The Subunit Structure of Human Thyroxine-binding Prealbumin*

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SUMMARY

Prealbumin was isolated from human plasma by ammonium sulfate fractionation, chromatographic separations on diethylaminoethyl-Sephadex, and gel filtrations. The highly purified protein was subjected to quantitative amino acid analyses, which showed a minimum of 140 amino acid residues per prealbumin molecule. By determination of the minimum in the curves for the fraction of maximum deviation it was found that the minimum molecular weight for prealbumin was 15,500. Molecular weight determinations by sedimentation equilibrium ultracentrifugations gave a value of 62,500 ± 2,200. These results indicate that prealbumin is composed of four subunits. Molecular weight determinations by gel chromatography in 6 M guanidine hydrochloride gave values of about 16,000 for prealbumin. By this method it was shown that the subunits are held together by noncovalent bonds.

A trypsin digest of prealbumin was examined by peptide-mapping techniques and the number of peptides (15 to 19) detected was in good agreement with the total number of lysine and arginine residues calculated from the amino acid composition of a prealbumin tetramer consisting of four identical chains. The NH₂-terminal sequence of the protein was shown to be uniquely Gly-Pro.

Prealbumin, reduced and ¹³C-carboxymethylated, was subjected to trypic digestion, and the radioactivity was used to trace and isolate the cysteine-containing peptides. Only one peptide contained radioactivity, and analysis of this peptide revealed the unique sequence Gly-Pro-Ser-Met-Val-Cys(Cm)-Lys. These data strengthen the view that prealbumin is composed of identical subunits.

The mode of dissociation of the prealbumin tetramer was investigated by sedimentation equilibrium ultracentrifugation in various concentrations of guanidine hydrochloride. Determinations of local weight and number average molecular weights were consistent with three species being involved in chemical equilibrium, i.e. monomers, dimers, and tetramers of the prealbumin subunits.

EXPERIMENTAL PROCEDURE

Materials

Plasma—Human plasma was obtained from outdated blood. The blood cells were removed by centrifugation and the plasma thus obtained was stored at −20°C until further processed.

Proteins—The retinol-binding protein was isolated from serum by affinity chromatography on a Sepharose column to which prealbumin had been covalently attached. Details of the procedure have been described (8).

Antisera—Antisera against separately purified samples of human prealbumin were produced in three rabbits. The immunization procedure has been described (12). Details of the procedure have been described (8).

Antiserum—Antisera against separately purified samples of human prealbumin were produced in three rabbits. The immunization procedure has been described (12). All antisera produced single precipitin lines against human serum when tested on Ouchterlony immunodiffusion plates or by immunoelectrophoresis.

Special Materials—Sephadex G-200, G-25, and DEAE-Sephadex A-50 were obtained from Pharmacia Fine Chemicals AB.

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The abbreviations used are: RBP, retinol-binding protein; dansyl, 1-dimethylaminonaphthalene-5-sulfonyl.

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Uppsala, and prepared according to the instructions supplied. Guanidine hydrochloride was obtained from Sigma and used without further purification. It was, however, noted that the lot used (600-1530) was of insufficient purity to be used in experiments on the alkylation of prealbumin. Such experiments were therefore performed with specially purified guanidine hydrochloride (British Drug Houses, Toronto). D2O was purchased from Norsk Hydro (Norway). Trypsin (code TRTPCK) was obtained from Worthington Biochemical Corporation. [14C]-Iodoacetate acid (specific activity 20 mCi per ~.mole) was obtained from the Biochemical Centre, Anerham. Polyamide layer sheets were obtained from Cheng-Chin Trading Company, Taiwan. Phenylisothiocyanate (Pierce Chemical Company, Rockford, Illinois) was used according to the manufacturer's instructions. Trifluoroacetic acid (Eastman) was refluxed over CrO3 and subsequently distilled at 72.4° before use. Pyridine (Mal- 

linckrodt Chemical Works) was distilled at 114–116° after refluxing over KOH. Butyl acetate (Merek) was distilled from K2CO3 at 126.5°. All other chemicals were of the highest quality available and were used without further purification.

Methods

Concentration of Proteins—Concentration of proteins during the isolation procedure was accomplished by ultrafiltration (13) with use of 3/8 inch Visking dialysis tubing (Union Carbide Corporation, Chicago, Illinois) as the ultrafiltration membrane. The recoveries of prealbumin after ultrafiltration always exceeded 90%. After the last purification step, prealbumin was dialyzed exhaustively against distilled water and lyophilized.

Polyacrylamide Gel Electrophoresis—Polyacrylamide gel electrophoresis in 0.4 m Tris-Cl buffer, pH 8.9, was carried out in slabs in an EC 474 apparatus (E-C Apparatus Corporation, Philadelphia, Pennsylvania) according to the instructions supplied by the manufacturer. The gels were prepared from Cyanogum-412 (E-C Apparatus Corporation, Philadelphia, Pennsylvania) with a concentration of 4% in the spacer gel and 8% in the running gel. Ammonium persulfate and tetramethylthylenediamine were added as catalysts.

Starch Gel Electrophoresis—Starch gel electrophoresis in 8 m urea was performed in formate buffer, pH 2.7. Gels of 1.8 mm thickness were run horizontally at a potential gradient of 25 volts per cm for 75 min in a water-cooled apparatus for thin layer electrophoresis (Desaga, Heidelberg). The details of this method have been outlined (7).

Immunochemical Methods—Immunodiffusion in gel according to Ouchterlony (14) was performed with a previously described microtechnique (15). Immunoelectrophoresis was carried out according to the Scheidegger micromethod (16).

The content of prealbumin in impure fractions was quantitatively estimated by the single radial immunodiffusion method described by Maneini, Carbonara, and Heremans (17). The details have been outlined (18). This technique was also used to monitor prealbumin during the purification procedure. Purified prealbumin was used as the reference in all determinations.

Ultracentrifugation—Molecular weights were determined at 20° in a Spinco model E analytical ultracentrifuge equipped with an RTIC temperature control unit and an electronic speed control. All samples were dissolved in the appropriate buffers and dialyzed in the cold against two changes of the solvent. Densities were determined by pycnometry. Six-channel Epon-filled Yphantis centriplexes were used in most experiments. Determinations performed in guanidine hydrochloride were conducted with use of 12-mm capillary cells. Sapphire windows were used throughout. Recordings were made with Rayleigh interference optics except at very low concentrations (0.1 mg per ml or less) when the photoelectric scanning system was employed.

The sedimentation equilibrium experiments were performed by means of the meniscus depletion technique of Yphantis (19). When guanidine hydrochloride was included in the solvent, the modification of the Yphantis technique described by Chervenka was employed (20). Speed settings were chosen in accordance with the suggestions of Yphantis (19). Equilibrium times were estimated from the time chart provided by van Holde (21). Fringe displacements at given z coordinates were measured on photographs taken at different time intervals. The experiments were discontinued when no fringe shift could be observed over a period of at least 3 hours.

Calculations—Calculations of apparent weight average molecular weights were computed from the following equation (22)

\[ M_w = 2NRT \left( \frac{d \mu C}{d \phi^2} \right) / (1 - \phi) \omega^2 \]

where the symbols have their usual meaning. The partial specific volume was calculated from parallel sedimentation-equilibrium runs in solvents with H2O and D2O, respectively, according to the equation given by Edelstein and Schachman (23)

\[ kM_w = 2NRT \left( \frac{d \mu C}{d \phi^2} \right) / (1 - \phi_{\text{protein}}/k) \omega^2 \]

where a value of 1.0155 for k was assumed (23). Local weight average molecular weights were obtained as described by Yphantis (19) over five equally spaced z coordinates. Local number average molecular weights were calculated from the equation (24)

\[ n = \frac{C}{C(r) \, dr^2} \]

where \( n = M(1 - \phi) \omega^2/2RT \). The integral was evaluated by summation of the integrals between adjacent points as outlined by Teller et al. (24).

Analytical Gel Chromatography—The molecular weight of prealbumin was estimated by gel chromatography on Sephadex G-200 equilibrated with 6 m guanidine hydrochloride in 0.02 m Tris-HCl buffer, pH 8.0. The details have been outlined elsewhere (9). The reference proteins were k, c?, and c? chains of human immunoglobulin (26), and light chains of human immunoglobulin (26).

Amino Acid Analyses—Quantitative amino acid analyses were performed on a Biochrom automatic amino acid analyzer (Bioal, Munich) with the two-column ion exchange chromatography method of Spackman, Stein, and Moore (27). Protein samples of 1 to 2 mg were hydrolyzed in 6 n HCl at 110° for 24 or 72 hours (28). Peptide samples (0.1 to 0.2 pmole) were hydrolyzed for 24 hours. Determinations of carboxymethylcysteine in alkylated samples of prealbumin were carried out after hydrolysis for 20 hours. Tryptophan in protein was estimated spectrophotometrically (29) and in peptides by measuring the fluorescence at 350 nm after excitation at 280 nm.

Peptide Mapping—Peptide mapping of prealbumin was performed on cellulose thin layers. Prealbumin, 0.5 mg dissolved

\[ \text{2 Cyanogum-41 consists of 95% acrylamide and 5% bisacrylamide.} \]
in 0.5 ml of 1.5 M Tris-HCl buffer, pH 8.0, containing 6 M guanidine hydrochloride and 10 mM EDTA, was reduced with 0.01 M dithiothreitol (Calbiochem) for 2 hours, and subsequently alkylated with 0.024 M iodoacetic acid (Schuchardt, Munich) in the dark. The reduced and alkylated protein was exhaustively dialyzed against distilled, deionized water and lyophilized. Preliminary experiments showed that this procedure did not effect complete denaturation of the protein. Prior to enzymatic digestion the prealbumin was therefore boiled in distilled water for 10 min and lyophilized. Tryptic digestion of prealbumin was carried out in 0.2 M NH_{4}HCO_{3} pH 8.2, at 37° for 3 hours at an enzyme to protein ratio of 1:50 (w/w). The digestion was stopped by lyophilization. Part of the tryptic digest (0.2 mg) was applied to a 0.5-mm cellulose (Whatman CC 41) thin layer plate (20 × 20 cm). Electrophoresis was conducted in pyridine acetic acid, pH 5.5.

After drying in an air stream, the plate was subjected to chromatography in the second dimension. The chromatographic system has been described by Waley and Watson (30). The dried plate was sprayed either with ninhydrin (0.2% in acetic acid), phenanthroline (31), Pauly reagent (32), or platinic iodide reagent (32). The phenanthroline-sprayed plates were inspected under ultraviolet light.

**NH_{2}-Terminal Analyses and Sequence Determinations—Quantitative NH_{2}-terminal amino acid analyses were performed with the Edman technique (33).** The phenylthiohydantoin amino acid derivatives were identified by chromatography on starch-impregnated silicic gel thin layer plates in the solvent systems recommended by Sjöquist (34). Quantitative estimation of the phenylthiohydantoin amino acid liberated was obtained by measuring the optical density at 269 nm in a Beckman DBG spectrophotometer.

Amino acid sequence determinations were accomplished with the dansyl end group method in conjunction with the Edman technique (35). Dansyl amino acids were identified by chromatography on polyamide thin layer sheets in the solvent systems recommended by Woods and Wang (36).

**Sulfhydryl Group Determinations and Alkylation of Prealbumin—**Prealbumin was alkylated with [^{14}C]iodoacetic acid of known specific activity, and the number of alkylated sulfhydryl groups in the protein could thus be estimated from the amount of radioactivity incorporated by the protein, provided no unspecific alkylation occurred.

Prealbumin was alkylated in different solvents both with and without prior reduction. For each analysis, 0.6 mg of prealbumin was used. The protein solutions were dialyzed for 72 hours against frequent changes of the various solvents. After dialysis, the protein concentrations were adjusted to 3 mg per ml. Dithiothreitol was added to a final concentration of 0.01 M, and the reduction was allowed to proceed for 30 min at room temperature. Alkylation was performed with [^{14}C]iodoacetic acid at a final concentration of 0.024 M. After 2 hours in the dark at +4°, the reaction was terminated by precipitation of the protein with 10 volumes of ice-cold 10% (w/v) trichloroacetic acid. The precipitates, collected by centrifugation, were washed twice with 10% trichloroacetic acid and finally dried from ether. The dry precipitates were dissolved in 0.1 M NaOH, and an aliquot from each was counted in a Beckman LS 250 liquid scintillation counter. The scintillation solution contained 5 g of diphenyloxazole and 100 g of naphthalene in 1 liter of dioxane. Corrections for quenching were made by the channel ratio method.

Another aliquot was diluted with 0.1 M NaOH, and the protein content was determined spectrophotometrically by the absorbance at 280 nm. The amount of alkylation could thus be computed from the radioactivity and absorbance measurements.

Prealbumin was also alkylated without prior reduction. In these experiments, [^{14}C]iodoacetic acid was added to the protein solutions to a final concentration of 0.010 M. The samples were then processed as described above.

Determinations of free sulphydryl groups were also performed with the 5,5'-dithiobis(2-nitrobenzoic acid) method, as outlined by Janstova, Fuller, and Hunter (37). The absorbance at 412 nm was recorded for 1 hour. To calculate the thiol content of the protein, a molar extinction coefficient at 412 nm of 1.36 × 10^{4} was used. The analyses were always performed at least in duplicate.

The method for the alkylation of prealbumin finally adopted in the basis of the results of the sulphydryl determinations was as follows. Lyophilized samples of prealbumin were dissolved in 0.75 M Tris-HCl buffer, pH 8.0, containing 3 M guanidine hydrochloride and 10 mM EDTA, and 10 mM dithiothreitol. After the reduction iodoacetic acid, in some experiments [^{14}C]iodoacetic acid, was added to a final concentration of 0.024 M. The alkylation was stopped after 2 hours in the dark at +4°, either by removing the excess reagents by gel chromatography on columns of Sephadex G-25 or by exhaustive dialysis against distilled water. All operations were carried out in the dark at +4°. The protein solutions were subsequently lyophilized.

**Isolation of ^{14}C-Labeled Prealbumin Peptides—**A lyophilized tryptic digest of ^{14}C-carboxymethylated prealbumin was dissolved in 0.2 M NH_{4}HCO_{3} and applied to a column of Sephadex G-25 (1.4 × 140 cm) equilibrated in the same solvent. The elution profile for the peptides was determined by measuring the absorbance at 220 nm. Peptides containing ^{14}C were traced by analyzing 25-μl aliquots from each fraction in a liquid scintillation counter. The radioactive fractions were pooled and lyophilized, and subsequently subjected to high voltage paper electrophoresis in cooled tanks (38) at 50 volts per cm on Whatman No. 3MM paper. Electrophoresis was performed in pyridine acetic acid buffer at pH 6.5. Further purification was obtained by electrophoresis at pH 3.5 of the eluted material from the pH 6.5 electrophoresis. Aspartic acid and N-ε-dinitrophenyllysine were applied as markers. The peptides were located by means of appropriate guide strips, which were assayed for radioactivity or sprayed with ninhydrin-cadmium reagent (39). Radioactive peptides were eluted from the papers with 0.1 M NH_{4}OH and lyophilized.

**Isolation of Human Prealbumin.** Prealbumin was isolated from 10 liters of outdated plasma. During the isolation procedure minute amounts of undigested thyroxine (Amersham-Searle) were added in order to facilitate the detection of the prealbumin. In some preparations, the purification of prealbumin was also followed by the single radial immunodiffusion technique.

In each preparation the starting material consisted of about 1.5 liters of plasma. The isolation procedure adopted was reproducible and gave highly purified, homogeneous prealbumin in a relatively good yield. The results of a typical isolation of prealbumin are summarized in Table I.

**Ammonium Sulfate Fractionation—**Prealbumin is a minor con-
taminating proteins and this fraction was therefore considered to be pure and accordingly not purified further (cf. text).

prealbumin present in plasma, i.e. free prealbumin and the prealbumin-RBP complex.

Total .. . . . . . . . . . . . 142~

First Sephadex G-200.. . 218~

First DEAE-Sephadex chromatography
Free prealbumin~ . . 103~
Complex~ . . . . . . 39~

Second DEAE-Sephadex chromatography
Free prealbumin~. . 80~
Complex~ . . . . . . 20~

Total . . . . . . . . . . . . 122~

Plasma (1200 ml) ......... 105,000~
Ammonium sulfate (40%) . . . . . . 67,000~

Table 1

| Fraction          | Total protein | Prealbumin $^a$ | Yield | Purity |
|-------------------|---------------|-----------------|-------|--------|
| Plasma (1200 ml)  | 105,000~      | 324             | 100   | 0.31   |
| Ammonium sulfate  | 67,000~       | 259             | 80    | 0.38   |
| First DEAE-Sephadex chromatography | 307~ | 201             | 62    | 65     |
| First Sephadex G-200 | 218~ | 126             | 42    | 77     |
| Second DEAE-Sephadex chromatography | 193~ | 78              | 20    |        |
| Complex $^b$      | 39~           | 2               | 20    |        |
| Total             | 122~          | 80~             | 40    | 91     |
| Second Sephadex G-200 | 32~ | 32              | 0     | 55~    |

$^a$ Measured by a single radial immunodiffusion technique.
$^b$ "Free" and "Complex" denote the two molecular forms of prealbumin present in plasma, i.e. free prealbumin and the prealbumin-RBP complex.
$^c$ Measured with the Folin technique.
$^d$ Denotes the two fractions obtained in this purification step.
$^e$ Estimated by reading the optical density at 280 nm.
$^f$ Total yield of prealbumin obtained in this (32 mg) and in the previous (80 mg) purification step.

Fig. 1. Fractionation of plasma proteins by ammonium sulfate. Aliquots of 100 ml of plasma, containing trace amounts of $^{125}$I, were adjusted to the desired concentration with solid ammonium sulfate. The supernatant, remaining after centrifugation, was assayed for prealbumin and albumin by a single radial immunodiffusion technique, and for $^{125}$I by counting in a well-type scintillation counter. The concentrations were adjusted for the increase in volume caused by the ammonium sulfate.

Fig. 2. Chromatography on DEAE-Sephadex of the supernatant obtained after precipitation of the plasma proteins with 40% ammonium sulfate. The column (50 X 8.5 cm) was equilibrated with 0.02 m Tris-HCl buffer, pH 7.4, containing 0.2 m NaCl. Prior to application, the sample (containing 60,800 mg of total protein and 221 mg of prealbumin) was dialyzed exhaustively against the same buffer. Elution was performed at pH 7.4 with a 5,000-ml linear gradient of NaCl from 0.2 to 0.6 m. Fractions of 32 ml were collected at a flow rate of 64 ml per hour. The distribution of prealbumin in the effluent was determined by Ouchterlony immunodiffusion analyses (horizontal bar). The fractions were pooled as indicated by the arrows.

volumes of plasma, containing trace amounts of $^{125}$I-labeled thyroxine, were adjusted to different concentrations of ammonium sulfate and left at +4°C overnight with continuous stirring. The precipitates were centrifuged off and the radioactivity in the supernatants was determined, as well as the amounts of prealbumin and albumin by immunological techniques. As can be seen from Fig. 1 the content of prealbumin in the supernatants was as high as in the original plasma sample even at an ammonium sulfate concentration of more than 40%. Accordingly 2-liter portions of plasma were adjusted with ammonium sulfate to a final concentration of 40%. The supernatants from the plasma samples obtained after centrifugation essentially contained only albumin and prealbumin, since most of the other plasma proteins had precipitated. The supernatants were diluted 1:1 with 0.02 m Tris-HCl buffer, pH 8.0, and subsequently concentrated by ultrafiltration.

First DEAE-Sephadex Chromatography—The concentrated supernatant from the ammonium sulfate fractionation step was exhaustively dialyzed against 0.02 m Tris-HCl buffer, pH 7.4, containing 0.2 m NaCl and thereafter subjected to ion exchange chromatography on a column of DEAE-Sephadex, equilibrated with the same buffer. The column was run as described in the legend of Fig. 2. As can be seen from the figure, a good separation was achieved between prealbumin and the bulk of the contaminating proteins. Prealbumin was eluted as a broad zone which contained both prealbumin and the complex between prealbumin and the retinol-binding protein, as will be shown below.

The fractions were pooled as indicated in the figure and concentrated by ultrafiltration.

Sephadex G-200 Chromatography—Preliminary experiments had shown that the prealbumin fraction obtained from the first DEAE-Sephadex chromatography step consisted of two molecular species of this protein, one species representing free prealbumin, the other the complex between prealbumin and the retinol-binding protein. These two species are of different size. Accordingly, this fraction was subjected to gel chromatography on a column of Sephadex G-200, equilibrated in 0.02 m Tris-HCl buffer, pH 8.0, containing 1.0 m NaCl. The result of a typical fractionation is shown in Fig. 3. It is evident from the figure...
that there was incomplete separation between the prealbumin-RBP complex and free prealbumin. The fractions containing free prealbumin and the prealbumin-RBP complex, respectively, were separately pooled and concentrated by ultrafiltration.

Second DEAE-Sephadex Chromatography—Immunoelectrophoretic analyses on the two fractions obtained from the Sephadex G-200 chromatography step showed that both fractions contained prealbumin, RBP, and also albumin. In the final purification steps a separation of prealbumin from albumin was effected. The two fractions containing free prealbumin and the prealbumin-RBP complex, respectively, were chromatographed separately on columns of DEAE-Sephadex, equilibrated with 0.02 M Tris-HCl buffer, pH 7.2, containing 0.25 M NaCl. The samples were applied after extensive dialysis against the same buffer and the columns were run as described in the legend of Fig. 4. The results of two typical chromatographic fractionations are shown in Fig. 4. In all fractionations material was eluted at three positions. A breakthrough peak, due to albumin, was demonstrated by the single radial immunodiffusion technique, and the zones were followed by two peaks emerging close together. The first peak was due to free prealbumin and the second to the complex between prealbumin and RBP as indicated by the absorbance of retinol at 330 nm. The fractions were pooled as indicated in the figure. The fraction containing free prealbumin was now considered to be pure (cf. Table I) and was therefore dialyzed against distilled, deionized water and lyophilized. The fraction containing the prealbumin-RBP complex was concentrated by ultrafiltration.

Second Sephadex G-200 Chromatography—Previously we have shown that the affinity between prealbumin and RBP is dependent on the ionic strength. At low ionic strength, the protein complex dissociates (9). Thus, the prealbumin-RBP-containing fraction was subjected to gel chromatography on a column of Sephadex G-200 equilibrated with 0.02 M Tris-HCl buffer, pH 8.0. Prior to application the sample was thoroughly dialyzed against the same buffer. Fig. 5 shows a typical elution pattern.
TABLE II

Molecular weight of prealbumin under physiological conditions

| Experiment | Concentration | Speed | Apparent Mₚ |
|------------|---------------|-------|-------------|
| 1          | 0.85          | 24,000| 63,200      |
| 2          | 0.75          | 28,000| 64,100      |
| 3          | 0.25          | 30,000| 61,800      |
| 4          | 0.10          | 20,000| 59,700      |
| 5          | 1.15          | 16,000| 63,300      |
| 6          | 0.00          | 30,000| 62,500      |
| 7          | 0.35          | 28,000| 63,600      |
| 8          | 0.12          | 24,000| 61,400      |

* The solvent used in Experiments 1 to 4 was 0.02 M Tris-Cl buffer, pH 8.0, containing 0.2 M NaCl, in Experiments 5 to 6 0.02 M phosphate buffer, pH 7.4, containing 0.2 M NaCl, and in Experiments 7 to 8, 0.02 M phosphate buffer, pH 6.8, containing 0.2 M NaCl.

It is evident that complete dissociation between prealbumin and RBP was obtained, as demonstrated by the absorbance of retinol at 330 nm. The fractions containing prealbumin were pooled, as indicated in the figure, dialyzed against distilled, deionized water and lyophilized. This material together with the fraction obtained from the previous purification step gave only a single arc of precipitation when subjected to immunoelectrophoresis, using antisera against whole plasma, RBP, and prealbumin. Polyacrylamide gel electrophoresis at pH 8.9 revealed a protein zone with high mobility, and only a single component was encountered. It is therefore concluded that the isolated prealbumin was of high purity and homogeneous.

RESULTS

Ultracentrifugations under Physiological Conditions—The purified prealbumin was subjected to analytical ultracentrifugation. Determinations of the molecular weight were performed in various buffers at about physiological pH and ionic strength. Eight different determinations are summarized in Table II. The measured molecular weight ranged from 59,700 to 64,100, and in all experiments linear relationships were obtained when In C was plotted versus r². Fig. 6 shows two experiments conducted at 24,000 rpm. Fig. 6A shows the results obtained in 0.02 M Tris-Cl buffer, pH 8.0, containing 0.2 M NaCl, and Fig. 6B shows the results obtained in the same buffer with D2O substituted for H2O. From the slope of the plot the molecular weight and the partial specific volume of the protein can be computed (23). By this procedure the partial specific volume of prealbumin was estimated as 0.742 ml per g, a figure in close correspondence with the value, 0.740, obtained from the amino acid composition (8).

Amino Acid Composition and Molecular Weight—Amino acid analyses were performed in quadruplicate on two separately purified samples of prealbumin to obtain data with sufficient accuracy for estimations of the minimum molecular weight. Determinations of minimum residue numbers are given in Table III. In the table the frequencies of the amino acid residues in prealbumin are arranged in increasing order of magnitude. A total of 140 residues per molecule was found and consequently a molecular weight of 15,657 was obtained.

The second method used was adopted from the procedure described by Black and Hugness (40). The calculations and the symbols used are the same as described by these authors. Fig. 7 shows a plot of the fraction of maximum deviation (f) versus molecular weight for prealbumin. It is evident that minimum values of f are obtained at two molecular weights, i.e. 15,500 and 31,000, with a slightly lower value for f at the lower molecular weight. The value 15,500 is in close agreement with the value 15,617 obtained by the other procedure. The integers closest to the calculated residue numbers for a molecular weight of 15,500, obtained by the last described procedure, are the same as those listed as minimum residue numbers for the first procedure (Table III).

Gel Chromatography in 6 M Guanidine Hydrochloride—The amino acid analyses indicated a minimum molecular weight of about 15,500 for prealbumin, whereas the ultracentrifugations performed under physiological conditions gave a molecular weight of about 62,000. In order to investigate the possibility of a covalently linked subunit structure for prealbumin, it was subjected to gel chromatography on columns of Sephadex G-100 or G-200 in 6 M guanidine hydrochloride. The columns were calibrated with reference proteins as described under “Experimental Procedure.” The prealbumin peak always appeared somewhat later than a with chains of haptoglobin under these conditions. From these experiments a molecular weight of about 16,000 was obtained for prealbumin, establishing that the subunits of prealbumin are held together by noncovalent bonds only. These data together with the results of the amino acid analyses clearly show that prealbumin is composed of four polypeptide chains of similar molecular weight.

NH₂-terminal Sequence of Prealbumin—In accordance with previous results (8) glycine was the only NH₂-terminal amino
TABLE III

Frequencies and minimum residue numbers for amino acids in prealbumin

| Amino acid residue, $i$ | Number of residues found$^a$ | Residue frequency$^b$ | Minimum residue number$^c$ |
|------------------------|-------------------------------|-----------------------|--------------------------|
| Half-cystine$^d$        | 0.99                          | 0.0071                | 1                        |
| Methionine              | 1.00                          | 0.0072                | 1                        |
| Tryptophane$^e$         | 2.90                          | 0.0215                | 3                        |
| Histidine               | 4.79                          | 0.0345                | 5                        |
| Arginine                | 4.81                          | 0.0346                | 5                        |
| Tyrosine                | 4.85                          | 0.0349                | 5                        |
| Phenylalanine$^f$       | 4.89                          | 0.0332                | 5                        |
| Isoleucine$^f$          | 0.14                          | 0.0442                | 6                        |
| Proline$^f$             | 7.14                          | 0.0514                | 7                        |
| Leucine$^f$             | 7.06                          | 0.0537                | 8                        |
| Lysine$^f$              | 8.90                          | 0.0647                | 9                        |
| Aspartic acid           | 9.89                          | 0.0712                | 10                       |
| Glucose                 | 9.99                          | 0.0719                | 10                       |
| Serine$^f$              | 10.88                         | 0.0763                | 11                       |
| Threonine               | 12.88                         | 0.0927                | 13                       |
| Alanine                 | 12.96                         | 0.0933                | 13                       |
| Glutamic acid           | 13.79                         | 0.0993                | 14                       |
| Valine$^f$              | 13.89                         | 0.1000                | 14                       |

$^a$ Except where noted, all figures are averages of values from four different preparations. On each preparation one 24-hour and one 72-hour hydrolysis was performed.

$^b$ Calculations based on the assumed presence of 1 residue of methionine.

$^c$ The minimum residue numbers, $n_i$, were calculated by assuming that the value for the amino acid with lowest frequency is 1 and then dividing the next larger frequency by integers and selecting $n_i$ as the integer yielding a quotient closest to the lowest frequency. This process was then continued using increasing frequencies, always matching the quotients for selection of a given $n_i$ with the mean of all previously determined values of $F_i/n_i$. The mean of all $F_i/n_i$ is 0.00714 and the range is 0.00709 to 0.00736, indicating a deviation from the integral $N_i$ of less than 3%.

$^d$ Half-cystine was determined as cysteic acid after performic acid oxidation. Determinations were performed on three different preparations of prealbumin.

$^e$ Determined spectrophotometrically.

$^f$ 72-hour hydrolysis value.

Values were obtained by extrapolation to zero time of hydrolysis.

tography on cellulose thin layer plates, 15 to 19 ninhydrin-positive peptide spots could be detected (cf. Fig. 8). Similar analyses performed at various pH values and in different chromatographic solvents did not increase the total number of ninhydrin-positive peptide spots. This number of peptides corresponds well with the total number (14) of lysine plus arginine residues in the subunit that is proposed for prealbumin. At the same time it should be recognized that this number of peptides is unlikely to correspond exactly with the actual number of unique sequences containing lysine and arginine in the protein chain. Thus some of the fainter spots may represent products of incomplete hydrolysis, while others could be due to chymotryptic-like cleavages. Moreover, the tryptic digest may contain large peptide fragments that stain poorly and remain immobile on the origin in the peptide maps. Nevertheless, despite these limitations, the results are consistent with the proposal that prealbumin is composed of four very similar if not identical protein chains. This conclusion received additional support when it was found that the number of peptides that stained specifically for histidine and tyrosine, arginine and sulfur-containing amino acids is also in accordance with the proposed amino acid composition of the subunit (Table IV).

Electrophoresis in Urea—To obtain further evidence for the assumed identity of the prealbumin subunits, starch gel electrophoresis in 8 M urea at pH 2.7 was carried out. Native, as well as reduced and alkylated prealbumin, exhibited a total of four protein zones under these conditions (Fig. 9). The main protein zone was accompanied by three minor protein zones which together seemed to represent less material than the major zone. Tentative explanations would therefore be either incomplete dissociation of the prealbumin tetramer into subunits in spite of the high urea concentration used (see below), or the presence of genetic polymorphism in human prealbumin.
Peptide maps of tryptic digests of reduced and alkylated prealbumin on thin layer cellulose plates. Protein (0.5 mg) in 50 μl of 0.2 M NH₄HCO₃ buffer, pH 8.2, was digested with trypsin for 3 hours at 37°C. The weight ratio of enzyme to substrate was 1:50. After digestion the samples were freeze-dried and redissolved in 10 μl of the electrophoresis buffer. The dissolved samples did not contain noticeable amounts of precipitate. Three microliters were applied to the thin layer plates. Glass plates (20 × 20 cm) were covered with a 0.5-mm layer of cellulose (Whatman, CC 41). Electrophoresis was carried out in pyridine-acetic acid-water (100:35:4865, v/v), pH 5.5 buffer at 300 volts for 75 min in a horizontal apparatus cooled with tap water. The plate was dried for 40 min at 45°C under modest laminar air flow. Ascending chromatography was performed in butanol-pyridine-acetic acid-water (15:10:3:12) at room temperature. The plate was equilibrated in the jar for 60 min before the start. After chromatography, the plates were dried for 30 min at 40°C and sprayed with the various staining reagents. A, peptide map developed with ninhydrin; B, composite peptide map after staining with the reagents noted in the figure.

**Table IV**

| Method                  | Number of peptides |
|-------------------------|--------------------|
|                         | Expected | Found   |
| Ninhydrin               | 15       | 15-19   |
| Chlorination            | 15       | 15-17   |
| Phenanthraquinoneb      | 5        | 5       |
| Paulyc                  | 10       | 6       |
| Platinic iodide d       | 2        | 1       |

* Number of peptides expected was based on the amino acid composition given in Table III, assuming a tetrameric structure with identical subunits for prealbumin.

1 Specific for arginine.
2 Specific for histidine and tyrosine.
3 Specific for methionine and cysteine.

**Determinations of Free Sulfhydryl Groups in Prealbumin**—Free sulfhydryl groups were reacted with 5,5'-dithiobis(2-nitrobenzoic acid) both with and without denaturing agents as described under "Experimental Procedure." Free sulfhydryl groups could, however, only be demonstrated with this technique after exposure of the protein to denaturing solutions containing isopropanol (cf. Table V).

The properties of the assumed sulfhydryl groups were subsequently investigated by alkylation with [14C]iodoacetic acid. Several solvents of various composition were used (cf. Table V). As can be inferred from the table, the free sulfhydryl groups were only slightly reactive if isopropanol was excluded from the solvent. Even heating the protein at 70°C for 12 hours in 6 M guanidine hydrochloride prior to alkylation did not render the sulfhydryl groups completely reactive. These results indicate that the sulfhydryl groups of prealbumin are normally inaccessible to modification. However, in isopropanol about 0.8 mole of sulfhydryl groups per mole of prealbumin subunit could be demonstrated. Reduction prior to alkylation did not increase the number of alkylated sulfhydryl groups. It is thus concluded from these results that each prealbumin subunit contains a free
sulfhydryl group located in an extremely hydrophobic environment in the tertiary structure of the native protein.

Isolation and Amino Acid Sequence Determination of Carboxymethylated Cysteine-containing Tryptic Peptide of Prealbumin—A tryptic digest of ¹⁴C-carboxymethylated prealbumin was subjected to gel chromatography on a Sephadex G-25 column as can be seen in Fig. 10 only one radioactive peak was found comprising 96% of the radioactivity incorporated into the protein. The fractions constituting this peak were pooled and lyophilized. The lyophilized material was subjected to high voltage electrophoresis at pH 6.5 in pyridine acetic acid. Of the radioactive zones detected only a single zone contained radioactivity. The material in this zone was eluted, lyophilized, and on part of the material the amino acid composition was determined.

The amino acid composition of the peptide is given in Table VI. As can be seen the peptide contained seven amino acids in approximately equivalent amounts. Except for lysine, the amino acids are uncharged and rather hydrophobic. The peptide is neutral at pH 6.5, as the positive charge of lysine is neutralized by the negative charge of the carboxymethylcysteine, which was the only carboxymethylated amino acid found in an acid hydrolysate of carboxymethylated prealbumin.

The covalent structure of the peptide was determined by the Edman procedure together with dansylation. The terminal amino acid residue was glycine, in accordance with the color of the peptide after staining with the ninhydrin-cadmium reagent. Five amino acids could unequivocally be identified, giving the sequence shown in Fig. 11. As the peptide was isolated from a tryptic digest, lysine should be present in the carboxyl-terminal position, and therefore the penultimate residue from the carboxyl-terminal would be carboxymethylcysteine.
Subunit Structure of Prealbumin

FIG. 11. Amino acid sequence of the 14C-carboxymethylated peptide isolated from a tryptic digest of prealbumin, alkylated with [14C]iodoacetic acid. Amino acids identified as their dansyl derivatives after each step of Edman degradation are underlined by the arrows.

FIG. 12. The concentration dependence of the $M_w$ and $M_n$ of prealbumin in 0.5, 4, and 6 M guanidine hydrochloride, respectively. The values were obtained by sedimentation equilibrium ultracentrifugation. The protein was dialyzed with two changes of the solvent. The speed was 30,000 rpm for samples in 2 M and 4 M guanidine hydrochloride and 34,000 for the sample in 6 M guanidine hydrochloride. Initial protein concentrations varied between 0.5 and 0.6 mg per ml. The symbols appearing in the order from top to bottom in the figure, are (O), $M_w$ and $M_n$, respectively, at 2 M, 4 M, and 6 M guanidine hydrochloride.

Ultracentrifugations of Prealbumin in Guanidine Hydrochloride—Equilibrium ultracentrifugations of prealbumin in 2, 4, and 6 M guanidine hydrochloride were performed to investigate the mode of dissociation of the tetramer into free subunits. Prealbumin displayed a heterogeneous behavior at all concentrations of guanidine hydrochloride, as can be seen in Fig. 12. In the figure, local weight and number average molecular weights are plotted against the prealbumin concentration. It can be seen that this protein in 2 M guanidine hydrochloride exhibits a molecular weight distribution simulating a tetramer-dimer dissociation, whereas in 4 M guanidine hydrochloride the molecular weight distribution seems to indicate a dissociation of prealbumin into monomers. In 6 M guanidine hydrochloride the $M_w$ and $M_n$ data exhibit only a slight concentration dependence, with the main species being the monomer. On the basis of the $M_w$ and $M_n$ data obtained in different concentrations of guanidine hydrochloride, the dissociation of the prealbumin tetramer seems to proceed to monomers, with a molecular weight of about 15,500, with dimers as intermediary species.

DISCUSSION

In an earlier report we showed that, on chromatography on Sephadex equilibrated with high concentrations of guanidine hydrochloride, prealbumin dissociates into subunits (9). Evidence for a subunit structure of prealbumin has also been obtained by studies of its genetic polymorphism (10) and by x-ray crystallographic examination (11). No detailed information has, however, been presented for the nature and size of the prealbumin subunits. The present study has been concerned with these aspects.

The molecular weight estimated under dissociating conditions by sedimentation equilibrium ultracentrifugation and gel chromatography is about 16,000. It is thus established that prealbumin is a tetrameric protein composed of subunits of identical size. The amino acid analyses indicated a minimum molecular weight of about 15,500, in good agreement with the physical estimates.

Prealbumin is known to bind 1 mole of thyroxine (2) and 1 mole of RBP (6–8) per mole of protein, suggesting that the prealbumin subunits are nonidentical, as it is hard to interpret 1:1 stoichiometry for a symmetrical protein. Analyses by peptide mapping, with the use of various staining reagents specific for different amino acids, did not reveal more than one type of subunit, although minor amino acid exchanges could have escaped detection. The determinations of the amino-terminal amino acid sequence established the unique sequence Gly–Pro, also in accordence with the existence of identical subunits. Electrophoresis of prealbumin on uren-formate starch gels, pH 2.7, revealed, however, multiple protein zones. The interpretation of this result is obscure but in view of the reported existence of a genetic polymorphism for prealbumin in rhesus monkeys the heterogeneity of human prealbumin may reflect a similar phenomenon.

It was not possible to establish the presence of sulfhydryl groups in prealbumin by means of alkylation under physiological conditions despite the fact that the amino acid composition clearly indicated that each subunit of prealbumin should contain one cysteine provided the polypeptide chains are identical. Inaccessibility of the sulfhydryl groups to alkylation was also noted under denaturing conditions in accordance with the results of Tomita, Shiratori, and Goodman (41). In 6.5 M isopropanol under denaturing conditions, however, the sulfhydryl groups became accessible to alkylation. By use of 5,5'-dithiobis(2-nitrobenzoic acid), [14C]iodoacetic acid, and by determinations of carboxymethylcysteine after acid hydrolysis of alkylated prealbumin it was shown that approximately 0.8 mole of sulfhydryl group per mole of polypeptide chain could be demonstrated. It therefore seems obvious that each prealbumin subunit contains a single residue of cysteine. Further support for this contention was obtained by the exploration of the amino acid sequence around the cysteines of prealbumin. A single radioactive tryptic peptide was thus obtained from 14C-carboxymethylated prealbumin. The amino acid composition revealed the presence of one mole of carboxymethylcysteine per mole of peptide. The unique sequence of the radioactive heptapeptide supports the findings that prealbumin is composed of four very similar if not identical polypeptide chains, each containing 1 cysteine residue. The hydrophobic environment required for alkylation of the cysteines may partly be explained by the amino acid sequence around cysteine which comprises several hydrophobic amino acids.

The assumed identity of the prealbumin subunits prompted us to reinvestigate its thyroxine-binding ability, and in the accompanying paper, evidence is presented for four thyroxine-binding sites in prealbumin (42). These additional data would further support the contention that prealbumin is composed of identical subunits.

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