Journal of Clinical Investigation
Vol. 42, No. 2, 1963

OBSERVATIONS CONCERNING THE BINDING OF THYROID HORMONES BY HUMAN SERUM PREALBUMIN *

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(Submitted for publication July 6, 1962; accepted October 10, 1962)

There is general agreement that thyroxine (T_4)[1] is the major component of the thyroid hormone in blood, where it exists almost entirely in a dissociable complex with certain plasma proteins. From the numerous studies that followed the discovery of the T_4-binding (α)-globulin of plasma (TBG) in 1952, it first appeared that the binding of T_4 in plasma could be mainly ascribed to binding sites of high avidity but low concentration, contributed by TBG. A secondary role was ascribed to binding sites of low avidity but almost unlimited concentration, contributed by albumin. These conclusions, based almost entirely on data obtained by electrophoretic separation of serum or plasma proteins on filter paper in barbital buffer at pH 8.6, have been considered in several recent reviews (1, 2).

In 1956, during efforts to purify TBG, an additional T_4-binding protein was observed in subfractions of human plasma proteins. Subsequent experiments revealed that a T_4-binding protein of similar electrophoretic mobility could be consistently demonstrated in unfractionated normal human serum when electrophoretic separations were performed in a buffer containing tris(hydroxymethyl)aminomethane (Tris) and maleic acid, rather than barbital. Because of its rapid anodal migration at pH 8.6, this protein was termed the thyroxine-binding prealbumin (TBPA) (3). It avidly bound T_4, but failed to bind significant quantities of 3,5,3′-triiodothyronine (T_3). It was suggested that TBPA is a protein distinct from TBG, with which it might share a significant role in the transport of T_4. Although several subsequent reports have suggested the contrary, the present report will describe in detail data indicating that TBPA is native to plasma, is distinct from TBG, and participates in the transport of T_4 under physiologic conditions.[2]

METHODS AND RESULTS

A previous communication has fully described the Tris-maleate buffer and electrophoretic apparatus employed in the present studies, as well as the techniques used in storing I^{131}-labeled compounds, in determining their distribution after electrophoresis, and in calculating hormonal binding capacities of TBG and TBPA from filter paper electrophoretic analysis of serum or plasma fractions (6). Unless significantly modified, detailed descriptions of these techniques will not be included here.

Interrelations of TBG and TBPA in plasma fractions. Lyophilized fractions of plasma proteins [3] prepared by Method 6 of Cohn and his colleagues (7) were dissolved in isotonic NaCl. After removal of insoluble residue by centrifugation, protein concentrations in the supernatant fluids were determined by the biuret reaction (8) and solutions were adjusted with physiological saline to a concentration of 3.0 g protein per 100 ml. I^{131}-labeled T_4 was added to each solution to a final concentration of 2 μc per ml (approximately 4 μg T_4 per 100 ml). One sample of each solu-

* Investigation supported in part by research grant A-267 from the National Institute of Arthritis and Metabolic Diseases, Bethesda, Md., and in part by the Medical Research and Development Board, Office of the Surgeon General, Department of the Army, under contract DA-49-007.

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[1] Unless otherwise indicated, wherever reference is made to optically active compounds, the levorotatory isomer is meant.

[2] Certain of the data presented herein have been described before meetings of the New York Academy of Sciences and the Laurentian Hormone Conference, and have been briefly referred to in the published transactions of these organizations (4, 5).

[3] Kindly supplied by Dr. Robert Pennell, Protein Foundation, Jamaica Plain, Mass.

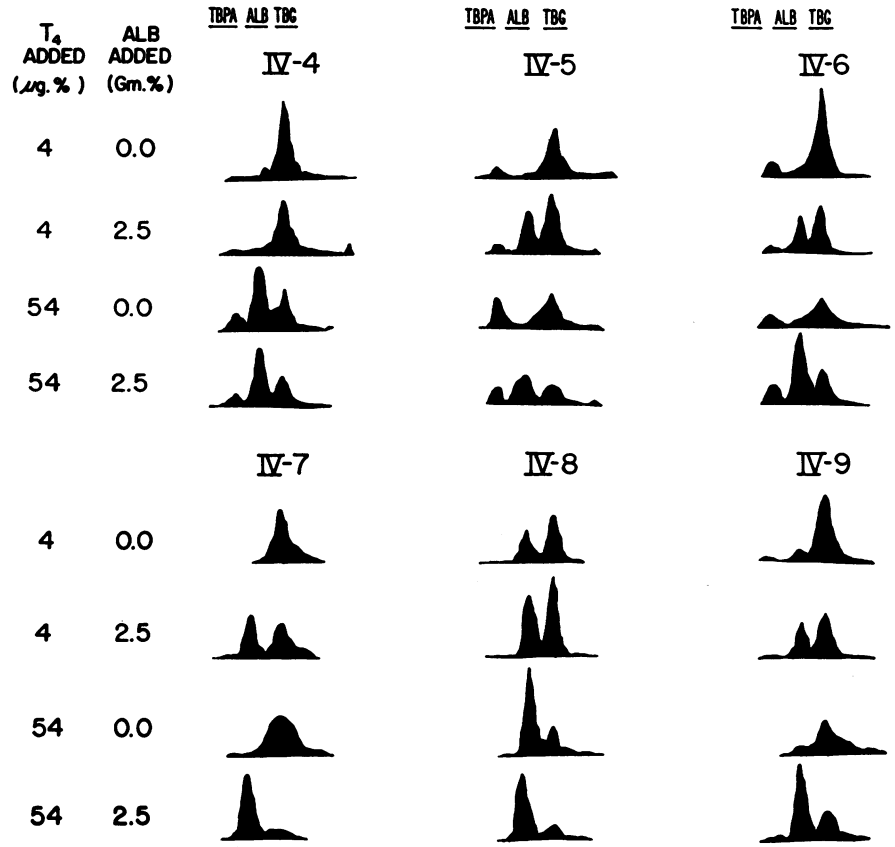

FIG. 1. BINDING OF I^{131}-LABELED THYROXINE IN COHN FRACTIONS OF PLASMA DURING FILTER PAPER ELECTROPHORESIS IN TRIS-MALEATE BUFFER, pH 8.6. Dark areas represent radioactive scans of individual electrophoretograms.

tion was left unaltered, while others were enriched with either 3.0 g per 100 ml of dried human serum albumin (HSA), or 150 μg per 100 ml of stable T_4, or both.[4] The resulting samples of each plasma fraction were then subjected to electrophoresis in Whatman no. 3 filter paper with Tris-maleate buffer at pH 8.6. This technique afforded a semiquantitative assessment of the binding activity of TBPA in each of the plasma fractions tested. Samples containing high concentrations of stable T_4 made possible demonstration of hormonal binding to TBPA in fractions rich in TBG and poor in TBPA. In fractions poor in TBG, HSA provided a binding protein with which TBPA could compete. Results are depicted in Figure 1. TBG was present to a variable extent in all fractions tested. TBPA, however, could be demonstrated only in Fractions IV-4, IV-5, IV-6, and to a very slight extent, in Fraction IV-9.[5]

Plasma Fraction IV-4 (7) was further refined by precipitation with ammonium sulfate, followed by adsorption on and elution from diethylaminoethyl(DEAE)-cellulose (acetate cycle). These procedures employed in purifying TBG and TBPA, to be described in detail in a later communication, yielded a "resin eluate" fraction highly enriched in these proteins and free of albumin. TBG and TBPA were enriched with I^{131}-labeled T_4 and were separated from each other by zonal electrophoresis of resin eluates in cellulose columns (9), with either Tris-maleate or 0.06 M barbital buffer at pH 8.6. After electrophoresis, proteins were eluted by passing through the col-

[4] The albumin employed in this experiment, Fraction V of Cohn and his colleagues (7), was shown by electrophoresis to contain a trace of TBG, but no TBPA.

[5] Variation in the relative proportion of TBG and TBPA was encountered in other batches of the fractions tested.

umns a buffer similar to that employed during electrophoresis. Samples were collected into a fraction collector, and individual fractions were assayed for radioactivity and for concentration of protein by direct well-counting and by determination of optical density at 280 mμ, respectively.

When equal samples of the same resin eluate were studied in this manner, approximately equal quantities of a protein migrating more rapidly than albumin were eluted from the column, regardless of the buffer system employed during electrophoresis. Fractions containing prealbumin also contained appreciable radioactivity when prepared in barbital buffer; however, considerably more radioactivity was associated with prealbumin (and correspondingly less with TBG) when Tris-maleate buffer was used (Figure 2).

Central fractions from the prealbumin and TBG peaks were separately pooled and subjected to pressure dialysis at 25 inches of Hg and 4° C for 72 hours against 3 changes of 200 to 500 volumes of distilled water. Filter paper electrophoresis with barbital buffer at pH 8.6 of samples of the two concentrated pools revealed only prealbumin, in one case, and TBG plus variably small proportions, estimated to be from 10 to 30 per cent, of α_1- and α_2-globulins, in the other. In addition, subsequent analyses indicated that prealbumin prepared in this manner was homogeneous during

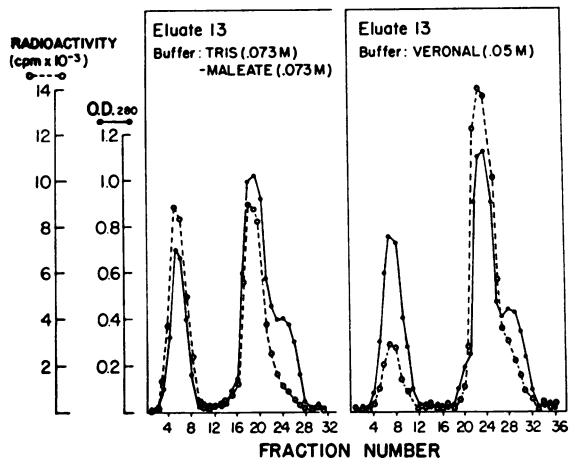

FIG. 2. CELLULOSE COLUMN ELECTROPHORESIS OF I^{131}-LABELED THYROXINE IN SOLUTIONS OF HUMAN PLASMA FRACTIONS RICH IN PREALBUMIN AND THYROXINE-BINDING GLOBULIN (TBG): EFFECT OF BUFFER MEDIUM, pH 8.6. The first radioactive peak eluted represents prealbumin and the second TBG. Veronal = barbital.

both free electrophoresis and ultracentrifugation. Little or no loss of radioactivity (0 to 12 per cent) occurred from either the TBG or the prealbumin pools during dialysis, regardless of whether Tris-maleate or barbital buffers had been employed in their preparation. Ascending filter-paper chromatography (butanol–dioxane–2N-ammonia, 4:1:5) of butanol extracts of these concentrated fractions revealed that the I^{131}-labeled material associated with them was still T_4.

Fractions rich in TBG were prepared from resin eluates by column electrophoresis in barbital buffer and were subjected to re-electrophoresis in either filter paper sheets or cellulose columns, with Tris-maleate buffer. In neither supporting medium did detectable quantities of added radioactive T_4 or of protein appear in the prealbumin zone.

Effects of buffer ions on TBPA. As previously reported (3), binding of labeled T_4 by TBPA could regularly be demonstrated when normal human serum was subjected to electrophoresis in filter paper with Tris-maleate buffer at pH 8.6. In order to evaluate the possible effects on binding of varying concentrations of these buffer ions, T_4-binding capacities of TBG and TBPA were determined in the sera of 4 normal subjects and 4 patients with inflammatory disease. In the latter patients, the T_4-binding capacity of TBPA was subnormal when assessed by electrophoresis in standard Tris-maleate buffer. Comparative studies were performed in Tris-maleate buffer at one-fourth, one-half, and twice the usual, as well as at the usual, concentration. Variations in the concentration of Tris-maleate had no consistent effect on the T_4-binding capacity of TBPA (Figure 3). A tendency in some sera toward lower values for the binding capacity of TBPA at lower concentrations of buffer may have been related to the less adequate separation of TBPA from albumin that occasionally occurred in the more dilute buffer media.

In order to ascertain whether the Tris-maleate buffer system was necessary for demonstration of TBPA in serum (as opposed to plasma fractions where T_4-binding by prealbumin in a barbital buffer had already been found), samples of normal human serum, enriched with I^{131}-labeled and stable T_4, were subjected to filter paper electrophoresis in the following solutions, all at pH 8.4

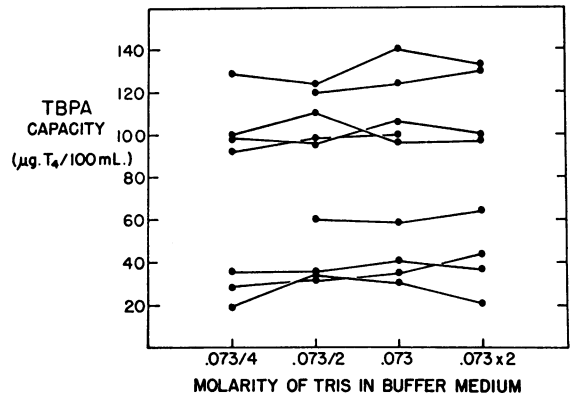

FIG. 3. BINDING CAPACITY OF THYROXINE-BINDING PREALBUMIN (TBPA) IN VARYING CONCENTRATIONS OF TRIS-MALEATE BUFFER, pH 8.6. Sera obtained from 4 normal subjects and 4 with acute inflammatory disease. Molarity of maleate equal to the molarity of Tris.

to 8.6: 0.06 M sodium phosphate, 0.05 M ammonium carbonate, 0.05 M alanine-0.05 M KCl, and 0.05 M glycine-0.05 M KCl. Clearly defined zones of migration of T_4, coinciding with those of TBPA as observed in the Tris-maleate system, were evident in each of the solutions employed. Sufficient observations to permit a quantitative comparison of the T_4-binding capacities of TBPA in the several systems were not made.

Dialysis systems were also employed to assess the effects of varying buffer anions upon T_4-binding by TBPA. Samples of serum were obtained from patients with hepatic cirrhosis. Sera selected for use were first shown to have a markedly decreased T_4-binding capacity of TBPA, as assessed in the Tris-maleate buffer system. Sera were then dialyzed for 24 hours against 100 volumes of the following solutions: 0.06 M phosphate, 0.073 M Tris-0.073 M maleate, 0.146 M Tris-0.146 M maleate, 0.05 M alanine-0.05 M KCl, 0.05 M glycine-0.05 M KCl, and 0.05 M ammonium carbonate, pH 8.4 to 8.6. After dialysis, protein concentrations were measured by the biuret reaction, and the total protein in each sample was adjusted with the appropriate buffer to a final concentration of 4.5 g per 100 ml. Purified prealbumin was dissolved in isotonic saline to a concentration of 3.0 g per 100 ml. I^{131}-labeled T_4 (100 μc per ml) was added, and samples were then diluted with each of the foregoing buffers to a final concentration of 50 mg per 100 ml. Two-ml samples of the solutions of prealbumin were then dialyzed against 12 ml of serum equilibrated with the corresponding buffer. Half-ml samples of the serum phase were withdrawn at regular intervals for 48 hours, were subjected to precipitation and washing with 10 per cent trichloroacetic acid (TCA), and the rate of increase of precipitable radioactivity in the dialyzed fraction was calculated for each buffer system. With the rate of dialysis of precipitable radioactivity in phosphate buffer as a reference (0.032 per cent per 24 hours), the following values for each of the solutions were obtained (mean of 2 experiments): phosphate, 1.00; 0.073 M Tris-0.073 M maleate, 0.91; 0.146 M Tris-0.146 M maleate, 0.89; alanine, 1.08; glycine, 1.02; ammonium carbonate, 0.96. Differences in dialysis rates of similar small degree were noted when HSA (4.5 g per 100 ml), rather than serum, was employed outside of the dialysis sac.

Binding of other iodophenols. Preparations of I^{131}-labeled tri- and tetraiodothyroacetic (TA_3 and TA_4) and tri- and tetraiodothyropropionic acids (TP_3 and TP_4), as well as D-thyroxine (D-T_4), were diluted in 1 per cent HSA to a concentration of 160 μc per ml immediately upon arrival from a commercial source.[6] Single or pooled specimens of serum obtained from normal volunteers were enriched with labeled iodophenol together with increasing, equimolecular concentrations of the corresponding unlabeled compound.[7] Filter paper electrophoresis of specimens was performed, with standard Tris-maleate buffer, and the binding capacities of TBG and TBPA for each of the compounds tested were determined. In most instances, binding capacities of TBG and TBPA for L-T_4 were assessed concomitantly in other samples of the same serum.

D-T_4 was bound little if at all by TBPA and was associated mainly with TBG and secondarily with albumin. Results obtained with different batches of I^{131}-labeled D-T_4 varied slightly, perhaps from contamination of D-T_4 with small amounts of L-T_4. In some batches, no binding of D-T_4 to TBPA could be discerned, even at the lowest concentrations employed (0.2 μc per ml, approximately 0.4 μg D-T_4 per 100 ml). In other

[6] Obtained in 50 per cent propylene glycol solution from Abbott Laboratories, Oak Ridge, Tenn.

[7] Kindly supplied by Dr. Wayne L. Ruddy, Warner-Chilcott Laboratories, Morris Plain, N. J.

batches, a small proportion of I^{131}-labeled D-T_4 was associated with TBPA. In these instances, labeled D-T_4 was displaced from TBPA by the addition of stable L-T_4 far more readily than was labeled L-T_4 itself (Figure 4). I^{131}-labeled D-T_4 was displaced from TBG onto albumin by the addition to serum of stable L-T_4. At all concentrations of stable L-T_4 employed, however, a slightly greater percentage of labeled D-T_4 than of L-T_4 was associated with TBG in paired samples of the same serum. Studies of the effects of enriching sera with stable D-T_4 upon the binding of I^{131}-labeled L- and D-T_4 were not performed.

Low concentrations of I^{131}-labeled TA_4 and TP_4 (0.4 μg per 100 ml and 4.0 μg per 100 ml, or approximately 5×10^{-3} and 5×10^{-2} μmoles per liter, respectively) were bound almost completely to TBPA during electrophoresis at pH 8.6 in Tris-maleate buffer. When increasing concentrations of the corresponding stable compounds were added, labeled material was progressively displaced onto albumin. Binding to TBG was not evident. The binding capacity of TBPA for TA_4 and TP_4 in several normal sera ranged between approximately 190 and 210 μg per 100 ml (2.5 and 2.8 μmoles per liter) and on a molar basis, consistently exceeded by 40 to 50 per cent the molar binding capacity of TBPA for T_4, measured concomitantly in the same sera (approximately 1.8 μmoles per liter, or 140 μg per 100 ml) (Figure 5).

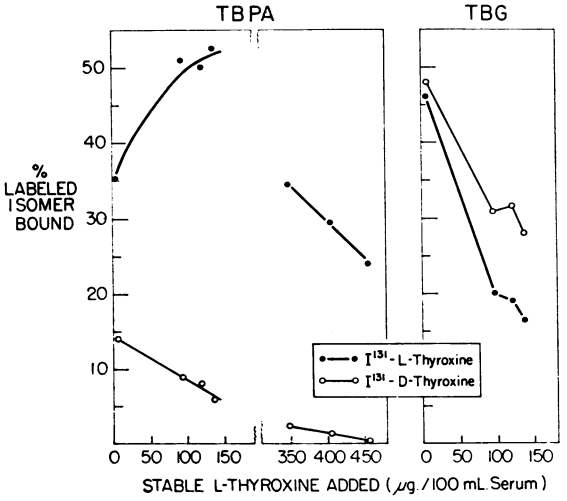

FIG. 4. BINDING OF I^{131}-LABELED D- AND L-THYROXINE TO TBPA AND TBG DURING FILTER PAPER ELECTROPHORESIS OF NORMAL HUMAN SERUM IN TRIS-MALEATE BUFFER, PH 8.6. EFFECT OF ADDING VARYING CONCENTRATIONS OF STABLE L-THYROXINE.

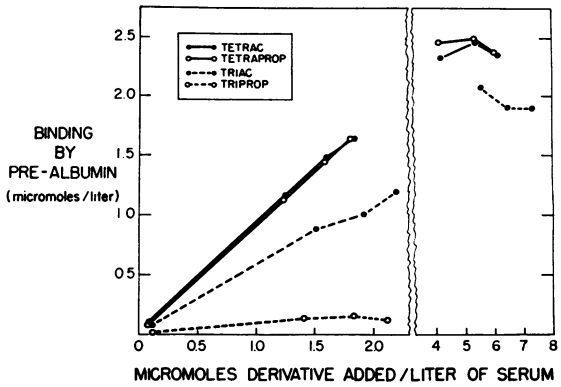

FIG. 5. BINDING CAPACITY OF TBPA IN NORMAL HUMAN SERUM FOR SEVERAL DEAMINATED DERIVATIVES OF THYROXINE AND 3,5,3'-L-TRIIODOTHYRONINE. Electrophoresis of serum in Tris-maleate buffer, pH 8.6. Binding capacity for thyroxine in the same serum was 1.8 μmoles per liter (139 μg per 100 ml). Molecular weights: thyroxine, 777; tetraiodothyroacetic acid, 748; tetraiodothyropropionic acid, 762; triiodothyroacetic acid, 622; and triiodothyropropionic acid, 636.

Low concentrations of TA_3 and especially TP_3 were less well bound by TBPA than their tetraiodinated analogues. At 0.4 μg per 100 ml (approximately 6×10^{-3} μmoles per liter), about 75 per cent of added labeled TA_3 was bound by TBPA, and the remainder with albumin. The molar binding capacity of TBPA for TA_3 was approximately the same as that for T_4. TP_3 was only weakly bound by TBPA. At a concentration of 0.4 μg per 100 ml (approximately 6×10^{-3} μmoles per liter), about one-fourth of the labeled compound was associated with TBPA, and the remainder with albumin. The percentage associated with TBPA declined rapidly as the concentration of stable TP_3 was increased, the apparent TP_3-binding capacity of TBPA being only 10 to 20 μg per 100 ml (0.15 or 0.31 μmole per liter).

Binding of the deaminated derivatives of T_4 and T_3 to TBG was not evident during scanning procedures, but a faint band of radioactivity could be seen in the TBG zone in radioautographs of the foregoing samples.

In additional experiments, sera containing 2 μc per ml of I^{131}-labeled TA_4, TA_3, TP_4, or TP_3 were enriched with standard concentrations of stable T_4. Typical results are presented in Figure 6. T_4 readily displaced TP_3 from TBPA. TA_3 was

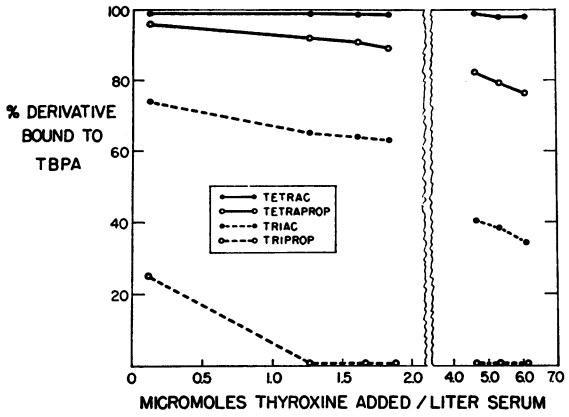

FIG. 6. DISPLACEMENT OF I^{131}-LABELED DEAMINATED DERIVATIVES OF THYROXINE AND 3,5,3′-L-TRIIODOTHYRONINE FROM TBPA BY VARYING CONCENTRATIONS OF STABLE L-THYROXINE. Electrophoresis of normal human serum in Tris-maleate buffer, pH 8.6. Molecular weights as given for Figure 5.

less readily displaced, whereas at concentrations of added T_4 ranging between 87 and 130 μg per 100 ml (approximately 1.1 to 1.7 μmoles per liter), TA_4 and TP_4 were displaced little if at all. At higher concentrations of T_4, slight displacement of labeled TA_4 and TP_4 from TBPA was evident, especially in the case of TP_4.

The ability of standard concentrations of the deaminated derivatives of T_4 and T_3 to displace tracer quantities of labeled T_4 from TBPA was compared with the ability of stable T_4 itself to effect this displacement. Electrophoretic studies were performed in samples of pooled normal serum enriched with 2.0 μc per ml of I^{131}-labeled T_4, together with increasing equimolecular concentrations of one of the derivatives or of T_4 itself. The effect of stable T_4 and of the derivatives on the distribution of labeled T_4 differed greatly. At concentrations sufficient to saturate T_4-binding sites on TBG but not on TBPA, stable T_4 displaced labeled T_4 from TBG to TBPA. Labeled T_4 was displaced from TBPA only when concentrations of stable T_4 sufficient to saturate T_4-binding sites on TBPA were approached or exceeded. As judged from the distribution of labeled T_4 in control specimens containing only endogenous stable hormone, TA_4 and TP_4 were even more effective than T_4 itself in displacing labeled T_4 from TBPA. At the two highest concentrations of derivative employed, displacement of labeled T_4 from TBPA was complete (Figure 7). Labeled T_4 displaced from TBPA became associated mainly with TBG, and to a far lesser extent, with albumin. This finding suggested that the deaminated derivatives did not significantly inhibit the binding of T_4 to TBG, and was consistent with the failure of appreciable quantities of labeled derivatives themselves to bind to TBG, as described above. Stable TP_3 had no effect, and TA_3 only a slight effect, in displacing labeled T_4 from TBPA.

Inhibitors of T_4-binding by TBPA. In view of the rarity with which T_4-binding by TBPA in normal serum can be demonstrated when paper electrophoresis is performed in barbital buffer, experiments were designed to determine whether barbital inhibits the binding of T_4 by this protein. Samples of normal human serum were enriched with I^{131}-labeled and stable T_4, and binding capacities for TBG and TBPA were determined in standard Tris-maleate buffer. Concomitantly, portions of the same samples were subjected to electrophoresis in Tris-maleate buffer to which had been added barbital in 0.005, 0.025, and 0.050 M concentrations.

At the two higher concentrations of added barbital, significant quantities of I^{131}-labeled T_4 could not be demonstrated in the TBPA zone. At the lowest concentration of barbital, binding of T_4 to TBPA was demonstrable, though decreased. In addition to its apparent inhibitory action on T_4-binding by TBPA, another effect of barbital was

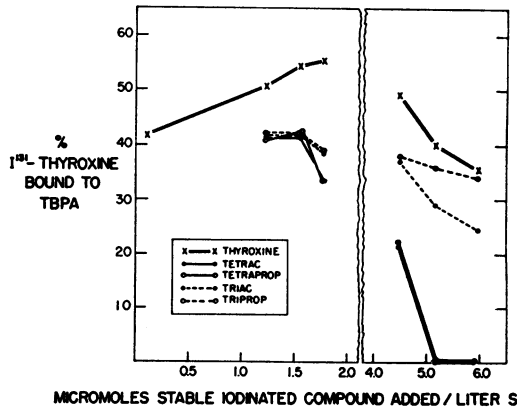

FIG. 7. DISPLACEMENT OF TRACER CONCENTRATIONS OF I^{131}-LABELED THYROXINE FROM TBPA IN NORMAL HUMAN SERUM BY ADDITION OF STABLE THYROXINE AND SEVERAL OF ITS DERIVATIVES. Electrophoresis of serum in Tris-maleate buffer, pH 8.6. Molecular weights as given for Figure 5.

noted. Both at the low concentration of added T_4 and at concentrations sufficient to saturate TBG, i.e., at 87 to 130 μg per 100 ml, inhibition of binding by TBPA was associated with an increase in the proportion of added T_4 migrating in the α-globulin zone and hence with an increase in the calculated T_4-binding capacities of proteins migrating in this area. Results similar to those depicted in Figure 8 were found in five additional experiments in which binding capacities of TBG and TBPA were determined after electrophoresis of normal sera in Tris-maleate buffer and in Tris-maleate buffer to which 0.05 M barbital had been added. In all, binding of T_4 by TBPA was abolished, whereas the binding capacity of proteins in or near the TBG zone was increased by an average of 69 per cent.

Although it was not discernible by radioactive scanning, a fine band of radioactivity was often evident in the α_1-globulin zone in radioautographs of sera subjected to electrophoresis in the Tris-maleate-barbital system. The proportion of labeled T_4 localized in this band was not determined. By inspection, however, it did not appear likely that this faint band of T_4 could account for all of the increase that barbital induced in the binding capacity of moieties in the α-globulin zone.

To clarify this finding further, similar studies were performed in serum virtually devoid of TBG (T_4-binding capacity less than 1 μg T_4 per 100 ml), but normal with respect to TBPA (6). As was the case in normal sera, addition of 0.05 M barbital to Tris-maleate buffer obliterated T_4-binding by TBPA. Only a very slight increase, however, was induced in the negligible binding of T_4 seen in control electrophoretograms in the α-globulin zone, and most of the labeled T_4 displaced from TBPA by barbital became associated with albumin.

Normal human serum, enriched with I^{131}-labeled and varying concentrations of stable TA_4, was subjected to electrophoresis both in Tris-maleate and in 0.05 M barbital buffer at pH 8.6. Binding of TA_4 by protein migrating anodally to albumin was readily demonstrable in barbital buffer, but a smaller proportion of the TA_4 was associated with TBPA than was the case in the Tris-maleate buffer control (Figure 9). Barbital did not induce binding of TA_4 to proteins localized in the α-globulin zone sufficient to be discernible by scanning procedures.

Dialysis systems afforded evidence that barbital also inhibits the binding of T_4 by purified prealbumin. Two-ml samples of solutions of puri-

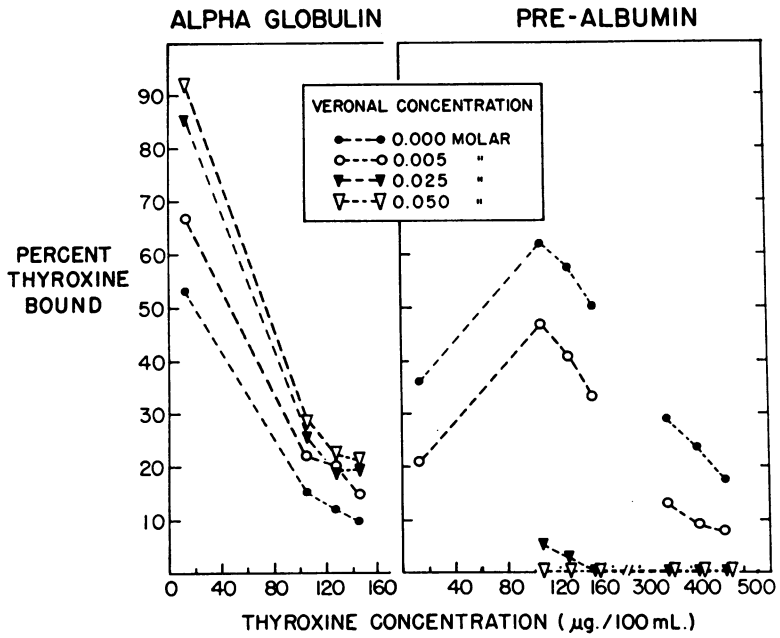

FIG. 8. THE EFFECT OF ADDING BARBITAL (VERONAL) TO TRIS-MALEATE BUFFER, pH 8.6, ON THE BINDING OF THYROXINE IN NORMAL HUMAN SERUM.

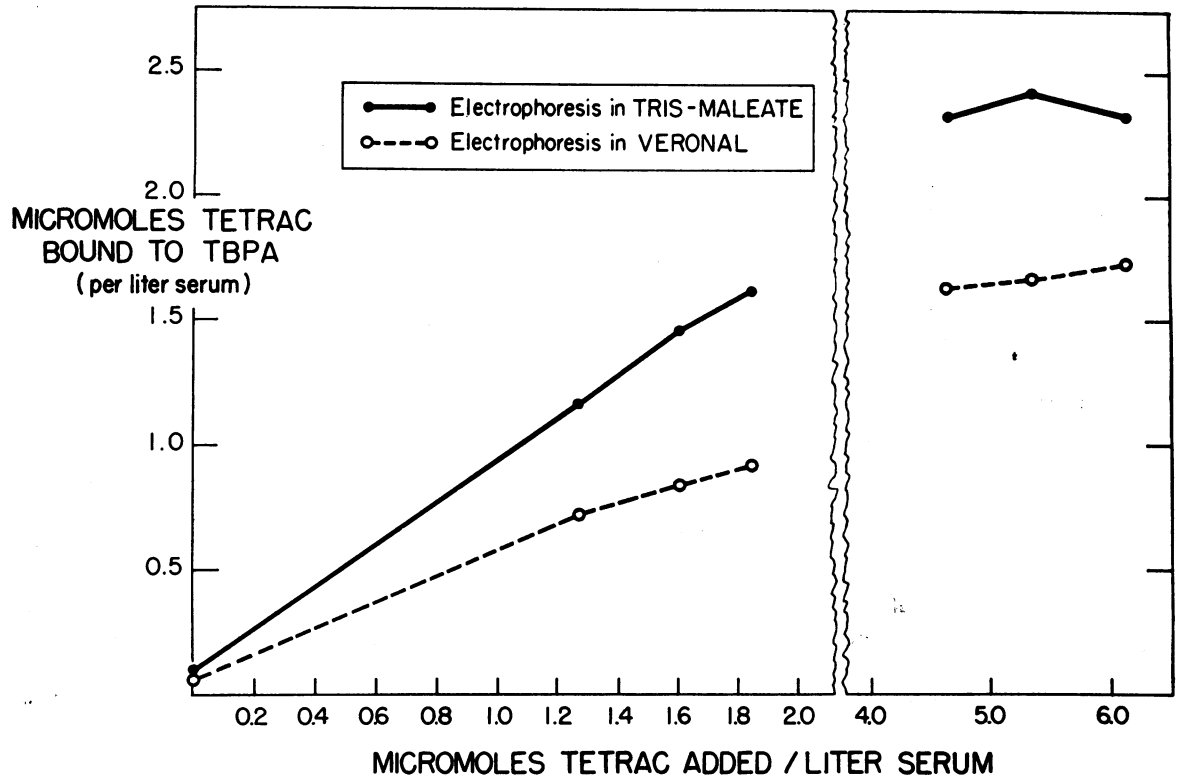

FIG. 9. DIFFERENCES IN THE BINDING OF TETRAIODOTHYROACETIC ACID TO TBPA IN NORMAL HUMAN SERUM DURING ELECTROPHORESIS IN TRIS-MALEATE AND IN BARBITAL (VERONAL) BUFFER, BOTH AT PH 8.6.

fied prealbumin (25 mg per 100 ml) containing I^{131}-labeled T_4 in 0.05 M phosphate, standard Tris-maleate, and 0.05 M barbital buffers were dialyzed for 48 hours against 12-ml samples of 5 per cent HSA previously equilibrated by dialysis against the corresponding buffer systems. Rates of dialysis of TCA-precipitable radioactivity were then assessed. With the rate of dialysis in phosphate buffer as a reference (0.047 per cent per 24 hours), rates of dialysis in duplicate experiments averaged: phosphate, 1.00; Tris-maleate, 0.96; and barbital, 8.20. At the completion of dialysis, the specific radioactivity of prealbumin in Tris buffer, in counts per minute per milligrams protein, was more than 2,500 times that of the albumin with which it had been equilibrated. In barbital buffer, the ratio of prealbumin to albumin specific activity was greatly reduced, but remained in excess of 300.

In view of reports that salicylates accelerate both the peripheral turnover of T_4 *in vivo* (10) and the rate of dialysis of T_4 from serum *in vitro* (11), experiments were performed to assess whether salicylate ion produced these effects by inhibiting the association of T_4 with either TBG or TBPA. Since it seemed possible that any bond between salicylate and a T_4-binding protein might be so weak as to be disrupted by either the dilution of salicylate or by the electromotive forces incident to electrophoresis, experiments were performed using Tris-maleate buffer enriched with salicylate at pH 8.6 to a concentration of 30 mg per 100 ml. This concentration of salicylate is similar to that achieved in serum by large therapeutic doses *in vivo* (10) and to that employed in the dialysis experiments reported by others (11). A typical result, comparing T_4-binding in standard Tris-maleate buffer to that obtained in Tris-maleate-salicylate, is shown in Figure 10. Salicylate induced a pronounced inhibition of T_4-binding by TBPA. As had been the case with barbital, this effect was associated with a distinct increase in the T_4-binding capacity of moieties in the α-globulin zone.

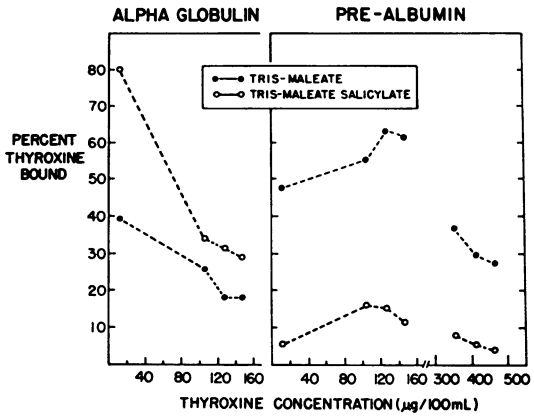

FIG. 10. THE EFFECT OF ADDING SALICYLATE (30 MG PER 100 ML) TO TRIS-MALEATE BUFFER, PH 8.6, ON THE BINDING OF THYROXINE TO PROTEINS IN THE α-GLOBULIN AND PREALBUMIN ZONES OF NORMAL HUMAN SERUM.

Similar though less striking inhibition of T_4-binding by TBPA was induced by enriching serum with 30 mg salicylate per 100 ml before electrophoresis in standard Tris-maleate buffer.

Since electrophoretic experiments were performed at pH 8.6, dialysis experiments were performed to obtain further evidence concerning the locus of inhibition of T_4-binding induced by salicylates at physiological pH. Advantage was taken of observations (see above) which indicated that, unlike L-T_4, D-T_4 is firmly bound by TBG, but is bound little if at all by TBPA. To normal serum was added one-tenth part of 0.5 M phosphate buffer at pH 7.4. A 2.0-ml sample of this mixture was enriched with stable L-T_4 and I^{131}-labeled L- or D-T_4 to a total concentration of 50 to 55 µg per 100 ml. This was dialyzed against 12 ml of the same serum-buffer mixture containing an equivalent concentration of stable L-T_4. Additional vessels were prepared in which the serum both within and outside of the dialysis sacs were enriched with salicylate (50 mg per 100 ml, pH 7.4). Vessels were placed on a rocking platform in a cold room at 4° C. Dialyzed samples were withdrawn periodically for 48 hours and TCA-precipitable radioactivity determined. As shown in Figure 11, L-T_4 was dialyzed from serum less rapidly than D-T_4. The rate of dialysis of L-T_4, however, was greatly increased by salicylate, whereas that of D-T_4 increased only slightly. Electrophoretic studies of the contents of the dialysis sacs revealed that none of the radioactivity introduced with the labeled D-T_4 was associated with TBPA in samples either containing or devoid of salicylate.

Barbital, 0.05 M at pH 7.4, when added to serum, produced the same relative effects on the rate of dialysis of D- and L-T_4 as did salicylate. When, however, salicylate was added in addition to barbital, no further increase in the rates of dialysis of the two isomers of T_4 occurred.

When comparable experiments were performed in a serum virtually devoid of TBG but with a normal binding activity of TBPA, L-T_4 was dialyzed from the serum more rapidly than normal, and salicylate greatly increased the rate. The rate of dialysis of D-T_4 in control sacs was as great as that of L-T_4 in the presence of salicylate; it was not further increased by salicylate.

Trypan blue has been reported to increase the uptake of I^{131}-labeled T_4 by erythrocytes suspended in serum diluted with buffer at physiological pH (12). Trypan blue had no effect when erythrocytes were suspended in buffer alone. This finding suggested that trypan blue might interfere with hormonal binding by one or more serum proteins. Samples of a pool of normal human serum were therefore enriched with trypan blue in concentrations of 50, 5, and 0.5 mg per ml. The highest concentration of trypan blue employed was that calculated to yield approximately the same concentration ratio trypan blue to protein as that achieved in the earlier experiments with erythrocytes (12). Portions of these samples

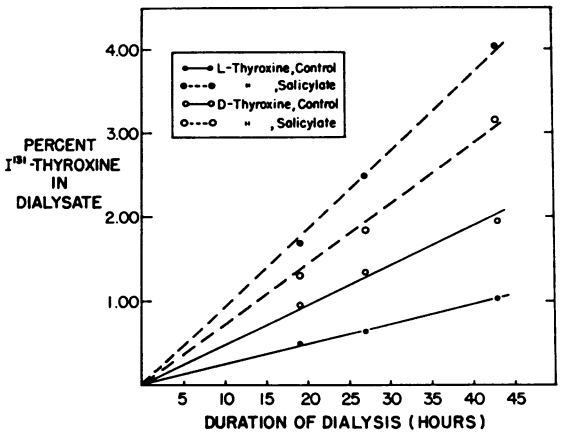

FIG. 11. THE EFFECT OF SALICYLATE (50 MG PER 100 ML) ON THE RATE OF DIALYSIS OF I^{131}-LABELED L- AND D-THYROXINE FROM NORMAL HUMAN SERUM BUFFERED TO PH 7.4.

were then enriched with I^{131}-labeled T_4 and with concentrations of stable T_4 ranging between 87 and 450 μg per 100 ml; binding capacities of TBG and TBPA were determined by electrophoresis in Tris-maleate buffer. At the highest concentrations of trypan blue employed, partial coagulation of proteins was noted; the electrophoretic pattern of serum proteins was distorted, with a preponderance remaining at the origin and a tail developing anodally. Identification of the usual electrophoretic zones was difficult, but a probable albumin zone was seen (Figure 12). A small proportion of labeled T_4 was localized anodally to this region. At the two lower concentrations of trypan blue, some protein remained at the point of application, but the usual electrophoretically separable groups of proteins were nevertheless clearly defined. Trypan blue in concentrations of 5 and 0.5 mg per 100 ml decreased the T_4-binding capacity of proteins in the prealbumin zone by an average of 80 per cent and 58 per cent, respectively. A slight increase in the T_4-binding capacity of moieties in the α-globulin zone was also noted.

Effect of pH on binding. As has recently been reported (13, 14), essentially no binding of labeled T_4 was noted in the area anodal to albumin when normal serum was subjected to filter paper electrophoresis in Tris-maleate buffer at pH 7.4.

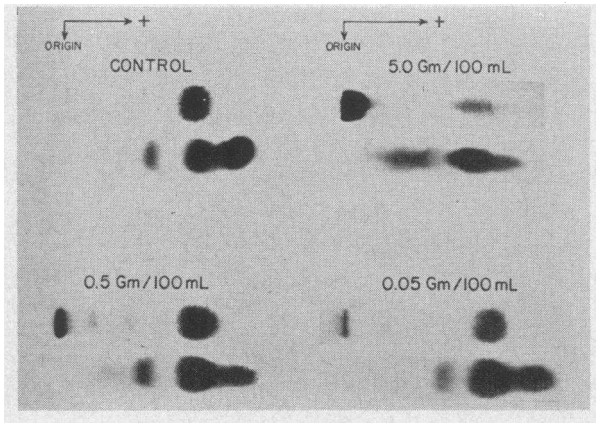

FIG. 12. THE EFFECT OF TRYPAN BLUE ON THE ELECTROPHORETIC MOBILITY OF THE PROTEINS AND THYROXINE-BINDING OF NORMAL HUMAN SERUM. Electrophoresis in Tris-maleate buffer, pH 8.6. Upper strip in each pair represents bromphenol blue stain of electrophoretogram and lower strip represents radioautograph of electrophoretogram before staining. Numbers indicate concentration of trypan blue in serum. For samples shown, stable thyroxine added to a concentration of 87 μg per 100 ml.

When resin eluates containing sufficient prealbumin and TBG to provide visible spots after bromphenol blue staining were studied by paper electrophoresis at pH 7.4, prealbumin retained its localization anodal to albumin. Binding of T_4 was localized, however, entirely to the TBG zone.

In electrophoretic studies performed at pH 7.4, I^{131}-labeled TA_4 added to normal serum in tracer concentrations of 0.4 μg per 100 ml was bound mainly to TBPA (75 per cent) and also to albumin (24 per cent). When the same samples of serum were studied in buffer at pH 8.6, TA_4 was bound almost entirely (> 95 per cent) to TBPA.

Dialysis experiments were performed in which 2 ml of a purified prealbumin solution, at 50 mg per 100 ml and containing tracer quantities of I^{131}-labeled T_4, was dialyzed against 12 ml of a 5.0 g per 100 ml solution of HSA. Rates of dialysis of TCA-precipitable radioactivity were ascertained for solutions prepared in Tris-maleate buffer, pH 8.6 and pH 7.4. These averaged 0.054 and 0.160 per 24 hours, respectively. After 72 hours of dialysis, the specific activity of prealbumin, in counts per minute per milligrams protein, was 1,120 times as great as that of albumin at pH 8.6. Although the relative T_4-binding activity of prealbumin was decreased at pH 7.4, the specific activity of prealbumin averaged 390 times that of albumin.

Electrophoretic experiments were also performed in an agar gel system (15, 16). Normal sera and solutions of HSA, both containing I^{131}-labeled T_4, were subjected to electrophoresis on glass slides in a film of agar gel (1.0 g per 100 ml), prepared in Tris-maleate buffer at pH 8.6, and in 0.05 M phosphate buffer at pH 7.4. After electrophoresis, slides were placed in apposition to no-screen X-ray film and radioautographs were prepared. No attempt was made to quantify the distribution of radioactivity among the various protein zones. Nevertheless, during electrophoresis of serum both at pH 7.4 and at 8.6, distinct binding of T_4 to TBPA, as well as to TBG and albumin, was clearly evident (Figure 13). The density of radioautographic darkening in the TBPA zone appeared greater at pH 8.6 than at 7.4.

Additional electrophoretic experiments were carried out in filter paper with 0.06 M phosphate

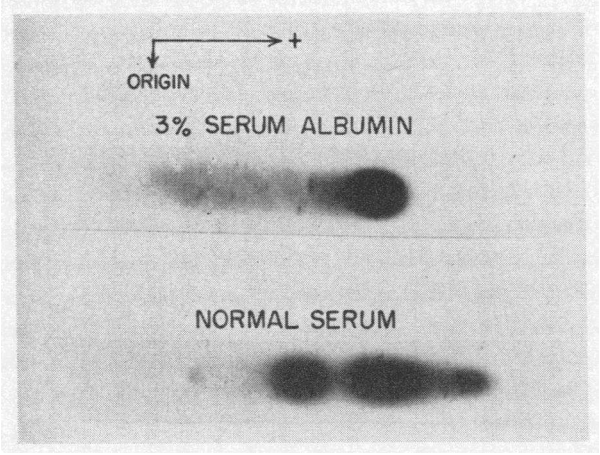

FIG. 13. BINDING OF I^{131}-LABELED THYROXINE (50 μG PER 100 ML) IN NORMAL HUMAN SERUM AND IN 3 PER CENT HUMAN SERUM ALBUMIN DURING ELECTROPHORESIS IN AGAR GEL, WITH 0.05 M PHOSPHATE BUFFER AT pH 7.4.

buffer at pH 7.4. Here, samples of unlabeled normal human serum were applied to a filter paper sheet in a vertical row. I^{131}-labeled T_4 dissolved in physiological saline was applied to the filter paper in a series of spots located at increasing distances lateral to the serum in the anodal direction. Electrophoresis was then performed in the standard manner. In this way, the serum proteins passed over the point of application of the labeled T_4 in the order of the rapidity of their anodal migration and were permitted to proceed for varying distances anodally beyond their point of encounter with the labeled hormone. At this pH, TBG and albumin were not well separated. Nevertheless, resolution was sufficient to allow several conclusions. T_4 that was not traversed by protein apparently remained bound to filter paper and was electrophoretically immobile. Even when all the major binding proteins had traversed the point of application, however, some labeled T_4 remained behind. Under these conditions, most of the T_4 affixed to the proteins was associated with TBG. In all samples, however, a proportion of the labeled T_4 (6 to 14 per cent) was associated with TBPA. The proportion of protein-bound T_4 migrating with TBPA decreased as the distance of migration of TBPA beyond its initial encounter with the labeled T_4 increased. In strips where both TBPA and albumin had passed over the labeled T_4, the radioactivity associated with TBPA was from 3 to 10 times as great as that associated with albumin.

Binding characteristics of prealbumin in cerebrospinal fluid. Pooled human cerebrospinal fluid was subjected to pressure dialysis against large volumes of distilled water. After lyophilization, proteins were dissolved in physiological saline solution to a concentration of 4.0 g per 100 ml. Solutions were enriched with tracer quantities of labeled T_4 or T_3, and were subjected to electrophoresis in standard Tris-maleate buffer and in the same buffer to which either 0.05 M barbital or 30 mg per 100 ml salicylate had been added. In all buffer systems, a faintly discernible bromphenol blue spot was noted in the prealbumin zone. In Tris-maleate buffer, T_4 was localized almost entirely in this area, but small proportions were associated with the albumin and α-globulin zones. T_3 was not localized in the prealbumin zone, was principally associated with albumin, and trailed cathodally toward the point of application. Addition of barbital or of salicylate diminished, but did not entirely inhibit, binding of T_4 in the prealbumin zone. T_4 displaced from prealbumin was bound mainly to albumin, but small proportions were also localized in the α- and β-globulin zones.

DISCUSSION

Since the discovery of TBG in 1952, a diversity of views has been held at one time or another concerning the number, nature, and role of the the proteins in plasma that bind T_4. These diverse views can perhaps be ascribed to possible artifacts introduced by available methods for studying the binding of T_4 to specific proteins. By exposing the interactants to electrical forces, to various supporting media, and to unphysiological conditions of ionic strength, composition, and pH, the zonal electrophoretic techniques almost always employed in studies of this subject may distort the protein-hormone interactions that obtain *in vivo*. As a result, findings in no single system can be considered as absolute, and conclusions are best drawn, as the present studies have attempted, from a correlation of findings obtained under a variety of experimental conditions and in several different systems.

Aside from TBG, the protein that has received greatest attention as a mediator in the transport of T_4 is one termed the thyroxine-binding prealbumin, TBPA. Consistent demonstration of

TBPA in normal human serum first came as the result of substituting, in a conventional filter-paper electrophoretic system at pH 8.6, Tris-maleate buffer for the barbital buffer almost universally employed in studies of T_4-binding to that time (3). It was suggested that TBPA and TBG are distinct proteins, sharing a significant role in the transport of T_4. Since then, however, various doubts concerning the nature and physiological import of TBPA have been expressed. The present report represents a compilation of observations supporting the initial view that TBPA is distinct from TBG and participates significantly in the transport of T_4

Since a number of the present observations concern the T_4-binding properties of a purified prealbumin, it is first necessary to consider whether this protein is identical with TBPA. Although a number of proteins have been noted that migrate rapidly toward the anode during electrophoresis of serum (17–19), the findings of Blumberg and Robbins (20) in the starch gel system indicate that in human serum, only the most rapidly migrating prealbumin, prealbumin 1 of Smithies (18) or A of Poulik and Smithies (19), binds T_4. The purified prealbumin described by Schultze, Schönenberger, and Schwick (21), shown by Tata to bind T_4 avidly (22), also appears to migrate to the position of prealbumin 1 in the starch gel system (23). The immunologic identity of the prealbumin of Schultze and co-workers with the T_4-binding prealbumin of cerebrospinal fluid and of whole serum has also been claimed (24). It is therefore relevant that, in the present studies, at least several properties were shared by the TBPA of serum, of cerebrospinal fluid, and the present preparation of purified prealbumin. All had similar electrophoretic mobility and strongly bound T_4, but not T_3. In all, the binding of T_4 was inhibited by barbital and by salicylate. These findings suggest that the T_4-binding moieties in all are either the same or very similar. Since starch gel electrophoresis of the present preparation of prealbumin has not been performed, it is not certain whether this preparation contains prealbumins other than those found in prealbumin 1. Nevertheless, it seems likely that TBPA is, if not the sole component of the present preparation of prealbumin, the major protein responsible for its T_4-binding properties.

The relation of Tris to the binding of T_4 by TBPA. Certain observations have suggested that the ability of TBPA to bind T_4 depends upon an interaction of the protein or hormone with Tris (25). It has been reported that when samples of TBPA, prepared by elution from starch gels after electrophoresis of serum in Tris buffer, were dialyzed free of Tris, their T_4-binding potency was lost. When the Tris was replaced, T_4-binding was restored, as shown by the ability of such preparations once again to inhibit the *in vitro* uptake of labeled T_4 by red blood cells. The uptake of labeled T_4 by red blood cells from whole plasma was also decreased when Tris was added. Since this report contained no data concerning the effects of Tris on the uptake of T_4 by red blood cells from protein-free media, the findings are difficult to interpret. In any event, the data seem inconsistent with the present or previous observations that *a*) the T_4-binding capacity of TBPA does not vary significantly with wide variations in the concentration of Tris-maleate buffer employed, *b*) preparations of purified prealbumin failed to lose significant quantities of labeled T_4 when dialyzed extensively against distilled water, *c*) Tris-maleate, when compared with several other buffer systems, did not alter significantly the rate of dialysis of labeled T_4 from solutions of purified prealbumin, and *d*) TBPA is readily demonstrable at pH 8.6 when buffers other than Tris but containing no barbital are employed in the electrophoresis of serum (26–28). Furthermore, binding of T_4 by TBPA is readily demonstrable in normal serum when electrophoresis is performed in a barbital buffer more dilute than usual (27, 29). Finally, as assessed by both electrophoretic and dialysis systems at pH 8.6, purified prealbumin displayed a binding potency far greater than that of albumin, even in the usual 0.05 M barbital buffer. These findings clearly indicate that the ability of prealbumin to bind T_4 avidly is not an artifact specifically induced by the Tris-maleate buffer system.

The effects of binding inhibitors. The foregoing observations indicate that the indemonstrability of TBPA during studies performed in normally constituted barbital buffer, rather than its demonstration during the use of other buffers, represents an artifactual situation. The present observations strongly support the initial hypothe-

sis that this artifact follows from the inhibition by barbital of the binding of T_4 by TBPA (3). Thus, although barbital is thought to inhibit the binding of T_4 by HSA (25), it greatly increased the rate of passage of labeled T_4 across a dialysis membrane from purified prealbumin to HSA. Furthermore, when added to Tris-maleate buffer in increasing concentrations, barbital progressively decreased T_4-binding by TBPA in serum; at the usual 0.05 M barbital concentration binding of T_4 was completely abolished. In contrast, comparable concentrations of barbital diminished, but did not abolish, the binding by TBPA of TA_4, a compound herein shown to be more avidly bound by TBPA than is T_4 itself. These findings are consonant with the interpretation that barbital and T_4 (and TA_4) compete for common binding sites on the TBPA molecule. This competition need not result, however, in a complete loss of the T_4-binding ability of prealbumin, as shown by the present electrophoretic and dialysis experiments, by previous studies of cerebrospinal fluid and of the serum of patients with nephrosis (30), and by studies of normal serum performed in dilute barbital buffer. The quantity or proportion of T_4 bound by prealbumin in the presence of barbital would appear to depend upon the relative concentration of these interactants and upon the availability of T_4-binding sites on other proteins within the mixture. Similar considerations would presumably apply in the case of other inhibitors of T_4-binding by TBPA, such as trypan blue, salicylate, and dinitrophenol (31).

In the present studies, the inhibition of T_4-binding by TBPA induced by addition of barbital, trypan blue, or salicylates to the Tris-maleate buffer was consistently associated with an increase in the T_4-binding capacity of proteins migrating in the α-globulin zone. The two-dimensional electrophoretic observations of Blumberg and his coworkers would suggest that barbital may cause a portion of the TBPA in serum to migrate in the α-globulin zone. In the present studies, radioautographs of sera that had undergone electrophoresis in Tris-maleate-barbital buffer did reveal a faint band of radioactivity in the α-globulin zone, but this did not appear sufficient to account for the increased α-globulin binding of T_4 that barbital induces. Furthermore, it seems unlikely that the electrophoretic mobility of more than a small fraction of TBPA could be altered by barbital, since both high concentrations of purified prealbumin and the protein in serum that binds TA_4 (presumably TBPA) retain their characteristic migration during electrophoresis in barbital buffer. It is, in addition, highly improbable that any TBPA whose mobility might be altered by barbital would continue to bind T_4 when T_4-binding by the remainder of the TBPA is completely inhibited by this agent. Thus, it is not clear why barbital increases T_4-binding in the α-globulin zone, although this effect may depend on the presence of TBG, since no appreciable increase was induced by barbital in a serum virtually devoid of TBG but normal with respect to TBPA.

The differing properties of TBG and TBPA. In 1958, Tata, on the basis of both immunologic and electrophoretic observations, suggested that TBPA is the principal T_4-binding moiety of serum, but that in its natural state, TBPA is bound in a complex to an α-globulin, giving rise to TBG (32). A previous communication from this laboratory pointed out the difficulties in interpreting the observations upon which this hypothesis had been based (4). Chief among these is the fact that all electrophoretic experiments had been performed in barbital buffer. Major functional differences between TBG and TBPA that rendered this hypothesis unlikely were also cited at that time and have been described in detail here. Among these is the differing affinity of the two proteins for several derivatives of T_4. Thus, T_3 is not bound by TBPA to a detectable degree (3). TA_4 and TP_4 are very firmly bound by TBPA, whereas TA_3 and TP_3 are bound progressively less well. On the other hand, TBG binds none of these deaminated derivatives to a significant extent. With regard to TA_4 and TP_4, the present findings agree with those of Tanaka and Starr (33). These authors, however, failed to note appreciable binding of TA_3 and TP_3 to TBPA, probably because electrophoretic studies of these less intensely bound compounds were performed in the inhibitory barbital buffer.[8] In general, the relative in-

[8] Tata, Widnell, and Gratzer have suggested that there exist in TBPA two varieties of binding sites, one of which binds T_4 and TA_4 and is inhibited by barbital, the other of which binds TA_4 but not T_4, and is not inhibited by barbital (34). Both these observations and those presently described, however, are equally consistent

tensity of the bond between TBPA and either T_4 or its derivatives, as herein described, bears a close inverse correlation with the rate of dialysis of these compounds from serum and with values for their *in vitro* uptake from plasma by erythrocytes (35).

A further striking difference between TBG and TBPA lies in their affinities for D-T_4. The present observations indicate that the binding of D-T_4 to TBG is at least as intense as that of L-T_4. Previous studies of this question have led to conflicting results. Since earlier electrophoretic observations were performed in barbital buffer, no information on the binding of D-T_4 to TBPA has been available. Some workers, however, have been led to suggest that D-T_4 is strongly bound by TBG (33, 36), while others have indicated that it is not (37). Tata's observations based on the T_4-stabilization method to assess binding indicate that Fraction IV-6 of Cohn and his colleagues binds D-T_4 avidly (22). In view of the present observations, this binding cannot be ascribed to the TBPA present in this fraction, but must be attributed to a firm bond between D-T_4 and TBG.

The differing responses to binding inhibitors and to changes in pH described here and elsewhere (13, 14, 31), as well as the independent variations in the T_4-binding capacities of TBG and TBPA that occur in diverse abnormal states (38), provide further evidence of the striking functional dissimilarities between the two proteins.

Finally, if TBG were the result of an interaction between TBPA and another serum protein, it would follow from the regularity with which TBPA can be demonstrated in Tris and other buffer systems that such buffers must dissociate the two proteins and liberate free TBPA. This seems unlikely, however, since values for the T_4-binding capacity of TBG in serum do not depend upon whether the buffer in which studies are performed does or does not permit the demonstration of TBPA. Furthermore, in the present experiments, fractions highly enriched in TBG and devoid of TBPA were prepared from other plasma fractions by electrophoresis in barbital buffer. No dissociation of TBPA from TBG could be demonstrated when such preparations were subjected to electrophoresis in a Tris-maleate system.

In view of these many considerations, it is very unlikely that TBPA is the prosthetic group of TBG, as Tata once suggested (32). In more recent publications, Tata has confirmed many of the foregoing findings and has provided additional evidence that leads him now to concur in the opinion that TBG and TBPA are distinct proteins (34, 39). Apparently, much of the earlier evidence that led him to the conclusion that TBG and TBPA are closely related was due, as we had suggested (4), to contamination by TBG of the prealbumin that he had employed.

The effects of pH on hormonal binding of TBPA. A third question raised concerning the importance of TBPA deals with the ability of the protein to bind T_4 at physiological pH. During conventional filter-paper electrophoresis of normal serum at pH 7.4, virtually no binding of T_4 by TBPA can be demonstrated (13, 14). The present studies indicate that this finding cannot be ascribed to a change in the electrophoretic mobility of TBPA at pH 7.4, since at this pH deaminated derivatives of T_4 continue to bind to proteins migrating anodal to albumin, albeit less intensely. Furthermore, in protein fractions or in serum sufficiently enriched with prealbumin to permit determination of its electrophoretic mobility by protein staining, prealbumin at pH 7.4 retains its characteristic location anodal to albumin.

The absence of demonstrable binding of T_4 by TBPA during electrophoresis of serum at pH 7.4 might also be explained by the observation that the binding avidity of TBPA is far less intense at pH 7.4 than at pH 8.6. Although prealbumin remained far more potent in direct binding competition and on a unit weight basis than albumin at pH 7.4, it might be that the greater binding potency of TBG and the far greater concentration of albumin in plasma would make TBPA a negligible factor in the transport of T_4 *in vivo*. Considerable light was shed upon this question by the observation of Hollander, Odak, Prout, and Asper that when electrophoresis of serum at pH 7.4 is carried out in agar gel rather than filter paper, a considerable proportion of labeled T_4 is associ-

with the interpretation that T_4 and its derivatives are bound by common binding sites on TBPA, but with varying intensity. The common inhibitor, barbital, would therefore variably affect their binding. Additional evidence bearing upon these possibilities will be discussed in a later communication.

ated with TBPA (16). Indeed, in agar gel, these workers noted very little difference between the proportion of labeled T_4 bound by TBPA at pH 7.4 and pH 8.6. The electrophoretic demonstration of TBPA at pH 7.4 in agar gels has been confirmed in the present studies. In addition, data has been obtained that may explain the discrepant findings in the two electrophoretic systems. It seemed possible that the binding activity of TBPA at pH 7.4 is sufficiently reduced so that during its relatively lengthy electrophoretic migration, TBPA gives up its bound T_4 as a result of a binding competition with filter paper. More slowly migrating proteins, following in the track of TBPA, and migrating less far, would be able to compete for T_4 with the filter paper, either readsorbing the hormone or appearing to do so because of the radioactive trail left by TBPA. The present experiments in which labeled T_4 was applied to the filter paper at varying distances anodal to the points of application of serum support this hypothetical sequence. Under these conditions, binding of T_4 by TBPA at pH 7.4 was readily seen, and the fraction of hormone bound by TBPA did decrease as the distance traversed by the TBPA-T_4 complex lengthened. Furthermore, much of the T_4 remained affixed to the filter paper at its point of application, even when traversed by albumin and TBG. This suggests that T_4 is strongly bound by filter paper at this pH. Finally, in those samples in which both TBPA and albumin had migrated across the point of application of T_4, TBPA bound at least 3 to 10 times the amount of T_4 bound to albumin. Presumably, the T_4-binding affinity of the agar gel is less intense than that of filter paper, and this permits TBPA to be demonstrable at pH 7.4 in the former supporting medium.

The physiological role of TBPA. The direct demonstration that TBPA in serum binds a significant proportion of T_4 at physiological pH provides a prerequisite for the suggestion that TBPA plays a role in the transport of the hormone under physiological conditions. Other data support this view. In the case of TBG, general acceptance of its physiological importance is based mainly on a large body of inferential evidence. Thus, in a number of abnormal states, it has been possible to correlate changes in the concentration and rate of turnover of T_4 *in vivo* with changes in the T_4-binding avidity of whole serum *in vitro*. These alterations, in turn, can be correlated with changes in the T_4-binding activity of TBG, as assessed electrophoretically at pH 8.6.[9] Specific instances in which these correlations occur have recently been reviewed (2, 5).

In a comparable manner, considerable data support the inference that TBPA contributes significantly to hormonal transport in nonelectrophoretic systems for the assessment of binding, as well as *in vivo*. Thus, the decreased T_4-binding by serum proteins at pH 7.4 that is induced by trypan blue (12) appears to correlate with the present electrophoretic evidence that trypan blue specifically inhibits binding of T_4 by TBPA at pH 8.6.

In vivo, salicylates promptly increase the fractional peripheral turnover of T_4 (10). *In vitro*, they speed the dialysis of labeled hormone from serum buffered at pH 7.4 (11). Although electrophoretic studies in barbital buffer revealed no effect of salicylates on the binding of T_4 by TBG (10), the presently described studies and those of other workers (31) indicate that salicylates inhibit T_4-binding by TBPA. This effect is most readily demonstrable when the buffer medium, rather than the serum, is enriched with the inhibitory agent. Despite such evidence that salicylates inhibit T_4-binding by TBPA at pH 8.6, it seemed possible that at pH 7.4 salicylates might inhibit binding of T_4 by other proteins, perhaps TBG. The present dialysis experiments, however, suggest that it is TBPA, not TBG, whose T_4-binding is inhibited at physiological pH. The greater effect of salicylates on the dialysis of L- than of D-T_4 from normal serum,[10] the enhanced dialysis of L-T_4 induced by salicylates in a serum virtually devoid of TBG, and the failure of salicylates to increased further the dialysis of L-T_4 from serum already enriched with barbital are all consonant with this conclusion.

Other data also indicate that changes in the

[9] Although it seems likely that changes in the binding activity of TBG under these conditions would also be demonstrable at pH 7.4, studies directed to this point have not been reported.

[10] The slight increase in dialysis of D-T_4 induced by salicylates in the experiments shown in Figure 11 can probably be ascribed to displacement of D-T_4 from TBG by the L-T_4 released by salicylate from TBPA.

binding of T_4 by TBPA, as measured at pH 8.6, are associated with alterations in the total binding of T_4 in serum at physiological pH. The decrease in electrophoretically demonstrable binding of T_4 by TBPA that occurs in the serum of some patients with acute or chronic illness is closely correlated with an increase in the *in vitro* uptake from such sera of labeled T_4 and T_3 by red blood cells (38). Dowling, Hutchinson, Hindle, and Kleeman have, furthermore, reported that a decrease in T_4-binding by TBPA in the serum of patients with the complications of pregnancy is associated with a greater rate of dialysis of labeled T_4 from such sera than from the serum of normally pregnant women (40). Finally, since dinitrophenol accelerates the *in vivo* turnover (41) and *in vitro* dialysis of T_4 (11), recent experiments indicating that this agent specifically inhibits the binding activity of TBPA at pH 8.6 (31) and at pH 7.4 (42) provide yet another indication that this protein participates in the transport of T_4 *in vivo*.

Altogether, the observations described or cited herein may be considered to indicate that TBG and TBPA are the major mediators of T_4 transport in normal serum; albumin would appear to contribute little. The relative importance of TBG and TBPA in the regulation of T_4 metabolism, however, cannot be assessed with certainty. Most observations indicate that TBG is responsible for binding considerably more T_4 than is TBPA. Thus, in the static sense, TBG would be the major protein determinant of the proportion and concentration of free or unbound T_4 in plasma. Accordingly it would follow, as is indeed the case, that changes in the binding activity of TBG would be associated with alterations in the fractional turnover of T_4 *in vivo*, and secondarily, with changes in the concentration of hormone in the plasma. In this sense, TBG would serve as a reservoir of thyroid hormone within the circulation, and the hormone bound to it would be metabolically inert.

The suggestion that TBPA binds only a small fraction of the circulating hormone would be consistent with the finding that decreased hormonal binding by this protein, such as that which occurs in some acutely ill patients (38), is not always associated with appreciable reduction in the concentration of hormone in the blood.[11] Nevertheless, it does not necessarily follow that TBPA exerts little influence on the metabolism of T_4. Among other factors, the relative ease with which TBG and TBPA penetrate to the extracellular fluid and hence to the cell membrane would ultimately determine which protein more profoundly influences hormonal turnover. Furthermore, the weaker binding affinity of TBPA than of TBG may give TBPA a metabolic significance beyond that expected from the proportion of T_4 in the blood that it may actually bind. For example, the lability of T_4-binding by TBPA to decreases in pH may afford a mechanism for the transcapillary passage of T_4 or for its delivery to areas of increased metabolic expenditure, such as exercising muscle. This concept, which suggests that TBG is the "savings account" and TBPA the "checking account" of thyroxine economy, although speculative at present, would serve both to reconcile the majority of available data and to provide a teleological rationale for the existence in plasma of two major T_4-binding proteins.

SUMMARY

Electrophoretic and dialysis techniques have been employed to determine the binding properties of the thyroxine-binding prealbumin (TBPA) of human plasma and purified preparations thereof. In normal serum, the thyroxine-binding activity of TBPA and purified prealbumin was demonstrable in a variety of alkaline inorganic and organic buffers, and was neither dependent on the presence nor significantly influenced by the concentration of the buffer, tris(hydroxymethyl)-aminomethane (Tris).

Barbital inhibited the binding of thyroxine by both TBPA and purified prealbumin. In 0.05 M barbital, the concentration usually employed in electrophoretic buffer media, thyroxine-binding by TBPA was completely abolished, but purified prealbumin retained a binding avidity far greater

[11] Although salicylates and dinitrophenol inhibit binding of T_4 by TBPA, their ability to accelerate the peripheral turnover of T_4 (10, 41) and to lower protein-bound iodine (10, 41, 43) need not result from the alteration in binding, but rather may arise from the hypermetabolism which they induce. Furthermore, both agents are capable of slowing thyroidal release of hormone (44, 45), and this, too, would tend to lower protein-bound iodine.

than that of serum albumin. Barbital inhibited the binding of those derivatives of thyroxine that are also bound by TBPA; the extent of inhibition varied inversely with the intensity of the TBPA-derivative interaction.

Electrophoretic observations at pH 8.6 indicated that trypan blue and salicylate also inhibit thyroxine-binding by TBPA, whereas, in the case of salicylates, dialysis experiments indicated that such inhibition also occurs at pH 7.4.

The inhibition of thyroxine-binding by TBPA induced by barbital, trypan blue, and salicylate was accompanied by an unexplained increase in thyroxine-binding by proteins migrating in the α-globulin zone.

Striking differences between the affinities of the thyroxine-binding globulin (TBG) and of TBPA for several derivatives of thyroxine have been demonstrated. D-Thyroxine was bound by TBG at least as avidly as L-thyroxine, but was bound little if at all by TBPA. 3,5,3'-L-Triiodothyronine was bound by TBG, but not by TBPA. Deamination of thyroxine and 3,5,3'-L-triiodothyronine yielded compounds whose affinity for TBG was virtually lost, but whose affinity for TBPA was increased. The affinity of TBPA for these derivatives was greater for the tetraiodinated than the triiodinated, and for the acetic acid rather than the propionic acid, analogues.

Previous demonstrations of thyroxine-binding by TBPA during electrophoresis of serum in agar gels at pH 7.4 have been confirmed. Evidence has been presented which suggests that thyroxine-binding by TBPA is not demonstrable during paper electrophoresis at pH 7.4 because of a decreased avidity of TBPA for thyroxine and an increase in the binding of thyroxine by filter paper at this pH.

From these and previous observations, it is concluded that TBPA is a protein native to human plasma and distinct from TBG. Its affinity for thyroxine is not an artifact induced by specific buffers, including Tris. TBPA transports a significant proportion of the thyroxine in plasma at physiological pH, and it may therefore play a special role in the peripheral metabolism of the hormone.

ACKNOWLEDGMENTS

The author is indebted for valuable technical assistance during portions of this work to Miss Ellen Cavenaugh, Mrs. Nancy Cramer, and Misses Lillian Eoyang, Patricia Eveleth, Rebecca Feretos, and Cevina Targum.

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