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A method for obtaining highly purified thyroxine-binding globulin (TBG) from whole human serum is presented. The method employs relatively simple procedures of step-wise ammonium sulfate precipitation followed by column chromatography on DEAE cellulose and DEAE Sephadex. The final product produces a single protein band on disc electrophoresis. The sedimentation constant of the TBG thus purified is 3.91 and its calculated mol wt is 54,000. An antiserum to the highly purified TBG produced a single arc on immunoelectrophoresis. When the antiserum was reacted against normal human serum or against serum from subjects deficient in TBG, each produced two arcs—one identical with that produced by the antigen alone. The second arc is probably the result of a contaminating protein in the antigen, present in too low a concentration to be detectable by disc gel electrophoresis. It is concluded that some persons with TBG "deficiency" have a circulating protein, immunologically indistinguishable from TBG, which is defective in its ability to bind thyroxine.

Studies on Human Thyroxine-Binding Globulin (TBG)

I. PURIFICATION OF TBG AND IMMUNOLOGIC STUDIES ON THE RELATIONSHIP BETWEEN TBG FROM NORMAL PERSONS AND THOSE WITH TBG "DEFICIENCY"

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ABSTRACT A method for obtaining highly purified thyroxine-binding globulin (TBG) from whole human serum is presented. The method employs relatively simple procedures of step-wise ammonium sulfate precipitation followed by column chromatography on DEAE cellulose and DEAE Sephadex. The final product produces a single protein band on disc electrophoresis. The sedimentation constant of the TBG thus purified is 3.91 and its calculated mol wt is 54,000. An antiserum to the highly purified TBG produced a single arc on immunoelectrophoresis. When the antiserum was reacted against normal human serum or against serum from subjects deficient in TBG, each produced two arcs—one identical with that produced by the antigen alone. The second arc is probably the result of a contaminating protein in the antigen, present in too low a concentration to be detectable by disc gel electrophoresis. It is concluded that some persons with TBG "deficiency" have a circulating protein, immunologically indistinguishable from TBG, which is defective in its ability to bind thyroxine.

INTRODUCTION

It has been estimated (1–4) that 99.95% of circulating thyroxine in normal human serum is bound to the three carrier proteins, thyroxine-binding globulin (TBG), albumin, and thyroxine-binding prealbumin (TBPA). Of these, TBG is present in trace amounts, estimated to be 1–2 mg/100 ml (5), but nevertheless it binds about 40% of the circulating thyroxine in vitro (6) in normal human serum. More recent studies indicate that TBG may bind a considerably greater percentage of circulating thyroxine in vivo (7). Since TBG thus acts as an important reservoir for circulating thyroid hormone, further characterization of its physical and chemical properties might provide insight into its physiologic function.

TBG has recently been obtained in a highly purified state by Giorgio and Tabachnick (8), who utilized column electrophoresis, ammonium sulfate fractionation, gel filtration, and preparative disc electrophoresis. They reported values for the sedimentation constant, the molecular weight, and the amino acid content of TBG.

The literature contains several reports of families of persons with TBG "deficiency." These have been identified by either the combination of an abnormally low serum protein-bound iodine concentration and an abnormally high resin sponge uptake of radiotriiodothyronine or by electrophoretic demonstration of absence of radioactivity in the α-globulin region after addition of ^{131}I-thyroxine to their serum. Genetic studies of some of these families indicated that the "deficiency" of TBG is transmitted as an X-linked dominant characteristic (9, 10).

The purpose of this paper is to present (*a*) a relatively simple method for separating TBG from human serum in a highly purified state, and (*b*) evidence that in hereditary TBG "deficiency" a protein immunologically indistinguishable from TBG is present which is unable to bind thyroxine.

METHODS

Materials

Dowex 2-×10 was obtained in the prewashed form (AG2-×10) from BioRad Laboratories, Richmond, Calif. It was prepared for column chromatography as previously described (11).

Diethylaminoethyl (DEAE) cellulose, Whatman DE 52, was obtained from H. Reeve Angel, Inc., Clifton, N. J.

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DEAE Sephadex A-50 was obtained from Pharmacia Fine Chemicals, Piscataway, N. J.

Radioactive thyroxine (T_4-^{131}I) was obtained from Abbott Laboratories, North Chicago, Ill. Radioactive thyroxine (T_4-^{125}I) was obtained from Mallinckrodt Nuclear Corp., St. Louis, Mo.

Human blood was obtained by venipuncture and allowed to clot overnight at 4°C. Sera were separated by centrifugation, pooled, and stored at −60°C before use. Serum from TBG-"deficient" persons was obtained from affected members of the family previously reported (9).

Methods

Purification of TBG. All operations were done at 4°C in a cold room, except where noted. Human serum, usually 2000–3000 ml, was adjusted to approximately 40% saturation with powdered ammonium sulfate, sedimented at 36,000 *g* for 1 hr, and the supernatant solution dialyzed exhaustively against distilled water according to conditions previously described (12). This 40% supernatant solution was percolated at room temperature through a column of Dowex 2-×10 anion exchange resin, 15 × 110 cm, equilibrated with 0.06 M Tris-chloride[1] buffer, pH 7.3. The main band of unadsorbed protein emerging at the void volume of the column contained TBG as determined by cellulose acetate electrophoresis by a method previously reported from this laboratory (13), and was saved for further processing. The protein content of this fraction was adjusted to 10–12 OD units/ml at 280 mμ by dilution with 0.06 M Tris-chloride buffer, pH 7.3. Powdered ammonium sulfate was added with constant stirring to 60% saturation (390 g/liter) and the solution allowed to stand for 48 hr. At this time the undissolved protein formed a floating zone which was difficult to centrifuge; accordingly, it was filtered through fluted filter paper (No. 12 Whatman filter paper). The clear yellow filtrate was brought to 65% saturation by addition of 34 g ammonium sulfate/liter and the resulting suspension was allowed to settle over a period of 48–72 hr. Most of the clear supernatant was carefully decanted, and the remaining concentrated suspension of 65% precipitate was sedimented for 1 hr at 36,000 *g*. The sedimented precipitate was redissolved in a minimal volume of cold distilled water and dialyzed against 10–20 volumes of 0.06 M Tris-chloride buffer, pH 7.3, with two changes of buffer 24 hr apart. This fraction, labeled "dialyzed 65% ppt," was the starting material for chromatography on DEAE cellulose. T_4-^{125}I was added in tracer amounts to this fraction to facilitate location of TBG in subsequent purification steps.

DEAE cellulose column chromatography

DEAE cellulose was prepared for equilibration according to the manufacturer's recommendations and equilibrated with 0.06 M Tris-chloride buffer, pH 7.3. A 50% (v/v) slurry of cellulose and buffer was packed into a 2.5-cm diameter glass column at a buffer flow rate of 70 ml $hr^{-1}cm^{-2}$ to a height of 40 cm. The volume of dialyzed 65% precipitate applied to the column ranged between 35 and 50 ml, representing material derived from 400–500 ml of serum. Elution was carried out at a constant flow rate of 150 ml/hr, first with equilibration buffer until all the unadsorbed protein was washed from the column and finally with a linear concentration gradient of sodium chloride. The gradient limit was 2 liters of 0.15 M NaCl dissolved in equilibration buffer; the mixing vessel contained 2 liters of equilibration buffer stirred magnetically. Fractions of 10–20 ml were collected volumetrically in plastic counting tubes. The radioactivity of all tube contents was determined in a well-type scintillation counter. Contents of tubes comprising the peak of radioactivity were pooled, concentrated by pressure dialysis to about 5 ml, and dialyzed for 24–48 hr against 1 liter of 0.06 M Tris-chloride buffer, pH, 8.6, with two changes of buffer.

[1] Trishydroxymethylaminomethane.

DEAE Sephadex column chromatography

DEAE Sephadex A50 was prepared according to the manufacturer's instructions and was equilibrated with 0.06 M Tris-chloride buffer, pH 8.6. A stirred 20% (v/v) slurry of DEAE Sephadex in equilibrating buffer was packed by gravity flow into a 0.9-cm diameter column to a height of 25 cm. The dialyzed sample from the DEAE cellulose column was applied and elution carried out with a linear concentration gradient of sodium chloride (limit = 0.2 M), as described before. The volume in each gradient reservoir was 500 ml. Elution was carried out under gravity flow at a rate of 10–20 ml/hr. Fractions of 5-ml were collected in glass counting tubes. All tubes were counted for radioactivity and the OD at 280 mμ was determined for each fraction. Those fractions comprising the ascending side of the radioactive peak were pooled and concentrated at a final volume of 1–2 ml by pressure dialysis.

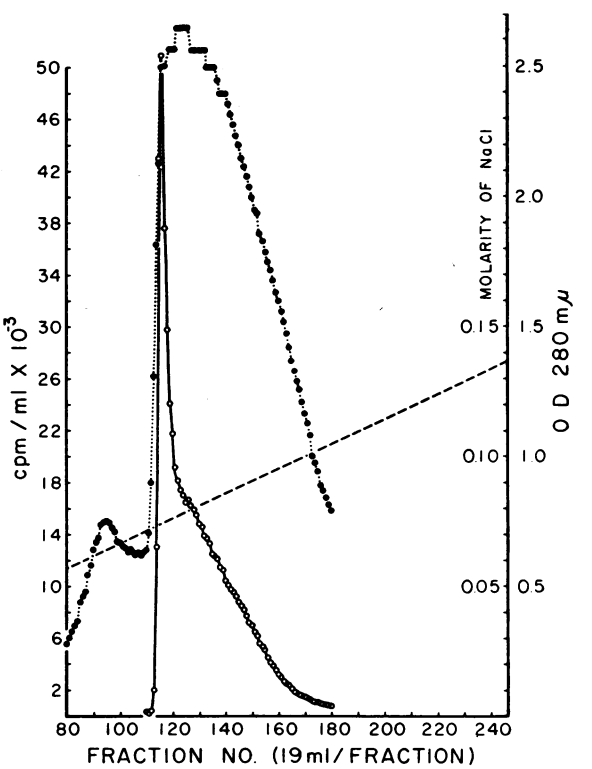

FIGURE 1 Portion of chromatogram showing gradient elution of TBG from DEAE cellulose. Column dimensions, 2.5 × 40 cm; sample volume, 35 ml. Thyroxine-^{125}I (T_4-^{125}I) radioactivity, O——O; optical density at 280 mμ, ● · · · ●; molarity of sodium chloride, – – –. The gradient was started at tube 40. Other conditions as in text.

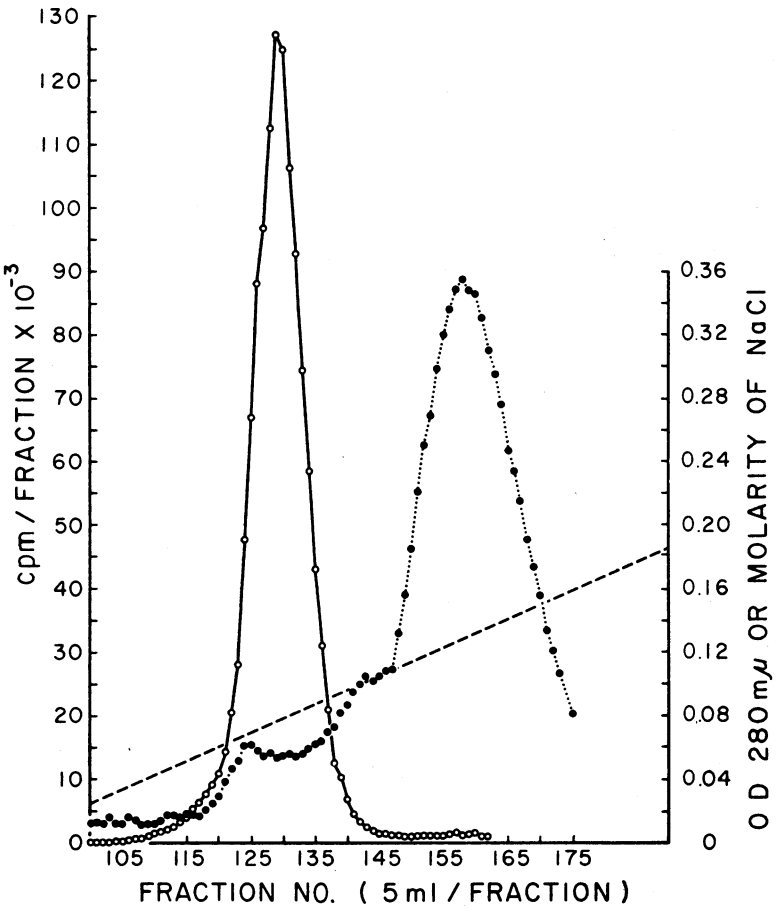

FIGURE 2 Portion of chromatogram showing gradient elution of TBG from DEAE Sephadex. Column dimensions, 0.9 × 25 cm; sample volume 4.0 ml. T_4-^{125}I radioactivity, O——O; optical density at 280 mμ, ● · · · ●; molarity of sodium chloride, – – –. The gradient was started at tube 17.

Cellulose acetate electrophoresis

Cellulose acetate electrophoreis was initially carried out in a Buchler electrophoresis cell by a method previously described (13). Later electrophoreses were carried out in a Beckman Microzone cell. The buffer used in the Beckman cell was 0.25 M Tris acetate, pH 8.9 (13). After electrophoresis, the cellulose acetate membrane was dried in an oven for 20 min at 90°C. Identification of the presence of TBG in each of the purification steps was done by radioautography, which was carried out as previously described (13).

Disc gel electrophoresis

Analytical disc gel electrophoresis was carried out by the method of Davis (14), using Tris glycine buffer, pH 8.2–8.4.

Immunoelectrophoresis

Immunoelectrophoresis was carried out by the method of Scheidegger (15), using electrode and gel buffers of veronal–calcium lactate as described by Hirschfeld (16). Radioautography of precipitin lines was accomplished by adding T_4-^{131}I to the well samples. Residual protein and radioactivity were removed by washing with 1% NaCl. After drying, slides were exposed to X-ray film for a period of approximately 2 wk.

Antibody preparation

New Zealand albino rabbits weighing about 2.5 kg were immunized with highly purified TBG eluted from the DEAE Sephadex column. The initial injection was made intravenously in Freund's complete adjuvant followed by a second and third subcutaneous injection of antigen alone at 4-day intervals. A fourth dose of antigen was injected subcutaneously 10 days after the third injection. 5 days after the last injection, bleeding was carried out. All injections contained about 0.5 mg of antigen protein.

Ultracentrifugal analysis

Sedimentation constants in sucrose density gradients were determined by ultracentrifugation, as described by Martin and Ames (17). After centrifugation the contents of the tube were fractionated into 40 samples of 10 drops each in counting tubes. The radioactivity of each sample was counted

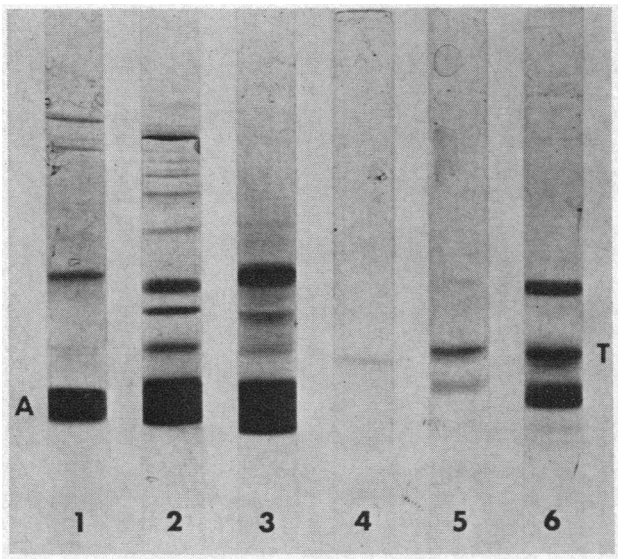

FIGURE 3 Analytical disc gel electrophoresis of successive steps in TBG purification. *1*, unconcentrated fraction unadsorbed by Dowex 2-×10 column; *2*, dialyzed 65% ammonium sulfate precipitate; *3*, radioactivity peak from DEAE cellulose column, shown in Fig. 1; *4*, tubes 120–125 DEAE Sephadex, Fig. *2*; *5*, tubes 127–132, DEAE Sephadex, Fig. *2*; *6*, tubes 133–137, DEAE Sephadex Fig. 2. Samples 2–6 were concentrated about 20-fold. Direction of migration was toward the bottom. *A*= albumin; *T* = TBG.

and then the sample was diluted with 3 ml distilled water and the OD at 210 mμ determined (18).

Protein content

Protein content of serum fractions was determined by measuring absorption in 1-cm cells at 280 mμ in a Zeiss PMQII spectrophotometer or by the method of Lowry, Rosebrough, Farr, and Randall (19). In some cases the absorption at 210 mμ of protein solutions was determined (18).

RESULTS

Purification of TBG. Chromatography of dialyzed 65% precipitate on DEAE cellulose, as depicted in Fig. 1, typically resulted in a biphasic peak of bound T_4-^{125}I in which the first, sharper portion emerging contained TBG and the second was attributable to albumin. The contents of fractions in the first $\frac{1}{2}$–$\frac{2}{3}$ of the TBG peak were pooled and concentrated for further chromatography on DEAE Sephadex. A typical chromatogram of material recovered from a DEAE cellulose column and applied to a DEAE Sephadex column is shown in Fig. 2. It was possible to remove all contaminating albumin and all but a trace of other contaminating proteins by this procedure. The purest TBG was invariably contained in the earliest (first one-third) of the radioactive peak emerging from the DEAE Sephadex column. Fig. 3 shows disc gel electrophoretograms of proteins from several steps in the purification and the purest TBG obtained from DEAE Sephadex chromatography. The final material appeared homogeneous by disc gel electrophoresis. The electrophoretic mobility of purified TBG and its ability to bind T_4-^{131}I were confirmed by radioautography of samples labeled with T_4-^{131}I after electrophoresis on cellulose acetate. The absence of T_4-^{131}I–binding in persons with TBG "deficiency" was also confirmed. A photograph of these radioautographs is shown in Fig. 4.

It was not possible to calculate exactly the yield or degree of purification of TBG resulting from the procedures employed because of contamination of all but the final fraction by albumin-bound radioiodinated thyroxine and losses associated with selection of fractions with the highest purity. However, on the basis of protein recovered in the final fraction, assuming the TBG content to be 2 mg/100 ml of serum (5), the final yield of TBG was approximately 10–20%.

Physical characterization of TBG. The small amount of pure TBG available made studies on its sedimentation behavior in the analytical ultracentrifuge unfeasible; however, it was possible to obtain a value for its sedimentation constant by ultracentrifugation of radiothyroxine-labeled TBG in a sucrose density gradient by the method of Martin and Ames (17). The results of such a centrifugation are shown in Fig. 5. The calculated $s_{20,w}$ of TBG in this experiment was 3.91, using purified human serum albumin as a marker protein and taking the value for its sedimentation constant as 4.6 (20). The value obtained corresponds very closely to that found by Giorgio and Tabachnick (3.92) for their purified TBG in the analytical ultracentrifuge (8). The mol wt estimated from the sedimentation constant (21) was

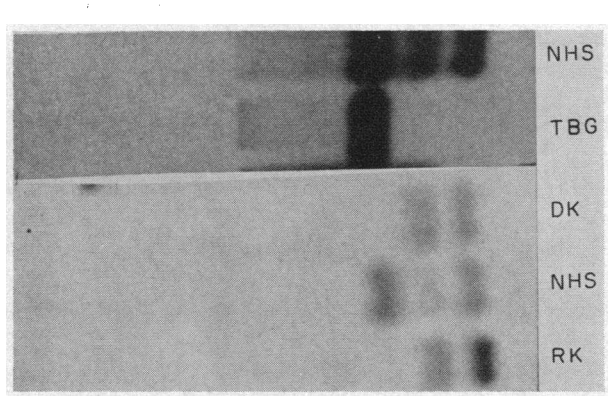

FIGURE 4 Radioautographs of cellulose acetate electrophoretograms. All samples enriched with T_4-^{131}I. Direction of migration was to the right. NHS = normal human serum, TBG = highly purified thyroxine-binding globulin, RK and DK = two affected brothers from a family with TBG "deficiency." The top two samples and bottom three samples were run separately under similar conditions.

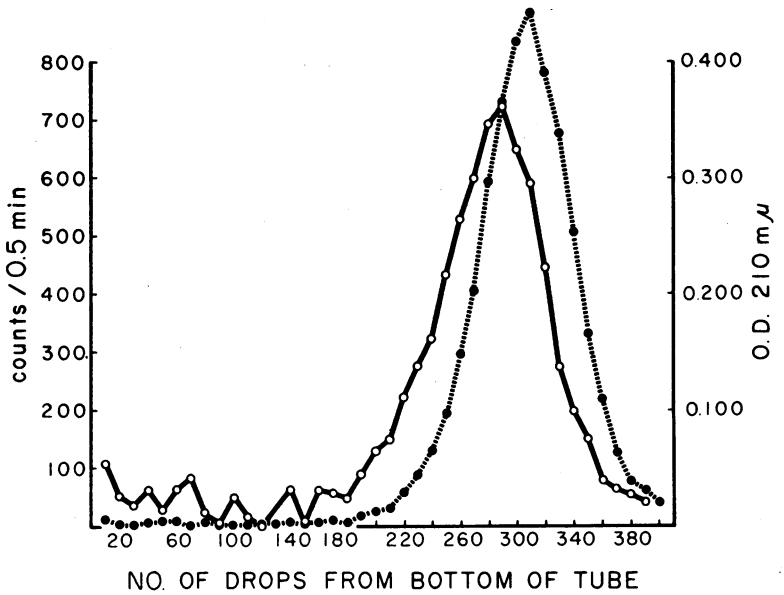

FIGURE 5 Density gradient ultracentrifugation of TBG. The tube contained 30 μg of T_4-^{125}I TBG and 500 μg of purified human serum albumin in a volume of 0.15 ml. Centrifugation for 17 hr at 35,000 rpm in a 5–20% linear sucrose gradient at 2°C. Radioactivity per 10-drop sample, ●---●; optical density at 210 mμ of same sample after dilution with water to 3 ml, ○——○. The calculated $s_{20,w}$ was 3.91.

54,000, in fair agreement with the figure of 58,000 obtained by Giorgio and Tabachnick.

Immunological characterization. Immunodiffusion experiments, using the most highly purified TBG as antigen and rabbit antiserum to TBG (see Methods), showed that the rabbit antiserum formed two precipitin lines with normal human serum and a single line with the TBG antigen. Furthermore, under these conditions,

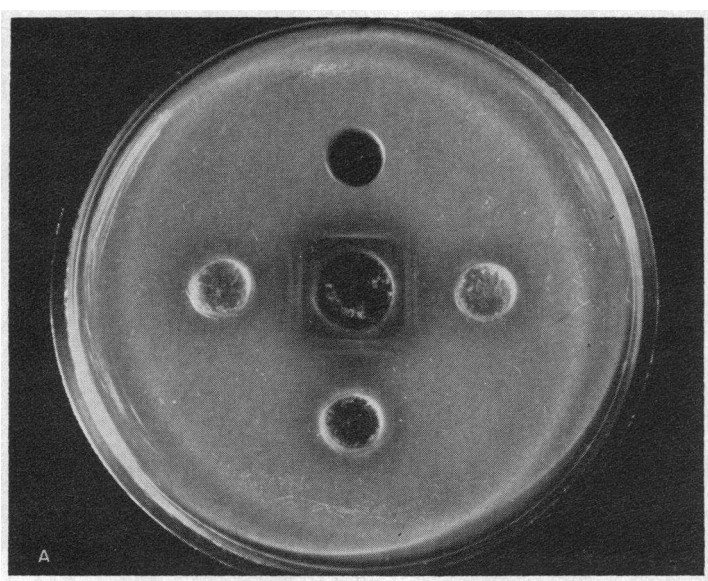

FIGURE 6 A Double diffusion in agarose. Top well contained purified TBG; each side well contained serum from a person "deficient" in TBG; the bottom well contained normal human serum, and the center well was filled with rabbit antiserum to TBG.

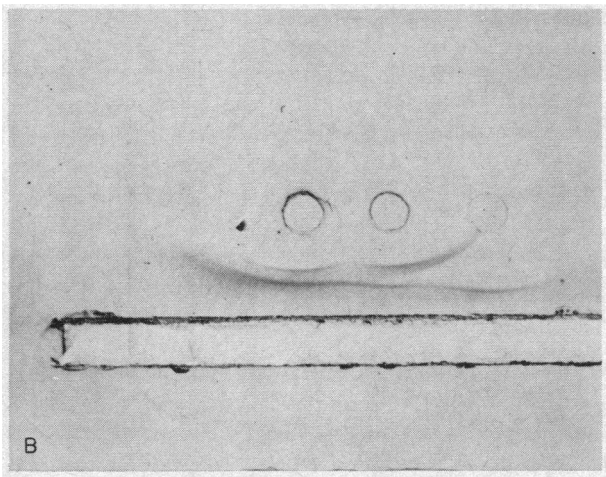

FIGURE 6 B Double diffusion in agarose. Left well contained TBG "deficient" serum, center well contained normal human serum, and the right well contained purified TBG. Rabbit antiserum to TBG in bottom trench. Diffusion for 24 hr at room temperature.

serum from individuals, shown to have no thyroxine-binding protein in the TBG position by radioautography, produced two precipitin lines that formed a pattern of complete antigenic identity with those of normal serum. A photograph of a double diffusion experiment showing these results is given in Fig. 6 A and B.

Similar findings were made when purified TBG, normal human serum, and TBG-"deficient" serum were compared in immunoelectrophoresis, using rabbit anti-TBG in the antiserum trench. A photograph of immunoelectrophoresis slides showing these results is given in Fig. 7 A. It is clear from the foregoing experiments that rabbit antiserum to highly purified TBG contains antibody which reacts with TBG and one other protein in serum and that the same proteins in normal and TBG-"deficient" serum react, i.e., TBG in normal and "deficient" serum are immunologically indistinguishable.

Radioautography of immunoelectrophoresis experiments described above was carried out by adding T_4-^{131}I to the contents of the wells (Fig. 7 B). A single arc of radioactivity was seen when both normal human serum and the highly purified TBG antigen were reacted against rabbit antiserum to TBG. This radioactive arc corresponded to the arc in the stained preparations. When immunoelectrophoretic radioautography was carried out with TBG-"deficient" serum reacted against the antiserum, no arc of radioactivity appeared.

The physical properties and the immunologic characteristics of the protein we have isolated indicate its close correspondence to the most highly purified TBG isolated by Giorgio and Tabachnick (8). In this regard we have confirmed their finding that, in a mixture of TBG and albumin, TBG emerges before albumin from a Sephadex G100 column, leading to an erroneously high value for its molecular weight. The method we have presented offers the advantage of relative simplicity in the purification of TBG directly from human serum.

DISCUSSION

Our interest in the prevalence of TBG "deficiency" in large populations has required a convenient assay for TBG. The availability of highly purified TBG and a specific antibody to it should facilitate the development of an immunoassay for this protein.

Antiserum to highly purified TBG when reacted against the antigen produces a single precipitin line, but when reacted against normal human serum or serum from TBG-"deficient" persons produces a second line. The presence of this second precipitin line indicates that

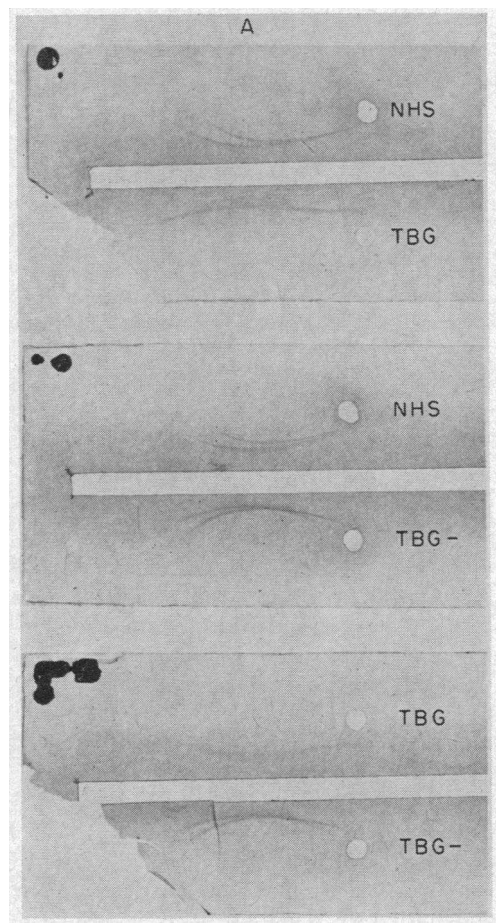

FIGURE 7 A Immunoelectrophoresis of highly purified thyroxine-binding globulin (TBG), normal human serum (NHS), and TBG-"deficient" serum (TBG^-) against rabbit antiserum to TBG. The well contents are as labeled; troughs contained rabbit antiserum to TBG in all cases. Anode is to the left.

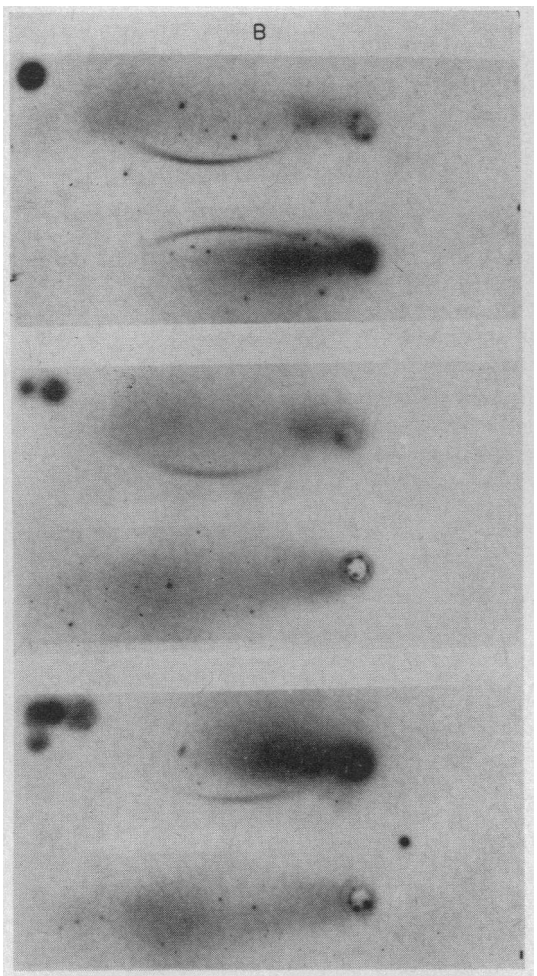

FIGURE 7 B Radioautographs of slides in Fig. 7 A. T_4-^{131}I added to well samples which are the same as in Fig. 7 A.

the highly purified TBG used for immunization probably contains a contaminating protein in such small concentration as to be undetectable by analytic disc gel electrophoresis at the concentration employed. Since the second precipitin line is not associated with radioactivity when T_4-^{131}I is present, it does not possess thyroxine-binding ability (Fig. 7 B).

Binding capacities for the highly purified TBG for thyroxine have not been determined in view of the probability that our purest product is not homogenous. At the present stage of purification of TBG, maximum binding of thyroxine could be influenced either by nonhomogeneity of the protein or by denaturation. The relative importance of these factors could not be assessed at this point. Giorgio and Tabachnick (8) have encountered this dilemma, since their highly purified TBG demonstrates only 25% of the expected binding capacity for thyroxine, assuming one binding site/molecule of protein. While they have stressed denaturation of their protein as the probable cause for this finding, their data do not exclude nonhomogeneity of their final product as an alternative cause.

The highly purified antigen, normal human serum, and TBG-"deficient" serum all react to TBG antiserum with a line of identity indicating that persons with TBG "deficiency" have in their serum a protein immunologically indistinguishable from TBG in normal serum. The abnormality in TBG "deficiency" appears to reside in this inability of the defective protein to bind thyroxine. Preliminary immunodiffusion experiments, using serially diluted normal human serum and TBG-"deficient" serum, indicate that the amount of immunoreactive TBG in the two serums is approximately the same. Although these findings are true for the two affected members of the family previously reported (9) and presumably true for the other affected members of this family, the generalization that this occurs in all persons with TBG "deficiency" should not be made.

Thus, TBG from individuals with TBG "deficiency" is a serum protein bearing a genetically controlled biochemical defect that does not affect its antigenicity. These data support the view that the antigenic and thyroxine-binding sites on the TBG molecule are different. Another example of such a protein is the nonfunctional variant of C'1 esterase inhibitor (22) found in some cases of hereditary angioneurotic edema.

The availability of highly purified TBG will permit studies on the characteristics of the binding site for thyroxine. Such studies are now in progress.

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