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Binding of Thyroxine and Thyroxine Analogs to Human Serum Prealbumin†

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ABSTRACT: The interaction of human serum prealbumin with thyroxine and thyroxine analogs was examined by equilibrium dialysis and ultraviolet absorption. Iodothyronines had a single binding site with the following association constants at pH 7.4, 0.1 M NaCl, 25°: L-thyroxine: 1.3×10^8 M^{-1}; 3,5,3′-triiodo-L-thyronine: 1.2×10^7 M^{-1}; 3,5-diiodo-3′,5′-dinitro-L-thyronine: $\geq 10^7$ M^{-1}. Iodotyrosine analogs were studied at pH 8.6, 0.1 M NaCl, 25°, and had the following values: 4-hydroxy-3,5-diiodobenzaldehyde: $n = 2$, $K = 5.5 \times 10^7$ M^{-1}; 3-(4-hydroxy-3,5-diiodophenyl)propionic acid: $n = 1$, $K = 2.5 \times 10^8$ M^{-1}; 4-hydroxy-3,5-diiodocinnamic acid: $n = 1$. The intensities of the circular dichroic bands due to bound ligands were consistent with one binding site for thyroxine and two for 4-hydroxy-3,5-diiodobenzaldehyde. The findings showed that thyroxine is bound more strongly to prealbumin than recently reported, that triiodothyronine is bound somewhat less strongly, and that iodotyrosine analogs are bound with similar affinities to either one or two sites. The spectra of thyroxine and the various analogs showed red shifts in 50% dioxane–water mixtures. When bound to human serum prealbumin they showed blue shifts except for 3,5-diiodo-3′,5′-dinitro-L-thyronine which showed a red shift.

Thyroxine-binding prealbumin is a serum protein with a molecular weight of 54,000 which is composed of four apparently identical subunits (Branch *et al.*, 1971). The conformation of the polypeptide chain has been determined recently by X-ray studies and the amino acid sequence and residue positions should be known soon (Blake *et al.*, 1971; Morgan *et al.*, 1971; Gonzales and Offord, 1971). Prealbumin is of considerable biological interest in that it transports both a hormone and a vitamin in serum. Thyroxine (T_4)[1] is bound directly to prealbumin whereas retinol (vitamin A) is bound by virtue of the binding of its protein carrier, retinol binding protein (Kanai *et al.*, 1968). The binding of hormone and protein is independent and therefore prealbumin must contain five binding sites for these two ligands since it binds 1 mol of thyroxine and 4 of protein (van Jaarsveld *et al.*, 1973a).

The interaction between T_4 and human prealbumin has been studied recently in two laboratories. Raz and Goodman (1969) measured the binding by equilibrium dialysis at pH 7.4 and reported a single site for T_4 with an association constant of 1.6×10^7 M^{-1}. Nilsson and Peterson (1971) using the rate of dialysis method of Colowick and Womack (1969) and the quenching of tryptophanyl fluorescence reported a single, strong binding site at pH 7.4 with $K = 1.1 \times 10^7$ M^{-1}, and three sites of much lower affinity.

In studies seeking to determine the functional groups of T_4 which are responsible for its high free energy of binding to

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[1] The following abbreviations are used: T_4, thyroxine, L form; T_3, triiodothyronine, L form.

prealbumin, it was observed that T_4 was bound much more strongly than reported previously with purified prealbumin. It has also been observed that prealbumin can bind ligands in two modes, exhibiting either a single or two identical sites. This report presents a reevaluation of the binding constant of T_4 as well as the stoichiometry and binding constants of several analogs.

Methods and Materials

Human prealbumin was obtained from Behring Diagnostics and purified by preparative polyacrylamide gel electrophoresis as described previously (Branch *et al.*, 1971). Additional samples of prealbumin were prepared from fresh human serum by chromatography on DEAE-Sephadex A-50 followed by preparative polyacrylamide gel electrophoresis (van Jaarsveld *et al.*, 1973a). Prealbumin concentrations were determined spectrophotometrically at 280 nm ($E^{1\%}_{1\,cm}$ = 14.1) (Raz and Goodman, 1969).

L-Thyroxine and 3,5,3′-L-triiodothyronine (T_3) were obtained from Calbiochem. Purity was confirmed by gas–liquid partition chromatography (glpc) (Funakoshi and Cahnmann, 1969). 4-Hydroxy-3,5-diiodobenzaldehyde, 3-(4-hydroxy-3,5-diiodophenyl)propionic acid, and 4-hydroxy-3,5-diiodocinnamic acid were prepared by the method of Matsuura and Cahnmann (1959) and their purity was confirmed by glpc as above. 3,5-Diiodo-3′,5′-dinitro-L-thyronine was a gift from Glaxo Laboratories, Ltd. The dinitro compound was recrystallized as described by Barnes *et al.* (1953). Purity was confirmed by glpc and also by thin-layer chromatography (tlc) on silica gel in ethyl acetate–pyridine–acetic acid–H_2O (5:5:1:3).

^{125}I-Labeled T_4 and T_3 (in 50% propylene glycol) were purchased from Abbott Laboratories. Their purity was checked by tlc and autoradiography as described by Ogawara and Cahnmann (1972). Iodide was usually the only contaminant. Further purification, if required, was performed by column chromatography on Dowex 50 described by Cahnmann (1972).

4-Hydroxy-3,5-[^{125}I]diiodobenzaldehyde. ICl was exchange labeled by adding 1.5 mCi of carrier-free $^{125}I^-$ to 0.2 ml of a 1.2 mM solution of ICl in 1 M acetic acid and allowing the solution to stand at room temperature for 1 hr. Ten microliters of a 10 mM solution of 4-hydroxybenzaldehyde in glacial acetic acid was then added and the reaction was allowed to proceed overnight at room temperature. Excess ICl was destroyed by addition of saturated aqueous SO_2. The solution was applied to a 1.5 × 15 cm column of Sephadex LH-20 previously equilibrated with 0.1 M ammonium acetate (pH 6.0). Two-ml fractions eluted with the same solvent were collected at a flow rate of 1 ml/min. Following elution of $^{125}I^-$, the eluent was changed to 50% methanol. When radioactivity started to emerge from the column the eluent was changed to 10 mM NH_4OH in 50% methanol. 4-Hydroxy-3,5-[^{125}I]diiodobenzaldehyde was eluted followed by 4-hydroxy-3-[^{125}I]iodobenzaldehyde. Fractions were analyzed by tlc with CH_2Cl_2 and autoradiography. Fractions containing only 4-hydroxy-3,5-[^{125}I]-diiodobenzaldehyde were pooled, lyophilized, and stored at 4° in 50% methanol containing 0.1% 2-mercaptoethanol.

3-(4-Hydroxy-3,5-[^{125}I]diiodophenyl)propionic Acid. This compound was prepared by the iodination method described in the preceding paragraph starting with 3-(4-hydroxy)phenylpropionic acid. Eluates from the Sephadex LH-20 column, when examined by tlc in $CHCl_3$–methanol (19:1) and benzene–dioxane–acetic acid (90:25:4), showed contamination by $^{125}I^-$ and a second radioactive impurity. Further purification was achieved on a 1.5 × 16 cm column of QAE-Sephadex A-25 equilibrated with 50% methanol. Following application of the compound, the column was washed with one volume of 10 mM NH_4OH in 50% methanol, followed by two volumes of 2 mM acetic acid in 50% methanol, and finally with 20 mM acetic acid in 50% methanol. Fractions containing only 3-(4-hydroxy-3,5-[^{125}I]diiodophenyl)propionic acid were pooled, lyophilized, and stored in 50% methanol containing 0.1% 2-mercaptoethanol.

Equilibrium Dialysis. The method used was modeled after that of Raz and Goodman (1969) with several modifications. Dialysis bags were prepared from no. 20 cellulose dialysis tubing (Union Carbide) which had been boiled for 30 min in 0.2 M Na_2CO_3–1 mM EDTA and washed extensively with deionized, distilled water. The dialysis bags, filled with 1.5 ml of the protein solution, were placed in either cellulose nitrate tubes (Beckman No. 302236) or polyallomer tubes (Beckman No. 326823) containing 5 ml of the appropriate buffer (see below). The tubes were covered and shaken in a Dubnoff metabolic shaker at room temperature (25 ± 1°) for 22–24 hr.

Preliminary experiments performed both in the presence and absence of protein showed that complete equilibrium of T_4 was achieved under these conditions. Adsorption of T_4 to the dialysis bags and to the cellulose nitrate or polyallomer tubes was negligible since 97–102% of the radioactivity was recovered. The relative amount of radioactivity present as $^{125}I^-$ at the end of the dialysis in the T_4 and T_3 experiments was determined by the method of Sterling and Brenner (1966) and amounted to 1–3.5%. Correction of the ligand binding data was based on the assumption that there was no appreciable binding of I^- to prealbumin.

Two procedures for the dialysis experiments, in which ligand was initially present either inside or outside the dialysis bag, gave comparable results. In a given series of experiments the concentration of prealbumin (ranging from 0.4 to 0.9 μM) inside the dialysis bags was constant.

After dialysis, 1-ml aliquots of both the inside and outside solutions were counted in a well-type scintillation counter. Calculations of the concentration of thyroxine in both solutions and other computations were carried out as described by Raz and Goodman (1969).

Other Methods. The concentration of the iodinated compounds were determined from the absorption of stock solutions which were made up in 0.01 M NaOH. The molar extinction coefficients are given in Table I except for T_3 which had a value of 4660 at 320 nm (Cahnmann, 1972).

Spectra and absorption were measured in a Cary 14 spectrophotometer. Difference absorption was obtained by using four cuvets, two for the reference and two for the sample, *i.e.*, the tandem cell technique.

Two buffers were used depending on the pH of the experiment: (a) pH 7.4, 0.05 M potassium phosphate–0.1 M NaCl–1 mM EDTA, and (b) pH 8.6, 0.05 M Tris-HCl–0.1 M NaCl–1 mM EDTA. Stock solutions of the iodinated compounds were prepared in 0.01 M KOH or NaOH.

Results

Iodothyronines. THYROXINE AND TRIIODOTHYRONINE. The binding of T_4 was measured by equilibrium dialysis at pH 7.4 and 0.05 M phosphate–0.10 M NaCl–1 mM EDTA at 25 ± 1°. The data are plotted in Figure 1 according to the Scatchard form of the mass action equation. The slope and intercept were determined by the method of least squares and give one

TABLE I: Wavelength Maxima (λ) and Molar Extinction Coefficients (ε).

Compound	H_2O[a]		50% Dioxane[b]		Prealbumin Complex		50% Dioxane–H_2O Δλ	Prealbumin Complex–H_2O Δλ
	λ (nm)	ε	λ (nm)	ε	λ	ε		
4-Hydroxy-3,5-diiodobenzaldehyde	342	20,200	348	22,300	336	19,800[c]	+6	−6
4-Hydroxy-3,5-diiodocinnamic acid	328	20,900	331	21,200	316	20,900[c]	+3	−12
3-(4-Hydroxy-3,5-diiodophenyl)propionic acid	310	5,500	316	5,930	308	6,100[c]	+6	−2
L-Thyroxine	325	6,180	331	6,370	323	6,300[c]	+6	−2
3,5-Diiodo-3′,5′-dinitro-L-thyronine	466	6,600	482	7,600	482	9,400[d]	+16	+16

[a] 10 mM NaOH. [b] 50% dioxane (v/v)–5 mM NaOH. [c] 0.05 M Tris–0.1 M NaCl–1 mM EDTA (pH 8.6). [d] 0.05 M phosphate–0.1 M NaCl–1 mM EDTA (pH 7.4).

binding site with an association constant of 1.3×10^8 M^{-1} (Figure 1). Equilibrium dialysis experiments were also performed with T_3. The same number of binding sites was found ($n = 1.05$) but the affinity was 10-fold less than obtained with T_4, *i.e.*, 1.2×10^7 M^{-1} (Figure 2).

The absorption spectrum of bound T_4 was determined at high ratios of protein to T_4 since changes in absorption can be used to measure binding if they are sufficiently intense. The bound form of T_4, compared to free T_4 at pH 8.6, showed only a very small blue shift in peak absorption, a slightly smaller band width at half the peak height and almost no change in molar extinction coefficient of the absorption maximum (Table I).

3,5-Diiodo-3′,5′-dinitro-L-thyronine. In this compound two polar nitro groups replace the two relatively nonpolar iodo groups in the phenolic ring of T_4. The stoichiometry of binding was determined by utilizing the intensification and the 16-nm red shift of the absorption spectrum of this compound when it binds to prealbumin (Table I). The maximum of the *difference* spectrum was at 494 nm and its molar extinction coefficient was 3300 (Figure 3). The increase in difference absorption with increasing concentrations of analog at pH 7.4, 0.05 M phosphate–0.10 M KCl–1 mM EDTA, 25 ± 1°, is shown in Figure 4. The difference absorption increased linearly between 0 and 0.90 mole ratio of analog to prealbumin and then remained constant at higher mole ratios. The combining ratio is therefore the same as for T_4 and T_3 unless a second molecule of this analog is bound without any change in absorption. The calculated curve for a binding constant of 10^6 is significantly different from the experimental data. The data fit theoretical curves with binding constants of 10^7 or greater. Lower protein concentrations could not be used conveniently since the total difference absorption change becomes too small to measure the binding constant precisely.

Iodotyrosine Analogs. To determine whether the iodothyronine moiety was necessary for binding, several analogs of iodotyrosine were evaluated. In order to work in a pH region

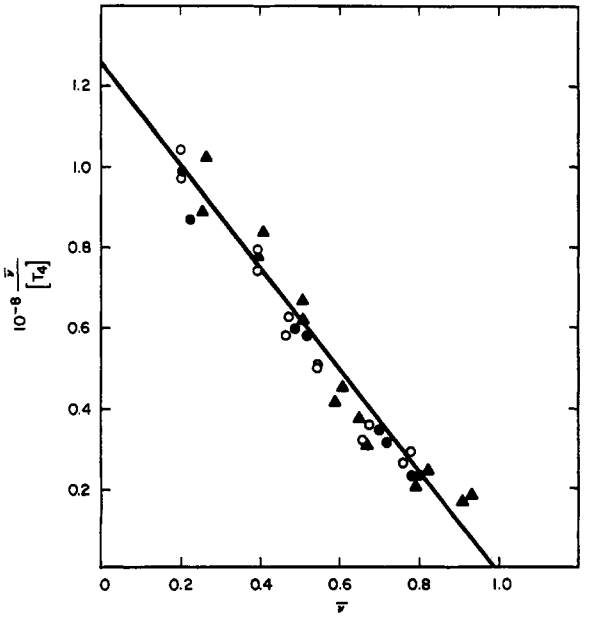

FIGURE 1: Scatchard plot of the binding of T_4 to prealbumin. Data obtained by equilibrium dialysis. Solvent: pH 7.4, 0.05 M phosphate–0.10 M NaCl–1.0 mM EDTA; $T = 25 \pm 1°$. Prealbumin concentration was between 0.38 and 0.45 μM. Different symbols represent different series of experiments.

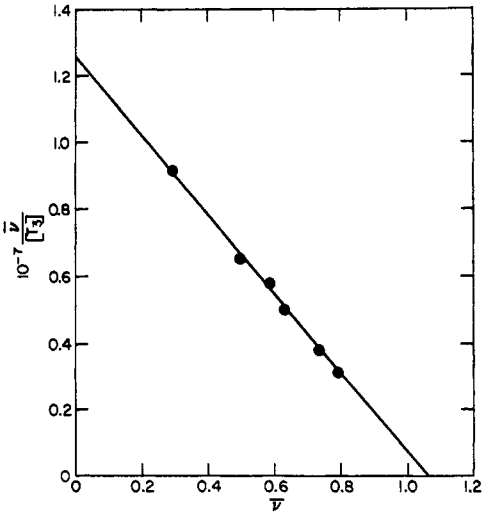

FIGURE 2: Scatchard plot of the binding of T_3 to prealbumin (0.77 μM). Data obtained by equilibrium dialysis. Solvent: pH 7.4, 0.05 M phosphate–0.1 M NaCl–1.0 mM EDTA; $T = 25 \pm 1°$.

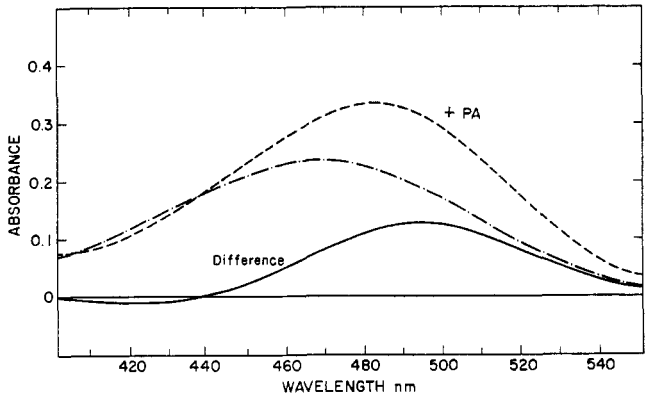

FIGURE 3: Absorption spectra of 3,5-diiodo-3′,5′-dinitro-L-thyronine (36 μM) in the presence (---) and absence (- · · -) of prealbumin (56 μM) and the resulting difference spectrum (—). Solvent: pH 7.4, 0.05 M phosphate–0.10 M NaCl–1 mM EDTA; $T = 25 \pm 1°$.

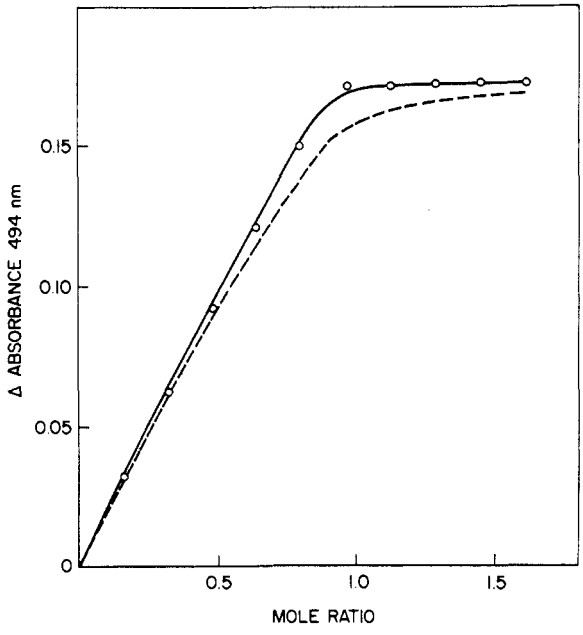

FIGURE 4: Titration of prealbumin (58 μM) with 3,5-diiodo-3′,5′-dinitro-L-thyronine. The difference absorption peak at 494 nm was measured as a function of the mole ratio of 3,5-diiodo-3′,5′-dinitro-L-thyronine to prealbumin. Same solvent as in Figure 3. The lines are theoretical binding curves calculated for $n = 0.9$ with $K = 10^6$ M^{-1} (---) and $K = 10^7$ M^{-1} (—). Points are experimental.

where the absorbance changes due to ionization of the phenolic group were eliminated, the experiments with the iodotyrosine analogs were performed at pH 8.6 in Tris buffer. There does not appear to be much difference in T_4 binding between pH 7.4 and 8.6 since the binding constant is only about three times larger at pH 8.6 than at 7.4.[2]

4-HYDROXY-3,5-DIIODOBENZALDEHYDE. The binding curve, as measured by equilibrium dialysis using the labeled compound, is shown in Figure 5. The number of moles bound is almost twice that found with the thyronine derivatives, *i.e.*, 1.7. The value of the binding constant, 5.5×10^7 M^{-1}, is between that found for T_4 and T_3.

The number of binding sites was established independently by difference absorption. The absorption of 4-hydroxy-3,5-diiodobenzaldehyde is blue shifted when it is bound to prealbumin. The peak of the difference absorption curve is at 357 nm and has a molar extinction of 7500 (Figure 6). At the concentration of prealbumin used in the experiment shown in Figure 7, all of the prealbumin sites are saturated before a significant fraction of ligand accumulates free in solution. The intersection of the two lines in Figure 7 gives 1.75 mol bound per mol of prealbumin, a value in accord with that found by equilibrium dialysis.

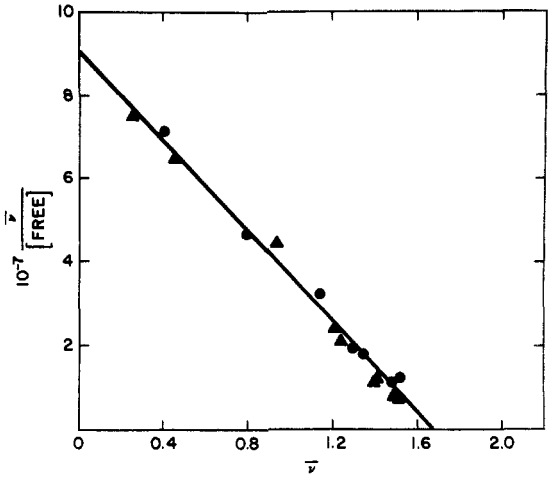

FIGURE 5: Scatchard plot of the binding of 4-hydroxy-3,5-diiodobenzaldehyde to prealbumin (0.88 μM). Data obtained by equilibrium dialysis at pH 8.6. Solvent: pH 8.6, 0.05 M Tris–0.10 M NaCl–1.0 mM EDTA; $T = 25 \pm 1°$. The two sets of points represent two different series of experiments.

3-(4-HYDROXY-3,5-DIIODOPHENYL)PROPIONIC ACID. The binding characteristics of a second diiodotyrosine analog

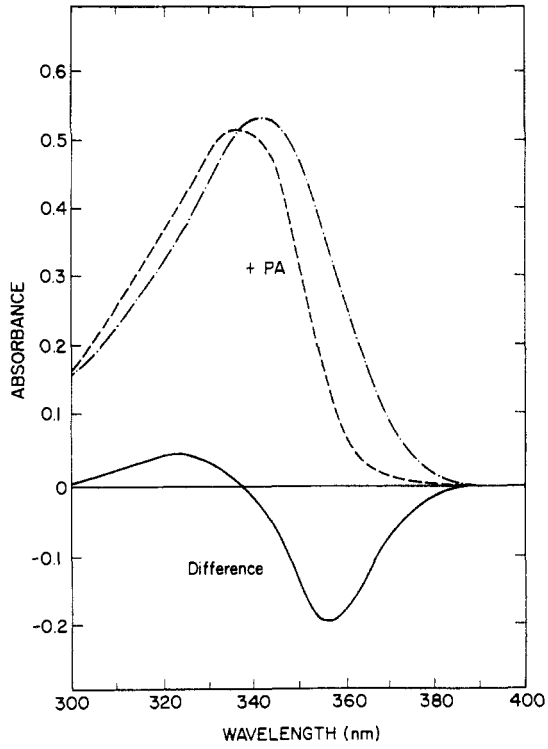

FIGURE 6: Absorption spectra of 4-hydroxy-3,5-diiodobenzaldehyde (28 μM) in the presence (---) and absence (- · · -) of prealbumin (19 μM) and the resulting difference spectrum (—). Solvent: pH 8.6, 0.05 M Tris–0.10 M NaCl–1.0 mM EDTA; $T = 25 \pm 1°$.

[2] Unpublished experiments of R. Ferguson, R. Pages, and W. Branch.

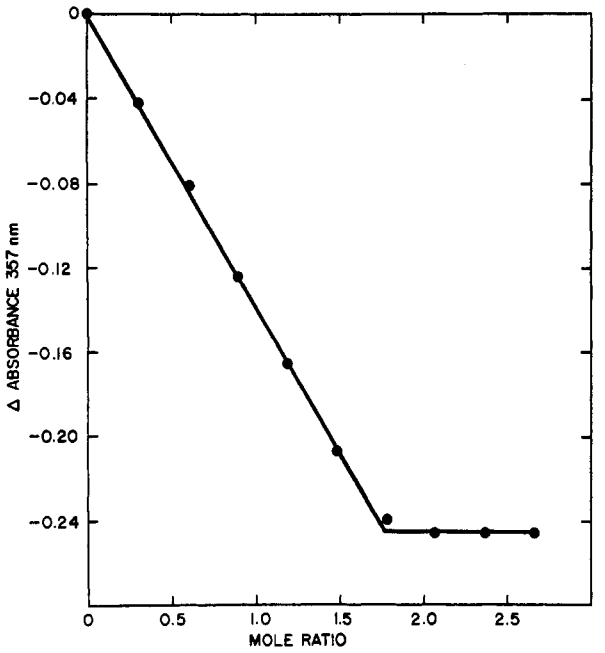

FIGURE 7: Titration of prealbumin (19 μM) with 4-hydroxy-3,5-diiodobenzaldehyde. The difference absorption peak at 357 nm was measured as a function of the mole ratio of 4-hydroxy-3,5-diiodobenzaldehyde to prealbumin. Solvent: pH 8.6, 0.05 M Tris-0.10 M NaCl-1.0 mM EDTA; $T = 25 \pm 1°$.

with a longer side chain than the aldehyde group of 4-hydroxy-3,5-diiodobenzaldehyde was also evaluated. The results of equilibrium dialysis of 3-(4-hydroxy-3,5-diiodophenyl)-propionic acid at pH 8.6 in 0.05 M Tris-0.10 M NaCl-1.0 mM EDTA, $T = 25 \pm 1°$, are shown in Figure 8. The data give 1.05 mol of ligand bound per mol of prealbumin with an affinity of 2.5×10^8 M^{-1}. It appears that the diphenyl ether structure is not necessary for strong binding.

4-HYDROXY-3,5-DIIODOCINNAMIC ACID. This compound differs from 3-(4-hydroxy-3,5-diiodophenyl)propionic acid

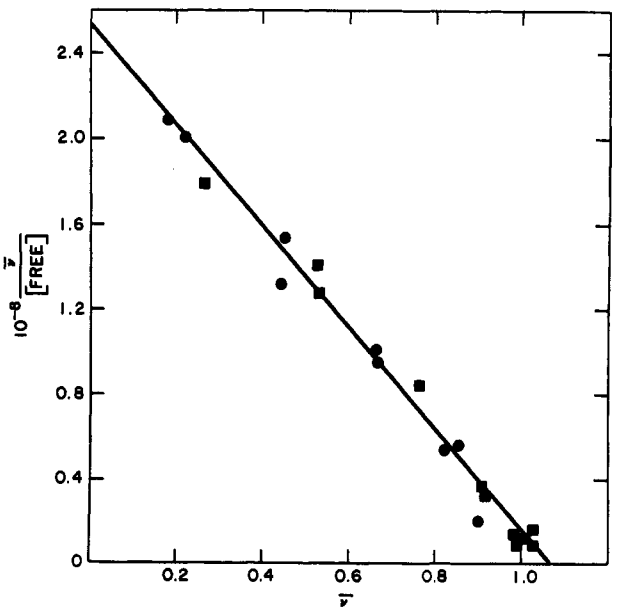

FIGURE 8: Scatchard plot of the binding of 3-(4-hydroxy-3,5-diiodophenyl)propionic acid to prealbumin by equilibrium dialysis at pH 8.6. Solvent: pH 8.6, 0.05 M Tris-0.10 M NaCl-1.0 mM EDTA; $T = 25 \pm 1°$. (●) [PA], 0.88 μM; (■) [PA], 0.82 μM.

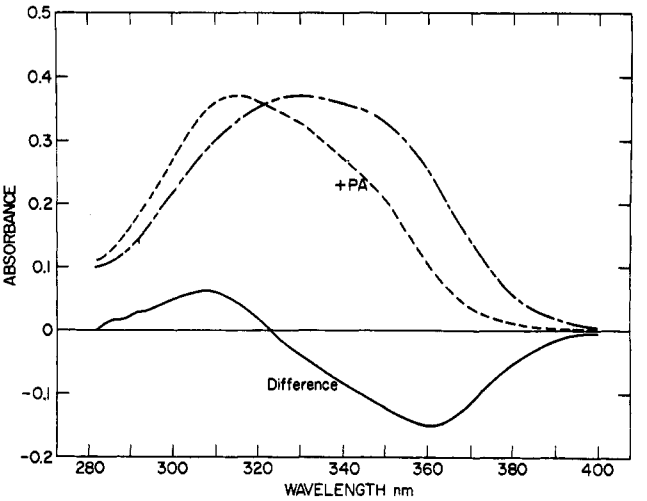

FIGURE 9: Abosrption spectra of 4-hydroxy-3,5-diiodocinnamic acid (17 μM) in the presence (---) and absence (-···-) of prealbumin (19 μM) and the resulting difference spectrum (—). Solvent: pH 8.6, 0.05 M Tris-0.10 M NaCl-1.0 mM EDTA; $T = 25 \pm 1°$.

only by a double bond, which results in an absorption as strong as that of 4-hydroxy-3,5-diiodobenzaldehyde. It also gives a blue-shifted absorption spectrum when bound to prealbumin with a difference peak near 360 nm which has a molar extinction coefficient of 8700 (Figure 9). A combining ratio of 0.95 mol of 4-hydroxy-3,5-diiodocinnamic acid was obtained by measuring the decrease in difference absorption with increasing amounts of ligand (Figure 10).

Solvent Effects. The effect of reducing the polarity of the solvent on the absorption properties of the various ligands was assessed in order to evaluate the spectral shifts observed when ligands bound to prealbumin. The effect of 50% dioxane on the absorption of the iodinated ligands in 0.01 M NaOH was determined. All five compounds in Table I showed red shifts in 50% dioxane-3,5-diiodo-3′,5′-dinitro-L-thyronine giving the greatest shift and 4-hydroxy-3,5-diiodocinnamic acid the least. The changes in molar extinction coefficient at the wavelength maximum were small for all the compounds except 3,5-diiodo-3′,5′-dinitro-L-thyronine.

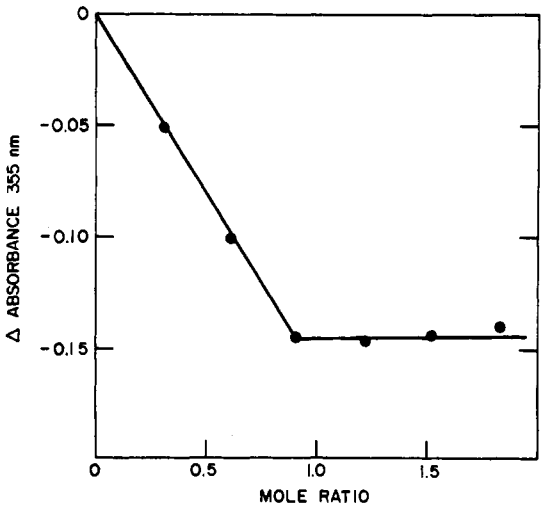

FIGURE 10: Titration of prealbumin (19 μM) with 4-hydroxy-3,5-diiodocinnamic acid. The difference absorption peaks at 355 nm were measured as a function of the mole ratio of 4-hydroxy-3,5-diiodocinnamic acid to prealbumin. Solvent: pH 8.6, 0.05 M Tris-0.10 M NaCl-1.0 mM EDTA; $T = 25 \pm 1°$.

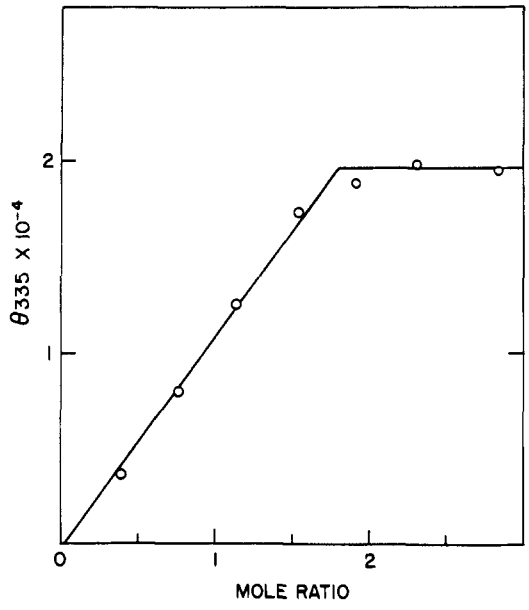

FIGURE 11: The molecular ellipticity at 335 nm of 4-hydroxy-3,5-diiodobenzaldehyde bound to prealbumin (39 μM) as a function of the mole ratio of the ligand to prealbumin. Solvent: pH 8.6, 0.05 M Tris–0.10 M NaCl; $T = 25°$.

The shifts of the absorption maxima of the various ligands when bound to prealbumin are also given in Table I. These were obtained from Figures 3, 6 and 9, or by adding an excess of prealbumin to aqueous solutions of the ligand at pH 8.6, 0.05 M Tris–0.01 M NaCl–1 mM EDTA.

Optical Activity of Bound Ligands. When certain chromophores are bound to their active sites in enzymes and proteins they show induced optical activity. When 1 mol of T_4 was bound to prealbumin at pH 7.4, 0.05 M phosphate–0.10 M NaCl, a new circular dichroic band with a peak at 245 nm resulted from the bound T_4. No further increase was observed with higher mole ratios of T_4 to prealbumin. 4-Hydroxy-3,5-diiodobenzaldehyde also becomes optically active when bound to prealbumin. It showed an ellipticity peak at 335 nm which is in good agreement with the absorption peak of the bound form. The binding sites of prealbumin were titrated from the change in ellipticity at 335 nm. It is clear from Figure 11 that 2 mol of ligand are bound per mol of prealbumin. At the concentration of prealbumin used (39 μM) it can be calculated that the ligand will be completely bound since the equilibrium constant is 5.5×10^7 M^{-1}.

Discussion

The binding constant of T_4 is about 10-fold greater than the values previously reported by Raz and Goodman (1969) at pH 7.4 in a phosphate–NaCl–EDTA buffer similar to that used presently. It is not clear why the present value differs since these authors also used equilibrium dialysis to obtain the binding constant. We have repeated the dialysis experiments with the same preparation of prealbumin used by Raz and Goodman (1969) and obtained the same binding data as reported in Figure 1. The low values reported by Nilsson and Peterson (1971) were found by indirect methods which do not directly measure the concentrations of bound and free ligand. A value of 2.3×10^8 M^{-1} for T_4 binding to prealbumin was reported by Woeber and Ingbar (1968) based on the distribution of T_4 between the binding proteins in serum. A similar value (3.56×10^8 M^{-1}) has been reported by Oppenheimer and Surks (1964).

It is of interest that T_3 is bound to prealbumin with a rather high affinity since many previous studies had indicated no transport of T_3 by prealbumin in serum (Robbins and Rall, 1967). More recent work is consistent with the present finding by demonstrating an interaction between T_3 and prealbumin (Nilsson and Peterson, 1971; Davis *et al.*, 1972; Larsen, 1972).

An unexpected observation of this study is the different number of prealbumin binding sites available for different ligands. Since there is only a single site for T_4 it seems likely that T_4 is bound in the interface of the four subunits where a large channel has been found by X-ray diffraction studies (Blake *et al.*, 1971). The binding of 1 mol of 2,3-diphosphoglyceric acid by deoxyhemoglobin is different since its binding site is formed by two β subunits (Arnone, 1972). There is no evidence of cooperative effects of prealbumin since the binding of 2 mol of 4-hydroxy-3,5-diiodobenzaldehyde takes place with the same binding energies for both sites. It has also been observed that 2 mol of the fluorescent probes, 1-dimethylaminonaphthalene-5-sulfonylglycine and 1-anilinonaphthalene-8-sulfonate, are bound with affinity constants of about 2×10^5 and 3×10^5 M, respectively.[2] Each molecule is presumably bound in the interface between two subunits since crystalline prealbumin shows twofold pseudosymmetry (Blake *et al.*, 1971). It would be surprising to find cooperative interactions between ligands bound to prealbumin as is found in hemoglobin in view of the very strong interactions that exist between prealbumin subunits. It has been shown that prealbumin is stable in 8 M urea–0.1 M sodium dodecyl sulfate, and dissociates very slowly in 6 M guanidine hydrochloride (Branch *et al.*, 1971, 1972). Independence of binding is also found with the two natural ligands of prealbumin since the binding of T_4 does not affect the binding of 4 mol of retinol binding protein (van Jaarsveld *et al.*, 1973b). The latter are apparently bound on the four subunits of prealbumin. The binding of retinol binding protein to prealbumin also has no effect on T_4 binding (Raz and Goodman, 1969).

A major difference between 4-hydroxy-3,5-diiodobenzaldehyde and 3-(4-hydroxy-3,5-diiodophenyl)propionic acid or 4-hydroxy-3,5-diiodocinnamic acid is its shorter side chain. Evidently two extra carbon atoms in the side chains of the two latter compounds are enough to cover a part of the second site and inhibit its occupation. The two fluorescent probes that are bound to prealbumin must interact with the sites somewhat differently since there is enough space to bind two molecules which are at least as large as the analogs for which only one site exists. The much lower binding energies also support this point of view. It is likely that the binding sites of prealbumin contain hydrophobic residues since the two fluorescent probes, T_4, and the analogs are rather nonpolar molecules.

The spectral shifts observed with the various iodinated compounds may be understood in terms of nonspecific solvent effects and hydrogen bonding of the iodophenolate moiety with hydrogen donor groups available either in the solvent or in the binding site of the protein. It should be remarked that the phenolate ion is a strong hydrogen acceptor group (Lees and Buraway, 1963). It appears to be a general rule that the spectral bands of $\pi \rightarrow \pi^*$ transitions are blue shifted when the group acts as an acceptor in forming a hydrogen bond and red shifted when acting as a donor (Baba and Suzuki, 1961; Ellis and Griffiths, 1966; Lees and Buraway, 1963).

The phenolic groups of all the compounds in Table I are

fully ionized at pH 8.6 in water.[2] Thus unless the pK values of the phenolic groups increase significantly when bound to prealbumin, the phenolate ion is the form that is bound. The absorption maxima of the four iodophenolate compounds are red shifted by 3–6 nm when dioxane is added to their aqueous solutions (Table I). The peak of the dinitrophenolate analog of thyroxine is shifted even more strongly to the red.

The shifts in the absorption wavelength maxima of the five compounds bound to prealbumin are more diverse than found with dioxane since two are blue shifted, one is red shited and two show relatively little change (Table I). The blue shifts found for 4-hydroxy-3,5-diiodobenzaldehyde and 4-hydroxy-3,5-diiodocinnamic acid do not arise from an increase in the pK of the phenolate groups since the wavelength maxima of their undissociated forms occur at considerably lower wavelengths, *i.e.*, 280 and 295 nm, respectively. The observed shifts are in the expected direction if the phenolate ion forms a stronger hydrogen bond in the nonpolar environment of the protein binding site than in water. The oxygen atom of the phenolate group could either remain in contact with the solvent since it is charged or form a hydrogen bond with a donor group of the protein.

The large red shift in the peak of 3,5-diiodo-3′,5′-dinitro-L-thyronine is the same when it is bound to prealbumin as when 50% dioxane is added to its aqueous solution. This similarity in behavior suggests that the polarity of environment of the dinitrophenolate group of the bound ligand is equivalent to that of the free ligand in 50% dioxane–50% water. The much weaker blue shifts found with T_4 and 3-(4-hydroxy-3,5-diiodophenyl)propionic acid could represent a balance between changes in hydrogen bonding and polarity effects.

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