Binding of Thyroid Hormones and Analogues to the Human Plasma Protein Prealbumin†

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ABSTRACT: The relative binding affinities to the human plasma protein prealbumin of the thyroid hormones, L-thyroxine and L-3,5,3′-triiodothyronine, and of 37 close structural analogues were measured by equilibrium dialysis. Analysis of the contributions of substituents to binding showed that all four iodine atoms contribute favorably. Addition of an iodine atom to an analogue contributes more favorably in the outer ring than in the inner ring. Halogen substituents in the 3, 5, and 3′ positions contribute more to binding than do alkyl groups with the same hydrophobic character in the same positions. This suggests a hydrogen-bonding and/or charge transfer interaction between the halogen and the protein. An electrostatic interaction between the carboxylate ion of the hormone side chain and the ammonium ion of Lys-15 accounts for the observed order in affinities: tetraprop > (L-thyroxine and D-thyroxine) > thyroxamine. L-Thyroxine binds with higher affinity than does D-thyroxine due to an interplay of electrostatic and steric forces involving the Lys-15, Leu-17, and Val-121 residues. The relative contributions of various structural features of the hormones in binding to prealbumin, thyroxine-binding globulin, and rat liver nuclear receptor were compared. Strong similarities were observed in the features of the 3 and 5 positions and in the side chains in contributing binding affinity to prealbumin and to the receptor. Binding to thyroxine-binding globulin and to prealbumin was influenced favorably by the same 3′ and 5′ substituents. In contrast, binding to the nuclear receptor was enhanced by 3′ alkyl and halogen substituents but was decreased by 5′ substitution.

A large body of structure–activity data pertaining to thyroid hormones and their analogues has been accumulated (Jorgensen, 1978). Initially, such studies focused on the structurally related variations of activity in vivo. Recently, however, in vitro tests have been developed measuring the binding of thyroid hormones and analogues to intact rat liver cell nuclei (Koerner et al., 1975) and to solubilized nuclear proteins (Samuels et al., 1974; Jorgensen et al., 1976). A high correlation was demonstrated between the affinities of thyroid hormone analogues to the nuclear proteins and their activities in vivo in the rat antigoiter assay. These observations supported other evidence (Oppenheimer et al., 1974) that the thyroid hormone receptor is localized in cell nuclei.

Fundamental to understanding the mechanism of hormone action is the development of a model at the molecular level for the binding interaction of hormones with their receptors. Presently, however, this is not possible due to the lack of availability of the pure receptor proteins, of their amino acid sequences, and of tertiary structures. It is possible, however, to determine the mechanism of analogue differentiation by a protein that binds thyroid hormones with appreciable affinity and whose detailed structure is known. Such a substance is one of the human plasma transport proteins for L-T_4,[1] prealbumin.

The structure of prealbumin in the solid state has been determined (Blake et al., 1974, 1978; Blake & Oatley, 1977). Prealbumin is a tetramer. The four identical subunits are tetrahedrally oriented in Cartesian space so that each of the three Cartesian axes is a C_2 symmetry axis. A central channel extends through the molecule and contains two crystallographically indistinguishable thyroxine-binding sites. The amino acids extending into the binding channel can be classified as polar–nonionic (Ser and Thr), polar–ionic (Lys, Glu, and His), and nonpolar (Val, Leu, Ala, and Met). The interaction of thyroxine with prealbumin has also been studied crystallographically and the results have been tentatively interpreted (Blake & Oatley, 1977) as follows. The 4′-hydroxyl group of L-T_4 is attached to the hydroxyl groups of Ser-117 and Thr-119 by hydrogen bonds through a well-defined water molecule. The 3′- and 5′-iodines are in contact with Leu-17 and Leu-110, and each of the 3- and 5-iodines fits into a pocket lined with methyl groups of Thr-106, Ala-108, and Val-121 and the β- and γ-methylenes of Lys-15. The α-ammonium and α-carboxylate groups of L-T_4 are in contact with Lys-15 and Glu-54. These assignments were tentative since low-resolution crystal data could only be analyzed for the approximate positions of the iodine atoms.

The binding of thyroid hormones and a small number of their analogues to prealbumin has been studied (Raz & Goodman, 1969; Pages et al., 1973; Ferguson et al., 1975; Cheng et al., 1977; Nilsson & Peterson, 1971; Nilsson et al., 1975). In spite of the crystallographic observation of two identical binding sites, it is generally accepted that there exists only one high-affinity binding site with an affinity constant in the range 10^7–10^8 M^{-1}. There is no general agreement on the number of low-affinity sites, although these appear to bind

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[1] Abbreviations used: L-T_4, L-thyroxine; L-T_3, L-3,5,3′-triiodothyronine; other abbreviations of hormone analogues are defined in Tables I and II; TBG, thyroxine-binding globulin; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; LHC and RHC, left- and right-hand chambers of the dialysis cell, respectively.

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L-T_4 with about one-tenth of the affinity of the higher affinity site. L-T_3 was found to have 10% and L-T_2 was found to have 0.6% the affinity of L-T_4 (Nilsson & Peterson, 1971; Nilsson et al., 1975; Pages et al., 1973). The acetic and propionic acid side-chain analogues of T_4, tetrac and tetraprop, were found to have affinities comparable to that of L-T_4 while the butyric acid side-chain analogue, tetrabut, showed lower affinity (Cheng et al., 1977).

In this study we (1) examine the contribution of 3, 5, 3′, and 5′ substituents to the binding to prealbumin and compare these data with contributions of the same substituents in binding to the nuclear proteins and to thyroxine-binding globulin, (2) examine the contribution of various side chains, and (3) explain the basis of the differentiation in binding of the D and L isomers of thyroxine by prealbumin.

Materials and Methods

Prealbumin. This protein was obtained from Dr. R. C. Hevey, Behring Diagnostics, Somerville, NJ, and had an immunological purity of 98%. It was used without further purification in a concentration of 50 nM on the basis of a molecular weight of 55 000.

Thyroid Hormones and Analogues. L-T_4, D-T_4, L-T_3, and D-T_3 were from Nutritional Biochemicals Co., Cleveland, OH. Triac was from Sigma Chemical Co., St. Louis, MO. L-T_0, L-3-T_1, L-3,3′-T_2, L-I_2Me_2, DL-Et_2I, and DL-Et_2I_2 were from Dr. Paul Block, Jr., University of Toledo, OH. L-3,3′,5′-T_3 was from Henning-Bulers Co., Berlin, Germany. Thyroxamine and DL-Br_2I_2 were from Dr. Rosalind Pitt-Rivers, University College, London. Triac, DL-I_2Et, L-I_2-*i*-Bu, and DL-I_2Ph were from Dr. Benjamin Blank, Smith Kline & French Laboratories, Philadelphia, PA; tetraform, tetrac, tetraprop, and tetrabut were from Dr. Robert Meltzer, Warner-Lambert Research Institute, Morris Plains, NJ. The remaining analogues were prepared in this laboratory. The purity of compounds was checked by TLC on silica gel plates using $CHCl_3$–MeOH–concentrated NH_4OH (20:10:1) and 2-propanol–concentrated NH_4OH (4:1). Preparative TLC was used when required to remove minor contaminants. [^{125}I]-L-T_4 was obtained from New England Nuclear Corp., Boston, MA. The specific activity was 750 μCi/μg.

Equilibrium Dialysis. All binding studies were carried out at 37 °C in 0.1 M Tris, 0.1 M NaCl, and 1 mM EDTA buffer at pH 8.0. This pH was chosen because of the limited solubility of some analogues at pH 7.4. Plexiglas dialysis units were used. The two chambers of each unit were separated by a cellulose dialysis membrane (Visking, Union Carbide Corp.) that had been previously hydrated in buffer for 30 min at room temperature. [^{125}I]-L-T_4 (40 μL; 3 μCi) was dissolved in 5.2 mL of buffer, 0.35 g of an anion-exchange resin (Iobeads, Technicon Co., Tarrytown, NY) was added to remove excess iodide, and the mixture was allowed to stand at room temperature for 30 min. The supernate was assayed by the method of Latham et al. (1976) and was found to contain less than 2% iodide.

Measurement and Analysis of L-T_4–Prealbumin Binding. To study the binding of L-T_4 to prealbumin, we added a series of 0.8-mL solutions (50 nM prealbumin, 10 pM [^{125}I]-L-T_4, and graded concentrations of L-T_4 in buffer) to the left-hand chamber (LHC) of the dialysis cell. To the right-hand chamber (RHC) was added 2.6 mL of buffer. Since the shapes of the two chambers were not identical, the use of 0.8 mL in the LHC and 2.6 mL in the RHC ensured equal heights and equal hydrostatic pressures. The units were incubated at 37 °C for 24 h, during which time equilibrium was established. A 0.5-mL aliquot from each chamber was counted in a γ scintillation counter to determine the concentrations of bound and free L-T_4. The data were fitted to Scatchard's model for two distinguishable and independent sites (eq 1; Tanford, 1961)

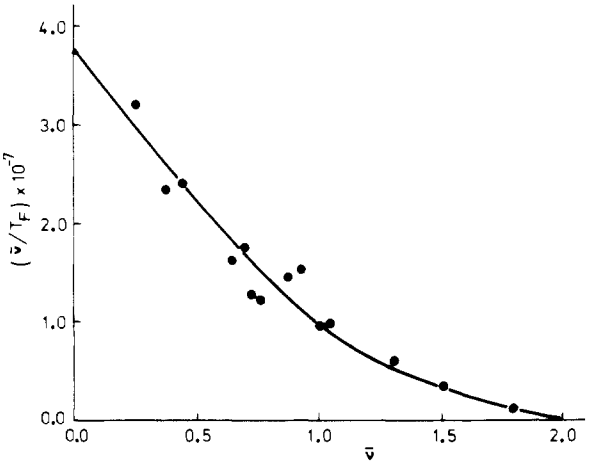


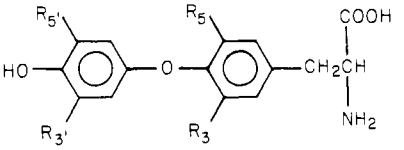
FIGURE 1: Scatchard plot of L-T_4–prealbumin binding by equilibrium dialysis in 0.1 M Tris, 0.1 M NaCl, and 1 mM EDTA buffer at pH 8.0 and 37 °C; 50 nM prealbumin. (●) Experimental points. Solid line is the best fit curve by using $N_1 = N_2 = 1$, $K_1 = 3.53 \times 10^7$ M^{-1}, and $K_2 = 2.57 \times 10^6$ M^{-1}.

$$\frac{\bar{\nu}}{T_F} = \frac{N_1K_1}{1 + K_1T_F} + \frac{N_2K_2}{1 + K_2T_F} \quad (1)$$

where $\bar{\nu}$ is the average number of L-T_4 molecules bound per prealbumin molecule, T_F is the concentration of free L-T_4 in moles per liter, N_1 and N_2 are the numbers of high- and low-affinity sites, respectively, and K_1 and K_2 are the affinity constants of L-T_4 at the high- and low-affinity sites, respectively. The fitting was done by the Marquardt–Levenberg procedure for nonlinear least squares using Public procedure FITFUN on the PROPHET computer system, Chemical/Biological Information Handling Program, National Institutes of Health, Bethesda, MD. The values of the model parameters as determined in 0.1 M Tris, 0.1 M NaCl, and 1 mM EDTA buffer at pH 8.0 and 37 °C were $N_1 = 0.93$ (±0.36), $N_2 = 1.17$ (±1.2), $K_1 = 3.72$ (±0.97) $\times 10^7$ M^{-1}, and $K_2 = 2.82$ (±6.53) $\times 10^6$ M^{-1}. When both N_1 and N_2 were assigned a value of 1.0, the corresponding values of the affinity constants were $K_1 = 3.53$ (±0.12) $\times 10^7$ M^{-1} and $K_2 = 2.57$ (±0.55) $\times 10^6$ M^{-1}. Figure 1 shows a Scatchard plot of the data and the best fit curve. The corresponding affinity constants measured by other investigators were $K_1 = 1.0 \times 10^8$ M^{-1} and $K_2 = 9.5 \times 10^5$ M^{-1} (Ferguson et al., 1975) and $K_1 = 1.6 \times 10^8$ M^{-1} and $K_2 = 2.5 \times 10^6$ M^{-1} (Cheng et al., 1977) in 0.05 M phosphate, 0.1 M NaCl, and 1 mM EDTA buffer at pH 7.4 and 25 °C. The differences in affinities between our measurements and those of other investigators are likely due to the differences in buffer, pH, and temperature.

Measurement and Analysis of the Competition of L-T_4–Prealbumin Binding by Analogues. The displacement of bound L-T_4 by analogues was studied in experiments identical with those above with one exception. The series of 0.8-mL LHC solutions had the following concentrations: 50 nM prealbumin, 10 pM [^{125}I]-L-T_4, 20 nM L-T_4, and graded concentrations of analogue. Dialysis was allowed to proceed at 37 °C for 24 h, after which time a 0.5-mL aliquot was withdrawn from each chamber and the radioactivity was measured in an automatic γ scintillation counter. This provided the concentrations of bound and free L-T_4; the initial concentration of analogue in the LHC was known. In order to determine the affinity constant of an analogue from these

Table I: Ring-Substiuted Analogues–Identification, Affinities, and Free Energies of Binding to Prealbumin at pH 8.0 and 37 °C in 0.1 M Tris, 0.1 M NaCl, and 1 mM EDTA



no.	compd (A)	DL	R_3	R_5	$R_{3'}$	$R_{5'}$	$C_1/K_1 \times 100$[a]	$\Delta G°(A) - \Delta G°$-(L-T_4)[b] (kcal/mol)
					Iodothyronines			
1	L-T_4	L	I	I	I	I	100 (0.88-1.13)	0.00 (0.17)
2	3,5,3′-T_3	L	I	I	I	H	9.2 (0.94-1.06)	1.47 (0.08)
3	3,3′,5′-T_3	L	I	H	I	I	32.9 (0.91-1.10)	0.69 (0.10)
4	3,5-T_2	L	I	I	H	H	0.32 (0.91-1.09)	3.55 (0.09)
5	3,3′-T_2	L	I	H	I	H	0.64 (0.94-1.07)	3.11 (0.09)
6	3′,5′-T_2	DL	H	H	I	I	3.3 (0.93-1.08)	2.10 (0.09)
7	3-T_1	L	I	H	H	H	0.014 (0.94-1.07)	5.49 (0.09)
8	3′-T_1	DL	H	H	I	H	0.068 (0.95-1.05)	4.49 (0.08)
9	T_0	L	H	H	H	H	<0.002	>6.6
					3′-Substituted 3,5-Diiodothyronines			
10	I_2Me	L	I	I	Me	H	0.44 (0.95-1.06)	3.34 (0.08)
11	I_2Et	DL	I	I	Et	H	0.43 (0.72-1.40)	3.35 (0.22)
12	I_2-*i*-Pr	L	I	I	*i*-Pr	H	0.83 (0.93-1.07)	2.95 (0.09)
13	I_2-*n*-Pr	L	I	I	*n*-Pr	H	0.69 (0.91-1.10)	3.07 (0.09)
14	I_2-*s*-Bu	L	I	I	*s*-Bu	H	6.0 (0.75-1.33)	1.73 (0.19)
15	I_2-*t*-Bu	L	I	I	*t*-Bu	H	1.3 (0.67-1.50)	2.67 (0.26)
16	I_2-*i*-Bu	L	I	I	*i*-Bu	H	2.2 (0.78-1.28)	2.35 (0.17)
17	I_2Ph	DL	I	I	phenyl	H	2.5 (0.55-1.82)	2.27 (0.38)
18	I_2Bz	L	I	I	benzyl	H	1.1 (0.68-1.48)	2.80 (0.25)
19	I_2Br	L	I	I	Br	H	1.6 (0.92-1.09)	2.54 (0.09)
20	I_2Cl	L	I	I	Cl	H	0.75 (0.85-1.18)	3.01 (0.13)
					3,5-Substituted Thyronines			
21	Me_2I	L	Me	Me	I	H	0.84 (0.84-1.19)	2.94 (0.13)
22	E_2I	DL	Et	Et	I	H	0.49 (0.93-1.08)	3.27 (0.09)
23	*i*-Pr_2I	DL	*i*-Pr	*i*-Pr	I	H	0.25 (0.89-1.12)	3.69 (0.10)
24	MeIIH	DL	Me	I	I	H	2.1 (0.81-1.23)	2.39 (0.15)
25	Me_2I_2	L	Me	Me	I	I	0.78 (0.88-1.14)	2.99 (0.11)
26	Et_2I_2	DL	Et	Et	I	I	2.5 (0.91-1.10)	2.27 (0.10)
27	*s*-Bu_2I_2	DL	*s*-Bu	*s*-Bu	I	I	0.56 (0.79-1.26)	3.19 (0.16)
28	$MeII_2$	DL	Me	I	I	I	8.0 (0.91-1.09)	1.55 (0.09)
29	Br_2I_2	DL	Br	Br	I	I	82 (0.70-1.44)	0.12 (0.24)
					3′,5′-Disubstituted 3,5-Diiodothyronines			
30	I_2Br_2	L	I	I	Br	Br	8.6 (0.97-1.03)	1.51 (0.08)
31	I_2Me_2	L	I	I	Me	Me	0.12 (0.90-1.12)	4.14 (0.10)

[a] $K_1 = 3.5 \times 10^7\ M^{-1}$. C_1/K_1 is the ratio of the affinity constant of the analogue to that of L-T_4 for the high-affinity site. The numbers in parentheses indicate uncertainty ranges, e.g., $C_1/K_1 \times 100 = 0.64$ (0.94–1.07) designates that the mean value of $C_1/K_1 \times 100$ falls in the range 0.64×0.94 to 0.64×1.07. [b] $\Delta G°(A) - \Delta G°(L\text{-}T_4) = -RT \ln (C_1/K_1)$. $R = 1.987 \times 10^{-3}$ kcal/(deg mol); $T = 310$ K. Numbers in parentheses are standard deviations calculated by eq A21 (Appendix).

data, we again assumed the presence of two independent and distinguishable sites. On the basis of this assumption, we derived expressions (see Appendix) for the initial LHC concentration of analogue (A_H; moles per liter) when the ratio of bound to free L-T_4 was at its half-maximal value. We also derived corresponding expressions for the initial LHC concentration of L-T_4 (T_H; moles per liter) when L-T_4 was used as an analogue. The ratio T_H/A_H was used to calculate the ratio of the association constant to the high-affinity site of the analogue (C_1) to that of L-T_4 (K_1) (Table I).

Contribution of Functional Groups. Contributions of various functional groups to the free energy of binding were analyzed as follows. The apparent standard free energy of binding, $\Delta G°$, was determined from the affinity constant K by $\Delta G° = -RT \ln K$. For a compound AXY which is made of a core "A" with substituents X and Y, neither of which is a hydrogen atom, the apparent standard free energy of binding is expressed as

$$\Delta G°(AXY) = \Delta G°(AHH) + \Delta G^1(X) + \Delta G^1(Y) + \Delta G^2(XY) \quad (2)$$

where $\Delta G°(AHH)$ is the binding free energy of compound AHH which is identical with AXY with the exception that X = Y = H, and

$$\Delta G^1(X) = \Delta G°(AXH) - \Delta G°(AHH) \quad (3)$$

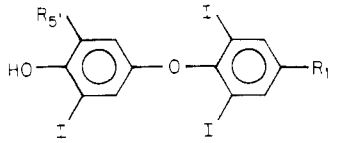
$$\Delta G^1(Y) = \Delta G°(AHY) - \Delta G°(AHH) \quad (4)$$

$$\Delta G^1(XY) = \Delta G°(AXY) - \Delta G°(AHH) \quad (5)$$

$$\Delta G^2(XY) = \Delta G^1(XY) - \Delta G^1(X) - \Delta G^1(Y) \quad (6)$$

The ΔG^1 term (eq 3 and 4) reflects the contributions of groups X and Y to the standard free energy of binding relative to the contribution of a hydrogen atom present in the same position when X and Y are present in different molecules. The $\Delta G^1(XY)$ term (eq 5) reflects the combined contribution of groups X and Y when present in the same molecule. The ΔG^2 term (eq 6) is the difference between the total contribution of groups X and Y when present in the same molecule and the sum of their contributions in separate molecules. Thus, it reflects the perturbing effects that groups X and Y have on each other's contribution.

Table II: Side-Chain Analogues–Identification, Affinities, and Free Energies of Binding to Prealbumin at pH 8.0 and 37 °C in 0.1 M Tris, 0.1 M NaCl, and 1 mM EDTA



no.	compd (A)	$R_{5'}$	R_1	$C_1/K_1 \times 100^a$	$\Delta G^\circ(A) - \Delta G^\circ(L\text{-}T_4)^b$ (kcal/mol)
32	triac	H	$CH_2CO_2^-$	19.7 (0.87–1.15)	+1.00 (0.11)
2	L-T_3	H	L-$CH_2CH(NH_3^+)CO_2^+$	9.2 (0.94–1.06)	+1.47 (0.08)
33	D-T_3	H	D-$CH_2CH(NH_3^+)CO_2^-$	0.25 (0.91–1.10)	+3.69 (0.10)
34	tetraform	I	CO_2^-	186 (0.98–1.02)	−0.38 (0.08)
35	tetrac	I	$CH_2CO_2^-$	676 (0.98–1.02)	−1.18 (0.08)
36	tetraprop	I	$(CH_2)_2CO_2^-$	298 (0.93–1.07)	−0.67 (0.09)
37	tetrabut	I	$(CH_2)_3CO_2^-$	267 (0.98–1.03)	−0.60 (0.08)
1	L-T_4	I	L-$CH_2CH(NH_3^+)CO_2^-$	100 (0.88–1.13)	0.00 (0.17)
38	D-T_4	I	D-$CH_2CH(NH_3^+)CO_2^-$	3.7 (0.93–1.07)	+2.01 (0.09)
39	thyroxamine	I	$(CH_2)_2NH_3^+$	0.37 (0.90–1.11)	+1.00 (0.10)

[a] See footnote *a*, Table I. [b] See footnote *b*, Table I.

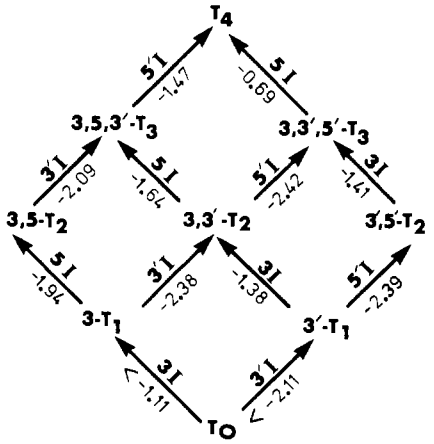


FIGURE 2: Diagrammatic representation of the effect on the free energy of binding to prealbumin produced by sequential additions of iodine atoms to thyronine. The number under each arrow corresponds to the change in binding free energy when the designated iodine atom replaces the corresponding hydrogen atom. The numbers are the $\Delta G^1(X)$ values for prealbumin from Table III.

Results and Discussion

Binding of Thyroxine Analogues to Prealbumin. Table I shows the apparent affinity constants (relative to L-T_4) of thyroid hormones and ring-substituted analogues measured in this study. Table II shows the relative affinities of analogues in which the L-alanine side chain has been varied. The data can be analyzed for the contribution of each of the following groups to binding: (1) 3-, 5-, 3'-, and 5'-iodine atoms, (2) other 3, 5, and 3' substituents, (3) 5' substituents, and (4) the various side chains.

(1) Contributions of the 3-, 5-, 3'-, and 5'-Iodine Atoms to Binding to Prealbumin and Comparisons with Their Contributions to Nuclear Receptor and TBG Binding. The relative binding affinities to prealbumin of L-T_4, of L-thyronine, and of the various tri-, di- and monoiodothyronines are shown in Table I (**1**–**9**).

The data of Table I and the binding affinity data for rat liver nuclear receptors (Koerner et al., 1975; Jorgensen & Andrea, 1975) and for thyroxine-binding globulin (TBG; Snyder et al., 1976) were recalculated as described under Materials and Methods (eq 3) to show the contributions of individual iodine atoms. The comparative values are listed in Table III.

Table III: Comparison of the Contribution of the Iodine Atoms to the Binding Free Energy to the Nuclear Receptors, Thyroxine-Binding Globulin, and Prealbumin

substituent X	parent compd[a,b] AXH	parent compd[a,b] AHH	$\Delta G^1(X)^b$ nuclear receptors[c]	$\Delta G^1(X)^b$ TBG[d]	$\Delta G^1(X)^b$ prealbumin[e]
3-I	3,3',5'-T_3	3',5'-T_2		−3.65	−1.41 (0.13)
	3,3'-T_2	3'-T_1		−2.48	−1.38 (0.12)
	3-T_1	T_0			<−1.11
5-I	L-T_4	3,3',5'-T_3	−2.97	−0.60	−0.69 (0.20)
	3,5,3'-T_3	3,3'-T_2	−3.26	−1.20	−1.64 (0.12)
	3,5-T_2	3-T_1		−0.21	−1.94 (0.13)
3'-I	3,5,3'-T_3	3,5-T_2	−3.58	−2.99	−2.08 (0.12)
	3,3'-T_2	3-T_1		−2.00	−2.38 (0.13)
	3'-T_1	T_0			<−2.11
5'-I	L-T_4	3,5,3'-T_3	+1.28	−1.48	−1.47 (0.19)
	3,3',5'-T_3	3,3'-T_2	+0.99	−2.08	−2.42 (0.13)
	3',5'-T_2	3'-T_1		−0.19	−2.39 (0.12)
	Me_2I_2	Me_2I			+0.05 (0.17)
	$MeII_2$	MeIIH			−0.84 (0.17)
	Et_2I_2	Et_2I			−1.00 (0.13)

[a] Parent compound abbreviations are defined in Table I. [b] $\Delta G^1(X) = \Delta G^\circ(AXH) - \Delta G^\circ(AHH)$. See eq 3 and associated text. [c] Calculated from the data of Koerner et al. (1975). [d] Calculated from the data of Snyder et al. (1976). [e] Calculated from the data in Table I. Numbers in parentheses are standard deviations calculated by eq A21 (Appendix).

Figure 2, based on the data of Table III, compares the contributions to the free energy of binding to prealbumin upon the addition of single iodine atoms to the inner and outer rings of partially iodinated thyronines. This figure shows that the addition of an outer-ring iodine atom (3'- or 5'-I) to a specific thyronine results in a greater gain in binding free energy than does the addition of an inner-ring iodine atom (3- or 5-I) to the same thyronine. This importance of the outer-ring iodine atom in binding to prealbumin may also be seen by the effect of iodine removal. Deletion of an outer-ring iodine from any compound of Figure 2 results in a greater loss in binding affinity than does deletion of an inner-ring iodine.

The data of Table III show the following. (a) The first iodine atom added to the inner ring (3-iodine) contributes favorably to the association with both prealbumin and TBG. In binding to TBG the 3-iodine atom contributes 2.5–3.6 kcal/mol. The contribution of the 3-iodine in binding to prealbumin is about 1.4 kcal/mol. Pairs of analogues nec-

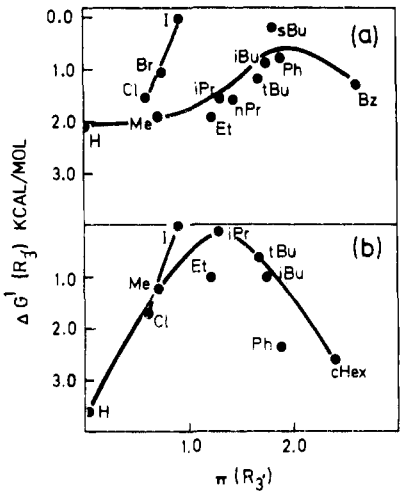


FIGURE 3: Binding of 3′-substituted 3,5-diiodothyronines to prealbumin (a) and to nuclear receptor (b). The contributions of 3′ substituents [$\Delta G^1(R_{3'})$] to the binding free energy are plotted against the Hansch π value (Fujita et al., 1964). The $\Delta G^1(R_{3'})$ values are calculated from the data of Table I by using eq 3, relative to 3′-I.

essary for estimating the 3-iodine contribution to nuclear receptor binding were not studied by Koerner et al. (1975). (b) The 5- and 3′-iodine atoms contribute favorably to the associations with nuclear receptor, TBG, and prealbumin. The contribution of the second iodine atom added to the inner ring (5-iodine) falls in the range of 0.2–1.9 kcal/mol for binding to TBG and to prealbumin and is clearly higher (3.0–3.3 kcal/mol) for the nuclear receptor. The latter high value suggests a combination of factors contributing to binding, which may include stabilization by 3,5-diiodo substitution of a minimum energy conformation for the two phenyl rings that places the rings and their substituents in close proximity to binding elements of the nuclear receptor (Andrea et al., 1979). The contribution of the 3′-iodine atom is approximately equivalent for binding to TBG and to prealbumin (2–3 kcal/mol) and somewhat higher for the nuclear receptor (3.6 kcal/mol). (c) The 5′-iodine atom contributes a repulsive interaction between the iodinated thyronine and the nuclear receptor (1–1.3 kcal/mol) whereas it contributes an attractive interaction with the transport proteins, TBG, and prealbumin (0.0–2.4 kcal/mol). Because of this, 3′,5′-diiodothyronines are more firmly bound to plasma transport proteins than are the corresponding 3′-iodothyronines, while the converse is true with the nuclear receptors. This feature of weaker transport protein binding and therefore greater availability of free hormone, which possesses higher affinity for the nuclear receptor, accounts for the higher hormonal activity of 3′-iodothyronines (e.g., L-T_3) as compared with the corresponding 3′,5′-diiodothyronine (e.g., L-T_4).

(*2*) *Contributions of 3, 5, and 3′ Substituents Other Than Iodine.* The binding affinities of various 3-, 5-, and 3′-substituted analogues are shown in Table I (compounds **10–29**). For 3,5-diiodothyronines substituted in the 3′ position by alkyl or aryl groups, or by halogen atoms (Table I, compounds **10–20**), regular trends are observed in binding affinities to prealbumin. The contribution of the 3′ substituent to binding (calculated by eq 3) is plotted against the Hansch π value (Fujita et al., 1964) which reflects the hydrophobic character of the substituent (Figure 3a). As hydrophobic character increases, the substituent contribution to binding affinity to prealbumin increases to a maximum and then falls for the excessive hydrophobic character or bulk of the benzyl substituent. For alkyl or aryl groups the predominant binding contribution would be expected to arise from hydrophobic factors, perhaps enhanced slightly by van der Waals interactions with the binding site. Figure 3a also shows that halogen atoms (Cl, Br, and I) in the 3′ position contribute much more to binding than is expected on the basis of their hydrophobic character, suggesting that other factors are involved in the interaction of the halogen atoms with the protein. As proposed by Blake & Oatley (1977) the 3′- (or 5′-) iodine atom is in the nonpolar environment of the Leu-17 and Leu-110 side chains which project into the binding cavity from one β sheet of prealbumin. In addition, the 3′-iodine is in proximity to the Ser-117 hydroxyl group and to its associated water molecule, which projects from the same β sheet as the leucine side chains. From the β sheet on the opposite side of the binding channel, a Thr-119 hydroxyl group and its associated water molecule are also close to the 3′- (or 5′-) iodine atom. In addition to hydrophobic associations with the Leu-17 and Leu-110 side chains, a 3′- (or 5′-) iodine atom could form either a hydrogen bond with the proton of a hydroxyl group or a charge transfer complex with the lone pair orbitals of an oxygen atom. An additional role for the 3′-iodine atom is its inductive effect in increasing the polarization and ionization of the neighboring 4′-hydroxyl group. This would enhance the interaction of the 4′-oxygen, through a crystallographically well-defined water molecule, with the hydroxyl groups of Ser-117 and Thr-119 as proposed by Blake & Oatley (1977). None of these three effects can be produced by a 3′-alkyl group. These features of a 3′-halogen atom account for its greater contribution to binding to prealbumin than those which are explained solely by its hydrophobic character.

Figure 3b is a similar graph for the binding of 3,5-diiodo-3′-substituted thyronines to the rat liver nuclear binding site, calculated from the data of Koerner et al. (1975). The primary difference between the curves of parts a and b of Figure 3 is that the 3′-alkyl residue curve in the nuclear receptor study peaks at a lower value of π, which also correlates with a smaller group size. The 3′-isopropyl group provides the best binding interaction with the nuclear receptor. Also, the differences in contributions to binding for alkyl groups and for halogen atoms are much smaller in the case of the nuclear receptor. This suggests that the primary contribution of the 3′ group in binding to the nuclear receptor involves hydrophobic factors, in contrast to the additional involvement of hydrogen bonding, charge transfer, and inductive forces in binding to prealbumin.

The present studies on binding to prealbumin were carried out at pH 8.0 while those on the nuclear receptor (Koerner et al., 1975) were carried out at pH 7.0. Since the pK_a of an *o*-halophenol is approximately 8, its phenolic hydroxyl group is approximately 50% ionized at pH 8 and only 10% ionized at pH 7. The corresponding *o*-alkylphenols have pK_a values of about 10, and, hence, at pH 7 and pH 8 they are essentially un-ionized. The higher ionization of the *o*-halophenols at pH 8 could partially account for the differences in the relative positions of the 3′-halogen curves and 3′-alkyl curves in parts a and b of Figure 3.

Figure 4a shows a similar analysis for the contributions of 3 and 5 substituents on the thyronine nucleus in binding to prealbumin. The binding data of Table I (compounds **21–29**) have been recalculated by using eq 5 to determine the combined contribution of 3 and 5 substituents. Alkyl groups show a typical "parabolic" curve, maximal with 3,5-diethyl substitution, when the binding contributions of 3 and 5 substituents are plotted against the summation of substituent group hydrophobic character (Hansch π). Compounds with both methyl and iodine substitution (Table I, **24** and **28**) show

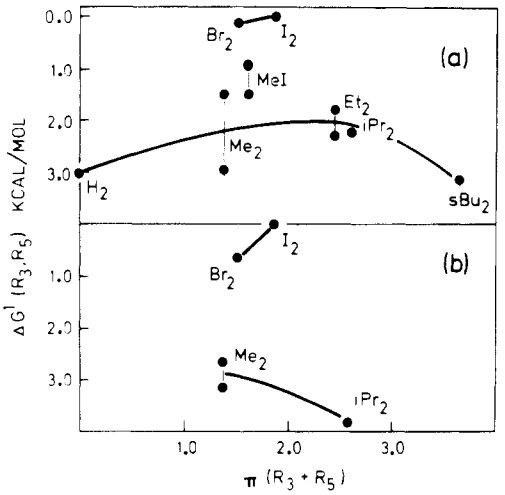


FIGURE 4: Binding of 3,5-substituted thyronines to prealbumin (a) and to nuclear receptor (b). The contributions of 3,5 substituents $[\Delta G^1(R_3, R_5)]$ to binding free energy are plotted against the sum of the Hansch π values of the two groups (Fujita et al., 1964). The $\Delta G^1(R_3, R_5)$ values are calculated from the data of Table I by using eq 5, relative to 3,5-I_2.

higher affinities than would be expected on the basis of their hydrophobic character alone, and the effect is more pronounced for 3,5-diiodo and 3,5-dibromo substituents (Table I, **1** and **29**). This suggests that halogen atoms in the 3 and 5 positions are exerting an effect that cannot be duplicated by alkyl groups in binding to prealbumin. This difference could be rationalized on the basis of a charge transfer and/or hydrogen-bonding interaction between the 3 and 5 iodine atoms with the hydroxyl group of Thr-106. Blake & Oatley (1977) describe the 3,5-iodines as fitting into a pocket lined with the methyl groups of Thr-106, Ala-108, and Val-121 and the β- and γ-methylenes of Lys-15. Our observations suggest the additional involvement of the hydroxyl group of Thr-106. This would occur in addition to the hydrophobic contributions of the 3,5-iodines.

Figure 4b is a similar analysis of the data calculated from the rat liver cell nuclear binding studies of Koerner et al. (1975). In spite of the fewer data points, it is clear that halogen atoms in the 3 and 5 positions contribute more favorably to binding than do alkyl groups with the same hydrophobic character. Snyder et al. (1976) have shown that 3,5-dibromo-3′,5′-diiodothyronine binds with high affinity to TBG (160% L-T_4) which identifies a strong binding contribution of 3,5-bromine substitution. In contrast, 3,5-dimethyl-3′-isopropylthyronine (0.05% L-T_4) binds with very low affinity to TBG relative to the corresponding 3,5-diiodo analogue (3.5% L-T_4), and 3,5,3′,5′-tetramethylthyronine (0% L-T_4) binds with low affinity relative to 3,5-diiodo-3′,5′-dimethylthyronine (0.29% L-T_4). Thus, halogen atoms contribute far more to binding than do alkyl groups in the same position. This suggests, by analogy with the more completely characterized prealbumin binding, that the 3 and 5 substituents contribute to nuclear receptor binding and to TBG binding by hydrogen bonding and/or charge transfer complexation, in addition to hydrophobic factors. This suggests that the binding environment for the 3,5 substituents may be similar in all three proteins.

(*3*) *Contributions of 5′ Substituents Other Than Iodine.* 3,5,3′,5′-Tetrasubstituted analogues (Table I, **30** and **31**), when compared with the corresponding 3,5,3′-trisubstituted analogues (**19** and **10**), show that the 5′-bromine atom greatly enhances binding to prealbumin, as it does with TBG (Snyder et al., 1976). The 5′-methyl group reduces binding affinity to prealbumin and has little effect on binding to TBG (Snyder et al., 1976). Any 5′ substituent reduces binding affinity to the nuclear receptor (Koerner et al., 1975). These data further support the multiple roles of 3′- and 5′-halogen atoms, involving possible hydrogen bonding, charge transfer complexation, and/or an inductive effect on the 4′-hydroxyl group, in binding to prealbumin and to TBG. These proteins have space to accommodate both 3′ and 5′ substituents, while binding to the nuclear receptor decreases in relation to the size of the substituent in the 5′ position (Dietrich et al., 1977).

(*4*) *Contributions of the Side Chain.* (*a*) *Charge and Side-Chain Length.* The binding affinities and calculated binding free energies relative to L-T_4 of side-chain analogues are listed in Table II. 3,5,3′,5′-Tetraiodo analogues, in which the side chains have only a negative charge (compounds **34**–**37**), have higher binding affinities than do L-T_4 (**1**) and D-T_4 (**38**), in which the side chains have a zero net charge. Thyroxamine (**39**), with only a positive charge in the side chain, has the lowest binding affinity. In the 3,5,3′-triiodo series, triac (**32**) with only a negative charge on the side chain binds with higher affinity than does L-T_3 (**2**) which has a zero net charge on the side chain. In this study using equilibrium dialysis and analogue displacement of [^{125}I]-L-T_4, maximal binding affinity occurs with the two-carbon acetic acid side chain. In the study of Cheng et al. (1977), using the quenching of 8-anilinonaphthalene-1-sulfonate fluorescence, the relative affinities to prealbumin of L-T_4, tetrac, tetraprop, and tetrabut were found to be 100, 80, 159, and 32, respectively.

These data are consistent with a predominant electrostatic interaction of the hormone side chain with the ϵ-ammonium group of Lys-15, which is present well within the entrance to the binding channel (Andrea et al., 1978). A negatively charged ligand side chain (COO^-) would provide maximal electrostatic interaction with the positively charged ammonium ion of Lys-15. The zwitterionic side chains (COO^-, NH_3^+) would undergo both attractive and repulsive interactions, and the positively charged side chain (NH_3^+) would experience only repulsive interactions with the Lys-15 ammonium ion. Theoretical calculations by Andrea et al. (1978) further implicate Lys-15 as the predominant ionic residue of prealbumin in the binding of the side chains of the thyroid hormones.

(*b*) *Differentiation in Binding of* L-T_4 *and* D-T_4. Both L-T_4 and D-T_4 carry a zero net charge in their zwitterionic side chains. However, the L enantiomer binds to prealbumin with 25-fold higher affinity than does the D enantiomer. A theoretical study of Andrea et al. (1978) indicates that this differentiation occurs by an interplay of electrostatic and steric forces. The predominant electrostatic interaction is that of the ϵ-ammonium group of Lys-15 with the carboxylate and ammonium ions of the hormone side chain. With L-T_4 occupying its binding site in the binding channel of prealbumin, the bulk of the Leu-17 side chain forces the α-carbon to point its smallest substituent, the hydrogen atom, toward this bulk. In so doing, the bulky carboxylate group of the L side chain is oriented closer to the Lys-15 ϵ-ammonium group than is the α-ammonium group, and the side chain experiences a net attraction. In this same configuration the D isomer would be forced to point its ammonium group closer to Lys-15 than its carboxylate group and would experience net repulsion in the interaction of the side chain with the protein. The bulky side chain of Val-121 exerts an identical effect of favoring the binding of the L isomer when the α-carbon atom of L-T_4 is in its proximity. All other charged amino acid side chains of prealbumin are too distant from the hormone side-chain

charges to exert an appreciable influence on these charges as compared to the influence of Lys-15. This is the first proposal of a specific molecular mechanism for the differentiation in binding of D and L enantiomers.

(*c*) *Comparison of the Side-Chain Contributions in Binding to Prealbumin, TBG, and Nuclear Receptor.* Triac and tetrac bind with higher affinities to the receptor sites of rat liver cell nuclei (Koerner et al., 1974) than do L-T_3 and L-T_4, respectively. In addition, L-T_3 binds to this receptor preparation with higher affinity than does D-T_3. These similarities in side-chain binding trends between prealbumin and receptor suggest that structural elements of the receptor in this binding region may closely resemble those of prealbumin. Specifically, these elements would appear to be a predominant positively charged amino acid(s) and structural constraints that favor binding of the L isomer to this positive charge.

Tetrac and tetraprop bind with very low affinities to TBG [1.7 and 3.6% of L-T_4, respectively (Snyder et al., 1976)]. Triac (0.3% L-T_4) also binds with low affinity relative to L-T_3 (9% L-T_4). D-T_4 binds with 54% the affinity of L-T_4. These results indicate that the side-chain binding site of TBG contains elements significantly different from those of prealbumin and of the nuclear receptor and suggest that this region of TBG contains a predominance of negative charge.

Acknowledgments

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Appendix

Independent-site models are well-known (Tanford, 1961). We derive the following equations in order to include the particular features of our experimental procedure. Mass conservation implies

$$P = P_F + PA_1 + PA_2 + PA_{1,2} + PX_1 + PX_2 + PX_{1,2} + PX_1A_2 + PX_2A_1 \quad (A1)$$

$$X = 4.25X_F + PX_1 + PX_2 + 2PX_{1,2} + PX_1A_2 + PX_2A_1 \quad (A2)$$

$$A = 4.25A_F + PA_1 + PA_2 + 2PA_{1,2} + PX_1A_2 + PX_2A_1 \quad (A3)$$

P, X, and A are the *initial* left-hand chamber (LHC) concentrations of prealbumin, L-T_4, and analogue, respectively. P_F, X_F, and A_F are the equilibrium concentrations of *free* prealbumin, L-T_4, and analogue, respectively. PA_1, PA_2, and $PA_{1,2}$ are the concentrations of prealbumin bound by analogue in the high- and low-affinity sites, "1" and "2", and bound by analogue at both sites 1 and 2, respectively. PX_1, PX_2, and $PX_{1,2}$ are the concentrations of prealbumin bound by L-T_4 at sites 1, 2, and 1 and 2, respectively. PX_1A_2 and PX_2A_1 are the concentrations of prealbumin bound by L-T_4 in site 1 and analogue in site 2 and vice versa, respectively.

The factor of 4.25 is a coefficient of X_F in eq A2 because of the unequal volumes of the dialysis chambers. Initially, 0.8 mL of X molar solution of L-T_4 is introduced into the LHC. At equilibrium, this has been redistributed into bound L-T_4 in the 0.8-mL LHC and free L-T_4 in the entire volume of the cell (3.4 mL). That is, $(0.8 \times 10^{-3})X = (3.4 \times 10^{-3})X_F + (0.8 \times 10^{-3})X_B$ where X_B is the sum of the protein-bound terms in eq A2. Equation A2 follows from this in a straightforward way. The factor of 4.25 in eq A3 has the same origin.

The mass action expressions for three of the protein-bound species are

$$PX_1 = K_1P_FX_F \quad (A4)$$

$$PX_{1,2} = K_1K_2P_FX_F^2 \quad (A5)$$

$$PX_1A_2 = K_1C_2P_FX_FA_F \quad (A6)$$

K_1 and K_2 are the affinity constants of L-T_4 at sites 1 and 2, and C_1 and C_2 are the affinity constants of analogue at sites 1 and 2. The mass action expressions for the remaining five protein-bound species are very similar to eq A4–A6. Substituting the mass action equations into eq A1 and rearranging gives

$$P_F = P/[(C_1A_F + 1)(C_2A_F + 1) + (K_1X_F + 1) \times (K_2X_F + 1) + \kappa A_FX_F - 1] \quad (A7)$$

$$\kappa = K_1C_2 + K_2C_1 \quad (A8)$$

$$R = \frac{PX_1 + PX_2 + 2PX_{1,2} + PX_1A_2 + PX_2A_1}{X_F} \quad (A9)$$

R is the ratio of bound to free L-T_4. Substituting the mass action equations into eq A9 and taking note of eq A7 gives

$$R = P(K_1 + K_2 + 2K_1K_2X_F + \kappa AF)/[(C_1A_F + 1) \times (C_2A_F + 1) + (K_1X_F + 1)(K_2X_F + 1) + \kappa A_FX_F - 1] \quad (A10)$$

The maximum concentration of protein-bound L-T_4 and the minimum concentration of free L-T_4 are attained in the absence of analogue. Thus, from eq A10

$$R_M = \lim_{A_F \to 0} R = \frac{K_1}{1 + K_1X_{Fi}} + \frac{K_2}{1 + K_2X_{Fi}} \quad (A11)$$

R_M is the maximal value for the ratio of bound to free L-T_4. X_{Fi} is the concentration of free L-T_4 when $R = R_M$, i.e., in the absence of analogue. By setting $R_M = (X - 4.25X_{Fi})/X_{Fi}$, we solved eq A11 numerically for X_{Fi}. The value of X_{Fi} thus calculated and the corresponding values of R_M and R_H (= $R_M/2$) are

$$X_{Fi} = 3.358 \times 10^{-9}\ M$$

$$R_M = 1.704$$

$$R_H = 0.852$$

X_{FH} (the concentration of free L-T_4 at $R = R_H$ when L-T_4 is used as an analogue) is calculated from

$$R_H = \frac{X - 4.25X_{FH}}{X_{FH}} \quad (A12)$$

Notice that X_{FH} is independent of the chemical nature of the analogue. A_{FH} (the concentration of free analogue when $R = R_H$) is calculated by applying eq A10 for $R = R_H$ ($X = X_{FH}$; $A = A_{FH}$) and rearranging to obtain

$$C_1C_2A_{FH}^2 + \left(C_1 + C_2 + \kappa T_{FH} - \frac{P\kappa}{R_H}\right)A_F - \gamma = 0 \quad (A13)$$

$$\gamma = \frac{P(K_1 + K_2 + 2K_1K_2X_{FH})}{R_H} - (K_1X_{FH} + 1)(K_2X_{FH} + 1) \quad (A14)$$

Note that, since R_H and X_{FH} are independent of the chemical nature of the analogue, γ is too. Equation A13 is a quadratic whose two roots are given by

$$A_{FH} = \frac{-\xi \pm (\xi^2 + 4C_1C_2\gamma)^{1/2}}{2C_1C_2} \quad (A15)$$

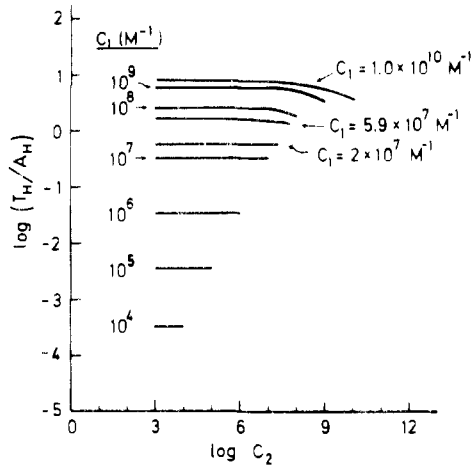

FIGURE A1: Model calculations of log (T_H/A_H) as a function of log C_2 for various values of C_1. See text for specification of the other parameters of the calculation.

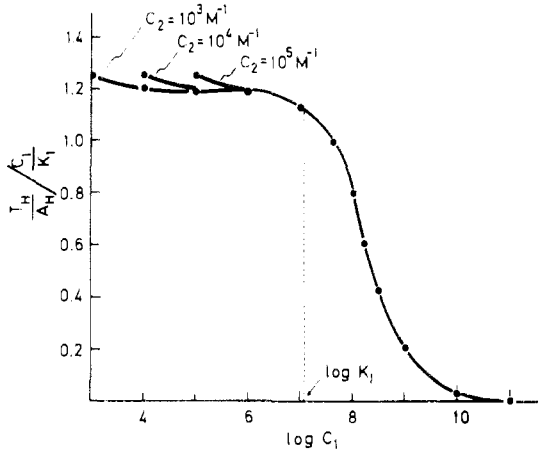

FIGURE A2: Model calculations of the ratio of $(T_H/A_H)/(C_1/K_1)$ as a function of log C_1 for various values of C_2.

$$\xi = C_1 + C_2 + \kappa T_{FH} - \frac{P_K}{R_H} \qquad \text{(A16)}$$

Note that, in eq A15, only the root with the positive sign was taken since it is the only physically acceptable root. The calculated value of $\gamma = 1.113$ (i.e., $\gamma > 0$) implies that $(\xi^2 + 4C_1C_2)^{1/2} > |\xi|$ which implies that the root with the negative sign gives the physically unacceptable negative value for A_{FH}. Finally, using the various mass action expressions for protein-bound terms in eq A3 and taking note of eq A7 gives

$$A_H = A_{FH}\left(4.25 + \frac{PC_1}{1 + K_1X_{FH} + C_1A_{FH}} + \frac{PC_2}{1 + K_2X_{FH} + C_2A_{FH}}\right) \qquad \text{(A17)}$$

Every variable on the right-hand side of eq A17 is either measurable or calculable with the exception of C_1 and C_2. This includes A_{FH}, whose value is calculable by eq A15 once C_1 and C_2 are specified. We can thus calculate A_H over every point of C_1,C_2 space (note that this space is characterized by $C_1 \geq C_2$). Moreover, setting $C_1 = K_1$ and $C_2 = K_2$ in eq A8 and A15–A17 gives T_{FH} and T_H.

Figure A1 shows that, for any particular value of C_1, log (T_H/A_H) does not vary substantially as C_2 changes when $C_1 \leq K_1$. The exact values of the slopes of log (T_H/A_H) vs. log C_2 curves in this regime of C_1 are very close to zero (the maximum absolute values of these slopes are 2×10^{-2}). Figure A2 examines the agreement between T_H/A_H and C_1/K_1. If the agreement is perfect, the ordinate of the figure should have a value of unity for all values of C_1. The calculated values of T_H/A_H, however, are $(1.1\text{–}1.25)C_1/K_1$ for $C_1 \leq K_1$ and $(0.8\text{–}1.1)C_1/K_1$ for $K_1 < C_1 \leq 3K_1$, and T_H/A_H becomes a poor estimator of C_1/K_1 for $C_1 > 3K_1$. In the latter two cases, however, one can still, at least qualitatively, discern that $C_1 > K_1$ without knowing the exact value of their ratio.

Table AI: Results of the Linear Least-Squares Fit of the Experimental Data of Thyroid Hormone Analogues to Equation A20

no.[a]	$-\ln A_H$[b]	$-\alpha$[b]	$\lvert r\rvert$[c]	n[c]
1	16.63 (0.12)	2.98 (0.69)	0.869	8
2	14.40 (0.06)	2.41 (0.40)	0.932	6
3	15.63 (0.09)	2.53 (0.41)	0.952	6
4	11.04 (0.09)	4.20 (0.40)	0.996	3
5	11.75 (0.07)	3.67 (0.29)	0.994	4
6	13.38 (0.08)	2.52 (0.18)	0.977	12
7	7.91 (0.07)	2.51 (0.27)	0.967	8
8	9.51 (0.05)	3.16 (0.16)	0.994	7
9	NC			6
10	11.38 (0.06)	6.00 (0.38)	0.998	3
11	11.35 (0.33)	3.64 (0.66)	0.954	5
12	12.00 (0.07)	3.63 (0.35)	0.969	9
13	11.82 (0.09)	4.95 (0.81)	0.929	8
14	13.98 (0.29)	5.22 (0.63)	0.952	9
15	12.46 (0.40)	3.33 (0.76)	0.931	5
16	12.98 (0.25)	3.48 (0.46)	0.967	6
17	13.10 (0.60)	4.98 (1.05)	0.922	6
18	12.25 (0.39)	3.55 (0.74)	0.959	4
19	12.67 (0.09)	4.53 (0.34)	0.986	7
20	11.91 (0.17)	3.21 (0.37)	0.939	12
21	12.02 (0.18)	4.63 (0.26)	0.998	3
22	11.49 (0.08)	2.41 (0.22)	0.964	11
23	11.80 (0.11)	2.42 (0.40)	0.951	6
24	12.92 (0.21)	2.79 (0.41)	0.949	7
25	11.94 (0.13)	2.63 (0.38)	0.943	8
26	13.11 (0.10)	2.58 (0.23)	0.976	8
27	11.61 (0.24)	3.09 (0.53)	0.958	5
28	14.26 (0.09)	1.99 (0.30)	0.913	11
29	16.47 (0.36)	5.22 (1.05)	0.896	8
30	14.33 (0.03)	1.88 (0.10)	0.991	9
31	10.08 (0.11)	2.81 (0.30)	0.994	3
32	15.14 (0.14)	2.40 (0.75)	0.916	4
33	10.81 (0.09)	2.63 (0.43)	0.974	4
34	17.15 (0.02)	0.67 (0.07)	0.995	3
35	17.95 (0.02)	1.19 (0.08)	0.996	4
36	17.48 (0.07)	1.20 (0.21)	0.958	5
37	17.41 (0.03)	1.41 (0.09)	0.994	5
38	13.56 (0.07)	1.76 (0.31)	0.957	5
39	11.21 (0.10)	2.51 (0.38)	0.973	5

[a] The numbers in this column correspond to the compound in column 1 of Tables I and II. [b] $\ln A_H$ and α are the intercept and slope of eq A20. Numbers in parentheses are their standard deviations. [c] $\lvert r\rvert$ is the absolute value of the correlation coefficient and n is the number of points.

In order to obtain the C_1/K_1 ratios for our various analogues, we set $C_2 = 0$ and calculated T_H/A_H as a function of C_1/K_1 and, from this, we determined which value of C_1/K_1 fit our experimentally observed T_H/A_H. The only analogues that had $C_1 > K_1$ were tetraform, tetrac, tetraprop, and tetrabut (Table II, compounds **34**–**37**). We can thus safely say that our reported C_1/K_1 ratios are quantitatively accurate except for these four analogues. Moreover, we can confidently say that these four analogues have higher affinities than L-T_4.

The reason for the loss the quantitative detail when $C_1 >> K_1$ can be most lucidly seen by taking the limit as $C_1 \to \infty$ while C_2 remains finite. From eq A16 and A15

$$\lim_{C_1 \to \infty} \xi = \eta C_1$$

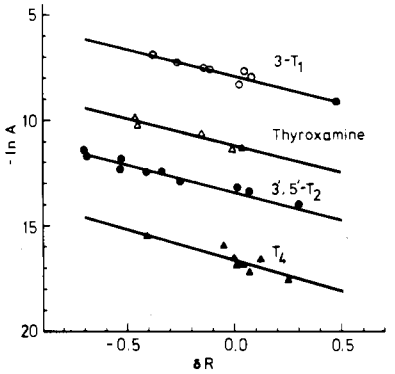

FIGURE A3: Experimental points and best fit lines (—) for the displacement of prealbumin-bound T_4 by various analogues according to eq A20. See Table I for abbreviations.

where η is some constant

$$\lim_{C_1 \to \infty} A_{FH} = \lim_{C_1 \to \infty} \frac{-\eta C_1 + (\eta^2 C_1^2 + 4\gamma C_1 C_2)^{1/2}}{2C_1 C_2} = 0$$

$$\lim_{C_1 \to \infty} A_H = P$$

Thus, in the limit of very high C_1, the initial LHC concentration of analogue required to cause half-maximal displacement approaches a constant value equal to the protein concentration. Since T_H itself is a constant independent of analogue, the ratio T_H/A_H approaches a constant value and does not change appreciably when C_1 increases any further.

Determination of A_H. In order to find the concentration of analogue at half-maximal displacement, we used the following procedure. It is clear that A (and, hence, ln A) is an analytic function of R. We thus expand ln A in a Taylor series about R_H. We define

$$\delta R = R - R_H \tag{A18}$$

and

$$\ln A = \ln A_H + \sum_{i=1}^{\infty} \left(\frac{d^i \ln A}{d\delta R^i} \right)_{\delta R=0} \frac{\delta R^i}{i!} \tag{A19}$$

The series may be expected to converge in its linear term for small R, i.e.

$$\ln A = \ln A_H + \alpha \delta R \tag{A20}$$

where $\alpha = (d \ln A/d\delta R)$ at $\delta R = 0$. We have thus accepted data for which $\delta R \leq 0.7$. For these data we performed a linear least-squares fit for ln A as a function of δR. No less than three experimental points were used for every analogue and in most cases there were 5–12 points. Table AI shows the slopes, intercepts, their standard deviations, the correlation coefficients, and the number of experimental points. Figure A3 shows the experimental points and the best fit curves for some of the analogues.

Error Analysis. If X and Y are independent variables, then the standard deviation $[\sigma(X - Y)]$ of their difference is given by

$$\sigma(X - Y) = [\sigma^2(X) + \sigma^2(Y)]^{1/2} \tag{A21}$$

where $\sigma(X)$ and $\sigma(Y)$ are the standard deviations in X and Y, respectively (Wonnacott & Wonnacott, 1972). Table AI shows that the standard deviations in the experimentally determined values of ln A_H are in the range 0–0.1 for 21 analogues, 0.1–0.2 for 8 analogues, 0.2–0.3 for 5 analogues and 0.3–0.6 for 4 analogues corresponding to a standard deviation in ln (T_H/A_H) of 0.12–0.16, 0.16–0.23, 0.23–0.32, and 0.32–0.61, respectively. From our model calculations, the almost 1 to 1 correspondence between ln (T_H/A_H) and ln (C_1/K_1) for $C_1 \leq K_1$ suggests that the error in our final accepted values of the latter is of the same order as the uncertainty in the former. Finally, if ln $C_1/K_1 = \mu \pm \sigma$, then the range of $C_1/K_1 = [\exp(-\sigma)$ to $\exp(+\sigma)][\exp(\mu)]$. Moreover, since $|RT| = 0.62$, the standard deviation in the corresponding free energy is 0.62σ. Thus, for $\sigma = 0.1$, 0.2, 0.3, and 0.5, the corresponding $[\exp(-\sigma), \exp(\sigma), 0.62\sigma] = (0.9, 1.1, 0.62)$, $(0.82, 1.22, 0.12)$, $(0.74, 1.35, 0.19)$, and $(0.61, 1.65, 0.31)$, respectively.

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