine present, since different iodine-containing compounds have widely different catalytic action on the ceric sulfate–arsenious acid mixture. The results just described provide an example that the catalytic activity of thyroxine and 3′,3,5-triiodo-L-thyronine may be substantially increased by another substance, which itself produces but a very faint reaction with ceric sulfate. In accord with this interpretation is the quantitative analysis as shown in Table I. Of the added thyroxine, 1.45 ng thyroxine-iodine (or 23%) was bound, but none of the 3′,3,5-triiodo-L-thyronine (Sigma). Corresponding values for added 93 ng 3′,3,5-triiodo-L-thyronine from Warner-Lambert of the amount bound were: 1.9 ng 3′,3,5-triiodo-L-thyronine-iodine (or 4%) for the dialysate extract from 0.2 ml serum H.

In each fading test one-third of the amount given in Table I was used. The smallest amount in a fraction, significantly different from zero when determined in duplicate, was 0.15 ng iodine. The fractions X and thyroxine had sufficiently different R_F values to demonstrate a shift between the amounts of iodine on both sides of the cut at R_F = 0.58, from a ratio equal to 0.91 for thyroxine alone to 1.83 for extract + thyroxine. The cuts were made at R_F values read from the paired strip that was stained, differing up to 2% and in one instance 4% from the average values given in Table I. The large proportion of iodine found outside the region designated for the thyroxine spot in the stained strip may be attributed to a slight upward shift of the thyroxine on the unstained strip, caused by irregularity in the filter paper.

The intensification of the spot at R_F = 0.55–0.60 in the chromatogram of serum dialysate on addition of thyroxine or 3′,3,5-triiodo-L-thyronine was observed with sera from seven healthy individuals.

Analytical Dialysis. In Figure 3 the amounts of thyroxine (measured as iodine) are shown as they increase in the outside fluid after the specified times of dialysis of 0.20 ml thyroxine solution inside against 5.00 ml solvent outside. In the first experiment thyroxine only was used; in the second and third experiments, chromatographic fractions (one containing the thyroxine-binding material and the other all material with smaller R_F values) were present in addition to thyroxine. The *p*H rose from 8.15 at the beginning of the dialysis to between 9.0 and 9.1 during the last hour. These changes in *p*H were reproducible from one experiment to the other. The proportion of thyroxine that escaped during the first 45 minutes was most indicative of a possible change in the escape rate coefficient, since the greatest difference in iodine concentration was to be expected at that time, as judged from the shape of the curves (Figure 3). During this period the *p*H stayed within the range 8.2–8.7. The reason the dialysis was not carried out close to the more physiological *p*H of 7.4 was the tendency of thyroxine to be adsorbed to glass at neutral *p*H and the necessity to introduce more buffer salt and to renounce the presence of bicarbonate ion. While adsorption of thyroxine on glass was found to be negligible, adsorption on the membrane had to be taken into account. A portion of thyroxine disappeared from the salt solution within a few minutes, and probably was adsorbed on the membrane both reversibly and nonreversibly. It is reasonable to assume that the amount of reversibly adsorbed thyroxine (Craig and Ansevin, 1963) decreased, and that more thyroxine was adsorbed nonreversibly on the part of the membrane exposed to the outside solution only. The amount of thyroxine ad-

TABLE I: Iodine Content of Chromatographic Fractions of Serum Dialysate Extract, with and without Added Thyroxine (Sigma) or 3′,3,5-Triiodo-L-thyronine (Sigma).[a]

Serum D

Fraction	R_F on Pilot Strip	Strip Cut at R_F	Blank Strip	Extract from 0.4 ml Serum	Extract from 0.4 ml Serum + 26 ng[b] Thyroxine	26 ng[b] Thyroxine	Corr.[c]
D + M + I^-	0.05–0.22	0.46	0.38	1.97	2.10	1.05	0.42
Substance X	0.55	0.58	0.29	1.64	5.71	6.63	2.62
Thyroxine	(0.62)[d]	0.76	0.29	1.30	3.53	7.31	2.89
3′,3,5-Triiodo-L-thyronine		1.00	0.25	1.01	0.92	1.05	0.42
Total			1.21	5.92	12.26	16.24	6.35

Amount bound: 1.45 ng (23% of iodine added as thyroxine).

Serum H

Fraction	R_F on Pilot Strip	Strip Cut at R_F	Blank Strip	Extract from 0.3 ml Serum	Extract from 0.3 ml Serum + 24 ng[b] 3′,3,5-Triiodo-L-thyronine	24 ng[b] 3′,3,5-Triiodo-L-thyronine	Corr.[c]
D + M	0.02	0.23	0.66	0.45	0.78	0.00	0.00
I^-	(0.37)[d]	0.39	0.29	0.82	1.15	0.08	0.08
Substance X	0.52	0.60	0.29	0.25	0.86	0.62	0.58
3′,3,5-Triiodo-L-thyronine (+ Thyroxine)	(0.78)[d]	1.00	0.37	0.74	6.58	6.94	6.45
Total			1.61	2.26	9.37	7.64	7.11

Amount bound: 0.00

[a] The values are the ng (10^{-9} g) contained in the fraction. The designations on the left indicate the location, not necessarily the presence, of the compounds diiodotyrosine (D), monoiodotyrosine (M), iodide (I^-), thyroxine, and 3′,3,5-triiodo-L-thyronine. [b] A portion is lost on the outside of the 2-μl pipet with which the thyroxine or the 3′,3,5-triiodo-L-thyronine is transferred to the tip of the pipet containing the extract, or to the filter paper strip. [c] Corrected for total thyroxine (or 3′,3,5-triiodo-L-thyronine) found to equal total (extract + thyroxine) minus total (extract). [d] No spot, or a spot too weak to be located. The value was taken from corresponding experiment at higher concentration.

sorbed was estimated by sampling the solution before contact with the membrane, as well as within 1 minute after introduction into the dialysis chamber (from which all excess fluid had been removed), and after further 5-minute agitation with no fluid outside. The dialysis was timed from the moment of immersion of the membrane in 5.0 ml salt solution. Samples of 0.2 ml were withdrawn for iodine analysis from the outside fluid at 15, 45, 85, 125, and 165 minutes or at times as specified in Table II. The amount adsorbed, calculated as the difference between the amount added and that present at the end inside and outside, plus the amounts withdrawn, is given in column (6) of Table II. Ranging from 69 to 89 ng, it agreed within the limits of error with the amount calculated from the difference between the amount added and that found after 5 minutes' agitation in air, if correction was made for the dilution by the solvent available within and on the surface of the mem-

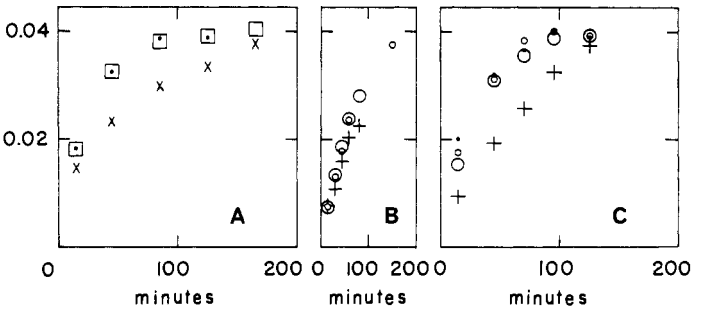

FIGURE 3: Iodine content of 0.2-ml portions outside fluid withdrawn during dialysis. (A) and (C) large membrane with 3.8 cm² dialysis area (Visking tubing supplied by A. H. Thomas Co.); (B) small membrane with 1.5 cm² dialysis area (Visking tubing supplied by National Instrument Laboratories). The scale of the abscissa has been reduced by a factor of 0.4 the ratio of the two membrane areas. (•), thyroxine or 3',3,5-triiodo-L-thyronine in salt solution; 5 minutes preliminary agitation with no fluid outside; (◦), thyroxine or 3',3,5-triiodo-L-thyronine in salt solution without preliminary agitation; (□), chromatographic fraction I + thyroxine; 5 minutes preliminary agitation; (×), chromatographic fraction II + thyroxine; 5 minutes preliminary agitation; (+) chromatographic fraction II + thyroxine, or fraction II + 3',3,5-triiodo-L- thyronine; (○), thyroxine or 3',3,5-triiodo-L-thyronine in salt solution with the concentrations of thyroxine and 3',3,5-triiodo-L-thyronine matched to the preceding (+) experiments. For ease of comparison, the ordinates are expressed as the fraction of the total amount of nonadsorbed thyroxine or 3',3,5-triiodo-L-thyronine accounted for at the end of dialysis.

brane.[3] Adsorption of thyroxine from the NaOH-NaCl solution amounted to 110 ng at 165 minutes and 96 ng at 85 minutes.

Calculation of Escape Rate Coefficients. The underlying assumption was that the amount of solute transferred per unit time is proportional to the concentration difference between inside and outside:

$$-\frac{dq_1}{dt} = \lambda\left(\frac{q_1}{v_1} - \frac{q_2}{v_2}\right) \qquad (2)$$

where q_1, q_2, v_1, and v_2 are the amounts of thyroxine (or 3',3,5-triiodo-L-thyronine) and volumes for the inside and outside, t is the time, and λ is the escape-rate coefficient. The latter was calculated under two simplifying assumptions: (a) that the portion of thyroxine (or 3',3,5-triiodo-L-thyronine) not recovered at the end of dialysis had been irreversibly adsorbed from the beginning, and (b) that the amount of nonadsorbed thyroxine (or 3',3,5-triiodo-L-thyronine) on the membrane was negligible (see Appendix).

If the total amount of thyroxine recovered at the end of each period, $q_1 + q_2$, is equal to k, then $q_1 = k - q_2$, and $dq_1 = -dq_2$. Substituting in equation (2) and integrating, we have

$$\lambda = -\frac{v_1 v_2}{v_1 + v_2} \frac{\Delta \ln\left(1 - \frac{v_1 + v_2}{k v_2} q_2\right)}{\Delta t} \qquad (3)$$

In Table II (part A) are given the values for λ/(membrane area) calculated with $v_1 = 0.220$ and v_2 decreasing from the average value of 4.991 to 4.026 as 0.200-ml portions were withdrawn at the stated intervals. The volume of outside fluid lost by evaporation during 165 minutes ranged from 0.15 to 0.23 ml with an average of 0.19 ml, and it was assumed to have been constant per unit time. The volume v_2 used was the average volume for the period, obtained by subtracting from 5.0 ml the volume that had evaporated at the midpoint of the period and the volume withdrawn from analysis up to that time.

In the absence of fraction II, approximately 90% of the thyroxine had dialyzed out after 85 minutes. Therefore the λ values for the two following periods were not calculated because, with the system so close to equilibrium, small errors in the iodine determinations and losses by adsorption interfered unduly. For these experiments, the k values for 85 minutes were calculated with the amount inside taken at 1.69 times that found at 165 minutes or 1.46 times that found at 125 minutes. The two factors had been computed from equation (3). Iodide iodine in the thyroxine preparation amounted to 6 or 7%, and total iodine in the dialysate extract fraction I to 9% of the total iodine in solution after addition of thyroxine. Both were neglected in the analysis. The iodine in fraction II probably derived from serum thyroxine, bound to the same material which interacted with the added thyroxine, and it contained 14% of the total iodine in solution (Tables I and II [A]).

In Table II(B) are shown the results of experiments with a smaller membrane and for a shorter period of time. The iodine content of fraction II, obtained from pooled extracts from four sera, equivalent to 2.5 ml serum, was 2.7 ng, or 1.3% of the total iodine in solution after addition of thyroxine. In spite of the large relative error in the value for λ/(membrane area) for

[3] An estimate of the volume of solvent on and in the membrane was obtained from experiments with salicylic acid under the assumption that adsorption on the membrane is negligible.

TABLE II: Escape Rate Coefficients and Adsorption of Thyroxine (Sigma) and of 3′,3,5-Triiodo-L-thyronine (Sigma) in Dialysis through Visking Cellulose Membrane.

Material Dialyzed[a]	Amount Added (1)	Amount in 0.20 ml Solution Tested within 1 min (2)	Tested after 5 min Agitation (3)	Column (3) Corrected for Dilution[b] (4)	Amount Adsorbed Calcd from Value Found at Start (5)[c]	Amount Adsorbed Calcd from Value Found at End (6)	Escape Rate Coefficients λ/(membrane area) × 10^3 cm/min					Ratio q_1/q_2 at End of Dialysis (min)
							Time (min)					
							0–15	15–45	45–85	85–125	125–165	
(A) Thyroxine	254	203	142	164	90	89	2.3	1.9	1.5			0.095 (125)
Chromat. fraction I + thyroxine	254	182	159	183	71	69	2.3	2.0	1.4			0.084 (165)
Chromat. fraction II + thyroxine	169	107	76	87	82	72	1.8	0.8	0.6	0.5	0.7	0.185 (165)
Thyroxine in 0.01 N NaOH 0.05 N NaCl	331	280				110	1.8	2.3	1.9			0.060 (165)
							0–15	15–30	30–45	45–60	60–80 or 60–150	
(B) Thyroxine	276	256				98	1.3	1.6	1.6	2.4	1.7	0.23 (150)
Chromat. fraction II + thyroxine	208	175				96	2.1	0.9	1.7	1.6	0.6	1.08 (80)
Thyroxine	155	142				45	1.9	1.9	1.8	2.2	1.6	0.63 (80)
							0–15	15–45	45–70	70–95	95–125	
(C) 3′,3,5-Triiodo-L-thyronine	320		208	239	81	148	2.5	1.6	1.6	1.0		0.079 (125)
3′,3,5-Triiodo-L-thyronine	279	247				125	2.2	1.9	3.4	2.4		0.099 (125)
Chromat. fraction II + 3′,3,-5-triiodo-L-thyronine	162	117				79	1.0	0.7	0.8	1.2		0.24 (125)
3′,3,5-Triiodo-L-thyronine	175	138				86	1.8	1.8	1.4	2.5		0.106 (125)

[a] (A) Effects of chromatographic fractions from serum H extract. Area available for dialysis 3.8 cm^2. (B) Effect of chromatographic fraction II from pooled serum extracts. Area available for dialysis 1.5 cm^2. (C) Effect of chromatographic fraction II from pooled serum extracts. Same membrane as in (A). All experiments except the fourth are with salt solution initially at *p*H 8.15. The experiments with values missing in columns (3), (4), and (5) were started without the 5-minute agitation. [b] By 30 μl solvent on and in membrane. [c] (5) = (1) − (4).

each individual interval, the lowering in the presence of fraction II is evident.

In Table II(C) and Figure 3C are shown the results of experiments with 3′,3,5-triiodo-L-thyronine in which the same membrane was used as in the experiments with thyroxine given in Table II(A). 3′,3,5-Triiodo-L-thyronine behaved similarly to thyroxine. The fraction II was obtained from the same pooled serum extracts as used in the experiment given in Table II(B).

An alternative method of calculating the rate coefficient was made possible by the NIHOMR-9B21 computer program[4] for compartmental analysis (see Appendix). The values for λ/(membrane area) $\times 10^3$ cm/min $\pm$ standard deviation thus obtained are, in the order in which they appear in Table II: (A) 2.3 $\pm$ 0.11, 2.2 $\pm$ 0.12, 1.0 $\pm$ 0.15, 1.9 $\pm$ 0.27; (B) 1.8 $\pm$ 0.04, 1.4 $\pm$ 0.08, 1.9 $\pm$ 0.02; (C) 2.1 $\pm$ 0.19, 2.1 $\pm$ 0.10, 0.9 $\pm$ 0.04, 1.8 $\pm$ 0.07. In these calculations, all data, including those for 125 and 165 minutes in the thyroxine experiments, have been used. The standard deviations are a measure of the consistency with equation (2) within any one dialysis experiment and they do not reflect the whole variability between dialysis experiments.

This experiment is to demonstrate under conditions approaching a physiological environment the presence of a factor interacting with thyroxine. The retarding effect of fraction II containing the binding factor was not attributable to interference by the iodine-containing material therein as, judging from the short-time preparative dialysis of serum, the dialysis-rate coefficient of any material in this fraction could not have been much, if at all, smaller than that of thyroxine in the presence of the binding factor. It should be noted that the molarity of thyroxine used in the escape rate experiments was about 10^{-4} of that used by Craig and Ansevin (1963) for amino acids in escape-rate studies.

Discussion

Oppenheimer and co-workers, (Oppenheimer *et al.*, 1963; Oppenheimer and Surks, 1964), in their work concerned with the determination of free thyroxine in human serum, emphasize the necessity of the assumption that the partition of thyroxine as demonstrated by paper electrophoresis is an accurate representation of the distribution of thyroxine among serum proteins. If thyroxine binding under conditions of paper electrophoresis, chromatography, or equilibrium dialysis were to imply similar binding under physiological conditions, the additional assumption has to be made that there is no dialyzable material in serum which, by interaction with thyroxine, could increase the proportion not bound to protein. The experiments reported were carried out to test for such interaction with thyroxine in an aqueous solution of the electrolyte composition of plasma, but with Ca and Mg omitted.

It is much more difficult to obtain evidence of thyroxine binding for dialyzable material than it is for macromolecules because membrane equilibrium methods cannot be used. Chromatography and measurements of rates of diffusion thorugh cellophane were chosen for this purpose. Because of recent improvements made in this laboratory in precision of analysis, the chemical determination of iodine was employed in preference to radioactive counting which so far has been the only method used in binding studies of thyroxine and 3′,3,5-triiodo-L-thyronine. The pronounced effect of serum dialysate in stained chromatograms which, at first, appeared to indicate strong 3′,3,5-triiodo-L-thyronine and thyroxine binding had to be discarded as far as evidence for 3′,3,5-triiodo-L-thyronine binding was concerned; by means of quantitative iodine analysis no increases were found in iodine content of the fraction containing the binding factor. The increase in iodine content of this fraction by 23% of the amount added as thyroxine was much less than the intensity of the spot at the location of the binding factor would have suggested, but too large to be attributed to a contaminant in the thyroxine preparation.

Interaction with thyroxine in the presence of butanol and at high *p*H during chromatography is no proof that such interaction would occur at a *p*H of 7.4 and in the absence of organic solvents. However, quantitative dialysis studies in an aqueous medium simulating physiological conditions showed a reduction of the escape-rate coefficient for thyroxine and 3′,3,5-triiodo-L-thyronine in the presence of the isolated fraction containing the chromatographically demonstrated thyroxine-binding factor (Fraction II). The chromatography experiment was presented because it showed a marked enhancement of the iodine reactivity in both thyroxine and a contaminant of 3′,3,5-triiodo-L-thyronine by the serum factor. One may speculate about the possibility of a physiological significance of such a change in the structure of thyroxine which renders the iodine more reactive.

The technique of obtaining an estimate of the escape-rate coefficient by periodic withdrawal of samples from a relatively small volume of outside fluid, in combination with a small (inside volume)/(membrane area) ratio, of 0.053 cm in the present case, is more economical with the test material than the technique used previously (Hoch and Turner, 1960) and less time consuming.

It may be of interest to compare the escape-rate coefficient per unit membrane area of a substance not containing iodine and of small molecular weight, which was measured by the same technique as that used for thyroid hormones. This substance, phenylacetic acid, was not adsorbed on the membrane and gave a value in 0.01 N HCl or in 0.01 N NaOH of 6.9×10^{-3} cm/min or about three times as great as that for thyroxine or 3′,3,5-triiodo-L-thyronine.

Comparison of Figure 2B with Figure 2A and C suggests a difference in porosity between the Visking cellophane from the two sources. That the flow rate of water under 30 cm liquid head at 25° was larger (1.3 μl/hr/cm^2) for the membrane used in the experiment of Figure 2B

[4] The senior author is greatly indebted to Dr. Mones Berman and to Mrs. Marjory F. Weiss for instruction and help in the use of their program.

than for the other membrane (1.0 μl/hr/cm^2) may in the present situation indicate that the pore size was greater, if one can assume equal number pores per unit area and equal thickness and tortuosity.

Appendix

Influence of Solvent Inside Membrane. If q_m and v_m are the amount of thyroxine and the volume of available solvent in the membrane, so that the total amount $k = q_1 + q_2 + q_m$ (2a), then, with assumption[5] that $q_m/v_m = (q_1/v_1 + q_2/v_2)/2$, one obtains $k = (1 + v_m/2v_1)q_1 + (1 + v_m/2v_2)q_2$, from which $q_1 = k/(1 + v_m/2v_1) - (1 + v_m/2v_2)q_2/(1 + v_m/2v_1)$, and $dq_1 = -[(1 + v_m/2v_2)/(1 + v_m/2v_1)]dq_2$. Substituting in equation (2) and integrating, we have

$$\lambda = -\frac{v_1(v_2 + v_m/2)}{v_1 + v_2 + v_m} \frac{\Delta \ln\left(1 - \dfrac{v_1 + v_2 + v_m}{kv_2} q_2\right)}{\Delta t} \quad (4)$$

From the volume of the cellophane and the weight and the specific gravity of cellulose, an estimate of v_m can be made. The value for the solvent in 3.8 cm^2 cellophane, thus calculated, was 11 μl. The influence of including v_m in the calculation of λ depends chiefly on the difference between the ratios $(v_1 + v_2)/(v_1 + v_2 + v_m)$ and $(q_1 + q_2)/(q_1 + q_2 + q_m)$. Initially this difference is largest, but the influence is zero because q_2 in equation (4) is zero. As equilibrium is approached, the influence is also zero, because the two ratios become equal. The effect occurs in the intermediate stage. Computations with both equations (3) and (4) showed that under the present conditions v_m may be disregarded.

Fitting of Equation (2) *by the Method of Least Squares.* In terms of the symbolism used by Berman *et al.* (1962a,b) in the coding for their program (NIH-OMR computer program 9B21), equation (2) is represented by:

where the circles are "compartments" and the λ_{ij}'s are "fractional turnover rates." In the present case, $\lambda_{21} = \lambda/v_1$ and $\lambda_{12} = \lambda/v_2$. The "initial condition" (I C) for compartment 1 is the total amount of solute recovered at the end of dialysis, and the initial condition for compartment 3 is the ratio v_1/v_2. There is only one variable, λ_{21}, and λ_{12} is made dependent on λ_{21} by: $\lambda_{12} = (v_1/v_2)\lambda_{21} = f_3\lambda_{21}$ (this is called "*f*-dependence" in the Berman-Weiss Manual for the program). At every time of withdrawal of a sample, the change in v_1/v_2 is entered. In the present analysis all experimental points have been assigned equal statistical weight.

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[5] Actually, the average concentration in the membrane must be slightly higher. Even for an ideal solute, the concentration gradient close to the inside must be smaller than toward the outside for the amount of solute in the membrane to decrease during the dialysis.