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Preparation of Crystalline Thyroxine-binding Prealbumin from Human Plasma*

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ABSTRACT: A procedure is described for the large-scale preparation of highly purified thyroxine-binding prealbumin from human plasma. The protein is obtained in 10% yield and 150-fold purification from the starting material, Cohn fraction IV-6. The procedure employs the sequential application of ammonium sulfate fractionation, batch adsorption to DEAE-cellulose, DEAE-column chromatography, and preparative electrophoresis in Pevikon C-870. The protein can then be crystallized from ammonium sulfate solution.

In human plasma, L-thyroxine (T_4)[1] is transported in association with several proteins. The majority of data (cf. reviews by Robbins and Rall, 1960; Ingbar, 1960; Tata, 1964) suggests that two plasma proteins play a predominant role in T_4 binding. These are an α-globulin, termed the T_4-binding globulin (TBG), and a prealbumin, the T_4-binding prealbumin (TBPA), which has an electrophoretic mobility 30% greater than albumin when subjected to Tiselius electrophoresis at *p*H 8.6. Albumin and perhaps other proteins appear to serve as secondary carriers of T_4 in plasma.

The electrophoretic migration of TBPA in starch gel at *p*H 8.6 is the same as that of the most rapidly migrating protein of normal plasma (band I of Poulik and Smithies, 1958), and the two proteins are thought to be identical (Blumberg *et al.*, 1961; Squef *et al.*, 1963; Oppenheimer *et al.*, 1965). A more slowly migrating prealbumin in starch gel electrophoresis has been identified as an acidic α_1-glycoprotein (Poulik and Smithies, 1958) and apparently does not bind thyroxine.

A large-scale preparation of purified TBPA has not

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[1] Abbreviations used in this work: T_4, L-thyroxine; TBG, T_4-binding globulin; TBPA, T_4-binding prealbumin.

been reported. Schultze *et al.* (1956) isolated a prealbumin from serum by means of alumina gel adsorption, ammonium sulfate precipitation, and zone electrophoresis. This material was shown by Tata (1961) to bind T_4, but was found to be immunologically heterogeneous and to contain TBG. Tata (1961) and Bocci (1964), taking advantage of the electrophoretic properties of this protein, have employed preparative electrophoresis of serum to isolate a highly purified TBA. Oppenheimer *et al.* (1965) further refined this approach by using DEAE-cellulose to concentrate the initial eluates from the Porath column electrophoresis of 150-ml volumes of plasma or serum, and performing a second preparative electrophoresis in starch gel. Got and Bourrillon (1963) used phenol or ammonium sulfate precipitation of serum followed by zone electrophoresis to obtain a purified prealbumin, but, as in the preparation of Bocci, the ability of the preparation to bind T_4 was not assessed. The quantity of TBPA obtainable by these methods is small,[2] since this protein represents only about 0.3% of the total serum protein (Cheng and Steinberg, 1964).

As a prerequisite to studies of the chemical and physiological properties of this protein, a large-scale preparation of purified TBPA was necessary. For this purpose advantage was taken of the observation that fractions IV-5 and IV-6 prepared by ethanol fractionation of human plasma (Surgenor *et al.*, 1949) are rich in TBPA (Ingbar, 1963). Fraction IV-6 contains much less denatured protein after lyophilization than does fraction IV-5, and was selected as the starting material for the present work. By means of sequential application of ammonium sulfate fractionation, batch adsorption to DEAE-cellulose at *p*H 4.8, DEAE-column chromatography, and preparative electrophoresis in Pevikon C-870, substantial quantities of a highly purified TBPA have been obtained and crystallization of the protein has been achieved.

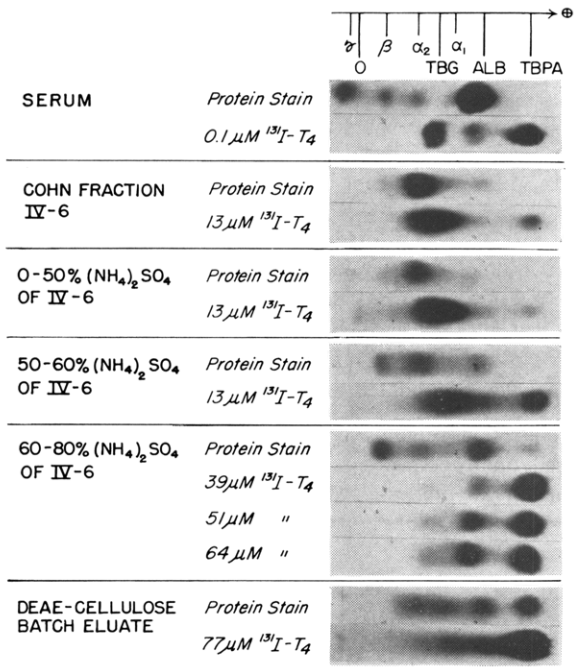

FIGURE 1: Electrophoretic patterns of protein fractions obtained during the purification of thyroxine-binding prealbumin. The paper electrophoresis was performed on each fraction containing the indicated concentrations of ^{131}I-labeled L-thyroxine (^{131}I-T_4). Radioautographs obtained after electrophoresis are shown below the protein stains of the respective fractions.

Materials

Cohn fraction IV-4 (Cohn *et al.*, 1946) was provided by the American Red Cross. Forty kg of paste in the frozen form, representing the material from about 2000 liters of plasma, was further fractionated (Surgenor *et al.*, 1949) by the Biologic Laboratories, Massachusetts Department of Public Health, to yield 2.14 kg of fraction IV-6 as the dry powder. The procedure of Gel and Coombs (1963) was used to prepare antisera for fraction IV-6 in rabbits. Human serum mercaptalbumin, crystallized four times as the mercury dimer, was prepared by the procedure of Hughes and Dintzis (1964).

DEAE-cellulose (Cellex-D, Bio-Rad Laboratories, Richmond, Calif.) with a capacity of 0.95 meq/g was screened to obtain 40–100 and 100–200 mesh sizes. The screened materials were separately cleaned by the procedure of Peterson and Sober (1956). The 100–200 mesh material was further washed with 0.01 M sodium acetate buffer (*p*H 4.80), and used in the batch adsorption process. The 40–100 mesh material, washed with 0.01 M potassium phosphate buffer (*p*H 7.4), was used for column chromatography.

The $(NH_4)_2SO_4$, enzyme grade, was obtained from Mann Research Laboratories, New York. L-Thyroxine (Sigma Chemical Co., St. Louis, Mo.) and ^{131}I-labeled thyroxine (Abbott Laboratories, Oak Ridge, Tenn.) were used without further purification. Tris-maleate buffers were prepared using Trizma base and maleic acid obtained from the Sigma Chemical Co.

Methods

The technique of starch-gel electrophoresis described by Smithies (1955) was used, employing a sodium borate buffer (*p*H 8.6) and the addition of a tracer amount of ^{131}I-labeled T_4 to the protein solution before electrophoresis. At the termination of the run the gel was bisected by a cut parallel to the largest face. One-half of the gel was stained for protein using Amido Black 10B. The thyroxine-binding proteins were located in the other half of the gel by the radioautographic technique of Blumberg and Robbins (1960). Immunoelectrophoresis was performed as described by Wunderly (1960), using 0.073 M Tris-maleate buffer (*p*H 8.6).

[2] Although no yields are given in the recent work of Oppenheimer *et al.* (1965), we have calculated a final recovery of about 45 mg of TBPA per liter of serum from their data.

TABLE I: Summary of the Purification of TBPA from Cohn Fraction IV-6.

Material	Protein[a] (g)	Thyroxine-Binding Capacity of TBPA (μmoles/g protein)[b]	Thyroxine-Binding Capacity of TBPA (μmoles/total protein)	Recovery (%)	Purification
Cohn fraction IV-6	1860.0	0.064	119.0	(100)	(1)
60–80% $(NH_4)_2SO_4$ fraction	27.3	1.57	42.9	36	25
DEAE-cellulose batch eluates	11.9	3.35	40.0	34	52
First DEAE-cellulose column eluates	5.49				
Second DEAE-cellulose column eluates	2.47				
Pevikon eluates	1.27	9.56	12.2	10	150

[a] Protein weights are those of the lyophilized salt-free materials except for Cohn fraction IV-6 which is the weight of the lyophilized powder. These weights were used for calculating the binding capacity of the total protein. [b] The binding capacity in μmoles/g protein was obtained using protein determinations by the Biuret method.

Pevikon-block electrophoresis was performed at 4° by a modification of the procedure of Bocci (1964, and references therein). Pevikon C-870 (Mercer Chemical Corporation, New York) as a slurry in 0.073 M Tris-maleate buffer (*p*H 8.6) was used to fill a 1.3 × 18 × 36 cm Lucite tray and the excess buffer allowed to drain off through Whatman 3MM filter paper wicks clamped vertically at the ends of the tray. A 1 × 15 cm trough cut 10 cm from one end of the tray was filled with a slurry composed of washed Pevikon and a 15% solution containing about 500 mg of protein. A dilute solution of brom phenol blue in human serum albumin was used as an electrophoretic marker at one end of the trough. Horizontal electrophoresis was performed with a potential of 210–225 v across the Pevikon block for 20–24 hours. The portion of the block containing protein which had migrated anodally beyond the albumin marker was then removed and packed into 4 × 11 cm nylon cylinders (3.2 cm id) which fitted into centrifuge tubes (No. 2807, International Equipment Co., Boston, Mass.). The cylinders were perforated at the bottom and the Pevikon was supported by a stainless steel screen below a circle of Whatman No. 42 filter paper. The protein solution in the Pevikon was separated by centrifugation at 2000*g* for 20 minutes.

Methods have been described previously (Ingbar, 1961) for paper electrophoresis using 0.073 M Tris-maleate buffer (*p*H 8.6) and for the determination of the maximal T_4-binding capacity of TBPA at various stages of purification. The basis of the latter method is the proportionately increased dissociation of the total ^{131}I-labeled T_4 from TBPA concomitant with the proportionately increased association of the T_4 with albumin and TBG as the TBPA-binding capacity is approached with increasing T_4 concentration (illustrated in Figure 1 for the 60–80% $(NH_4)_2SO_4$ fraction). The electrophoresis was performed on protein solutions adjusted to 2.5% (w/v) as measured by the Biuret method (Gornal *et al.*, 1949). Fraction IV-6 was added to the final TBPA preparation[3] to provide the necessary additional T_4-binding proteins. The additional TBPA introduced from fraction IV-6 represented less than 1% of the total TBPA present.

Results

Ammonium Sulfate Fractionation. The results of a preliminary $(NH_4)_2SO_4$ fractionation of 25 g/liter of fraction IV-6 in 36.5 mM Tris-maleate buffer (*p*H 8.6) are illustrated in Figure 1. The 0–50% and 50–60% fractions contained most of the α-globulins originally present in fraction IV-6. As judged from the distribution of a standard ^{131}I-labeled T_4 concentration among the various proteins and from measurements of binding capacity, the 0–50 and 50–60% fractions also contained most of the TBG. The 60–80% fraction was relatively enriched in albumin, α_2- and β-globulins, and was the only $(NH_4)_2SO_4$ fraction in which TBPA was visible by protein staining. The T_4-binding capacity of TBPA per g of protein in the 60–80% fraction was 25 times greater than fraction IV-6, 52 times greater than the 0–50% fraction, and 7 times greater than the 50–60% fraction. Although the 60–80% fraction contained only about a third of the TBPA from fraction IV-6, it was selected for further purification.[4]

Each 250 g of powdered fraction IV-6 is suspended in 10 liters of 36.5 mM Tris-maleate buffer (*p*H 8.6) at 4°. All subsequent operations are performed at 4°. The turbid solution which results is brought to 60% satura-

[3] From a measurement of the organic iodide content of this material, a maximum of 6.5×10^{-3} μmole of T_4 was present per g of TBPA.

[4] This procedure is also applicable to the preparation of TBPA from fraction IV-5. In this case, 500 g of fraction IV-5 is suspended in 10 liters of 36.5 mM Tris-maleate buffer (*p*H 8.6) and centrifuged at 4000*g* for 1 hour at 4°, and the supernatant was used for fractionation with $(NH_4)_2SO_4$ as described for fraction IV-6.

TABLE II: Summary of the Purification of TBPA using DEAE-Cellulose Batch Adsorption.

Material	Acetate Buffer (pH; M)	Initial Protein[b] (%)	Thyroxine-Binding Capacity of TBPA (μmoles/g protein)	Thyroxine-Binding Capacity of TBPA (μmoles/ total protein)
Starting material[a]		(100)	1.57	42.9
Final supernatant	4.5; 0.01	33.9	0.04	0.33
Combined washes	5.5; 0.01	15.8	0.01	0.02
Eluate 1	5.5; 0.10	5.3	1.04	1.51
2	5.5; 0.60	28.3	3.32	25.6
3	5.5; 0.30	8.6	3.36	7.90
4	5.5; 0.30	3.2	3.54	3.08
5	5.5; 0.60	3.6	3.18	3.12
Recovery		98.7		41.6

[a] The starting material was 27.3 g of the 60–80% $(NH_4)_2SO_4$ fraction. [b] The protein concentration was estimated by absorbance at 280 mμ. Values are the average from three separate batch purifications.

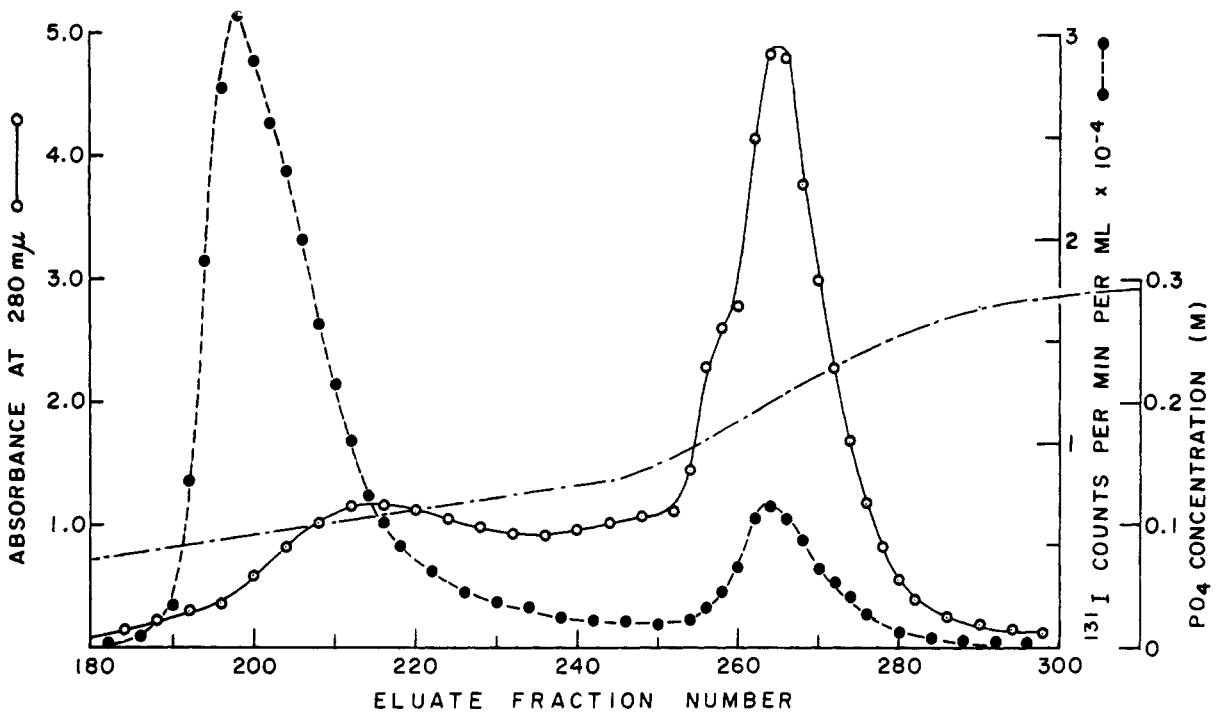

FIGURE 2: Initial column chromatography on DEAE-cellulose using protein obtained from DEAE-cellulose batch elution (Figure 1) to which 2 μcuries of ^{131}I-labeled L-thyroxine was added. See text for details. The protein concentration is shown as the ordinate on the left, and the concentration of radioactivity as the ordinate on the right. The total phosphate concentration of the eluate fractions is indicated by — - — -.

tion with 3.90 kg of $(NH_4)_2SO_4$ and allowed to stand overnight. The precipitate is separated by centrifugation at 1340*g* for 1 hour and is discarded. The supernatant is brought to 80% saturation with 1.43 kg of $(NH_4)_2SO_4$. After 12 to 16 hours the precipitate is obtained by centrifugation at 4000*g* for 1 hour. The precipitate is resuspended in about 50 ml of water, dialyzed against several large volumes of water, and lyophilized. The results of this procedure are summarized in Table I, and represent the fractionation of material from about 1740 liters of original plasma volume.

DEAE-Cellulose Batch Adsorption. TBPA has been found to be adsorbed to DEAE-cellulose, together

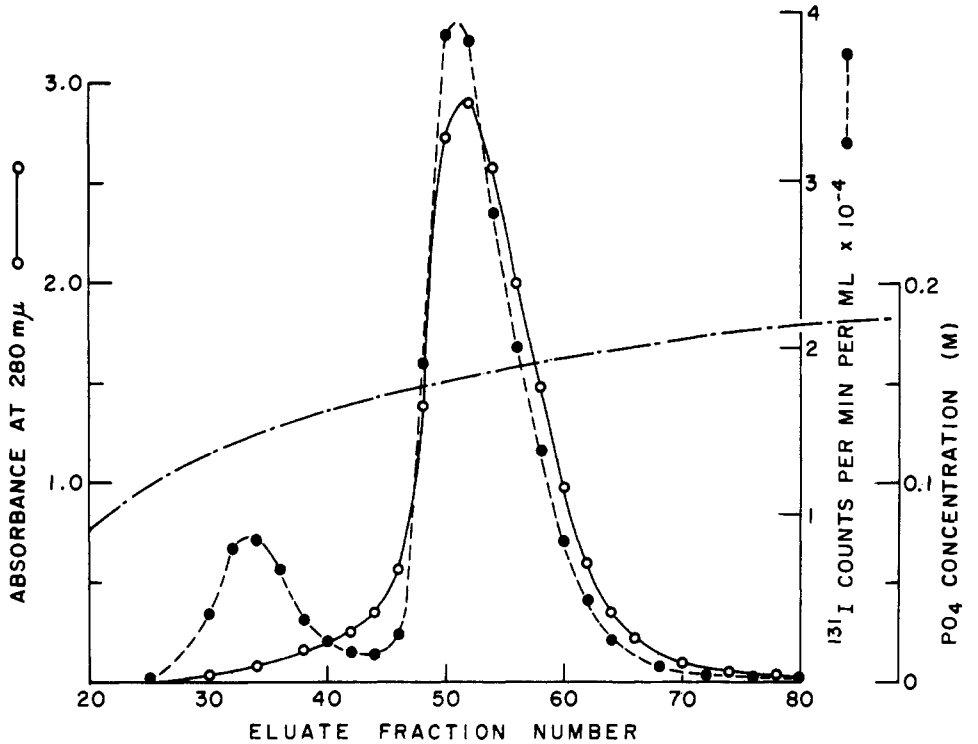

FIGURE 3: DEAE-cellulose column chromatography of the protein from eluate fractions 252–278 of Figure 2. See text and Figure 2 for details.

with TBG, in 0.01 M sodium acetate buffer (*p*H 4.5) (Ingbar, 1960). Elution of both TBG and TBPA was effected with acetate buffers of higher ionic strength and *p*H. When material from the 60–80% $(NH_4)_2SO_4$ fraction was similarly treated, only a small proportion of the total TBPA was adsorbed. By raising the *p*H to 4.8, however, adsorption of TBPA became virtually complete. The variation between the earlier work and the present procedure is probably due to a marked difference between the behavior of the two lots of DEAE-cellulose employed.

Each 10 g of the 60–80% $(NH_4)_2SO_4$ fraction is dissolved in 1 liter of 0.01 M sodium acetate buffer (*p*H 4.80). The *p*H is readjusted to 4.80 with 0.8 M sodium acetate buffer (*p*H 4.0). A slurry of 22 g of DEAE-cellulose in 225 ml of the 0.01 M acetate buffer is added to the protein solution and stirred for 45 minutes, and the DEAE-cellulose is separated by centrifugation at 1340*g* for 15 minutes. The supernatant is similarly treated twice more with 225-ml portions of this DEAE-cellulose slurry. The final supernatant contains about 3 g of protein but essentially no TBPA, and may be discarded. The three portions of DEAE-cellulose, containing adsorbed TBPA, are combined and suspended in 1 liter of 0.01 M sodium acetate buffer (*p*H 5.50). The suspension of DEAE-cellulose is stirred for 45 minutes and centrifuged for 15 minutes at 1340*g*. This procedure is repeated twice, and the supernatants, which contain mostly α-globulins and some albumin, are discarded. The washed DEAE-cellulose containing adsorbed TBPA is then suspended in eluting buffers of higher ionic strength, using first 500 ml of 0.10 M sodium acetate buffer (*p*H 5.50). The suspension is stirred for 45 minutes and centrifuged for 15 minutes at 1340*g*. The supernatant, termed eluate 1, contains mainly albumin, α-globulins, and TBG, but very little TBPA, and may be discarded. The DEAE-cellulose is resuspended in 500 ml of 0.60 M sodium acetate buffer (*p*H 5.50), stirred, and centrifuged as before. The supernatant, termed eluate 2, contains TBPA, albumin, α-globulins, and some TBG. Eluate 2 is saved. Eluates 3 and 4 are similarly obtained using 500 ml each of 0.30 M sodium acetate buffer (*p*H 5.50). The remaining TBPA adsorbed to the DEAE-cellulose is eluted with 500 ml of the 0.60 M buffer and is termed eluate 5. The compositions of eluates 2, 3, 4, and 5 are almost identical. Eluates 2 through 5 are combined, dialyzed overnight against large volumes of water, and lyophilized. The results of this DEAE-cellulose batch adsorption procedure are summarized in Table II. The composition of the final material is illustrated in Figure 1. As judged by staining of the proteins, this material contained a dense spot in the TBPA zone and areas of lesser density in the region of albumin, α_1- and α_2-globulins, and TBG. The DEAE-cellulose batch adsorption resulted in almost complete recovery of TBPA and a twofold purification (Table I).

DEAE-Cellulose Chromatography. The material from DEAE-cellulose batch adsorption is further purified by means of DEAE-cellulose column chromatography,

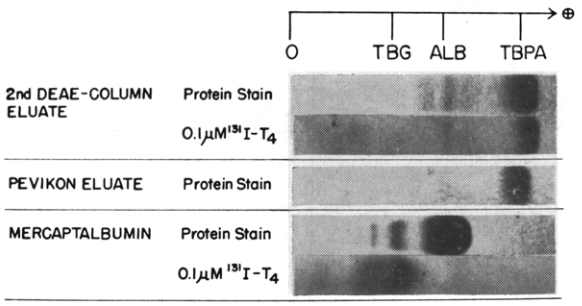

FIGURE 4: Electrophoretic patterns obtained in starch gel (Tris-maleate buffer, *p*H 8.6). Upper portion, eluate fractions 42–70 of Figure 3 plus ^{131}I-labeled L-thyroxine (^{131}I-T_4); middle portion, protein from above further purified by Pevikon-block electrophoresis; lower portion, four-times-crystallized human serum mercaptalbumin plus ^{131}I-T_4. Radioautographs are shown below the respective protein stains.

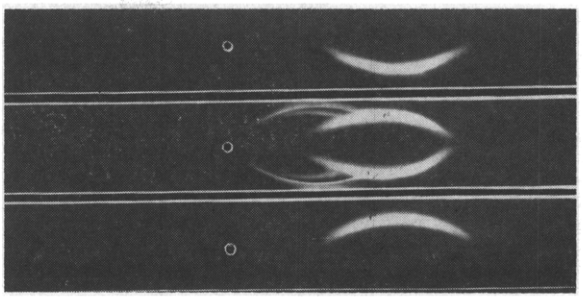

FIGURE 5: Immunoelectrophoretic study. The center well contained protein from eluate fractions 42–70 of Figure 3. The top and bottom wells contained 1 × and 2 × concentrations of the above protein after further purification by Pevikon-block electrophoresis. The troughs contained an antisera prepared in rabbits against the starting material, fraction IV-6.

a modification of the procedure of Fahey *et al.* (1958). Each 2.9 g is dissolved in about 50 ml of 0.01 M potassium phosphate buffer at *p*H 7.4 (buffer I) and dialyzed overnight against 2 liters of buffer I. After a tracer amount of ^{131}I-labeled T_4 (1–2 μcuries) is added to the dialyzed solution, it is applied to a 4.0 × 57 cm DEAE-cellulose column which is packed under 5 psi of nitrogen pressure and equilibrated with buffer I. A mixing chamber initially containing 3 liters of buffer I is used. The flow rate from the mixing chamber onto (and from) the column is 1.8 ml/min. Fractions of 16 ml are collected. The elution of proteins, consisting mainly of TBG, α-globulins, and albumin, is first carried out using a linear gradient of KH_2PO_4. A flow rate of 0.90 ml/min. of 0.40 M KH_2PO_4 into the mixing chamber is used until the first peak of radioactivity is eluted, as illustrated in Figure 2. Then a convex gradient is begun by increasing the flow rate of 0.4 M KH_2PO_4 into the mixing chamber to 1.8 ml/min. The TBPA is eluted from 0.15 to 0.25 M total phosphate concentration in the eluate fractions. The TBPA is associated with the position of the second peak of radioactivity. The ratio of ^{131}I-T_4 to absorbance at 280 mμ of this second radioactive peak is used to select the fractions (252–278) containing TBPA to be used in further purification. These fractions are combined, dialyzed against water, and lyophilized.

The material from the above DEAE-cellulose column chromatography contains mostly TBPA, as judged by paper and starch gel electrophoresis. Some impurities, consisting of albumin, α-globulins, and TBG, are removed by a second chromatography on a 2.5- × 30-cm DEAE-cellulose column. The protein is applied as previously described using buffer I. The protein is eluted with a convex gradient, formed by 0.20 M KH_2PO_4 flowing into a 750-ml constant volume mixing chamber, and collected in 21-ml fractions. The results of this chromatography are shown in Figure 3. There is a smaller radioactive peak containing TBG which precedes the elution of the main peak containing TBPA. The eluate fractions (47–60) with similar ratios of ^{131}I-labeled T_4 to absorbance at 280 mμ are combined, dialyzed against water, and lyophilized. This material is still heterogeneous as judged by starch gel electrophoresis (Figure 4) and immunoelectrophoresis (Figure 5). TBG has been removed but small amounts of albumin and α-globulin impurities are present. Radioautography performed after starch gel electrophoresis (Figure 4) and immunoelectrophoresis (not shown) show coincidence of ^{131}I-labeled T_4 radioactivity and the protein stain of TBPA. At this stage of purification TBPA has been prepared in approximately 20% yield from Cohn fraction IV-6. Its purity is seen from Figure 4 to be comparable to that of the three-times-recrystallized human serum mercaptalbumin which has been used as an electrophoretic marker.[5] Further chromatography on DEAE-cellulose failed to separate the remaining impurities from TBPA.

Pevikon-Block Electrophoresis. The TBPA from the second DEAE-cellulose column chromatography is further purified by horizontal zone electrophoresis using Pevikon C-870 as the supporting medium. This procedure yields an average total recovery of only 85% of the 500 mg of protein subjected to electrophoresis. By eluting the protein, which moves anodally well beyond the albumin marker, the purity of TBPA is increased, but the yield is considerably reduced. The eluate containing this protein is dialyzed against water, lyophilized, and stored at −5°. The protein from the region of the Pevikon block just anodal to the albumin marker is subjected to a second electrophoresis. The results of the purification achieved by this procedure are given in Table I.

By immunoelectrophoresis (Figure 5) the final TBPA preparation appeared to be homogeneous. The immunoelectrophoresis was performed with excess

[5] Radioautography reveals TBG to be present as a minor contaminant of this albumin preparation.

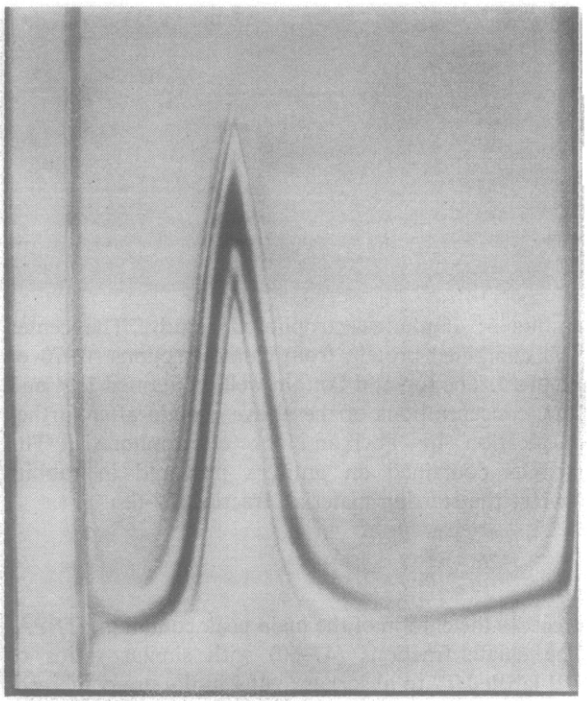

FIGURE 6: Sedimentation velocity pattern of thyroxine-binding prealbumin from Pevikon-block electrophoresis. Frame 70 min. after reaching maximum speed of 56,100 rpm; bar angle 45°; concentration 20 mg/ml in 0.1 M NaCl; $s_{20,w} = 3.8 \times 10^{-13}$ sec.

antigen to provide maximum opportunity for the detection of impurities. In starch gel electrophoresis (Figure 4) an apparent but very minor contaminant, not visible in the photograph, was barely detectable by protein staining. When starch gel electrophoresis in 7 M urea was performed by the procedure of Wake and Baldwin (1961), a single sharp band was observed with a mobility greater than albumin. The mobility of the protein was -8.8×10^{-5} cm^2 v^{-1} sec^{-1}, as measured by Tiselius electrophoresis in sodium diethylbarbiturate buffer (*p*H 8.6), ionic strength 0.1. In the ultracentrifuge pattern of TBPA, described in Figure 6, a single peak is seen which is not completely symmetrical due to the presence of a small amount of a faster sedimenting component[6] which did not separate from the main peak after 2-hours centrifugation.

Crystallization. A 100-mg quantity of TBPA from Pevikon-block electrophoresis is dissolved in 5 ml of 0.01 M potassium buffer (*p*H 7.4). Solid $(NH_4)_2SO_4$ is added in small increments until the solution becomes turbid, at approximately 50% saturation. After 1 hour, the small amount of flocculent amorphous material is removed by centrifugation. The $(NH_4)_2SO_4$ concentration of the supernatant solution is slowly increased until a crystalline sheen appears. The precipitate formed after standing overnight is entirely crystalline and has the electrophoretic and T_4-binding properties of TBPA. The crystals exhibit the property of double refraction of polarized light and are shown in Figure 7. These crystals are quite different in shape from the elongated dodecahedrons of human serum albumin obtained by Kendall (1941) from 50% saturated $(NH_4)_2SO_4$ solution.

[6] This minor component has been removed by a further electrophoresis in polyacrylamide gel.

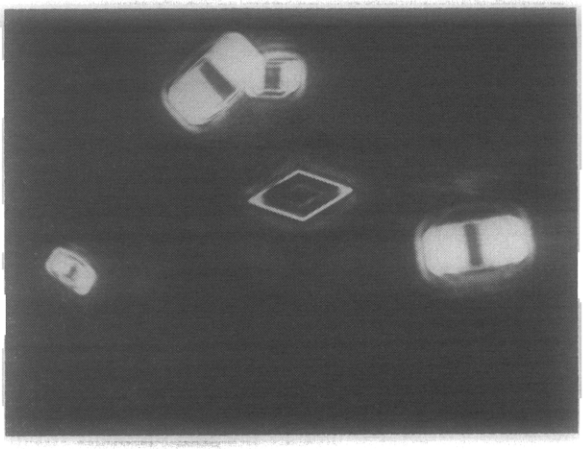

FIGURE 7: Crystalline thyroxine-binding prealbumin × 160 with polarized light.

Discussion

The method described above has made possible the isolation of a highly purified preparation of the thyroxine-binding prealbumin from human plasma, from which crystalline material has been obtained. As compared to the T_4-binding capacity of the starting material, fraction IV-6, that of the final preparation was increased 150-fold to a value of 9.56 μmoles of T_4/g of protein.[3] From an average of estimates of the concentration of TBPA in normal serum[7] (210 mg/liter) and the normal T_4-binding capacity of serum as measured in this laboratory (1.9 μmoles/liter), a rough value of 9.2 μmoles of T_4/g of TBPA can be calculated for the TBPA in whole serum. The agreement between these values is consistent with the high degree of purity which has been demonstrated for the isolated protein and also indicates that its T_4-binding ability has not been appreciably diminished during the preparative procedures.

Values for the T_4-binding capacity of TBPA given above should most certainly not be considered absolute. Although the paper electrophoretic method employed for their measurement is internally consistent in comparing T_4-binding capacities of fractions obtained throughout the purification procedure, the *p*H and

[7] Hoch and Chanutin (1953) reported 7–33 mg/dl from measurements by Tiselius electrophoresis. Aly and Niederhellman (1958) found 20–34 mg/dl by paper electrophoresis. Oppenheimer *et al.* (1965) obtained an extrapolated value of 16 mg/dl in a recovery study employing starch gel electrophoresis.

composition of the electrophoretic media, possible adsorption of TBPA to the filter paper, and interactions of T_4 and of TBPA with other proteins all may alter the values obtained. Indeed, on the basis of the molecular weight of 73,000 reported by Oppenheimer *et al.* (1965) and an assumed binding of 1 mole of T_4/mole of TBPA, the expected binding capacity of TBPA would be 13.7 μmoles of T_4/g of protein, a value considerably greater than that obtained by our electrophoretic technique. Oppenheimer *et al.* (1965) obtained an average value of 12.9 μmoles of T_4 bound/g of their purified TBPA using an electrophoretic procedure which employed a glycine-acetate buffer (*p*H 8.6). However, their protein concentrations were essentially based upon the Kjeldahl nitrogen content of 14.9% reported by Schultze *et al.* (1962) for TBPA, whereas our protein determinations were based upon the Biuret method using human serum mercaptalbumin as standard (16.3% N; Spahr and Edsall, 1964).

Considering the difficulty encountered in the present study in eliminating traces of contaminating proteins even by preparative electrophoresis, it would appear that rigid criteria must be applied in determining the absolute purity and precise chemical composition of TBPA preparations.

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