Thyroid Hormone Binding to Human Serum Prealbumin and Rat Liver Nuclear Receptor: Kinetics, Contribution of the Hormone Phenolic Hydroxyl Group, and Accommodation of Hormone Side-Chain Bulk†

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ABSTRACT: The kinetics of binding of the thyroid hormones, L-thyroxine (L-T_4) and L-3,5,3′-triiodothyronine (L-T_3), to human serum prealbumin were measured by a rapid gel filtration procedure to separate protein-bound from free hormone. The association rate constant for the L-T_4–prealbumin complex is comparable in magnitude to that of the L-T_3–receptor complex. A lower limit for the L-T_3–prealbumin association rate constant is 10^6 M^{-1} min^{-1}. The dissociation rate constant for the L-T_3–prealbumin complex is higher than that for the L-T_4–prealbumin complex. This is attributed to steric effects, due to the extra iodine atom of L-T_4, that are operative in L-T_4 dissociation from prealbumin. The dissociation rate constant for the L-T_3–prealbumin complex is much higher than that for the L-T_3–receptor complex, implying that the receptor binding site is in some sense more confined than the binding site of prealbumin. Analysis of the binding patterns of 4′-hydroxy and deoxy analogues shows that the 4′-hydroxyl group contributes negligibly to binding to prealbumin in contrast to its high contribution to binding to nuclear receptor. Whereas the 4′-hydroxyl group may be involved in hydrogen bonding with an aliphatic hydroxyl group(s) or a water molecule in the binding site of prealbumin, the above observation suggests that it may be involved in hydrogen bonding with strong hydrogen bond donating or accepting groups such as an ammonium cation or carboxylate anion, respectively, in the receptor. Binding studies were performed with analogues of T_4 and T_3 modified by the addition of hydrocarbon chains of varying length to the hormone α-amino group to further compare the side-chain binding regions in prealbumin and nuclear receptor. With receptor, both L and D enantiomers of *N*-acetyl-T_3, *N*-pentanoyl-T_3, and *N*-decanoyl-T_3 showed low affinities (<1%) relative to L-T_3. The affinities of D-*N*-acetyl-T_4 and D-*N*-pentanoyl-T_4 for prealbumin were much higher (24% and 69%, respectively) and were almost 2–4 times higher than that of the L enantiomers. The reversal observed in enantiomeric selectivity from that previously reported for analogues having the free α-amino group is rationalized by the interplay of steric and electrostatic effects introduced by the addition of side-chain bulk and elimination of positive charge. The data suggest that the region of the receptor binding site occupied by the hormone side chain is more confined and also less sensitive to side-chain asymmetry than this region in prealbumin.

High-affinity limited capacity thyroid hormone binding sites have been identified in nuclei (Samuels et al., 1974; Eberhardt et al., 1979), mitochondria (Sterling et al., 1977), and the cytoplasm (Surks et al., 1975; Davis et al., 1974) of thyroid hormone receptive tissues. The specific associations between thyroid hormones and thyroxine binding globulin (Nilsson & Peterson, 1975; Snyder et al., 1976) and thyroxine binding prealbumin (Andrea et al., 1980), the two serum proteins responsible for transport of the hormones to tissue sites, have been well-defined. Whereas the functions of the cytoplasmic and mitochondrial binders are still unclear, there is considerable evidence that the nuclear receptor is central to the initiation of hormonal activity; T_3[1] binding stimulates transcriptional events leading to the synthesis of proteins involved in the expression of hormonal effects (Tata & Widnell, 1966; Martial et al., 1977). However, little is known regarding the structure of the intracellular receptor or the nature of the signal transmitted to the DNA as a result of hormone binding. The receptor appears to be an asymmetrical acidic protein with a molecular weight of 50 500 (Latham et al., 1976). In contrast, prealbumin has been well characterized; crystallographic analysis at 1.8-Å resolution has revealed considerable detail regarding the tertiary and quaternary structure (Blake et al., 1978). The protein has a molecular weight of 55 000 and is composed of four identical subunits arranged with tetrahedral symmetry. High-resolution X-ray studies show that T_4 is bound to one of two identical sites in a narrow cylindrical channel running through the center of the long axis of the molecule (Blake & Oatley, 1977).

Prealbumin is an attractive model for studying the nature of hormonal interactions with the nuclear receptor. The two proteins appear to have common features: (a) The molecular weights are quite similar. (b) The receptor is a DNA-binding protein; X-ray analysis has shown that prealbumin has a putative DNA-binding site complementary to double-stranded DNA (Blake & Oatley, 1977). (c) Receptor from crude nuclear extract can be altered by further purification, acidification, or heating to a form more similar to prealbumin in that T_4 is bound with higher affinity than T_3 (Eberhardt et al., 1979). Furthermore, structure–activity relationship studies employing a large number of thyroxine analogues have identified common structural features contributing to binding. They have been discussed by Jorgensen (1978) and include (a) a positively charged region that can ion pair with the

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[1] Abbreviations: T_3, L-3,5,3′-triiodothyronine; T_4, thyroxine. Other abbreviations of hormone analogues are defined in Tables I–III.

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carboxylate ion of the hormone alanine side chain, (b) regions containing hydrophobic pockets that can accommodate the bulky hormone inner- and outer-ring iodine substituents, and (c) a site near the 5′-iodine that can hydrogen bond to the hormone 4′-hydroxyl group.

The binding of L-T_4 and L-T_3 to prealbumin has been studied mainly by equilibrium dialysis (Raz & Goodman, 1969; Nilsson & Peterson, 1971; Pages et al., 1973; Ferguson et al., 1975; Nilsson et al., 1975; Andrea et al., 1980). Hillier & Balfour (1971) determined the first-order rate constant for the dissociation of the L-T_4–prealbumin complex in whole plasma by using gel filtration. The kinetics of binding of these hormones to pure prealbumin have not been reported. Bolger (1977) and Bolger & Jorgensen (1980) studied the kinetics of binding of L-T_3 to solubilized rat liver nuclear receptor by gel filtration.

The affinities of side-chain, partially iodinated, and 3-, 5-, and 3′-halogen- and alkyl-substituted analogues to prealbumin have been studied and compared to their affinities to rat liver nuclear receptor (Andrea et al., 1980). The contributions of the 4′-hydroxyl groups to binding of a variety of analogues to nuclear receptor have been explored (Bolger, 1977; Bolger & Jorgensen, 1980). Analogous studies for binding to prealbumin have not been performed.

In this study, a gel filtration procedure is used to measure the binding of L-T_4 and analogues to prealbumin. In this technique, the separation of the hormone–prealbumin complex from free hormone can be carried out rapidly enough to study the binding kinetics. Comparison of these results to their counterparts in binding to the nuclear receptor allowed the delineation of the differences in the reaction profiles. The affinities of a variety of 4′-hydroxy and deoxy pairs of analogues were also measured, and the contributions of the 4′-hydroxyl groups to binding to prealbumin and to nuclear receptor were compared. Finally, to further compare structural features in the region occupied by the hormone alanine side-chain residue, we have studied the binding of a series of hormone analogues modified by the addition of hydrocarbon bulk to the amino group of the side chain. Amino group modification was chosen since deamino analogues demonstrated higher binding affinities to both receptor and prealbumin, suggesting that neutralization of charge through amide formation might result in compounds retaining high binding affinity.

Experimental Procedures

Materials. Thyroid Hormones, Chemicals, and Proteins. T_4 and T_3 were from Nutritional Biochemicals. [^{125}I]-L-T_4 and [^{125}I]-L-T_3 were purchased from New England Nuclear Corp. The specific activities were 800–1200 μCi/μg. The sources of other hormone analogues have been reported previously (Andrea et al., 1980; Bolger & Jorgensen, 1980). *N*-Hydroxysuccinamide, *N*,*N*′-dicyclohexylcarbodiimide, and acyl chlorides were obtained from Aldrich.

The solubilized nuclear receptor source was a crude nuclear extract prepared by the method of Latham et al. (1976). It was used without further purification. Prealbumin was obtained from Dr. R. C. Hevey, Behring Diagnostics, Somerville, NJ, and had an immunological purity of 98%. The assumed molecular weight was 55 000 (Blake et al., 1978).

Synthesis of N-Acylthyronine Analogues. All compounds with the exception of the hexadienylamide were synthesized by direct acylation of T_3 or T_4 with the appropriate acyl chloride. Preparative thin-layer chromatography was used to separate and purify products from starting materials. Eighty micromoles of D- or L-T_3 or T_4 (free acid or sodium salt) and 25 mg of $NaHCO_3$ were added to 2 mL of dimethylformamide. The mixture was chilled to 0 °C, and a 6 M excess of acyl chloride was slowly added with stirring. After 15 min, the solution was filtered and evaporated to dryness at 45 °C under vacuum. The residue was dissolved in methanol and applied to a PLQF-type preparative thin-layer chromatography plate (Whatman). The plate was briefly but thoroughly air-dried and developed in ethyl acetate–acetic acid (9:1). Since the neutral products migrated with an R_f of 0.3–0.6 while the unreacted amino acids remained at the origin, this method ensured that the products were not contaminated with starting material. The band containing product was located by brief exposure to ultraviolet light and immediately scraped off the plate and eluted with methanol by using a scintered glass funnel. The methanol was removed at 45 °C under vacuum, and solidification of the product was achieved by the addition of cold water to the resultant oil. The white solids were washed twice with 0.5-mL aliquots of cold water, collected by centrifugation, and lyophilized. The yields in the acylation step (estimated by adding [^{125}I]T_3 or [^{125}I]T_4 to control reactions) were 40–55%, and typical final yields of product were 30–40%.

N-Hexadienyl-T_3 was prepared by coupling T_3 with sorbic acid using the *N*-hydroxysuccinamide-activated ester of sorbic acid. A mixture of 75 mL of ethyl acetate and 25 mL of dimethylformamide was added to 2.24 g of sorbic acid and 2.3 g of *N*-hydroxysuccinamide with stirring. *N*,*N*′-Dicyclohexylcarbodiimide (5.3 g) was added under N_2. The solution was filtered after 2 h and again after 24 h, and the solvents were removed under vacuum at 50 °C. The oily crystalline solid was dissolved in hot ethanol and filtered and the filtrate washed twice with cold ether. The product, 2,4-hexadienoyl-*N*-succinamide, was crystallized from cold ethanol (yield = 50%).

For the coupling reaction, 80 μmol of D- or L-T_3 (free acid) and 85 μmol of the succinamide ester were dissolved in 2 mL of dimethyl sulfoxide. After 24 h, cold water was added, and the precipitate was collected by centrifugation and washed with water. The product was taken up in methanol and purified by preparative TLC as described above. The T_4 analogues of this compound could not be obtained as pure compounds after extraction from thin-layer plates.

Physical Methods. Melting points were determined in a Hoover apparatus. Microanalyses were performed by the Microanalytical Laboratory, Department of Chemistry, University of California, Berkeley, CA. Optical rotations were measured with a Perkin-Elmer Model 141 polarimeter.

Equilibrium Binding Assays. The gel filtration binding assay described by Latham et al. (1976) as modified by Bolger & Jorgensen (1980) was used to study L-T_3–receptor binding. In this assay, after equilibration of [^{125}]T_3 with the receptor, bound hormone (measured by γ spectrometry) is separated from free hormone at 4 °C by gel filtration. There is no significant dissociation of the complex in the time required for gel filtration since the dissociation constant (k_2) at 4 °C is about 5×10^{-4} min^{-1} (Bolger & Jorgensen, 1980). Employing this assay, our analysis of the binding of increasing concentrations of T_3 by the method of Scatchard (1949) revealed a single class of high-affinity sites with an affinity constant (K_1) of 2.2×10^9 M^{-1}. Values of 1.5×10^9 and 10^9 M^{-1} were reported by Bolger & Jorgensen (1980) and Latham et al. (1976), respectively.

A similar procedure was developed for studying L-T_4–prealbumin binding. The prealbumin assay mixture contained 10 nM prealbumin, approximately 0.2 nM [^{125}I]-L-T_4 containing approximately 0.15 μCi, and graded concentrations

of L-T_4 or hormone analogue in 0.1 M Tris-HCl buffer (pH 8.0) containing 0.1 M NaCl and 1 mM EDTA in volume of 0.5 mL. After incubation for 30 min at 4 °C, protein-bound [^{125}I]T_4 was isolated by gel filtration at 4 °C on Sephadex G-25 minicolumns (bed volume = 2.0 ± 0.05 mL) equilibrated with the assay incubation buffer. For gel filtration, 0.4 mL of the reaction was applied to the minicolumn followed by rinsing with an equal volume of buffer. The protein fraction was then collected with 0.8 mL of additional buffer. Mild pressure was applied to limit the elution time through the column to less than 30 s.

For Scatchard analyses, the concentration of unlabeled T_4 was varied from 5 to 800 nM. To messure the binding of unlabeled analogues to prealbumin, analogues were tested for their ability (relative to L-T_4) to displace [^{125}I]-L-T_4 from the high-affinity binding sites. In the analogue competition assays, all reactions contained 12.5 nM L-T_4. Stock solutions of analogues were prepared in methanol and diluted to final concentration in assay buffer. A PROPHET computer program (Bolger, 1979) was employed to analyze the binding data according to the method of Oppenheimer et al. (1974) using the equation

$$\frac{\text{free cpm}}{\text{bound cpm}} = 1/[K_1(T_4)(P_t)] + \frac{[K_1(A)/K_1(T_4)](\text{concn of analogue added})}{P_t}$$

where $K_1(T_4)$ and $K_1(A)$ are the affinity constants for L-T_4 (obtained from a Scatchard plot) and unlabeled analogue, respectively, and P_t is the concentration of hormone binding sites.

Kinetic Assays. The procedures used to follow the time course of the association and dissociation of the L-T_4–prealbumin complex were modifications of the equilibrium binding assay. Dissociation experiments were performed by using the equilibrium binding assay with prealbumin and L-T_4 present at 10 nM. Assay reaction mixtures were allowed to reach equilibrium at 4 °C, and 0.4-mL aliquots were filtered on Sephadex G-25 minicolumns to obtain protein-bound [^{125}I]T_4 as described above. The L-T_4–prealbumin complexes were then allowed to dissociate at 4 °C, and the concentration was determined by repeating the gel filtration step using 0.4-mL aliquots at various time intervals. Association experiments were performed with prealbumin present at 20 nM and L-T_4 at 5 nM. The concentration of bound L-T_4 was then determined at 4 °C as a function of time.

Kinetic Model for L-T_4-Prealbumin Binding and Data Analysis. We focus on the kinetics of L-T_4 binding to a single site of prealbumin. The justification for this is given below. The reaction is

$$L + M \rightarrow LM$$

where L, M, and LM designate the ligand, macromolecule, and ligand–macromolecule complex, respectively. The kinetic equation for this reaction is

$$\frac{dB}{dt} = k_1(P - B)(T - B) - k_2B \tag{1a}$$

with the initial condition

$$B(t = 0) = B_0 \tag{1b}$$

k_1 and k_2 are the kinetic constants for the forward and reverse reactions, respectively; P, T, B, and t are the concentration of binding sites (in this case one per macromolecule), total ligand, and bound ligand and time, respectively. The initial concentration of bound L-T_4 (B_0) is zero in the kinetic association experiment and is measured in the kinetic dissociation experiment.

The concentrations of the different species are subject to the following mass conservation conditions:

$$T = B + F \tag{2a}$$

$$P = B + P_F \tag{2b}$$

where F and P_F are the concentrations of free ligands and free sites (one per macromolecule). P, T, k_1, and k_2 are independent of time. Equation 1a can be rearranged into

$$\frac{dB}{dt} = \lambda - \eta B + \xi B^2 \tag{3a}$$

where

$$\lambda = k_1PT \tag{3b}$$

$$\eta = k_2 + k_1(P + T) \tag{3c}$$

$$\xi = k_1 \tag{3d}$$

λ, η, and ξ are constants (independent of t and B). The solution to eq 3a is

$$\int_{B_0}^{B(t)} \frac{dB}{\lambda - \eta B + \xi B^2} = t - \ln C \tag{4}$$

where $\ln C$ is the constant of integration. The value of integral in eq 4 is

$$\frac{1}{(\eta^2 - 4\lambda\xi)^{1/2}} \ln \frac{2\xi B - \eta - (\eta^2 - 4\lambda\xi)^{1/2}}{2\xi B - \eta + (\eta^2 - 4\lambda\xi)^{1/2}} \tag{5}$$

Defining

$$\zeta = (\eta^2 - 4\lambda\xi)^{1/2} \tag{6}$$

and substituting eq 5 and 6 into eq 4, after rearranging and taking antilogarithms, gives

$$B = \frac{\eta}{2\xi} + \left(\frac{C^\zeta + e^{\zeta t}}{C^\zeta - e^{\zeta t}}\right)\left(\frac{\zeta}{2\xi}\right) \tag{7}$$

Applying the initial condition (eq 1b) to eq 7 gives

$$C = \left(\frac{2\xi B_0 - \eta + \zeta}{2\xi B_0 - \eta - \zeta}\right)\frac{1}{\zeta} \tag{8}$$

Equations 7 and 8 together with the defining equations 3b–d and 6 are the solution to the initial value problem of eq 1a and 1b.

Applying eq 7 to the determination of rate constants k_1 and k_2 for binding to a single site of prealbumin requires knowledge of B, which is the concentration of singly occupied prealbumin molecules as a function of time. In the tracer techniques for concentration determination, the only measurable concentration is total bound L-$T_4(X)$ where $X = B + B_2$, with B and B_2 being the concentrations of L-T_4 bound to sites 1 (high affinity) and 2 (low affinity), respectively. At any time point, $B_2(t) < (B_2)_{eq}$ where $(B_2)_{eq}$ is the equilibrium concentration of B_2. Hence

$$X(t) \geq X(t) - B_2(t) = B(t) \geq X(t) - (B_2)_{eq}$$

The equality signs in these inequalities are to hold if the value of K_2 is zero or much smaller than K_1 (K_1 and K_2 are the affinity constants of L-T_4 to the high- and low-affinity sites, respectively). The measured values of K_1 and K_2 (see Results and Discussion) are 7.6×10^7 M^{-1} and 10^6 M^{-1}, respectively. In kinetic experiments, the concentration of free L-T_4 at equilibrium is (2–7) $\times 10^{-9}$ M and $(B)_{eq}$ and B_2 are (2.8–3.4)

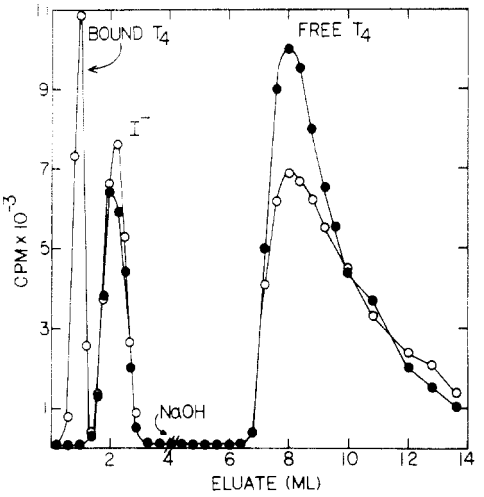


FIGURE 1: Separation of the T_4–prealbumin complex from unbound T_4 and free iodide on Sephadex G-25 at 4 °C. A 0.4-mL aliquot of an L-T_4–prealbumin binding assay mixture incubated with (●) or without (○) unlabeled T_4 (10^{-5} M) was filtered on Sephadex G-25 as described under Experimental Procedures. The elution buffer was changed to 0.25 N NaOH where indicated.

$\times 10^{-9}$ M and (9.7–9.9) $\times 10^{-11}$ M, respectively. Hence $(B_2/B)_{eq} \sim 0.032$. This implies that $(B_2)_{eq}$ and $B_2(t)$ are negligible fractions of $X(t)$. Thus, for the curve fitting purposes, we set $B(t) = X(t)$. $B(t)$ was evaluated by using eq 7 with the appropriate initial conditions and assumed values of k_1 and k_2. The values of k_1 and k_2, whose corresponding curves bracketed the observed $B(t)$ (from kinetic association and dissociation experiments), were determined.

Results and Discussion

L-T_4–Prealbumin Bindiing. The separation of the T_4–prealbumin complex from free T_4 by the gel filtration procedure used in the prealbumin binding assay is shown in Figure 1. After application of the sample, the T_4–prealbumin complex is eluted in a 0.8-mL volume following a 0.4-mL "wash" volume. When a large excess of unlabeled T_4 is included during incubation, virtually all of the bound, labeled T_4 is displaced. Therefore, essentially all of the binding measured in the assay appears to be "specific" in nature. Free, radioactive iodide (5–16% of the radioactivity in a typical commercial lot of [^{125}I]T_4 elutes directly after the protein peak. Free hormone binds tightly to the gel matrix and can be eluted by increasing the pH (Figure 1). The amount of bound [^{125}I]T_4 determined by the procedure was linearly proportional to the concentration of prealbumin included in the assay from 1 to 25 nM protein (data not shown).

The gel filtration step can be carried out in approximately 30 s. Since the half-life of the dissociation of the T_4–prealbumin complex measured at 4 °C was 13 min (see below), the extent of dissociation of [^{125}I]T_4 during gel filtration was minimal. Increasing the elution time through the column from 30 s to 1 min gave identical affinity constants (K_1) for L-T_4 and L-T_3 and also identical kinetic association and dissociation rate constants (k_1 and k_2) for L-T_4 (see below). Hence, any dissociation of the complex during elution was not a significant source of error in determining kinetic parameters.

Figure 2 shows a Scatchard plot of L-T_4–prealbumin binding using the equilibrium binding assay and gel filtration to separate bound from free hormone. The data were fitted to Scatchard's model for two distinguishable and independent sites as in Andrea et al. (1980). The best-fit parameters were $K_1 = 7.6$ (±0.25) $\times 10^7$ M^{-1}, $K_2 = 1.0$ (±1.3) $\times 10^6$ M^{-1}, $N_1 = 0.90$ (±0.25), and $N_2 = 0.80$ (±7.4) where K_1 and K_2 are

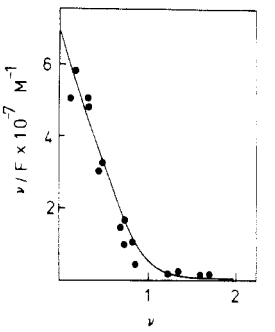


FIGURE 2: Scatchard plot of L-T_4–prealbumin binding. (●) Experimental points; (—) best fit curve for Scatchard's model for two distinguishable and independent binding sites using $K_1 = 7.6 \times 10^7$ M^{-1}, $K_2 = 1.0 \times 10^6$ M^{-1}, $N_1 = 0.903$, and $N_2 = 0.8$.

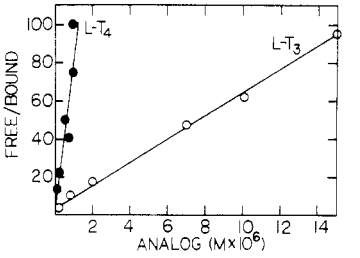
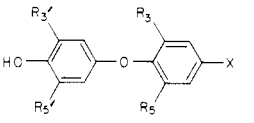


FIGURE 3: Equilibrium competition binding assay for hormone analogues. Unlabeled L-T_3 and L-T_4 were tested for their ability to displace [^{125}I]-L-T_4 from high-affinity binding sites as described under Experimental Procedures.

Table I: Binding of Thyroxine Analogues to Prealbumin



analogue	X	binding affinity [a] gel filt. [b]	equil. dial. [c]	ANS fluor. [d]	equil. dial. [d]
L-T_4	alanine	100	100	100	100
D-T_4	alanine	11.0	3.7		
L-3,5,3′-T_3	alanine	7.5	9.2	7.9	12.6
L-3,3′,5′-T_3	alanine	12.2	32.9		
DL-3,5-T_2	alanine	2.5	3.3		
T_4Fo	COOH	500	186		
T_4Ac	CH_2COOH	800	676	79.4	
T_4Pr	$(CH_2)_2COOH$	600	298	126	159
T_4EtA	$(CH_2)_2NH_2$	0.21	0.37		
L-*N*-Ac-T_4	*N*-acetylalanine	16.7		5.0	8.0

[a] Relative to L-T_4 = 100. [b] Using gel filtration with 0.1 M Tris-HCl, 0.1 M NaCl (pH 8.0); K_1(L-T_4) = 6.1 $\times 10^7$ M^{-1} (see Experimental Procedures). [c] From Andrea et al. (1980) using equilibrium dialysis with 0.1 M Tris-HCl and 0.1 M NaCl (pH 8.0); K_1(L-T_4) = 3.5 $\times 10^7$ M^{-1}. [d] From Cheng et al. (1977a) with 0.05 M potassium phosphate and 0.1 M NaCl (pH 7.4) using 8-anilinonaphthalene-1-sulfonate (ANS) fluorescence titration, K_1(L-T_4) = 3.2 $\times 10^8$ M^{-1}, or using equilibrium dialysis, K_1(L-T_4) = 1.6 $\times 10^8$ M^{-1}.

the affinity constants of the high- and low-affinity sites and N_1 and N_2 are their corresponding number of binding sites per protein molecule. When N_1 and N_2 were constrained to have values of 1, $K_1 = 6.6$ (±3.1) $\times 10^7$ M^{-1} and $K_2 = 1.0$ (±1.2) $\times 10^6$ M^{-1}. Other investigators [see Andrea et al. (1980) and references cited therein] reported values of $K_1 \approx 10^7$–10^8 M^{-1}, $K_2 \approx 10^6$ M^{-1}, and $N_1 \approx N_2 \approx 1$.

Table I compares the binding affinities we measured for unlabeled analogues (relative to L-T_4) using the gel filtration competitive binding assay to values reported by other workers

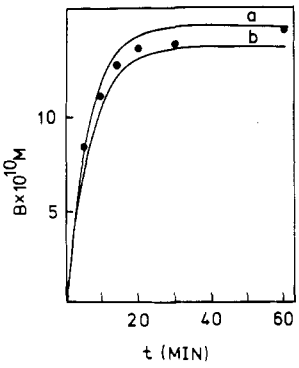


FIGURE 4: L-T_4–prealbumin association kinetics. (●) Experimental points; (—) calculated curves using k_2 = 0.11 min^{-1} and k_1 = 2.6 × 10^6 M^{-1} min^{-1} (a) or 2.3 × 10^6 M^{-1} min^{-1} (b).

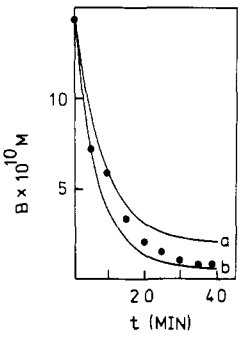


FIGURE 5: L-T_4–prealbumin dissociation kinetics. (●) Experimental points; (—) calculated curves using k_2 = 0.1 min^{-1} and k_1 = 2.0 × 10^6 M^{-1} min^{-1} (a) or 1.0 × 10^6 M^{-1} min^{-1} (b).

employing equilibrium dialysis or fluorescence techniques. The actual plots obtained in the present study for L-T_3 and L-T_4 are shown in Figure 3. The data in Table I verify the results reported by Andrea et al. (1980); the deamino side-chain analogues (T_4Fo, T_4Ac, and T_4Pr) show higher affinities than T_4 while binding of the decarboxylated analogue, thyroxamine, is very low. the K_1 measured for L-T_4 (6.1 × 10^7 M^{-1}) is very similar to the value we measured directly by Scatchard analysis. The K_1 for the L-T_3 (4.6 × 10^6 M^{-1}) is somewhat less than previously reported values [see Andrea et al. (1980)].

Kinetics of L-T_4 Binding to Prealbumin. Figures 4 and 5 show the kinetics of association and dissociation of the T_4–prealbumin complex measured by gel filtration. The binding of L-T_4 to the high-affinity site can be fitted by using k_1 = (1–2.6) × 10^6 M^{-1} min^{-1} and k_2 = 0.10–0.11 min^{-1}. Hillier & Balfour (1971) determined the rate constant for the dissociation of the L-T_4–prealbumin complex to be 1.13 and 7.59 min^{-1} at 20 and 37 °C, respectively. Using these two data points and the Arrhenius equation

$$\ln k_2 = A - \left(\frac{E_a}{R}\right)\left(\frac{1}{T}\right)$$

where A, E_a, R, and T are the intercept of the Arrhenius plot, activation energy, gas constant (1.987 × 10^{-3} kcal deg^{-1} mol^{-1}) and absolute temperature, respectively, one calculates A = 34.98 and E_a = 20.30 kcal/mol. The value of k_2 at 0–4 °C extrapolates to 0.08–0.15 min^{-1}, in close agreement to our values. The affinity constant K_1 calculated from the ratio k_1/k_2 is 9.1 × 10^6 to 2.6 × 10^7 M^{-1}, similar to the value obtained from our Scatchard analysis of the binding isotherms.

Attempts to determine the rate constants for the binding of L-T_3 to prealbumin revealed that the reaction was essentially at equilibrium within the first minute, and hence accurate values for the rate constants could not be calculated. In a kinetic association experiment, B_0 = 0 and eq 1a shows that the initial reaction rate is

$$R(0) = k_1PT \quad (9)$$

where $R(0)$ is the initial rate (dB/dt at t = 0) and T is the total concentration of L-T_3. Moreover, the value $B(1)$ (concentration of L-T_3 bound to the high affinity site after 1 min of reaction) sets a lower limit for the initial reaction rate, i.e.

$$R(0) \geq B(1) \text{ M } min^{-1} \quad (10)$$

Equations 9 and 10 can be combined to give a lower limit for k_1 as

$$k_1(\text{L-T}_3) \geq \frac{B(1)}{PT} \text{ } M^{-1} \text{ } min^{-1} \quad (11)$$

We determined that when P and T were 2 × 10^{-8} and 10^{-7} M, respectively, $B(1)$ = 6 × 10^{-9} M min^{-1} so that k_1(L-T_3) ≥ 3 × 10^6 M^{-1} min^{-1}. Using

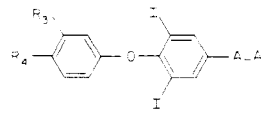
$$k_2 = k_1/K_1$$

where K_1 is the affinity constant of the high-affinity site (determined from Figure 3 to be 4.6 × 10^6 M^{-1} by treating L-T_3 as an analogue) gives k_2(L-T_3) ≥ 0.65 min^{-1}. Bolger (1977) measured the kinetics of binding of L-T_3 to solubilized nuclear receptor and found that at 4 °C, k_1 = 3.55 × 10^6 M^{-1} min^{-1} and k_2 = 5.16 × 10^{-4} min^{-1}.

These observations can be summarized as follows: k_1(L-T_3–prealbumin) ≥ 10^6 M^{-1} min^{-1}, k_1(L-T_4–prealbumin) ≈ k_1(L-T_3–receptor) ≈ 10^6 M^{-1} min^{-1}, k_2(L-T_3–prealbumin) > k_2(L-T_4–prealbumin) ≫ k_2(L-T_3–receptor). The higher activation energy for L-T_4 dissociation from prealbumin (as compared to that of L-T_3) is likely due to steric interactions between the 5′-iodine of L-T_4 and the "wall" of the binding channel of prealbumin. the activation energy for the dissociation of the L-T_3–receptor complex is much higher than the corresponding one for the dissociation of the L-T_3–prealbumin complex. Such effects are likely due to steric effects brought about by the receptor having either a narrower (or in some sense more restricted) binding site than prealbumin. Such steric effects are in accordance with previous observations (Bolger & Jorgensen, 1980; Andrea et al., 1980) that L-T_4 binds to the receptor with much lower affinity than to prealbumin.

Contribution of the 4′-Hydroxyl Group to Binding to Prealbumin and Nuclear Receptor. Table II shows the affinities and free energies of binding of 4′-hydroxy and deoxy 3′-substituted analogues to prealbumin as well as the contribution of the 4′-hydroxyl groups to binding of these analogues to prealbumin and nuclear receptor. Except in the case of the 3′-iodo substitution (2), 4′-hydroxyl groups contribute negligibly to binding to prealbumin. In the same series of 4′-hydroxy and deoxy 3′-substituted analogues, the hydroxyl group contributes significantly more strongly to binding to nuclear receptor than to prealbumin. The small contribution of the hydroxyl group to binding to prealbumin is not surprising in view of the fact that at the onset of binding, hydrogen bonds between solvent water molecules and the 4′-hydroxyl group or phenoxide anion of a hormone analogue molecule in solution must be broken. On binding, hydrogen bonds are re-formed between the 4′-hydroxyl or phenoxide and the crystallographically observed water molecule (Blake & Oatley, 1977) and/or other hydrogen-bonding groups (serine and threonine) in the binding site. If the number of hydrogen bonds to water broken and reformed are equal, then the contribution of the hydroxyl groups to the binding energy will be close to zero. It may be argued that the same is true for

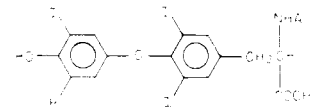
Table II: Contributions of the 4′-Hydroxyl Groups of T_3 Analogues to Free Energies of Binding to Prealbumin and Nuclear Receptor



		prealbumin					NR [a]
		$C_1/K_1 \times 100$ [b]		$\Delta G°(A) - \Delta G°(L\text{-}T_4)$ [c] (kcal/mol)		$\Delta G^1(OH)$ [d]	$\Delta G^1(OH)$ [a]
no.	R_3' [e]	$R_4' = OH$	$R_4' = H$	$R_4' = OH$	$R_4' = H$	(kcal/mol)	(kcal/mol)
1	H	0.01 (1.16)	0.01 (1.15)	4.92	4.92	0.00	−1.24
2	I	4.01 (1.10)	0.42 (1.11)	1.77	3.01	−1.24	−1.83
3	Br	0.49 (1.14)	0.54 (1.14)	2.93	2.87	+0.06	−2.48
4	Cl	0.80 (1.10)	0.31 (1.13)	2.66	3.18	−0.52	−2.04
5	F	0.07 (1.20)	0.05 (1.19)	4.00	4.17	−0.17	−1.56
6	Me	0.04 (1.10)	0.07 (1.11)	4.29	3.97	+0.32	−1.60
7	Et	0.31 (1.11)	0.13 (1.17)	3.18	3.66	−0.48	−2.03
8	*i*-Pr	0.41 (1.12)	0.29 (1.10)	3.03	3.22	−0.19	−2.54
9	*t*-Bu	0.81 (1.12)	0.45 (1.10)	2.65	2.97	−0.32	−1.95

[a] NR, nuclear receptor. $\Delta G^1(OH)$ calculated from the data of Bolger & Jorgensen (1980) using $\Delta G^1(OH) = \Delta G°(4'\text{-hydroxy}) - \Delta G°(4'\text{-deoxy})$ [see eq 3 and associated text in Andrea et al. (1980)]. [b] C_1/K_1 is the ratio of the affinity constant of the analogue (determined by analogue competition binding assays as shown in Figure 3) to that of L-T_4 for the high-affinity site. The numbers in parentheses indicate uncertainty ranges, e.g., $C_1/K_1 \times 100 = 0.01$ (1.16) designates that the mean value of $C_1/K_1 \times 100$ falls in the range 0.01/1.16 to 0.01 × 1.16. [c] $\Delta G°(A) - \Delta G°(L\text{-}T_4) = -RT \ln (C_1/K_1)$. $R = 1.987 \times 10^{-3}$ kcal mol^{-1} deg^{-1}, $T = 277$ K. [d] Calculated as in footnote *a*. [e] All alanyl side chains are L enantiomers except numbers 5 and 7 which are DL mixtures.

Table III: Physical Constants of *N*-Acyl Analogues of T_3 and T_4



				optical rotation [a] in deg	anal (%)								
					calcd				found				
name	R	A	DL	$[\alpha]^{28}D$	C	H	N	I	C	H	N	I	mp [b] (°C)
N-Ac-T_3	H	$COCH_3$	L	+24.0	29.46	2.04	2.02	54.95	29.23	2.09	2.02	54.90	95–100
N-Ac-T_3	H	$COCH_3$	D	−23.9	29.46	2.04	2.02		29.28	2.22	1.84		95–100
N-Ac-T_4	I	$COCH_3$	L	+16.8 [c]	24.93	1.60	1.71		24.63	1.70	1.62		149
N-Ac-T_4	I	$COCH_3$	D	−18.4	24.93	1.60	1.71	61.99	24.70	1.73	1.61	61.68	149
N-Pent-T_3	H	$CO(CH_2)_3CH_3$	L	+18.1	32.68	2.74	1.91	51.79	33.08	2.88	1.83	51.48	90–95
N-Pent-T_3	H	$CO(CH_2)_3CH_3$	D	−19.5	32.68	2.74	1.91	51.79	32.56	2.81	1.84	51.66	90–95
N-Pent-T_4	I	$CO(CH_2)_3CH_3$	L	+13.1	27.90	2.22	1.63	58.96	28.03	2.23	1.54	58.76	115–116
N-Pent-T_4	I	$CO(CH_2)_3CH_3$	D	−14.7	27.90	2.22	1.63	58.96	27.85	2.28	1.50	58.60	115–116
N-Dec-T_3	H	$CO(CH_2)_8CH_3$	L	+21.4	37.29	3.75	1.74	47.28	37.07	3.74	1.72	47.12	80
N-Dec-T_3	H	$CO(CH_2)_8CH_3$	D	−18.8	37.29	3.75	1.74	47.28	37.08	3.78	1.74	46.88	80
N-Dec-T_4	I	$CO(CH_2)_8CH_3$	L	+15.2	32.25	3.14	1.50		33.00	3.37	1.51		156
N-Dec-T_4	I	$CO(CH_2)_8CH_3$	D	−16.8	32.25	3.14	1.50		32.46	3.26	1.42		156
N-Hex-T_3	H	$CO(CH)_4CH_3$	L	+21.0 [c]	33.85	2.43	1.88		34.40	2.70	1.87		115–120
N-Hex-T_3	H	$CO(CH)_4CH_3$	D	−16.5 [c]	33.85	2.43	1.88		34.04	2.97	1.72		115–120

[a] Determined in ethanol. C = 1%. [b] All compounds melted with decomposition. [c] C = 0.4%

the entropy, since the vibrational entropy of the broken and re-formed hydrogen bonds are about the same. Hence, the net change in binding free energy when an inert 4′-hydrogen is replaced by a hydroxyl group would be close to zero. The high contribution of the 4′-hydroxyl group to binding to nuclear receptor is likely to be due to direct bonding, via its proton, to a strong hydrogen bond accepting group such as a carboxylate anion and/or, via its lone electron pairs, to a strong hydrogen bond donating group such as an ammonium cation on the receptor. The lowering of the free energy in the formation of such strong hydrogen bonds could more than compensate for its increase due to breaking the 4′-hydroxyl–solvent water hydrogen bonds at the onset of binding.

Accommodation of Hormone Side-Chain Bulk; Binding of N-Acyl Analogues to Prealbumin and Nuclear Receptor. Table III lists the appropriate physical constants and analytic data for the *N*-acyl compounds prepared for this study. The optical rotation ($[\alpha]^{28}D$) values indicated that acylation of the α-amino group proceeded with retention of molecular symmetry. All D enantiomers showed negative rotations, and the corresponding L enantiomers showed positive rotations. Even though the hexadienyl-T_3 analogues were synthesized by an alternative method (condensation of T_3 with an activated ester), the direction of optical rotation was consistent with that of the acylated series. Blank (1964) reported $[\alpha]^{25}D$ values of −16.6 for D-acetyl-T_4 and +14.1 for L-acetyl-T_4. These compounds were obtained by condensation of T_3 with acetic anhydride.

The thin-layer chromatographic properties of the amides determined on silica with three solvent systems are shown in Table IV. All the compounds were ninhydrin negative and were identified by their UV absorption. When chromatographed under alkaline conditions, the T_4 derivatives turned yellow shortly after exposure to ultraviolet light. As expected,

Table IV: Chromatographic Mobility of *N*-Acyl Analogues[a]

compd	solvent A	solvent B	solvent C
N-Ac-T_3	0.32	0.31	0.35
N-Ac-T_4	0.33	0.20	0.24
N-Pent-T_3	0.52	0.28	0.33
N-Pent-T_4	0.52	0.16	0.24
N-Hex-T_3	0.57	0.47	0.42
N-Dec-T_3	0.59	0.49	0.40
N-Dec-T_4	0.59	0.34	0.22

[a] Ascending chromatography was performed on LK6F silica plates (Whatman). Solvent A was ethyl acetate–acetic acid (9:1), solvent B was chloroform–methanol–concentrated NH_4OH (20:10:1), and solvent C was ethyl acetate–2-propanol–25% NH_4OH (55:35:20).

Table V: Binding Affinities of *N*-Acyl-T_3 and -T_4 Analogues to Prealbumin and Nuclear Receptor[a]

compd	LD	binding affinity: prealbumin (% L-T_4)	binding affinity: receptor (% L-T_3)
T_3, T_4	D	11.0 (1.02)	72.50 (1.10)
N-Ac-T_3, -T_4	L	16.7 (1.09)	0.24 (1.12)
	D	24.0 (1.08)	0.14 (1.07)
N-Pent-T_3, -T_4	L	17.7 (1.04)	0.28 (1.25)
	D	69.3 (1.12)	0.30 (1.10)
N-Hex-T_3	L		0.91 (1.27)
	D		1.17 (1.38)
N-Dec-T_3, -T_4	L	3.4 (1.26)	0.31 (1.25)
	D	3.4 (1.12)	0.46 (1.28)

[a] The T_3 analogues were tested with receptor and the T_4 analogues with prealbumin employing the competition binding assays described under Experimental Procedures. Values are expressed relative to L-T_3 or L-T_4. The numbers in parentheses indicate uncertainty ranges (defined in Table II).

the retention on silica in solvent system A decreased as a function of side-chain length (decreasing polarity).

The binding affinities of the T_3 analogues for the receptor and the corresponding T_4 analogues for prealbumin are shown in Table V. The low affinities of the T_3 analogues to the receptor are contrasted by the relatively high affinities observed for the T_4 analogues to prealbumin. Whereas acylation of L-T_4 led to a 6–30-fold decrease in binding to prealbumin, acylation of D- or L-T_3 led to a 100–700-fold decrease in binding to the receptor. The receptor showed no significant difference in selectivity for the D or L isomers of the amides. This lack of sensitivity to side-chain asymmetry has also been reported for T_3 activity in vivo (Oppenheimer et al., 1973) and in binding studies with whole cell nuclei (Koerner et al., 1974; DeGroot & Torresani, 1975). In contrast, both serum-binding proteins, thyroxine binding globulin, and prealbumin show a marked preference in affinity for L over D isomers. With thyroxine binding globulin, L isomers are bound 2–4 times more tightly (Hao & Tabachnick, 1971; Synder et al., 1976) and with prealbumin about 30 times more tightly than the corresponding D forms (Andrea et al., 1980). However, the data presented in Table V show that after acylation of the amino group of T_4, this enantiomeric selectivity with prealbumin is reversed. With the exception of the *N*-decanoyl analogues, where binding affinity is low and no isomeric preference is apparent, the D forms show almost 2–4 times the affinity of the L forms. Whereas acylation of L-T_4 decreased binding about 6-fold, acylation of D-T_4 increased binding 2–6-fold.

These results support our current view of the steric and functional features of T_4 binding to prealbumin (Figure 6). The side-chain carboxyl group is thought to play a major role

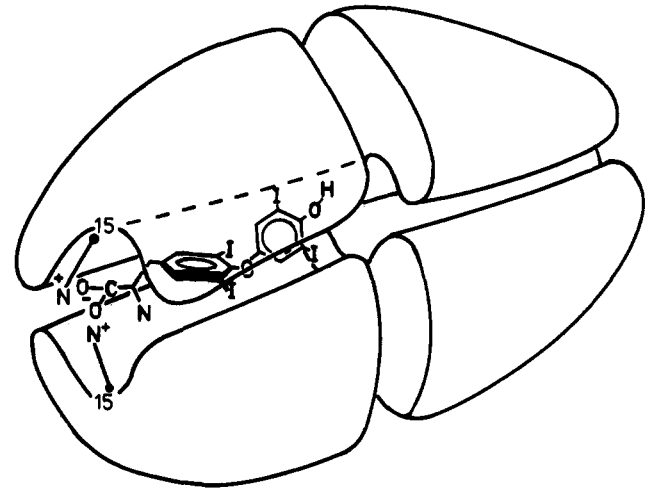


FIGURE 6: Schematic drawing of the four identical subunits of prealbumin associated to form a central channel. T_4 is shown bound with the carboxylate ion of the hormone side chain forming ion paired associations with α-ammonium groups Lys-15 residues of prealbumin which extend into the mouth of the channel. Drawn from the X-ray data of Blake et al. (1974).

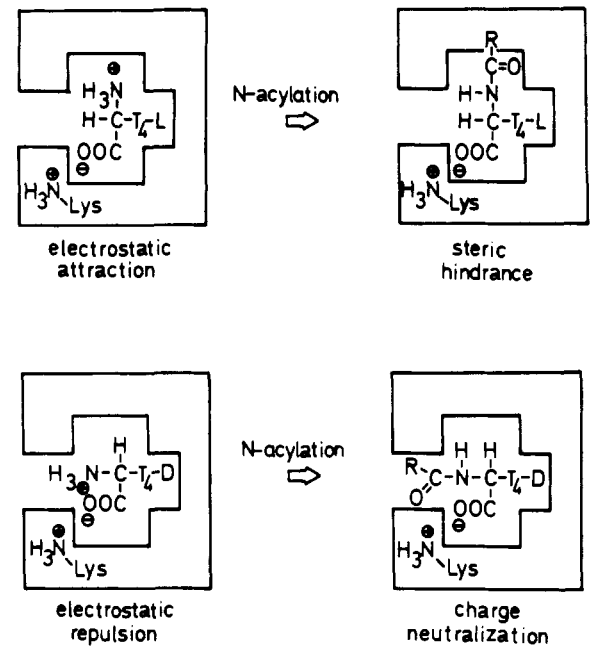


FIGURE 7: Schematic drawing depicting the side-chain interactions of the D and L enantiomers of T_4 and *N*-acyl-T_4 analogues with prealbumin. The enclosed areas represent protein structure of the tetramer forming the binding channel, and Lys-NH_3^+ represents the ammonium groups of lysine residues which extend into the mouth of the binding channel.

in binding due to ion pairing with the ammonium groups of protein Lys-15 residues. This was suggested from affinity-labeling studies with dansyl chloride and *N*-bromoacetyl-T_4 (Cheng et al., 1975, 1977b). The hormone ammonium group is believed to contribute a negative effect on binding due to electrostatic repulsion between it and the lysine ammonium groups (Andrea et al., 1978). Deamination of T_4 would then be expected to increase binding whereas decarboxylation should decrease binding. Indeed, Andrea et al. (1980) showed that the deamino T_4 analogues, T_4-formate, T_4-acetate, and T_4-propionate bind with higher affinities than T_4 whereas thyroxamine binds only very weakly. We have confirmed these results by using the binding assay described in the present report (Table I).

Computer modeling of the T_4–prealbumin complex suggests that the ability of the protein to differentiate between T_4 enantiomers is due to a combination of the aforementioned electrostatic forces and the additional constraints placed on orientation of the side chain to bulky Leu-17 and Val-121 groups (Andrea et al., 1978). Therefore, the observed reversal in the affinities of T_4 enantiomers after acylation can be explained as diagrammed in Figure 7 by the interplay of both modifications resulting from hormone derivatization: the addition of carbon bulk to the amino function and the elimination of the influence of charge.

The lower affinity of L-T_4 after acylation can be explained by steric hindrance due to the introduction of carbon bulk. This negative steric effect is greater than any positive effects that may appear from elimination of charge. The higher affinity for D-T_4 observed after acylation can be explained by elimination of repulsive effects due to neutralization of charge. The latter effect is more prominent than possible negative steric effects from the introduction of bulk. In contrast, any increase in binding to the receptor resulting from neutralization of charge appears to be strongly masked by the introduction of steric bulk. These results suggest that in the region of the binding channel occupied by the side-chain α carbon, the receptor is more tolerant than prealbumin with regard to the steric orientation of charge but much less accomodating than prealbumin to an increase in aliphatic bulk. That the receptor in crude nuclear extracts has a more restricted binding site than prealbumin is suggested by the kinetic analysis (discussed earlier) of the binding of T_3 and T_4 to these proteins. The data in Table V show that tolerance of prealbumin for carbon extension via the amino group decreases markedly after the introduction of five carbon atoms, since the binding of both enantiomers of *N*-decanoyl-T_4 is low. This decrease in binding may result from a steric confrontation with protein structure in the region approaching the mouth of the binding channel. The specific location of this proposed steric effect cannot be determined since the X-ray coordinates of residues near the mouth of the binding channel is uncertain and the configuration of the side chain of the bound analogue is also unknown. However, high-resolution X-ray studies of prealbumin–hormone complexes (Blake et al., 1974) support the view that such an effect would occur well within the realm of the binding channel. The channel takes the form of an eight-stranded cylinder about 21-Å long and 8 Å in diameter. T_3 and T_4 are bound oriented with their long axes lying approximately parallel to the channel axis and with the α carbon of the side chain located about 9 Å from the channel mouth. Therefore, even if the side-chain hydrocarbon arm of decanoyl-T_4 were fully extended in the bound form, the analogue would still appear to be entirely accommodated within the binding channel.

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