The Interaction of Thyroxine with Human Plasma Prealbumin and with the Prealbumin-Retinol-binding Protein Complex*

AMIRAM RAZ† AND DEWITT S. GOODMAN§
From the Department of Medicine, Columbia University College of Physicians and Surgeons, New York, New York 10032

SUMMARY

Prealbumin was isolated from human plasma by chromatography on columns of diethylaminoethyl Sephadex and Sephadex G-200, followed by preparative polyacrylamide gel electrophoresis. The prealbumin was homogeneous in the analytical ultracentrifuge with an $s_{20, w}$ of 3.7 S and with a molecular weight of about 50,000. Prealbumin formed a protein-protein complex with plasma retinol-binding protein in a molar ratio of 1:1.

The interaction of thyroxine with prealbumin was quantitatively studied by the method of equilibrium dialysis with $^3$H-labeled thyroxine. Studies were conducted at 24° and at 37° in phosphate buffer, pH 7.4, and in phosphate buffer containing 0.14 M NaCl. Prealbumin was found to possess a single binding site for 1 molecule of thyroxine. The association constant for the thyroxine-prealbumin interaction was approximately $1.6 \times 10^{10}$, and was only slightly affected by temperature change or by NaCl. The binding capacity and affinity of prealbumin for thyroxine were similar in the presence and absence of retinol-binding protein. Moreover, the binding of thyroxine to prealbumin did not interfere with the interaction of prealbumin with retinol-binding protein. The interaction of prealbumin with thyroxine appears to be independent of the prealbumin-retinol-binding protein interaction.

We have recently reported the isolation of human retinol-binding protein, the specific plasma protein responsible for the plasma transport of Vitamin A (10). Retinol-binding protein interacts strongly with plasma prealbumin, and circulates in plasma together with prealbumin in the form of a protein-protein complex.

The experiments reported here were designed to examine the possible effects of the interaction between prealbumin and RBP on the binding of thyroxine to prealbumin. Prealbumin was purified by column chromatography and polyacrylamide gel electrophoresis. The interaction of prealbumin with thyroxine was then directly and quantitatively studied, both in the presence and absence of RBP, by the method of equilibrium dialysis.

EXPERIMENTAL PROCEDURE

Purification of Plasma Prealbumin—Plasma in acid citrate dextrose anticoagulant was obtained from the blood bank and was concentrated approximately 2-fold by ultrafiltration through a UM-1 membrane (Diaflo apparatus, Amicon Corporation, Cambridge, Massachusetts). The plasma was then serially chromatographed on columns of DEAE-Sephadex A-50 and of Sephadex G-200 (Pharmacia) under conditions similar to those described previously (10). In a typical experiment, 1000 ml of plasma were concentrated to 500 ml and applied to a DEAE-Sephadex column (3 cm $\times$ 125 cm). Elution was carried out with 0.05 M phosphate buffer, pH 7.5, and a linear gradient of NaCl from 0 to 1.0 M. The fractions comprising the prealbumin-containing protein peak were pooled, concentrated by ultrafiltration, and dialyzed against the buffer solution to be used for gel filtration. After gel filtration on a column of Sephadex G-200 (5 cm $\times$ 125 cm) the prealbumin-containing peak was desorbed and chromatographed on a small (2.5 cm $\times$ 60 cm) DEAE-Sephadex column under conditions similar to those used in the initial chromatographic run.

Further purification of prealbumin was effected by preparative polyacrylamide gel electrophoresis and chromatography on a column of Sephadex G-100 as described previously (10).

Analytical Ultracentrifugation—Sedimentation velocity and equilibrium analyses were kindly carried out by Drs. W. Pollin and P. Feigelson in a Spinco model E ultracentrifuge equipped with a monochromator and photoelectric scanner. The absorbance of the cell contents at 280 m$\mu$ was determined at intervals as a function of distance from the center of rotation. Sedimentation

* The abbreviation used is: RBP, retinol-binding protein.

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§ Career Scientist of the Health Research Council of the City of New York under Contract I-399.
tion velocity studies were carried out on a prealbumin solution of 0.68 mg per ml in 0.1 M sodium phosphate buffer, pH 7.0. Centrifugation was conducted at 20° at a speed of 52,000 rpm, and the cells were scanned at 12-min intervals. The sedimentation coefficient was calculated for a solution of this protein concentration in water at 20° (ε20,ω). This value for the sedimentation coefficient should be very close to the value at infinite dilution (ε20,ω), because of the very dilute solution used for analysis. The molecular weight of prealbumin was determined by sedimentation equilibrium, by the method of Yphantis (11). A solution of prealbumin, 0.065 mg per ml in the above buffer, was subjected to centrifugation at 20° at 30,000 rpm for 20 hours. The molecular weight was calculated with the value of 0.74 as an estimate for the term Vp, and an assumed value of 1.0 for Vr. The resulting value of Vp was very close to the value for Vp which can be calculated from the amino acid composition, the partial molal volume for each amino acid, and the measured density of the solution. Hence it is felt that the assumed value for Vp did not introduce any major error in the estimation of the molecular weight.

Amino Acid Analyses—The amino acid composition of prealbumin was kindly determined by Dr. R. E. Canfield as described previously (10), according to the method of Spackman, Stein, and Moore (12). Half-cystine and methionine were determined as cysteic acid and methionine sulfone, respectively, by oxidation of a portion of prealbumin (approximately 5 mg) with performic acid according to the method of Hirs (13), followed by hydrolysis with HCl for 24 hours and amino acid analysis as described above. The observed numbers of micromoles of cysteic acid and methionine sulfone were compared to the observed numbers of micromoles of several amino acids including glutamic acid, aspartic acid, glycine, alanine, valine, isoleucine, and leucine. The number of residues of half-cystine and methionine per molecule of prealbumin were then estimated from the assigned values of the number of residues for the other amino acids.

The tryptophan content of prealbumin was estimated by determining the ratio of tyrosine to tryptophan according to the method of Goodwin and Morton (14), as described in detail previously (10). The tryptophan content was also determined according to the method of Barman and Koshland (15), as follows: 3.4 mg of prealbumin were dissolved in 1.0 ml of 10 M urea, pH 2.7, and incubated for 2 hours at 37°. 2-Hydroxy-5-nitrobenzylbromide, 5 mg, in dry acetone was then added, and the mixture was separated by gel filtration on Sephadex G-25. The 2-hydroxy-5-nitrobenzyl protein peak (first peak eluted from column) was pooled and precipitated with trichloracetic acid. The precipitate was washed twice with ethanol-HCl and dissolved in 1.0 ml of 11.2 N HCl. An aliquot (0.1 ml) was adjusted to pH 12.8 with 2 N NaOH and diluted to 1.0 ml and the absorbance was measured at 410 μμ. A blank sample containing water instead of protein was carried parallel through the entire procedure, and its absorbance at 410 μμ was subtracted from that of the sample.

Thyroxine Preparations—131I-Labeled thyroxine (60 μCi per μg) was obtained from Abbott and was purified just before use on a column (1 cm × 10 cm) of Sephadex LH-20 (Pharmacia) as described by Nauman, Nauman, and Werner (16). The purified 131I-thyroxine preparation was evaporated to dryness and dissolved in 0.01 N NaOH.

L-Thyroxine sodium pentahydrate was obtained from Mann. A stock solution of thyroxine (6 × 10−4 M) in 0.04 N NaOH was diluted to the desired thyroxine concentration in a final solution of 0.01 N NaOH. The concentration of the stock solution was verified by analysis for thyroxine which was kindly carried out by Dr. I. Radichevich according to the method of Nauman et al. (16).

Equilibrium Dialysis—The interaction of thyroxine with prealbumin was studied by the method of equilibrium dialysis. Dialysis bags were prepared from cellulose casing dialysis tubing (1 ½ inch flat diameter, obtained from Union Carbide) which had been treated with dilute nitric acid and washed with distilled water as described by Sterling, Rosen, and Tabachnik (17). Studies were conducted in two buffers: (a) 0.06 M phosphatc buffer, pH 7.4, and (b) a more "physiological" buffer of 0.14 M NaCl, 0.04 M KCl, and 0.01 M potassium phosphate, pH 7.4 (designated NaCl-P04 buffer). The solutions also contained EDTA, 7 × 10−4 M (17, 18).

In each study, a stock solution was prepared containing prealbumin, thyroxine-131I, buffer, and EDTA. An amount of dilute HC1 which would exactly neutralize the small amount of NaOH added with the thyroxine solutions was then added. To 1.8 ml of this stock solution, 0.2 ml of a specific thyroxine solution was added, and 1.5 ml of this mixture were placed inside a dialysis bag. The initial total thyroxine concentration inside the bags ranged from 1.6 × 10−3 to 4 × 10−4 M. The prealbumin concentration inside the bags ranged between 4.07 and 4.09 mg/100 ml. Each bag and its contents were placed in a polycollam tube, 1 inch × 3.5 inch (Spinco) containing 5 ml of buffer. The tubes were placed upright in a water bath incubator, and dialysis was performed by horizontal gentle shaking for 20 hours at either room temperature (24°) or at 37°. Preliminary experiments showed that complete equilibration of thyroxine between the inside and outside solutions, both in the presence and absence of protein, was achieved under these conditions. These equilibration experiments were carried out by comparing the results obtained with duplicate mixtures in which the labeled thyroxine was added either to the inside or to the outside solution.

After dialysis, 1-ml samples of both the inside and outside solutions were assayed for radioactivity in an autogamma spectrometer (model 3002, Packard). The total recovery of 131I was 98 to 100%. Of the recovered 131I, 3 to 5% was found adsorbed to the dialysis tubing; there was no significant binding by the walls of the polycollamer tubes. The relative amount of 131I not present as thyroxine at the end of the study was determined on selected samples by the addition of plasma to small portions of both the inside and outside solutions, followed by trichloracetic acid precipitation, as described by Oppenheimer et al. (19). 131I which was not trichloracetic acid-precipitable, presumably present as inorganic iodide, amounted to 2 to 4% of the total 131I in the system. Appropriate correction for this small amount of 131I was made, based on the assumption that there was no appreciable protein binding of iodide compared to thyroxine (18, 19).

The concentration of free and bound thyroxine in each inside solution was obtained as follows:

Concentration of free thyroxine = R × counts per min in 1 ml of outside solution
Concentration of bound thyroxine = R × counts per min in 1 ml of inside solution minus R × counts per min in 1 ml of outside solution
formed as described previously (10).

Three major peaks of protein were obtained. Peak A consisted mainly of $\gamma$-globulins, and Peak C of serum albumin. Ceruloplasmin was eluted in the latter portion of the descending limb of Peak C. The fractions comprising the smaller Peak D (cross-hatched area) were strongly fluorescent, and contained prealbumin and RBP as their major components.

where $R$ is the ratio of total moles of thyroxine added/total counts per min for each individual tube. The average number of thyroxine molecules bound per prealbumin molecule, $\tilde{v}$, was then determined by dividing the concentration of bound thyroxine by the concentration of prealbumin. For these calculations, the molecular weight of prealbumin was taken as 50,000 (see below).

Calculations—The binding data were analyzed by plotting $\tilde{v}/c$ against $\tilde{v}$ for each binding study (where $c$ is the molar concentration of free thyroxine). As indicated by Scatchard (20) and Scatchard, Scheinberg, and Armstrong (21), if the protein under study contains only a single class of identical binding sites this plot should describe a straight line according to the equation $\tilde{v}/c = kn - k\tilde{v}$ (where $k$ is the apparent association constant for each binding site and $n$ is the number of sites with association constant $k$). If the protein contains more than one class of binding sites this plot will describe a curve which is concave upward.

For each binding study the data were subjected to regression analysis, and the line which best fitted the data was computed by the method of least squares (22). In each case this analysis provided the values for the $y$-intercept ($kn$), the slope of the line ($-k$), and the correlation coefficient ($r$).

Other Procedures—Double diffusion in gel was performed by the method of Ouchterlony (23), with gels made of 0.7% agar in sodium barbiturate-glycine buffer, pH 7.6. Commercial rabbit antisera against whole human serum, prealbumin, albumin, ceruloplasmin, and $\alpha_1$-acid glycoprotein were obtained from Behringwerke (Hoechst Pharmaceutical Company, Kansas City, Missouri).

Analytical polyacrylamide disc gel electrophoresis was performed as described previously (10).

Absorption spectra and absorbances were usually measured in a Beckman DB spectrophotometer. In some instances, spectra were obtained with a Cary model 14 spectrophotometer.

The extinction coefficient ($E_{280}^1$) at 280 $\mu m$ was determined on a sample of lyophilized prealbumin which had been additionally dried overnight over P$_2$O$_5$, and then weighed and dissolved in water. The protein concentration of the same solution was determined by the method of Lowry et al. (24), with bovine serum albumin as a standard, and the result was used to obtain another estimate for the value of $E_{280}^1$.

Protein concentrations were then estimated from optical density measurements at 280 $\mu m$.

RESULTS

Isolation of Plasma Prealbumin

Plasma prealbumin was purified by a sequence of procedures similar to those previously described for the purification of retinol-binding protein (10), except for the elimination of Cohn fractionation as the initial procedure. In the first step, as used here, concentrated plasma was fractionated on a DEAE-Sephadex column with the resulting typical elution pattern shown in Fig. 1. Representative fractions from the entire elution pattern were examined by analytical disc gel electrophoresis. Proteins with anodic electrophoretic mobility greater than that of albumin were detected only in some of the fractions comprising Peak C and in the fractions comprising Peak D. The protein present in Peak C migrated only slightly in front of albumin; this protein was subsequently purified (see below) and shown to be identical with the plasma $\alpha_1$-acid glycoprotein. The more rapidly migrating plasma prealbumin was exclusively localized in Peak D, which also contained all of the RBP.

The fractions comprising Peak D were pooled and the prealbumin was purified further by the following sequence of procedures: gel filtration on Sephadex G-200; rechromatography on DEAE-Sephadex; preparative polyacrylamide gel electrophoresis; and gel filtration on Sephadex G-100. The behavior of prealbumin during each column chromatography was the same as reported in detail previously (10). The elution patterns obtained on repeat DEAE-Sephadex chromatography, on preparative polyacrylamide gel electrophoresis, and on the final gel filtration step with Sephadex G-100 were identical with those shown in Figures 3, 7, and 12, respectively, of Reference 10. The final purified prealbumin preparation showed a single band on analytical disc gel electrophoresis. It was estimated that the final preparation contained prealbumin purified approximately 250- to 400-fold as compared to whole plasma; the net recovery of prealbumin after purification was of the order of 50%.

Prealbumin

Sedimentation Velocity and Molecular Weight—Sedimentation velocity studies showed that prealbumin migrated as a single homogeneous protein with a sedimentation constant ($s_{20,\text{w}}$) of 3.70 S (for a 0.68 mg per ml solution). An estimate of the molecular weight from this $s_{20,\text{w}}$ value, with the empirical correlation

\[ s_{20,\text{w}} \approx \frac{1.62 \times 10^{-4} \times M_w^{1.3}}{1 + 1.30 \times 10^{-4} \times M_w} \]

\[ M_w \approx \frac{1.62 \times 10^{-4} \times s_{20,\text{w}}^2}{1 + 1.30 \times 10^{-4} \times s_{20,\text{w}}} \]

8. The concentration of prealbumin has been estimated to be approximately 20 to 30 mg/100 ml of plasma (1, 3, 25), comprising roughly 0.25 to 0.4% of the total plasma proteins. Hence the final purified prealbumin preparation can be estimated to have been purified 250 to 400 times as compared to whole plasma. The yield of purified prealbumin was usually 12 to 15 mg for each 100 ml of plasma which was processed, hence representing a recovery of the order of 50%.
described by Paetkau and Lardy (26), yielded a value of 47,000. With this value, the ideal speed and the time needed to reach equilibrium were estimated to be 30,000 rpm and 8 hours, respectively. Fig. 2 shows the results of a sedimentation equilibrium analysis after centrifugation at this speed for 20 hours. The prealbumin appeared to be homogeneous, as can be judged from the linearity of the data, with a molecular weight of approximately 49,400.

**Immunodiffusion**—Purified prealbumin did not react with commercial antiserum against human serum albumin, ceruloplasmin, or α1-acid glycoprotein. Prealbumin gave a single precipitin line against commercial antiprealbumin antiserum, and this line showed a reaction of identity with the line obtained by reacting whole human plasma against the same antiserum (Fig. 3). A single precipitin line was also obtained by testing prealbumin against an antiserum to whole plasma, and this line showed a reaction of identity with the line obtained against antiprealbumin antiserum.

**Amino Acid Composition**—The results of the amino acid analysis of prealbumin are given in Table I. The least abundant amino acid which was not destroyed during the standard analysis was arginine, which comprised 3.09% of the total amino acids (on a micromolar basis). The molecular weight of prealbumin calculated from the amino acid composition was closest to the value obtained by analytical ultracentrifuge analysis by assigning a value of 14.0 for the number of arginine residues per prealbumin molecule. The final column in Table I lists the estimated number of residues for each amino acid per molecule of prealbumin. The molecular weight calculated from these values is 50,400 to 50,800. The half-cystine and methionine contents were estimated by performic acid oxidation to be 3.96 residues (assuming 90% recovery of cysteic acid (13)), and 2.24 residues, respectively, per molecule of prealbumin. The probable number of residues of half-cystine and methionine is therefore 4 and 2 to 3, respectively.

The molar ratio of tyrosine to tryptophan as determined spectrophotometrically (14) was 2.35. This indicated that prealbumin contains approximately 8 tryptophan residues per molecule, since the amino acid analysis indicated the presence of 18 tyrosines per molecule. Chemical determination of tryptophan by the 2-hydroxy-5-nitrobenzylbromide color reaction (15) yielded a value of 6.83 residues per molecule of prealbumin. The number of tryptophan residues per molecule is probably 7 or 8. On a weight basis, tyrosine comprises approximately 5.54%, and tryptophan 2.54 to 2.76%, of the weight of the prealbumin.

**Extinction Coefficient**—The extinction coefficient (ε_1%_cm) of prealbumin at 280 nm was determined on a solution of concentration 0.50 mg per ml by weight; the value obtained was 14.13. Determination of the protein concentration of the same solution by the method of Lowry et al. (24) yielded an ε_1%_cm of 12.44. Dividing this value by 0.89, as previously suggested by Oppenheimer et al. (6) in order to correct for the effects of the relatively high tryptophan and tyrosine contents of prealbumin, yielded a corrected ε_1%_cm of 14.06. For the equilibrium dialysis studies a value of 14.1 was used for the ε_1%_cm.

**Complex Formation with RBP**—The capacity of the prealbumin preparation to form complexes with RBP was determined as described previously (10). The maximal binding capacity of
Amino acid composition of prealbumin

Five equal sized samples (~1 mg each) of prealbumin were analyzed: two for 24 hours, one for 48 hours, and two for 72 hours. The results of the duplicate 24- and 72-hour analyses were averaged, and the average values are tabulated below in the first and third columns.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Observed 24 hrs</th>
<th>Observed 48 hrs</th>
<th>Observed 72 hrs</th>
<th>Corrected distribution</th>
<th>Amount of each amino acid relative to arginine</th>
<th>Estimated no. of residues per prealbumin molecule</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histidine</td>
<td>6.30</td>
<td>7.75</td>
<td>6.40</td>
<td>3.37</td>
<td>15.3</td>
<td>15</td>
</tr>
<tr>
<td>Arginine</td>
<td>6.43</td>
<td>5.75</td>
<td>6.45</td>
<td>3.09</td>
<td>14.0</td>
<td>14</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>13.99</td>
<td>15.51</td>
<td>14.38</td>
<td>7.26</td>
<td>32.9</td>
<td>33</td>
</tr>
<tr>
<td>Threonine</td>
<td>18.51</td>
<td>18.36</td>
<td>16.09</td>
<td>9.34</td>
<td>42.3</td>
<td>42</td>
</tr>
<tr>
<td>Serine</td>
<td>16.60</td>
<td>15.46</td>
<td>13.24</td>
<td>8.96</td>
<td>40.1</td>
<td>40</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>20.74</td>
<td>21.35</td>
<td>19.72</td>
<td>10.22</td>
<td>46.3</td>
<td>46</td>
</tr>
<tr>
<td>Proline</td>
<td>16.43</td>
<td>15.42</td>
<td>15.57</td>
<td>8.04</td>
<td>36.4</td>
<td>36-37</td>
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<tr>
<td>Glycine</td>
<td>20.09</td>
<td>20.51</td>
<td>19.06</td>
<td>9.57</td>
<td>44.7</td>
<td>45</td>
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<tr>
<td>Alanine</td>
<td>17.86</td>
<td>18.92</td>
<td>17.50</td>
<td>8.99</td>
<td>40.7</td>
<td>41</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>8.12</td>
<td>8.39</td>
<td>7.74</td>
<td>4.01</td>
<td>18.2</td>
<td>18</td>
</tr>
<tr>
<td>Leucine</td>
<td>11.31</td>
<td>11.69</td>
<td>10.74</td>
<td>5.57</td>
<td>25.2</td>
<td>25</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>7.88</td>
<td>8.15</td>
<td>7.61</td>
<td>3.91</td>
<td>17.7</td>
<td>18</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>7.36</td>
<td>8.00</td>
<td>8.23</td>
<td>4.25</td>
<td>19.3</td>
<td>19</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>6.45</td>
<td>6.40</td>
<td>6.40</td>
<td>3.37</td>
<td>15.3</td>
<td>15</td>
</tr>
<tr>
<td>Total</td>
<td>198.96</td>
<td>203.20</td>
<td>189.35</td>
<td>100.00</td>
<td>465-468</td>
<td></td>
</tr>
</tbody>
</table>

* Half-cystine, methionine, and tryptophan were noted to be present, but because of their being present in small amounts or partial destruction (or both) during the acidic hydrolysis, these amino acids could not be quantitatively assayed. Quantitative determinations of these three amino acids were carried out on separate portions of prealbumin (see text), and the estimated number of residues of each amino acid per prealbumin molecule is listed in the last column.

Table I presents the values of n and k as computed from regression analysis of each set of data. The clearly linear relationship between \( \frac{v}{c} \) and \( \bar{v} \) in each study is confirmed by the high values observed for the correlation coefficients (0.95 to 0.97). For the study at 24° in phosphate buffer alone, the association constant \( k \) was computed as 1.5 × 10^4. An almost identical value was obtained for the study carried out at 37° in NaCl-PO4 buffer. This indicates that chloride ions have very little effect on the binding of thyroxine to plasma prealbumin. A slightly lower value of \( k \) (1.3 × 10^4) was obtained for the study carried strongly with RBP, and had the same maximal binding capacity for RBP (at a molar ratio, prealbumin to RBP, of approximately 1:1). Based on these results, the prealbumin appeared to be identical in the two preparations.

Comparison with Prealbumin Prepared by Porath Column Electrophoresis

Some of the properties of the prealbumin preparation were compared with those of a sample of prealbumin obtained by Porath column electrophoresis, under conditions as described elsewhere (27). The latter sample was kindly provided by Drs. H. Massari and S. C. Werner, and was shown by analytical disc gel electrophoresis to be almost pure, with a small trace of albumin. A mixture of the two prealbumin preparations migrated as a single band on analytical disc gel electrophoresis, and was eluted as a single component after gel filtration on Sephadex G-100. In addition, both preparations were able to complex prealbumin for RBP was identical with that seen before (10). It should be noted that, in our earlier work (10), a smaller proportion of the total plasma prealbumin was isolated during the course of RBP purification, since a significant part of the total prealbumin, mainly the prealbumin not joined in complex with RBP, was lost during the initial Cohn fractionation. The finding that the present and previous prealbumin preparations had identical binding capacities for RBP (at a molar ratio, prealbumin to RBP, of approximately 1:1). Based on these results, the prealbumin appeared to be identical in the two preparations.

Binding of Thyroxine to Prealbumin

Figs. 4 and 5 show the results of the equilibrium dialysis studies of the binding of thyroxine to prealbumin alone. Three studies were carried out: one in phosphate buffer alone, at 24° (Fig. 4), and two in NaCl-PO4 buffer, at 24° and at 37° (Fig. 5). In each of the three studies the plot of \( \frac{v}{c} \) against \( \bar{v} \) described a straight line with the x-intercept close to 1.0. The data therefore show that prealbumin contains a single binding site for thyroxine.

Table II presents the values of n and k as computed from regression analysis of each set of data. The clearly linear relationship between \( \frac{v}{c} \) and \( \bar{v} \) in each study is confirmed by the high values observed for the correlation coefficients (0.95 to 0.97). For the study at 24° in phosphate buffer alone, the association constant \( k \) was computed as 1.5 × 10^4. An almost identical value was obtained for the study carried out at 37° in NaCl-PO4 buffer. This indicates that chloride ions have very little effect on the binding of thyroxine to plasma prealbumin. A slightly lower value of \( k \) (1.3 × 10^4) was obtained for the study carried strongly with RBP, and had the same maximal binding capacity for RBP (at a molar ratio, prealbumin to RBP, of approximately 1:1). Based on these results, the prealbumin appeared to be identical in the two preparations.

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FIG. 4. The interaction of thyroxine with prealbumin, in phosphate buffer alone at 24° (see text for further details).

FIG. 5. The interaction of thyroxine with prealbumin, in NaCl-PO₄ buffer at 24° and at 37° (see text for further details).

TABLE II
Interaction of thyroxine with plasma prealbumin

<table>
<thead>
<tr>
<th>Protein</th>
<th>Buffer</th>
<th>Temperature</th>
<th>n</th>
<th>k</th>
<th>r</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prealbumin</td>
<td>Phosphate</td>
<td>24°</td>
<td>0.96</td>
<td>1.5 X 10⁶</td>
<td>0.97</td>
</tr>
<tr>
<td>Prealbumin</td>
<td>NaCl-PO₄</td>
<td>24°</td>
<td>1.00</td>
<td>1.6 X 10⁶</td>
<td>0.95</td>
</tr>
<tr>
<td>Prealbumin</td>
<td>NaCl-PO₄</td>
<td>37°</td>
<td>0.84</td>
<td>1.3 X 10⁶</td>
<td>0.96</td>
</tr>
<tr>
<td>Prealbumin + RBP</td>
<td>NaCl-PO₄</td>
<td>24°</td>
<td>0.86</td>
<td>2.0 X 10⁶</td>
<td>0.96</td>
</tr>
</tbody>
</table>
ported (10) there was no complex formation observed between the protein and thyroxine when studied by equilibrium dialysis under the same conditions described above. Furthermore, as previously reported (10) there was no complex formation observed between this protein and RBP.

FIG. 6. The interaction of thyroxine with the prealbumin-RBP complex (see text for further details).

DISCUSSION

It is now well established that thyroxine circulates in plasma in association with three plasma proteins, thyroxine-binding globulin, prealbumin, and albumin (1, 2). Of the three proteins, thyroxine-binding globulin has the highest affinity for thyroxine, and under normal conditions most of the plasma thyroxine circulates bound to thyroxine-binding globulin. The association constant for the interaction of thyroxine with thyroxine-binding globulin has been estimated indirectly to be of the order of $10^9$ (4, 5). Indirect estimates have also been derived for the affinity of prealbumin for thyroxine. Thus, Oppenheimer and Surks (4) estimated the association constant of prealbumin for thyroxine as $3.6 \times 10^9$, and Woeber and Ingbar (5) as $2.3 \times 10^9$. These estimates were based upon assumptions about the quantitative distribution of endogenous plasma thyroxine among the binding proteins. As discussed by Woeber and Ingbar (5), however, considerable uncertainty exists as to the precise quantitative role of prealbumin in the overall binding of thyroxine in plasma. Estimates of the proportion of endogenous thyroxine normally bound to prealbumin have varied widely; recent estimates have been in the range of 30% (1) or 15% (5).

Direct quantitative studies of the interaction of thyroxine with human serum albumin have been carried out by the method of equilibrium dialysis (17, 18, 30) or by fluorescence quenching (30). The results of these studies all indicate that albumin contains a single strong binding site for thyroxine, together with several sites with weaker affinity. The apparent association constant of the single strong site on albumin for thyroxine has been reported to be $5 \times 10^9$ (17), $1.4 \times 10^9$ (18), and $1.6 \times 10^9$ (19). It has also been reported (18) that chloride ions reduce the affinity of albumin for thyroxine by about 50%, as compared to the affinity in phosphate buffer alone.

In the experiments reported here, the interaction of purified plasma prealbumin with thyroxine was studied directly by the method of equilibrium dialysis. These studies showed that prealbumin contains a single binding site for thyroxine. The apparent association constant for the binding of thyroxine to prealbumin at 24° in "physiological" buffer (0.14M NaCl in 0.01M phosphate buffer) was calculated to be $1.6 \times 10^9$. The affinity of prealbumin for thyroxine was very slightly reduced when the experiments were repeated at 37°. Chloride ions did not significantly affect the affinity of prealbumin for thyroxine. It should, of course, be noted that the apparent association constants derived here are valid for the particular experimental conditions used, but differ from the so-called intrinsic association constants by a number of factors which deal with such matters as the effects of electrostatic interactions and competition with other ions and molecules. These factors have been discussed in detail previously for the interaction of human serum albumin with fatty acid anions (31).

The value of the association constant derived from the present experiments is approximately an order of magnitude less than the previously obtained indirect estimates of others. The reasons for this discrepancy are not apparent, but may reflect inaccuracies in the assumptions upon which the indirect estimates depend, or possibly unrecognized effects of the purification procedures used in the isolation of plasma prealbumin.

The prealbumin studied in the present experiments was isolated from whole plasma by a sequence of procedures which included chromatography on DEAE-Sephadex and Sephadex G-200, followed by preparative polyacrylamide gel electrophoresis. Prealbumin was obtained in an over-all yield of approximately 50%. The protein appeared to be a single pure component as judged by polyacrylamide disc gel electrophoresis, immunodiffusion, and analytical ultracentrifugation. The latter technique showed the presence of a single homogenous component with a sedimentation constant of 3.7 S and a molecular weight of about 49,400. These values are lower than those previously reported for prealbumin preparations isolated in other laboratories. Previous estimates have ranged between 3.8 and 4.6 for $s_{20,w}$ and 61,000 and 73,000 for the molecular weight (6-9, 32). No explanation is available for the lower molecular weight and sedimentation velocity values observed in the present study. The molecular weight estimate of about 50,000 for our preparation is, however, supported by the results of a recent study in our laboratory of the prealbumin-RBP complex. In this study (to be reported in detail elsewhere) the prealbumin-RBP complex (molar ratio 1:1) behaved on analytical ultracen-
trifugation as a single component with an $s_{20,w}$ value of 4.6 S and a molecular weight of approximately 70,000 to 71,000. Since the molecular weight of RBP is approximately 21,000 (10), the molecular weight of the prealbumin-RBP complex is consistent with a molecular weight of about 50,000 for prealbumin. Furthermore, the Scatchard plots of the binding of thyroxine to prealbumin (Figs. 4 and 5) gave values of $n$ near 1.0 (mean 0.93) when the prealbumin molecular weight was taken as 50,000. A mean value of exactly 1.0 for $n$ would have been obtained if the calculations had used a molecular weight of 64,000 for prealbumin. It is tempting to speculate that some of the previous prealbumin preparations may have been contaminated with RBP and hence contained, in part, the prealbumin-RBP complex.

The relative distribution of amino acids in prealbumin reported here is fairly similar to that reported by Schultz et al. (8), except for the fact that these authors did not detect half-cystine in their prealbumin preparation. The least abundant amino acids in prealbumin are half-cystine (4 residues per molecule) and methionine (2 to 3 residues per molecule). Prealbumin is relatively rich in tryptophan, containing 7 to 8 residues per molecule, equivalent to approximately 2.6 to 2.7% by weight. This value is similar to the 2.6% tryptophan content reported by Schultz et al. (8), but lower than the 3.15% reported by Oppenheimer et al. (6). Prealbumin also contains about 18 tryosine residues per molecule (6.8% by weight).

The observed extinction coefficient ($E_{1%}^{1%}$) at 280 $\mu$m of 14.1 was slightly higher than previously reported values (13.6 (6) and 12.2 (9)). It should be noted that RBP has a higher tyrosine and tryptophan content and a higher $E_{1%}^{1%}$ than does prealbumin (10).

The major goal of the studies reported here was to examine the effect of the interaction of prealbumin with RBP on its interaction with thyroxine. The interaction of prealbumin with RBP appeared to be independent of its interaction with thyroxine. Thus, the binding capacity and affinity of prealbumin for thyroxine were similar both in the presence and absence of RBP. These findings indicated that the formation of the prealbumin-RBP complex did not interfere with the ability of prealbumin to interact with thyroxine. Moreover, the addition of enough thyroxine to saturate prealbumin did not impair the ability of the prealbumin to interact with RBP. The studies showed that the prealbumin molecule could simultaneously interact with 1 molecule of thyroxine and with 1 of RBP. It thus appears that the protein-protein interaction between prealbumin and RBP involves a site on the prealbumin molecule which is separate and different from the site involved in the prealbumin-thyroxine interaction.

The interaction of prealbumin with RBP serves an important physiological function by preventing the glomerular filtration of the relatively small RBP molecule and hence the loss of RBP and vitamin A in the urine (10). Under normal circumstances, probably one-third or more of the plasma prealbumin molecules circulate in the form of a complex with RBP, since the usual concentration of RBP has been estimated as 3 to 4 mg/100 ml (10) and that of prealbumin as roughly 20 to 30 mg/100 ml (1, 3, 25). In contrast, less than 1% of the total plasma prealbumin circulates in the form of a complex with thyroxine (5). It would thus appear that the commonly used term, thyroxine-binding prealbumin, may not be the most appropriate designation for this protein.

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