Title
Thyroid hormone analogs

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Publication Date
1977

Peer reviewed|Thesis/dissertation

THYROID HORMONE ANALOGS: - SYNTHESIS, ANTIGOITER ACTIVITIES, DECOMPOSITION OF THE THERMODYNAMIC FUNCTIONS OF BINDING, CONFORMATIONAL POPULATION ANALYSIS AND BINDING TO PREALBUMIN

by

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DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

PHARMACEUTICAL CHEMISTRY

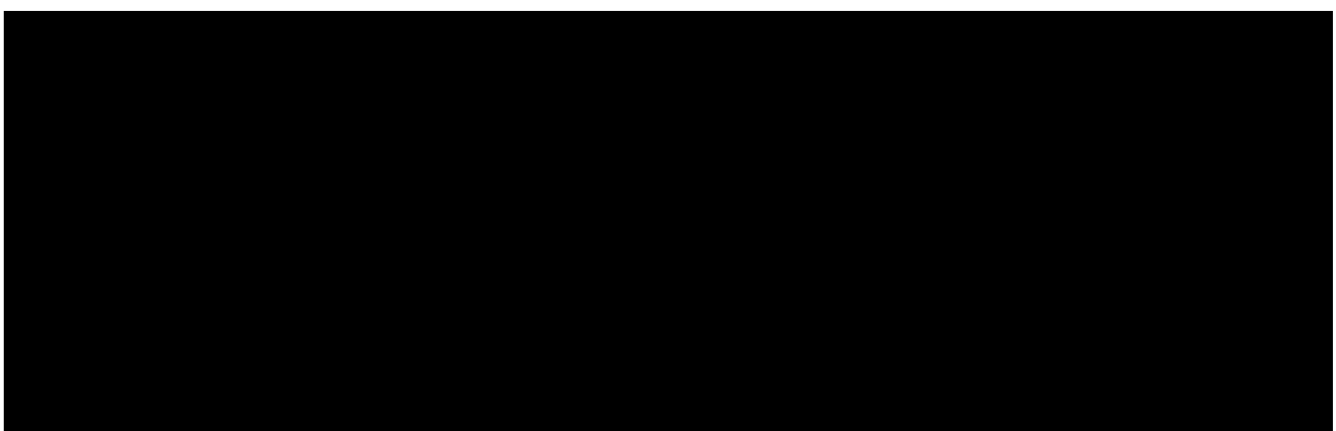
in the

GRADUATE DIVISION

(San Francisco)

of the

UNIVERSITY OF CALIFORNIA



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THYROID HORMONE ANALOGS: - SYNTHESIS, ANTIGOITER ACTIVITIES, DECOMPOSITION OF THE THERMODYNAMIC FUNCTIONS OF BINDING, CONFORMATIONAL POPULATION ANALYSIS AND BINDING TO PREALBUMIN.

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Ph.D. Dissertation

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ABSTRACT

Thyroid hormone analogs were designed and synthesized to test the possibility that lipid character can be transferred from one portion of the molecule to another without loss of activity. The results showed that such transfer of lipid character results only in partial retention of activity.

A theory is presented in which the thermodynamic functions of binding of a compound to a macromolecule is decomposed into contributions from a reference compound in addition to one, two ... N groups perturbation terms. A simplified version of the theory is presented and its use in selected cases is demonstrated.

Quantum mechanical and statistical mechanical studies were used to generate and analyze the potential energy surfaces of thyroid hormones and analogs. The free energy change (ΔG_{lock}) attending the process in which the entire population of molecules is locked into a fixed conformation

is defined and calculated from the potential energy surfaces. The results support a previous hypothesis that thyroid hormones exert their activity in a conformation in which the two phenyl rings are mutually perpendicular.

The binding of thyroid hormones and analogs to the plasma protein prealbumin was studied. The results demonstrate that:

(i) the outer ring iodine atoms contribute more strongly to binding than inner ring iodines, and

(ii) the side chain carboxylate anion strengthens the binding whereas the positive ammonium weakens it.

The latter observation supports the affinity labelling results of other investigators which show that the lysine 9 and lysine 15 are specifically labelled.

DEDICATION

To

Arthur Andrea

Angele Madik

Karen Andrea

ACKNOWLEDGMENTS

I wish to express my thanks and appreciation to the following:

Dr. Eugene C. Jorgensen for guidance, patience and support.

Dr. Peter A. Kollman for guidance and encouragement.

Dr. John D. Baxter for being on my research committee and for reading my dissertation.

Mike B. Bolger for his help on the Prophet System and for useful discussions in developing the binding models.

George M. Anderson for enlightening discussions.

Richard D. Tuck for writing the plotting algorithms.

The Department of Pharmaceutical Chemistry for a teaching assistantship.

The National Institute of Health for financial support (Traineeship).

The Graduate Division for financial support (Chancellor's Patent Fund and Earl C. Anthony Fund).

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Chapter 1

Introduction

In recent years there have been several developments in medicinal chemistry in general and in the thyroid area in particular. The former include the development of linear free energy type relationships that correlate biological activity with linear free energy parameters as well as the development of theoretical methods to study the conformational preferences in biologically active molecules. Among the latter developments are the discovery, in rat liver cells, of nuclear proteins that bind thyroid hormones with high affinity and the studies that have emerged on the binding of thyroid hormones and analogs to various plasma proteins. Finally, a closely related development has been the determination of the three dimensional structure of the plasma protein prealbumin in the solid state.

Among the most commonly used linear free energy type parameters is the Hansch π value for functional groups. This parameter measures the contribution of a functional group to the in vitro "lipid solubility" of a compound, and supposedly reflects the extent of the in vivo hydrophobic interaction between a biologically active molecule and its "receptor" as well as the contribution of a functional group to the ease of passage across alternating lipid and aqueous compartments. The question came up (see chapter 2) as to whether it is possible to transfer a fixed amount of lipid character from one portion of a thyroid hormone analog to another portion with complete retention of biological activity. A set of compounds were designed and synthesized to test this idea.

The discovery of nuclear proteins which seemed to be "thyroid hormone receptor(s) encouraged the application of thermodynamic principles to understanding the structural requirements for thyroid hormone

action. The utility of these discoveries lies mainly in the fact that in vitro measurements of binding affinities avoids complicating factors like metabolism, distribution, excretion etc. that are operative in in vivo measurements. A general outline for the analysis of the thermodynamic functions of binding in terms of groups contribution is presented in chapter 2.

Molecular orbital methods have been used in the past for studying conformations of biologically active molecules. Statistical mechanical approaches to these problems have been infrequently used. In chapter 3 we present an analysis of the conformation of thyroid hormones and analogs using both of these approaches.

The determination of the crystal structure of prealbumin provided us with an incentive for studying the variation of binding affinities with the structure of a systematically varied series of thyroid hormones and analogs. This study was aimed towards understanding the nature of the interaction between thyroid hormone analogs and prealbumin, as well as testing some of the ideas developed for the analysis of the thermodynamic functions of binding. These results are discussed in Chapter 5.

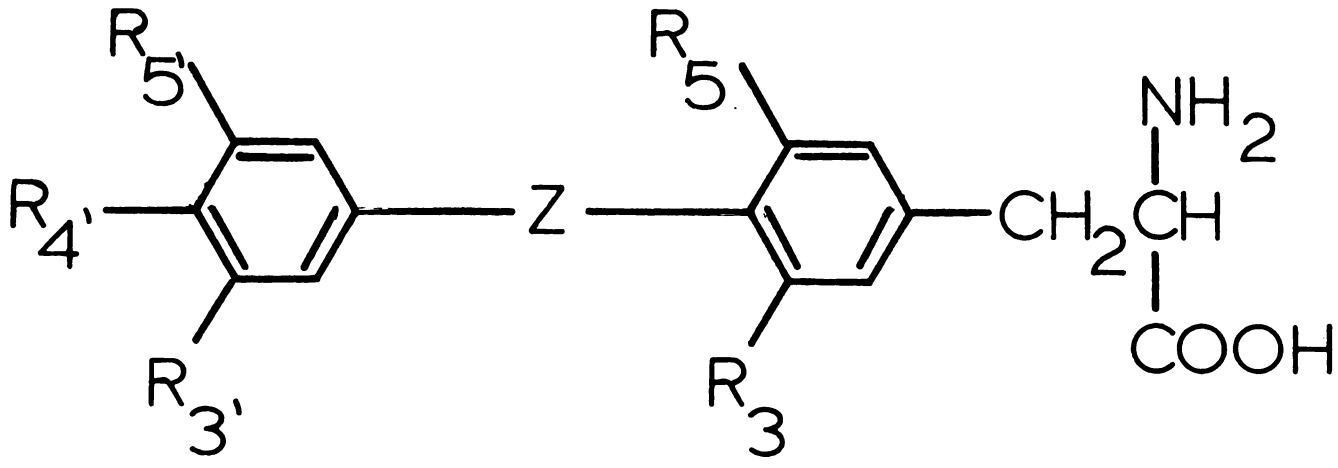
Chapter 2

Synthesis and Biological Activities of Thyroid Hormone Analogs

I. INTRODUCTION

The structure activity relationships of thyroid hormones and analogs have been discussed in detail by Jorgensen (1,2). The observations that are relevant to this study can be summarized as follows: (a) The activity of 3,5-diiodo-3'-alkyl-L-thyronines increases as the alkyl groups changes from methyl to ethyl. It peaks at the isopropyl and decreases for higher homologs. (b) Analogs in which all halogen atoms are replaced by alkyl groups retain activity. (c) No diastereomeric compound with an asymmetric 3'-substituent has been tested.

In recent years, hydrophobic "forces" have been implicated in drug action. A measure of the contributions of a functional group to the lipid solubility of a compound is the Hansch π value (3). Iodine has a higher π value than a methyl group. The π value increases with the number of carbon atoms in a homologous series. It is known (a) that 3,5-diiodo-3'-isopropyl-L-thyronine (2-1a) is the most active analog in the 3,5-diiodo series (it is also the most active known analog), and (b) that its 3,5-dimethyl analog (2-1b) is the most active in the 3,5-dimethyl series (2,4). This led Jorgensen to suggest that it might be possible to remove a fixed amount of lipid solubility from the inner ring of (2-1a) (say by replacing the 3 and 5 iodine atoms by methyl groups) and add it to the outer ring (by replacing the 3'-isopropyl group by a group which has a higher π value) and obtain a compound which is equiactive to (2-1a) and more active than (2-1b). We decided to choose a 3'-sec-butyl group to replace the 3'-isopropyl. This group has the added advantage that it has an asymmetric carbon and hence the 3,5-diiodo-3'-sec-butyl-L-thyronine (2-1c) and its 3,5-dimethyl analog (2-1d) have diastereomers that can be used in aswering questions concerning the



(2-1) General structure of thyroid hormone analogs.

(a)	I_2iPr	: R_3 = I	R_5 = I	$R_{3'}$ = iPr	$R_{4'}$ = OH	$R_{5'}$ = H
(b)	Me_2iPr	: R_3 = CH_3	R_5 = CH_3	$R_{3'}$ = iPr	$R_{4'}$ = OH	$R_{5'}$ = H
(c)	4'-O-Me-I_2iPr	: R_3 = I	R_5 = I	$R_{3'}$ = iPr	$R_{4'}$ = OCH_3	$R_{5'}$ = H
(d)	I_2sBu	: R_3 = I	R_5 = I	$R_{3'}$ = sBu	$R_{4'}$ = OH	$R_{5'}$ = H
(e)	Me_2sBu	: R_3 = CH_3	R_5 = CH_3	$R_{3'}$ = sBu	$R_{4'}$ = OH	$R_{5'}$ = H
(f)	4'-O-Me-I_2sBu	: R_3 = I	R_5 = I	$R_{3'}$ = sBu	$R_{4'}$ = OCH_3	$R_{5'}$ = H
(g)	T_4	: R_3 = I	R_5 = I	$R_{3'}$ = I	$R_{4'}$ = OH	$R_{5'}$ = I

stereospecific nature of the active site. Finally, it was advantageous to have the 3,5-diiodo-3'-sec-butyl-4'-O-methyl-L-thyronine (2-1c) to answer some questions pertaining to the type of involvement of the 4'-hydroxyl group in receptor and/or plasma protein binding.

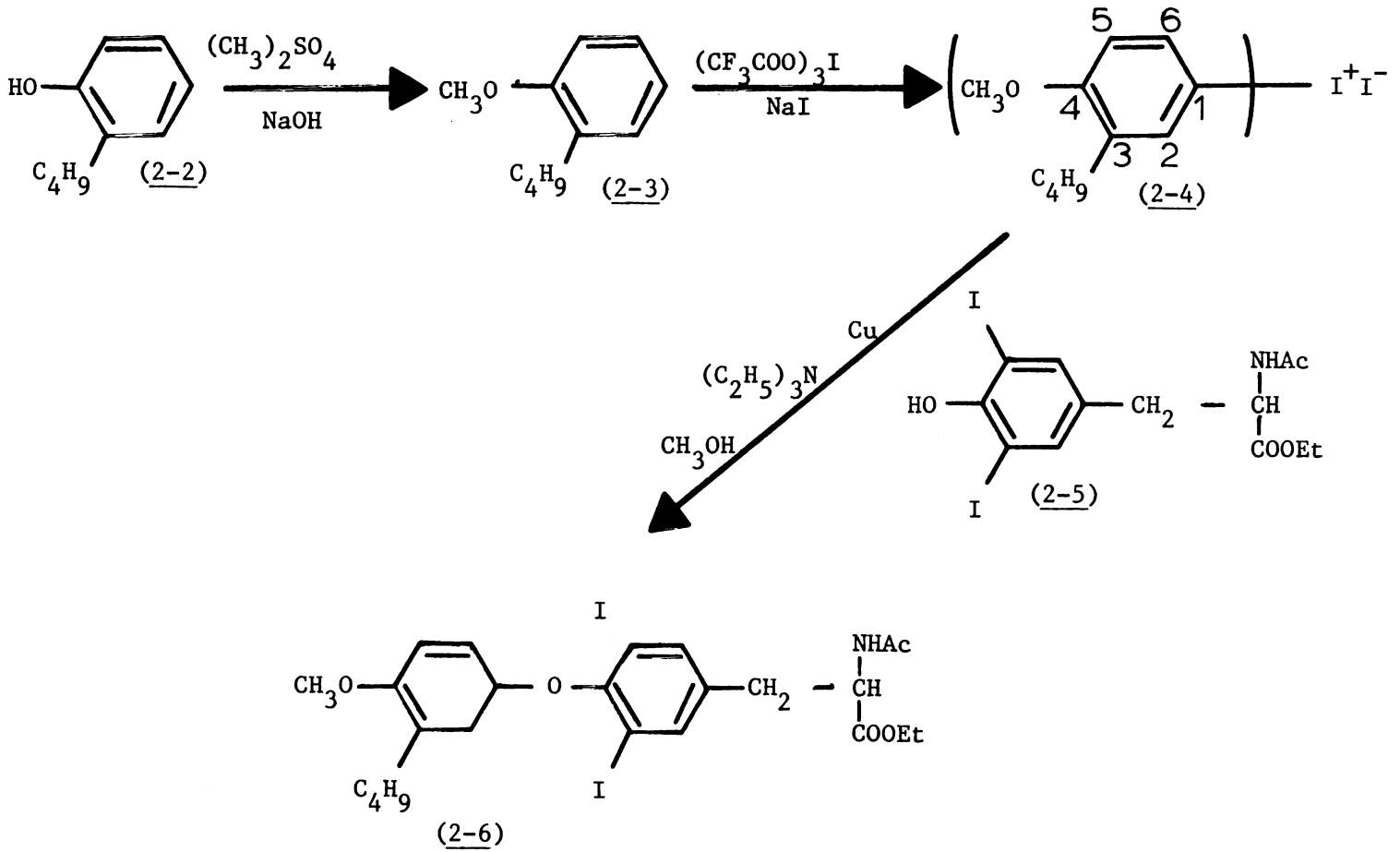
II-SYNTHESIS

a. Outline

The synthetic outline for the preparation of the compounds of interest is a combination of the schemes of Blank, et al. (5) and Jorgensen, et al. (4). The phenolic hydroxyl group of 2-DL-sec-butyl-phenol (2-2) is protected by methylation to the corresponding anisole (2-3). Two anisole molecules are coupled to form the reactive iodonium derivative (2-4). 3,5-Diiodo-3'-DL-sec-butyl-4'-O-methyl-N-acetyl-L-thyronine ethyl ester (2-6) is prepared by coupling (2-4) with 3,5-diiodo-N-acetyl-L-tyrosine ethyl ester (2-5). Hydrolysis of (2-6) under strong conditions gives 3,5-diiodo-3'-DL-sec-butyl-L-thyronine (2-7), or under mild conditions, its 4'-O-methyl analog (2-8). The 3 and 5 iodine atoms of (2-6) are replaced by cyano groups to give (2-9) which can be reduced to the 3,5-dimethyl protected analog (2-10) by catalytic hydrogenation. Hydrolysis of (2-10) to the 3,5-dimethyl-3'-DL-sec-butyl-L-thyronine (2-11) is carried out under acidic conditions.

It is possible to obtain the four diastereomers of each of compounds (2-7) and (2-10) by first resolving (2-2) into its two enantiomers then following the synthetic scheme outlined above for each of these enantiomers with each of the two enantiomers of (2-5). In order to resolve (2-2), the method of Hawthorne, et al. (6) was followed. D-Camphor-10-sulfonyl chloride (2-13D) is prepared by reacting D-camphor-10-sulfonic acid

Synthetic Outline



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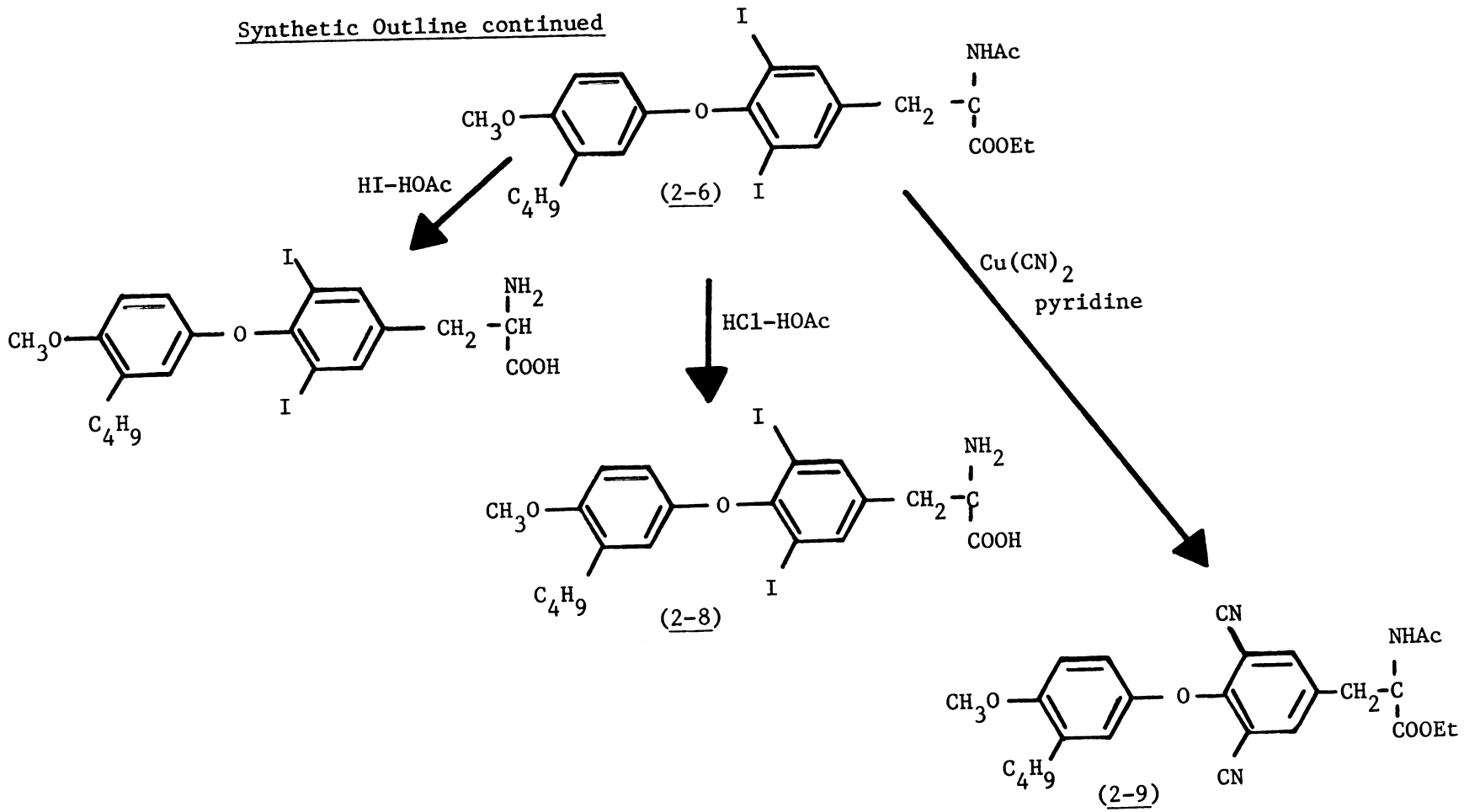

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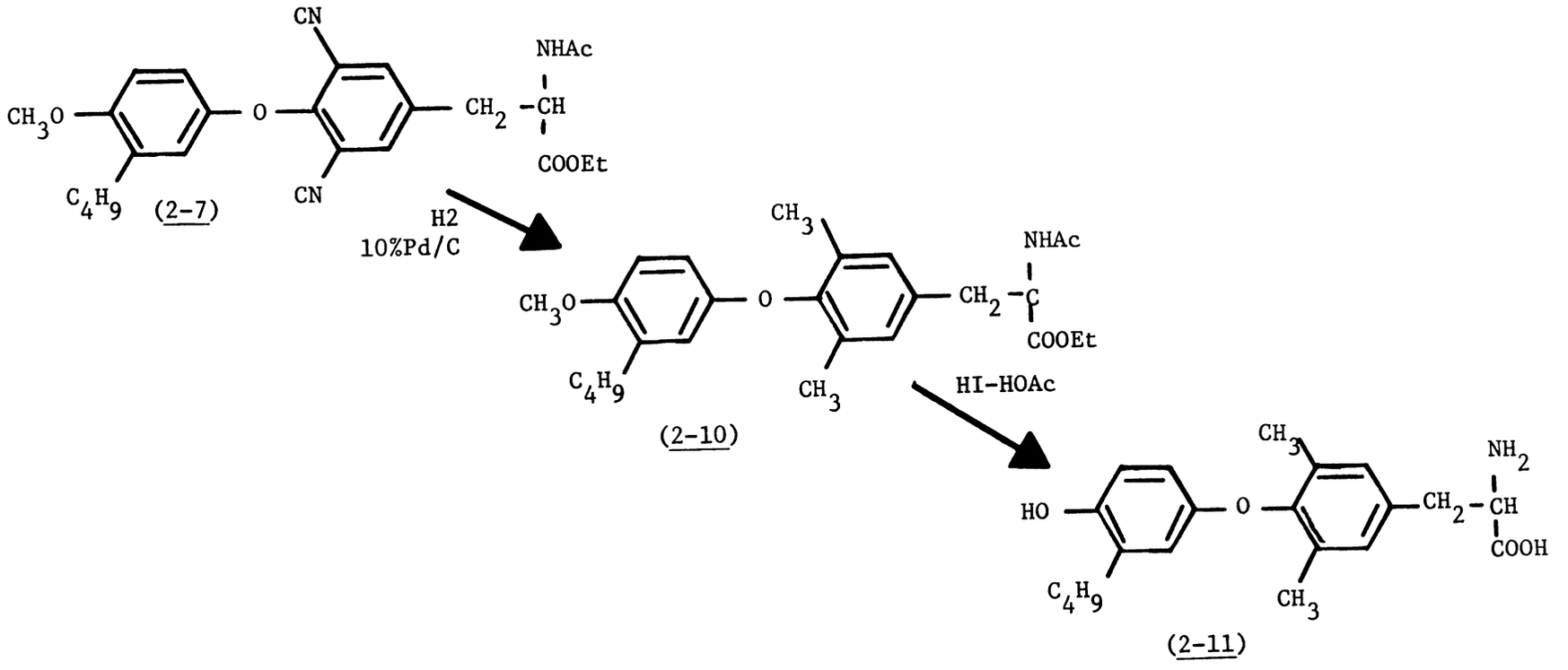

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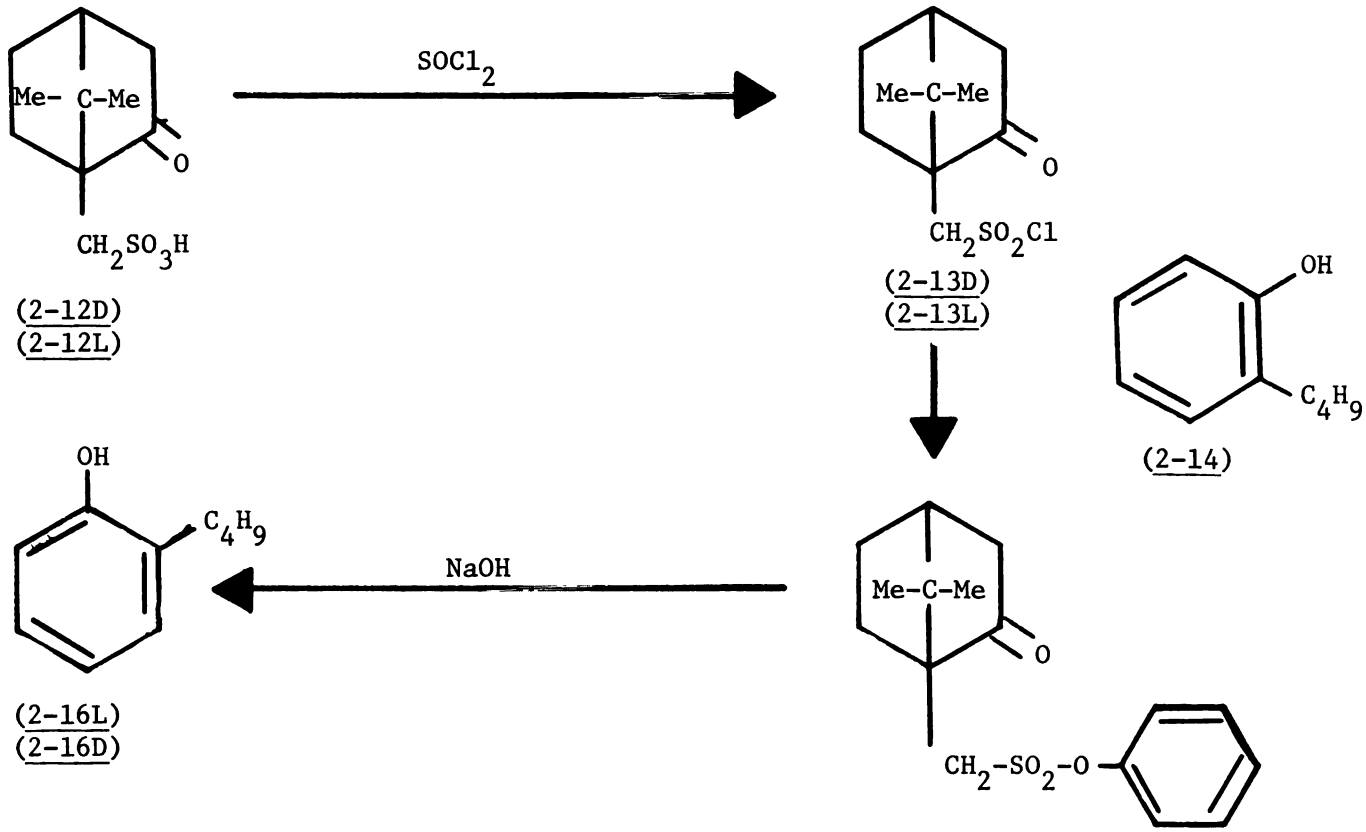


(2-12D)
(2-12L)

(2-13D)
(2-13L)

(2-14)

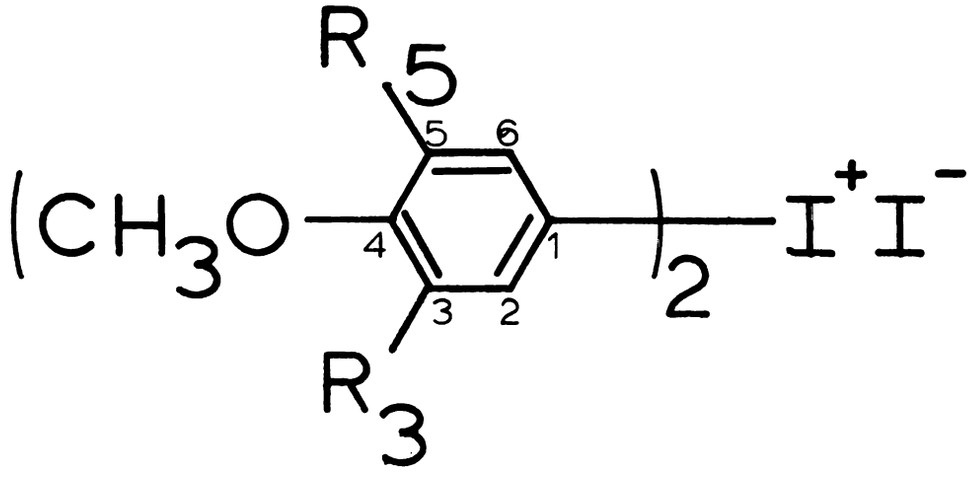
(2-16L)
(2-16D)

(2-15a) O-DL-sec-butylphenyl -D-camphor-10-sulfonate

(2-15b) O-DL-sec-butylphenyl -L-camphor-10-sulfonate

(2-12D) with thionyl chloride. 2-DL-sec-Butylphenol (2-14) is reacted with (2-13D) to give the diastereomeric mixture of the corresponding ester (2-15a). The LD diastereomer of (2-15a) is crystallized (leaving a mother liquor rich in the DD diastereomer), purified and hydrolyzed to give the L-phenol (2-16L). The solution rich in the DD diastereomer is hydrolyzed to give a DL (rich in D) mixture of the phenol. This is then reacted with the L-camphor-10-sulfonyl chloride (2-13L) to generate the diastereomeric mixture (2-15b). The DL diastereomer of (2-15b) is crystallized and hydrolyzed to yield the D-phenol (2-16D).

In the course of synthesis we found out that the intermediate di-(3-DL-sec-butyl-4-methoxyphenyl) iodonium iodide (2-4) could not be synthesized under the conditions currently described in the literature (5, 7, 8, 9). All attempts to synthesize it resulted in brownish-black oils from which the desired product could not be salvaged. Attempts by one of our colleagues (S. W. Dietrich) to synthesize the di(3-n-propyl-4-methoxyphenyl)iodonium iodide (2-17a) and di-(3,5-diisopropyl-4-methoxyphenyl) iodonium iodide (2-17b) were equally unsuccessful. Beringer, et al. (10,11) showed that iodonium compounds decompose relatively slowly (first order rate constant about 10^{-4} - 10^{-2} hr^{-1} at 60°C) when dimethyl formamide is used as a solvent and that the rate of decomposition is enhanced in less polar solvents (e.g. benzene) which enhance ion pair formation. The decomposition was also enhanced by electron attracting groups and decreased by electron donating groups. They concluded that decomposition was occuring by a nucleophilic attack by the free halide anion on C-1 (see 2-4) of the ring. Thus, it became necesssary to modify the (then) currently available reaction conditions.



(2-17)

(a) R_3 = n-propyl, R_5 = H.

(b) R_3 = R_5 = i-propyl

b. Experimental Section

Melting points were determined with a Thomas-Hoover Uni-Melt stirred oil capillary tube melting point apparatus. Proton Magnetic Resonance (PMR) spectra were determined on a 60 mHz spectrometer (Varian model A-60A). Chemical shifts are expressed in δ units (parts per million downfield from TMS). For the presentation of PMR data, the following conventions are used: s=singlet, d=doublet, t=triplet, q=quartet, dd=doublet of doublets, m=multiplet, cm=complex multiplet, J=coupling constant, Jo=ortho coupling constant, Jm=meta coupling constant. Infra red spectra were recorded as liquid film using irtran discs with a Perkin Elmer model 337 grating infrared spectrometer. Microanalyses were performed by Microanalytical Laboratory, University of California, Berkeley, California. Optical rotations were measured with Perkin Elmer model 141 polarimeter. Thin layer chromatography on silica gel plates was used routinely as a check for purity of samples as well as in determining the progress of reactions.

2-DL-sec-butylanisole(2-3). 2-DL-sec-butylphenol (2-2) (100.00 g, .67 mole) was stirred in 200 mls solution of .7N sodium hydroxide. Dimethyl sulfate (86.70 g, .67 moles) was added gradually with stirring. The mixture was refluxed for 3 hours at 70-75°C, cooled, extracted with five (50 ml) portions of ether. The ether layer was then washed with five (50 ml) portions of sodium hydroxide solution and then with two (50 ml) portions of water and dried over anhydrous sodium sulfate. Evaporation of the ether and subsequent distillation in vacuo yielded the desired anisole (74.5 g, 68%). Boiling point 63-66° at 0.2 mm Hg (Lit (12) 83-84° at 9 mm Hg). IR 3500 cm^{-1} (phenolic OH) present in (2-2) but absent in (2-3). PMR ($CDCl_3$) δ : 0.83 (t, J=7Hz, 3H, sBu CH_3-C-C-C),

1.18 (d, J=7, 3H, C-C-C-CH_3), 1.3 - 2.0 (cm, 2H, sBu CH_2), 2.8 - 3.4 (cm, 1H, sBu CH), 6.7-7.4 (cm, 4H, Ar-H), 3.73 (s, 3H, OMe: present in (2-3) but absent in (2-2)), 5.47 (s, 1H, OH: concentration dependent, present in (2-2) but absent in (2-3)).

Di-(3-DL-sec-butyl-4-methoxyphenyl)iodonium iodide (2-4).

The procedure presented here is based on the procedure of Beringer et al (8). Acetic anhydride (7 ml) in a two necked (100 ml) round bottom flask equipped with a thermometer, magnetic stirrer and a dropping funnel was cooled to 5°C. Nitric acid (specific gravity 1.50, 2.7 ml) was added dropwise with stirring at 10-15°C. The solution was cooled to 5°C, the stirring stopped, and finely pulverized iodine (2.5 g, 19.6 mol) was added. Trifluoroacetic acid (4.7 ml) was quickly added and the stirring was resumed at 23-25°C until the iodine particles were completely dissolved. The solution was transferred to a single necked (250 ml) round bottom flask and the brownish fumes were removed in vacuo using an aspirator for 5-7 minutes followed by a vacuum pump (0.1-0.5 mm Hg) for 20-25 minutes at 20-25°C. After the fumes were completely removed (the solution was orange-yellow) acetic acid (15 ml) was added and the reagent was transferred to two necked round bottom flask equipped with a magnetic stirrer, thermometer and a dropping funnel. The reagent was cooled to -20°C, whereupon its color changed to lemon yellow. 2-DL-sec-butylanisole (2-3) (6.65 g, 40 mmoles) dissolved in acetic anhydride (15 ml) and trifluoroacetic acid (3 ml) was added at (-10)-(-20)°C. The color of the solution gradually turned to green then greenish-brown. After the addition was complete, stirring was continued for 20 minutes as the solution warmed to room temperature. The solution was added onto a mixture of solid sodium iodide (5.42 g) and sodium bisulfite (5.00 g)

and stirred for 2 minutes. Water (200 ml) was added and the mixture stirred vigorously for 5 minutes, whereupon a brown oil heavier than water was formed. Benzene (a quantity just sufficient to dissolve the brown oil forming a solution lighter than water) was added. The benzene layer was separated and washed with four (200 ml) portions of water. Heptane was added until the desired product precipitated. The product was recrystallized by dissolving in benzene and precipitating with heptane. Yield (6.95 g, 60%). M.P. 157-158. PMR ($CDCl_3$) δ :0.77 (t, J=7, 3H, sBu CH_3-C-C'-C), 1.2 - 1.8 (cm, 2H, sBu CH_2), 2.8 - 3.3 (cm, 1H, sBu CH), 3.80 (s, 3H, OMe), 6.81 (d, Jo=9, 1H, Ar-5H), 7.68 (d, Jm=2, 1H, Ar-2H), 7.86 (dd, Jo=9, Jm=2, 1H, Ar-6H). Analysis: $C_{22}H_{30}O_2I_2$: Calculated C, 45.54, H, 5.21, I, 43.47; Found C, 45.39, H, 5.06, I, 43.56.

3,5-Diiodo-N-acetyl-L-tyrosine Ethyl Ester (2-5). This compound was prepared by the method of Barnes et al. (13). Yield (78%, lit (13) 88%). M.P. 154-155 (Lit (13). 154-155).

3,5-Diiodo-3'-DL-sec-butyl-4'-O-methyl-N-acetyl-L-thyronine Ethyl Ester (2-6). The general procedure of Blank, et al. (5) was used. A mixture of di-(3'-DL-sec-butyl-4-methoxyphenyl) iodonium iodide (2-4) (5.22 g, 9.00 mmoles), 3,5-diiodo-N-acetyl-L-tyrosine ethyl ester (2-5) (2.52 g, 5.01 mmoles), triethyl amine (.75 ml), copper powder (50 mg) in anhydrous methanol (60 ml) was stirred for 24 hours and filtered. The solvent was evaporated at 50°C and reduced pressure. The syrupy fluid was dissolved in benzene (200 ml), extracted with six 50 ml portions of dil HCl (conc HCl:H_2O 1:9 by volume), six 50 ml portions of 5% NaOH, washed with six 50 ml portions of water and dried over anhydrous sodium sulfate. The benzene was removed under reduced pressure to yield a brownish oil. The desired product was obtained either by elution with chloroform on a silica

gel column followed by evaporation of the chloroform or by dissolving in ether followed by precipitation with heptane. Yield (1.12 g, 34%). M.P. (105-107). $[\alpha]_D^{24}$: + 39.25 (c, 2, $CHCl_3$). PMR δ : 0.83 (t, J=7, 3H, sBu, CH_3-C-C-C), 1.16(d, J=7, 3H, sBu C-C-C-CH_3), 1.3 (t, J=7, 3H, ester CH_3) 1.3-1.7 (Cm, sBu CH_2) 2.05 (s, 3H, acetyl CH_3), 2.8-3.3 (cm, 3H, sBu CH and alanyl CH_2), 3.78 (s, 3H, OCH_3), 4.24 (q, J=7, 2H, ester CH_2), 4.7-5.2 (cm 1H, alanyl CH), 6.2-6.9 (cm, 3H, Ar 2', 5' and 6'H), 7.66 (s, 2H, Ar 2 and 6H). Analysis: $C_{24}H_{29}I_2NO_5$. Calculated C, 43.33; H, 4.39; I, 38.15; Found C, 43.26; H, 4.25; I, 38.32.

3,5-Diiodo-3'-DL-sec-butyl-L-thyronine (2-7). The general procedure of Blank et al.(5) was used. A mixture of 3,5-diiodo-3'-DL-sec-butyl-4'-O-methyl-N-acetyl-L-tyrosine ethyl ester (2-6) (1.00 g, 1.5 mmoles) in a solution (20 ml) made of equal volumes of glacial acetic acid and hydroiodic acid was heated under reflux for 4 hours, cooled and poured into ice water (80 ml). The pH was adjusted to 5.00 with aqueous sodium hydroxide and the precipitated solid was filtered, washed with water, recrystallized from EtOH/H_2O, filtered and dried in vacuo. Yield (0.62 g, 72%). M.P. 214-215° (decomp.) $[\alpha]_D^{24}$= + 26.31 (c, 1, EtOH: 1N HCl/3:1 by volume). TLC Rf: 0.63 (n-BuOH:HOAc:H_2O 4:5:1 by volume, upper layer), 0.36 (iPrOH:conc NH_4OH 4:1 by volume), 0.27 ($CHCl_3$:MeOH:conc NH_4OH 20:10:1 by volume). Analysis: $C_{19}H_{21}I_2NO_4$: Calculated C, 39.27; H, 3.64; I, 43.67; Found C, 39.08; H, 3.77; I, 43.64.

3,5-Diiodo-3'-DL-sec-butyl-4'-O-methyl-L-thyronine.H_2O (2-8). A mixture of 3,5-diiodo-3'-DL-sec-butyl-4'-O-methyl-N-acetyl-L-thyronine ethyl ester (2-6) (0.65 g, 0.98 mmoles), conc HCl (13 ml) and glacial HOAc

(5 ml) was heated under reflux for 4 hours, cooled and poured into ice water (40 ml). TLC showed presence of (2-7). The precipitate was filtered and recrystallized 3 times by dissolving in a hot solution made of equal volumes of hydrochloric acid and glacial acetic acid followed by precipitation with water and filteration. The final product was obtained at the isoelectric point (pH $\sim$ 5.5) and was dried *in vacuo*. Yield (0.45 g, 71%), M.P. 189-191 (decomp.) $[\alpha]_D^{24}$= + 26.00 (*C*, 1, EtOH: 1*N* HCl/3:1 by volume). TLC Rf 0.65 (n-BuOH:HOAc:H_2O 4:5:1 by volume, upper layer), 0.40 (iPrOH: conc NH_4OH 4:1 by volume). Analysis: $C_{20}H_{23}I_2NO_4.H_2O$: Calculated C, 39.17; H, 4.11; N, 2.28; I, 41.39; Found C, 39.38; H, 4.06; N, 2.30; I, 41.50.

3,5-Dicyano-3'-DL-sec-butyl-4'-O-methyl-N-acetyl-L-thyronine Ethyl Ester (2-9). The general procedure of Barnes, *et al*. (14) as used by Jorgensen, *et al.* (4), was utilized. A mixture of 3,5-diiodo-3'-DL-sec-butyl-N-acetyl-L-thyronine ethyl ester (2-6) (1.50 g, 2.26 mmoles), cuprous cyanide (1.00 g, 11.17 mmoles) and pyridine (10 ml) were refluxed for 14 hours. The reaction mixture was poured onto ice (50 g). The resulting solid was filtered, washed with cold water and stirred for 30 minutes in a mixture of 2N NH_4OH (50 ml) and chloroform (35 ml). After filtration through filter aid, the chloroform layer was separated and washed successively with 2N NH_4OH, water, 2N HCl and water and was dried over sodium sulfate. The chloroform was removed under reduced pressure and the resulting oil was purified by elution with chloroform on a silica gel column. Evaporation of the solvent gave the desired product (.529 g, 50%). M.P. 107-109. $[\alpha]_D^{24}$= + 48.00 (*c*, 2, $CHCl_3$). The PMR spectrum of (2-9) is identical to that of (2-6) with the exceptions

that the 2',5' and 6' aromatic protons multiplet at 6.7-6.9 in (2-6) is replaced by a multiplet at 6.7-6.9 in (2-9) and that the NH resonance occurs at 6.0-6.4 in (2-9). Analysis $C_{26}H_{29}N_3O_5$: Calculated C, 67.37; H, 6.31; N, 9.07; Found C, 67.19; H, 6.49; N, 8.94.

3,5-Dimethyl-3'-DL-sec-butyl-4'-O-methyl-N-acetyl-L-thyronine Ethyl Ester (2-10). The procedure of Block, et al. (15) as used by Jorgensen, et al. (4) was utilized. p-Cymene was purified as described by Block, et al. (15). A three necked flask (100 ml) was fitted with a thermometer, gas dispersion tube, and a reflux condenser, the tip of which led to a second dispersion tube immersed beneath the surface of water (100 ml) containing methyl red indicator. 3,5-Dicyano-3'-DL-sec-butyl-4'-O-methyl-N-acetyl-L-thyronine Ethyl Ester (2-9) (1.00 g, 2.16 mmoles) was dissolved in freshly purified p-cymene (50 ml) containing 10% Pd/C (0.6 g), the reaction system having been flushed with nitrogen gas. Hydrogen was bubbled through while the temperature was maintained at 150-160°C. The evolved ammonia was absorbed and titrated against 0.1 N HCl. The reaction was stopped when 97% of the theoretical amount of ammonia was titrated. The catalyst was removed by filtration through filter aid while hot and washed with acetone. The solvent was removed in vacuo (using the aspirator until all the acetone was removed and then using the vacuum pump at 2 mm Hg) at 60-65°C. The oily product was purified on a silica gel column (eluting with chloroform). The chloroform was removed and the resulting oil was stored overnight covered with petroleum ether. The crystals were filtered and dried. Yield (0.75 g, 79%). M.P. 74-75°. $[\alpha]_D^{24}$ = + 54.67 (c, 2, $CHCl_3$). PMR ($CDCl_3$) chemical shifts identical (± .05 ppm) to (2-9) with the following exceptions: The peaks at : 2.05 (s, 3H, acetyl CH_3 and 7.66 (s, 2H, Ar 2, 6H) in (2-6) are shifted upfield to 2.00 and 6.83

respectively in (2-10). In addition (2-10) showed a peak at 2.08 (s, 6H, 3,5-$(CH_3)_2$). Analysis: $C_{26}H_{35}NO_2$: Calculated C, 70.72; H, 7.99; N, 3.17; Found, C, 70.66; H, 7.89; N, 3.22.

3,5-Dimethyl-3'-DL-sec-butyl-L-thyronine. 1/2 H_2O (2-11). The procedure of Jorgensen, et al. (4) was followed with slight modification. 3,5-dimethyl-3'-DL-sec-butyl-4'-O-methyl-N-acetyl-L-thyronine ethyl ester (2-10) (287 mg, 0.65 mmoles) was dissolved in glacial acetic acid (10 ml). Nitrogen gas was bubbled through the solution for 15 minutes. The solution was heated under reflux in a nitrogen atmosphere while hyroiodic acid (5 ml) was added. The solution was refluxed for 4 hours, and the solvent was removed in vacuo at 50-60°C. The product was dissolved in 1-1.5 ml of a solution made of (H_2O:HOAc:conc HCl 6:5:0.5 by volume). 10 N NaOH was added to a pH of 5.5. The precipitated product was filtered, washed with water and dried. Yield (216 mg, 93%). M.P. 193-194 (decomp.) $[\alpha]_D^{24}$= + 18.57 (c, 0.5, EtOH;1N HCl/3:1 by volume). TLC Rf 0.52 (n-BuOH:HOAc:H_2O 4:5:1 by volume), 0.30 (iPrOH:conc NH_4OH 4:1 by volume). Analysis: $C_{21}H_{27}NO_4$.1/2 H_2O: Calculated C, 68.83; H, 7.70; N, 3.82; Found C, 68.74; H, 7.74; N, 3.39.

D-Camphor-10-sulfonyl Chloride (2-13D). An elaboration on the methods of Smiles, et al. (16) and Read, et al. (17) was used. D-camphor-10-sulfonic acid (2-12D) (10.00 g, 43.1 mmoles) was placed in a three necked round bottom flask fitted with a thermometer, condenser (with a drying tube at the outlet), and an overhead stirrer. Thionyl chloride (16.30 g, 137 mmoles) was added. The oily mixture was stirred at 20-30°C for 30 minutes then at 64°C for one hour. The reaction mixture was poured onto water (200 ml) and stirred until the oil crystallized, and was filtered.

Purification was effected by three successive triturations (in a mortar) with water followed by filteration. Yield (7.30 g, 70%). M. P. 64-65 (lit (16) 67-68; lit (17) 70).

O-L-sec-butylphenyl-D-camphor-10-sulfonate (LL diastereomer of 2-15a)

An elaboration of the procedure of Hawthorne, et al. (6) was utilized. 2-DL-sec-Butylphenol (2-14) (67.00 g, .45 mmoles) was dissolved in pyridine (148 mol) and D-camphor-10-sulfonyl chloride (2-13D) (83.00 g, .33 moles) was added. The mixture was shaken to effect solution, stoppered and allowed to stand at room temperature for 48 hours. The mixture was then poured onto ice and 6N HCl (500 ml), the oil was taken up in ether, washed twice with water, 1N NaOH and finally again with water. The ether layer was dried and the crude ester (the diastereomeric mixture 2-15a) was obtained by evaporation of the solvent on a steam bath. Yield (55.00 g, 91%; lit (6) 95%). The LD diastereomer could not be crystallized by the methods of Hawthorne, et al. (6). Moreover, these investigators could not supply us with seed crystals (18,19). Thus the crude crystals of the LD diastereomer were obtained as follows: The oily ester mixture was dissolved in absolute ethanol (100 ml) by heating on a steam bath. The solution was left uncovered. After the ethanol evaporated, crystals formed in two weeks. All subsequent recrystallizations were done by dissolving the crystals in hot absolute ethanol, and storing at 0°C with seed crystals. After five recrystallizations the desired product was obtained. Yield (34.05 g, 57%; lit (6) 35%). MP 68-70° (lit (6) 71-72.5). $[\alpha]_D^{23}$ = + 42.3 (c, 6.0, benzene) (lit (6): $[\alpha]_D^{23}$ = + 42.4° (c, 6.0, benzene))

III. BIOLOGICAL ACTIVITIES

The rat antigoiter activities were determined for the three thyroid hormone analogs newly synthesized for this study. The binding affinities to solubilized rat hepatic nuclei were measured by Bolger (20,21). The reversal of rat fetal goiter was measured by Comite (22,23).

The rat antigoiter assay was developed by Dempsey and Astwood (24) and modified by Jorgensen and Slade (25). Animals are given thiouracil (.3%) in their feed. This blocks the biosynthesis of the thyroid hormones T_3 and T_4. The lowering of the levels of circulating hormone reduces the negative feed back to the pituitary and hypothalamus causing release of thyrotropin (TSH) which leads to an increased vascularization and hyperplasia of the thyroid gland. This condition is called goiter. The analogs to be tested are administered subcutaneously in graded doses. The relative activity of an analog is estimated (based on a standard log dose-response curve) by the molar dose of the analog, relative to that of the standard (T_3 or T_4) required to cause 50% reversal of the thiouracil induced goiter.

The outline of the procedure is as follows. An arbitrary molar ratio value of 1.00 was assigned to the dose of L-T_4 containing 1 μg of L-T_4 per 100 g of rat body weight or to the dose of L-T_3 containing 0.25 g of L-T_3 per 100 g of rat body weight. Solutions were prepared so that the dose administered to 100 gm rat was confined in 0.125 ml of solution. Solutions of the compounds were made in a solvent which contained 0.9% NaCl and 0.1 N NaOH. Alternatively, compounds that were insoluble in this solvent were dissolved in ethanol and diluted with normal saline.

These solutions were decanted into 50 ml vials which were fitted with a septum, capped, and stored in the refrigerator when not in use. Male Long-Evans or Sprague Dewley rats (Simonsen Laboratories, Gilroy, California) weighing between 70 and 100 g were obtained and housed three to a cage. Groups of six rats were used for each dose level of each analog, as well as the normal controls and thiouracil controls. The normal and thiouracil controls were injected with solvent. The volume of solution to be injected into each rat was determined on the basis of daily weighing as follows:

Rat Weight (g)	Volume of Solution (ml)
60-79	.075
80-99	.100
100 - 119	.125
120 - 139	.150
140 - 159	.175
160 - 179	.200
180 - 199	.225
200 - 219	.250

The animals were weighed and sacrificed (by chloroform - ether inhalation) after 10 days of injection. The thyroid glands were excised, kept moist with saline, cleaned of extraneous tissue under a dissecting microscope and weighed to the nearest 0.1 mg. The thyroid weights were converted to mg/100 g body weight and the means and standard deviations were calculated for each dose level.

IV. RESULTS AND DISCUSSION

The results of the two assays are presented in tables 2-1 and 2-2. The corresponding log dose-response curves are shown in figures 2-1 and 2-2. The analog activities are shown in table 2-3.

The sum of the π values (3) for the 3,5 and 3' groups of I_2iPr, and Me_2sBu (2-1a and 2-d) are 3.14 and 3.18, respectively. If the argument (section I above) that removing a fixed amount of lipid character from the inner ring and adding it to the outer ring leads to full retention of activity is correct, the I_2iPr and Me_2sBu are expected to have almost identical biological activities. Table 2-3 however, shows that I_2iPr is about 30 times as active as Me_2sBu. This is a clear demonstration that such transfer of lipid character is not acceptable. In addition, our calculations in chapter 4 suggest that it is not the lipid character alone of the 3 and 5 substituents that contributes to binding but rather their ability to lock the rings into a mutually perpendicular conformation.

Table 2-3 also shows that the activity of the 4'-O-Me-I_2sBu is about 30% as active as the corresponding 4'-hydroxy compound (I_2sBu) indicating the importance of the phenolic proton in imparting biological activity.

Table 2-1. Results of the First Rat Antigoiter Assay

Compound[a] Injected	Daily Dose (μg/100 g body weight	Molar Ratio	Mean Thyroid Weight (mg/100 g body weight)
Normal Control	---	---	9.48 ± 1.15
Thiouracil Control	---	---	29.96 ± 2.54
L-T_4	0.600	0.600	28.57 ± 4.30
	1.000	1.000	26.37 ± 5.39
	1.667	1.667	20.08 ± 4.17
		1.75[b]	
L-I_2iPr	0.064	0.100	29.19 ± 4.40
	0.128	0.200	27.88 ± 6.90
L-Me_2iPr	1.545	4.000	28.35 ± 4.45
	3.091	8.000	18.51 ± 3.26
		7.5[b]	
L-Me_2sBu	1.608	4.000	29.51 ± 3.37
	3.217	8.000	24.04 ± 4.75
		∿11[b]	
L-4'-O-Me I_2iPr	0.981	1.500	21.11 ± 8.03
		1.65[b]	
L-4'-O-Me I_2sBu	1.004	1.500	30.13 ± 3.79
		>1.5[b]	

[a]For structures see (2-1a) to (2-1g)

[b]Molar dose ratio required to cause 50% reversal of thiouracil induced goiter.

Table 2-2. Results of the Second Rat Antigoiter Assay

Compound[a] Injected	Daily Dose (μg/100 g body weight)	Molar Ratio	Mean Thyroid Weight (mg/100 g body weight)
Normal Control	---	---	9.26 ± 2.00
Thiouracil Control	---	---	29.68 ± 6.78
L-T_4	1.000	1.000	26.16 ± 5.45
	1.590	1.590	22.09 ± 1.55
	2.520	2.520	12.80 ± 4.11
	4.000	4.000	8.34 ± 2.01
		1.60[b]	
L-I_2iPr	0.128	0.200	25.81 ± 3.22
	0.221	0.346	12.78 ± 7.14
	0.383	0.600	9.17 ± 5.70
		0.268[b]	
L-I_2sBu	0.131	0.200	25.31 ± 2.24
	0.226	0.346	21.80 ± 6.77
	0.392	0.600	16.25 ± 6.09
		0.362[b]	
L-Me_2iPr	1.545	4.000	26.05 ± 10.25
	3.090	8.000	18.36 ± 5.31
	3.662	9.480	17.96 ± 11.75
	6.181	16.000	9.37 ± 6.68
		7.34[b]	
L-Me_2sBu	2.413	6.000	25.98 ± 3.44
	4.403	10.95	22.55 ± 6.91
	8.042	20.00	9.35 ± 4.78
		9.94[b]	

[a]For structures see (2-1a) to (2-1g).

[b]Molar dose ratio required to cause 50% reversal of thiouracil induced goiter.

Table 2-3. Activities of Thyroid Hormones and Newly Synthesized Analogs in the Rat Antigoiter Assay.

Compound	Activity
L-T_4	100
L-I_2iPr	600
L-I_2sBu	442
L-Me_2iPr	23,22[a]
L-Me_2sBu	16,16[a]
L-4'-O-Me-I_2iPr	106
L-4'-O-Me-I_2sBu	<117

[a]Results from the two assays.

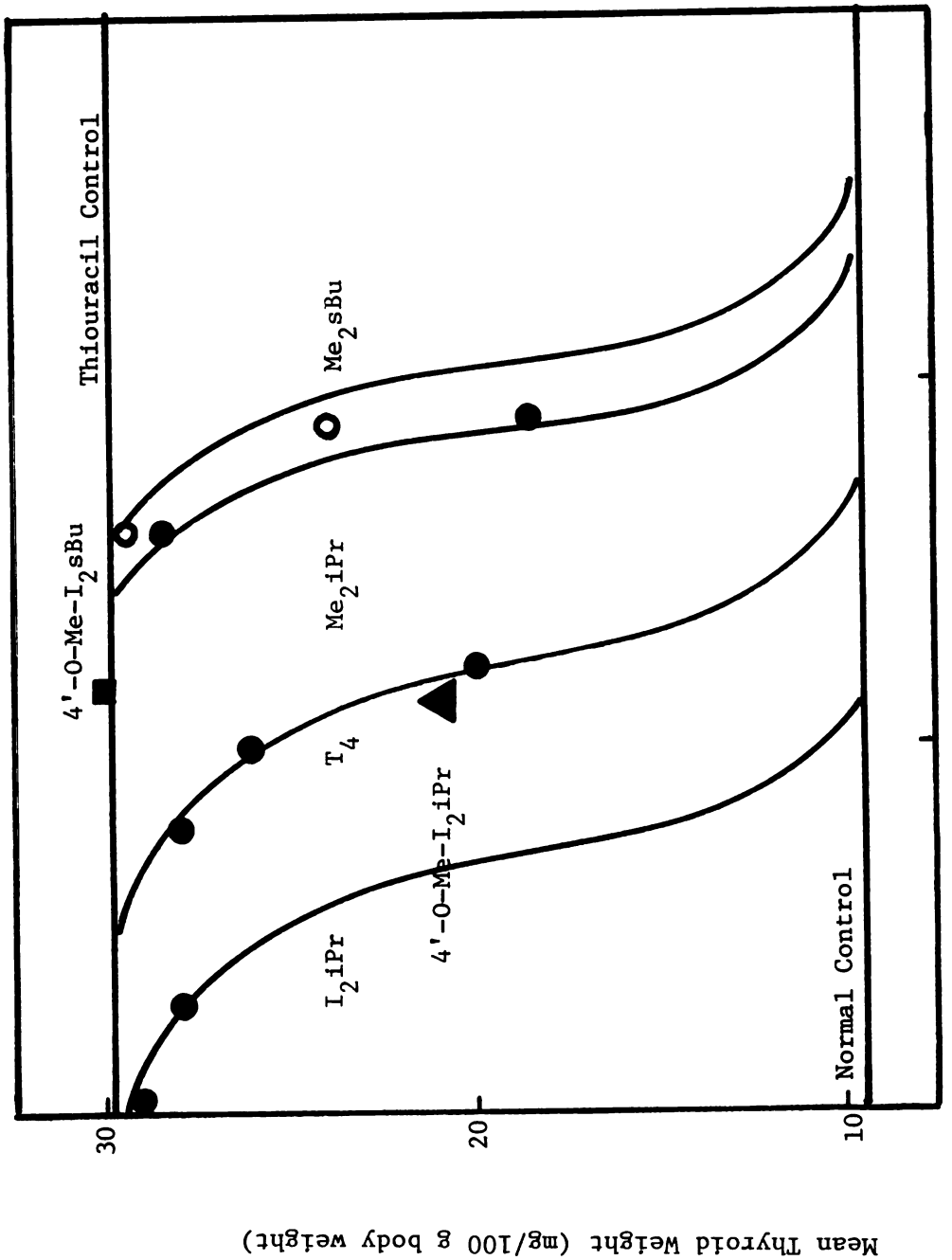


Figure 2-1. Log dose-response curves for the first rat antigoiter assay.

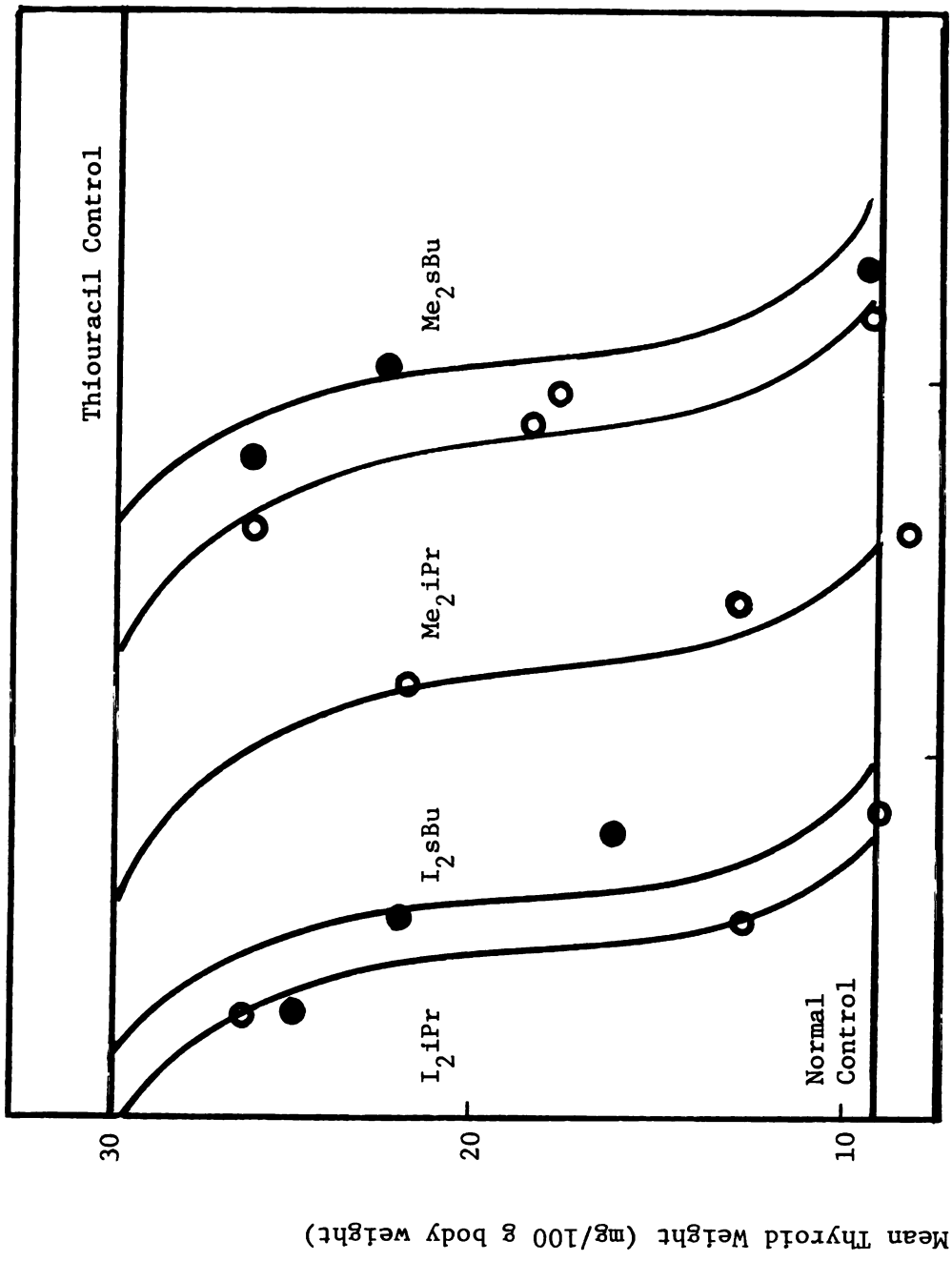


Figure 2-2. Log dose-response curves for the second rat antigoiter assay.

Chapter 3

The Analytical Decomposition of the Free Energy of Binding

I- INTRODUCTION

Research in medicinal chemistry, like any other science, can be classified as theoretical and applied. Applied research is manifested in attempts to develop novel medicinal compounds as well as to improve already available ones. The theoretical component on the other hand manifests itself in attempts to define chemical features that are essential in the expression of biological activity and to understand the variation of biological activity upon structural modification. The theoretical approaches can be more succintly described as attempts to understand biological activity as a manifestation of physical forces operating at a molecular level as well as to predict the variation of these forces upon structural modification.

Medicinal chemists typically think and express their results in terms of the variation of biological activity when one functional group is replaced by another. The question that immediately imposes itself is: how does one interpret the variation of biological activity in an intact organism in terms of the variation in chemical structure and other physical properties? A meaningful answer requires a thorough knowledge of the totality of processes that are operative between the introduction of a compound into the biological system and the measurement of the response. One can think of at least three such processes: (i) The partitioning of the compound into reversible storage compartments and irreversible sites of loss. (ii) The biotransformation of the compound into metabolites that may or may not contribute to the measured response. (iii) The interaction of the compound with the biological receptor(s) and the ensuing events. The relative significance of each of these processes is moderately understood in certain classes. One would like

to think that the variation of biological activity with structure reflects the effectiveness of interaction with a specific receptor. The alteration of a functional group on a compound, however, may alter the biological activity by its effect on any one of the these three processes. There is no a priori reason to believe that all analogs are affected negligibly or equally by the first two processes. Neither is there reason to believe that a macroscopically observed response is a consequence of the interaction of a biologically active molecule with a single receptor. Thus it may be misleading to make inferences about the physical chemical nature of the interaction between the biologically active molecule and its receptor(s) on the basis of the variation of biological activity with functional group alteration.

All this leads us to believe that application of first principles to whole organism responses is a path beset with uncertainties. Is the situation totally hopeless? Can one make any progress along these lines? Let us elaborate on point (iii) in the previous paragraph. Our present logic strongly enforces upon us that a biologically active molecule must at some point interact with a cellular component, probably (but not necessarily) a macromolecule, thereby starting the process of expression of response. It is also conceivable that in some cases the response is a function of the affinity of the biologically active molecule to the macromolecule. These ideas have permeated the thought processes in medicinal chemistry and constitute a working hypothesis. Hence it is useful to understand the physical origin of the modulation of binding affinities of small molecules to a macromolecule by functional group alteration.

What is needed is a set of measures of the variation of the thermodynamic functions of binding with the structure. Such measures should have the property of focusing attention on single or few individual groups at a time rather than whole molecules. In addition, these measures can form the basis of systematic studies of group contributions as well as the basis for the interpretations of these contributions from first principles. The definitions, theorems, lemmas and proofs that appear in the next sections materialize these ideas in a self consistent fashion.

II- HISTORICAL INTRODUCTION

Examining the data of Koerner, et al., (26) that the binding constant of T_3 to intact rat hepatic nuclei is 6.1×10^8 M^{-1} at 37°C (binding free energy = -12.46 kcal/mole) it seemed reasonable that if each of the three iodine atoms, the charged groups on the side chain and the phenolic hydroxyl group contributed about -2 kcal/mole then the summation of these contributions would be approximately equal to the free energy of binding of the whole hormone molecule. This idea can be quantified by defining the contribution ($\Delta\Delta G(X)$) of a substituent group X (relative to hydrogen in the same position) to the free energy of binding by

$$\Delta\ \Delta G(X) = \Delta G(AX) - \Delta G(AH) = -RT\ \ln \frac{K_{AX}}{K_{AH}}$$

where AX is an analog with group X at a certain position and AH is an analog identical to AX in every respect except that the group X is replaced by hydrogen, K_{AX} and K_{AH} are the binding constants of AX and AH respectively. $\Delta G(AX)$ and $\Delta G(AH)$ are the binding free energies of AX and

and AH respectively. $\Delta\Delta G(X)$ is the contribution of a group X at a certain position (relative to hydrogen at the same position) to the free energy of binding.

Bolger realized that if the contributions of the 3'-methyl and 4'-hydroxy groups in figure 3-4 were independent, then one would expect that

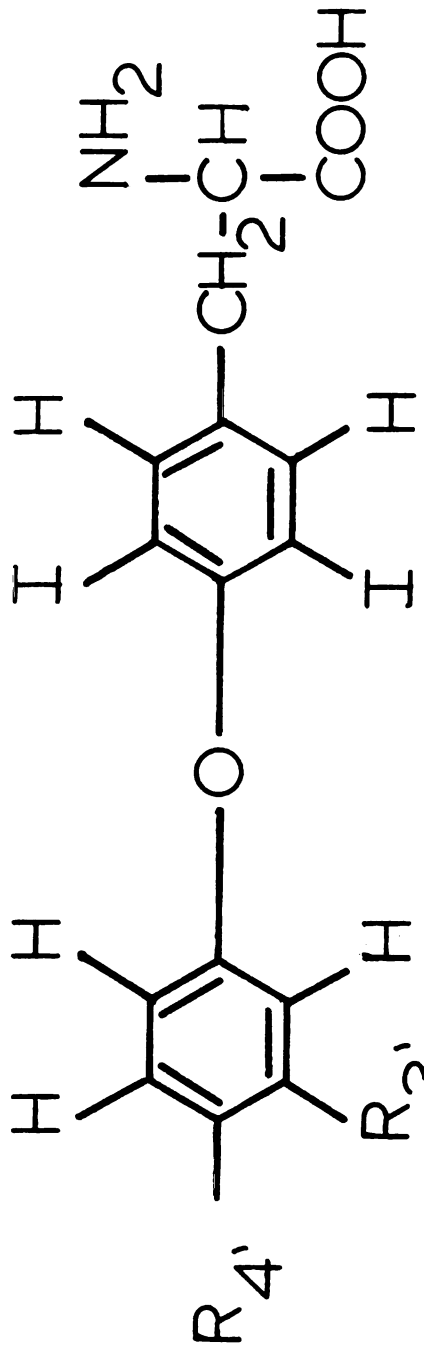
$$\Delta\Delta G(3'\text{-}CH_3) + \Delta\Delta G(4'\text{-}OH) = \Delta\Delta G(3'\text{-}CH_3,4'\text{-}OH) \qquad (3\text{-}0)$$

where

$$\Delta\Delta G(3'\text{-}CH_3) = \Delta G(3\text{-}2) - \Delta G(3\text{-}1)$$
$$\Delta\Delta G(4'\text{-}OH) = \Delta G(3\text{-}3) - \Delta G(3\text{-}1)$$
$$\Delta\Delta G(3'\text{-}CH_3,4'\text{-}OH) = \Delta G(3\text{-}4) - \Delta G(3\text{-}1)$$

Upon actual measurement of the binding free energies of compounds (3-1) - (3-4), he found equation (3-0) to be not exact. He decided to introduce an interactive term to the left hand side of equation (3-0) to make it equal to the right hand side. He also realized that this interactive term reflects the perturbation that the 3'-methyl and 4'-hydroxyl groups have on each other's contribution. Moreover the correction term was found to be smaller than $\Delta\Delta G(3'\text{-}CH_3)$ and $\Delta\Delta G(4'\text{-}OH)$.

The development up to this point strongly suggested to us the possible usefulness of generalizing the above approach to any number of groups and to all thermodynamic functions.



(3-1) R_3' = H R_4' = H

(3-2) R_3' = CH_3 R_4' = H

(3-3) R_3' = H R_4' = OH

(3-4) R_3' = CH_3 R_4' = OH

III- THEORY OF THE DECOMPOSITION OF THE BINDING FREE ENERGY

We found it necessary to resort to the formal methods in order to ascertain internal consistency and generality of the theory. We will develop the theory in terms of the Gibbs free energy. We consider the formalism equally applicable to the following thermodynamic functions: Helmholtz free energy, entropy, enthalpy, and energy of binding. We will first introduce a set of definitions. Based on these, the decomposition theorem as well as some useful lemmas will be proved.

Definition 1: The symbol X_i designates the i^{th} functional group attached to a specified position of a certain molecule. The position of group X_i on the molecule is implict in the symbol X and is not related logically to the subscript i. For example 1-hydroxy-3-methyl-5-iodobenzene has three functional groups: X_1 = 1-hydroxy, X_2 = 3-methyl, and X_3 = 5-iodo. The symbol Y_i is defined in precisely the same way.

Definition 2: $AX_1X_2...X_N$ is a compound which has a moiety A, to which are attached the N functional groups $X_1X_2...X_N$ whose contribution to the free energy of binding is to be determined relative to the N functional groups $Y_1Y_2...Y_N$. Any one or more (but not all) of the N X_i groups in $AX_1X_2...X_N$ may be the same as the corresponding Y_i.

Definition 3: $AY_1Y_2...Y_N$ is a compound identical in every respect to $AX_1X_2...X_N$ with the exception that all the $X_1X_2...X_N$ functional groups are replaced by $Y_1Y_2...Y_N$.

Defintion 4: $\Delta_0G(AX_1X_2...X_N)$ is the standard free energy of binding of the compound $AX_1X_2...X_N$ as determined by

$$\Delta_0G(AX_1X_2...X_N) = -RT \ln K\ (AX_1X_2...X_N)$$

where $K(AX_1X_2...X_N)$ is the affinity constant of the compound $(AX_1X_2...X_N)$, T is the absolute temperature and R is the gas constant. $\Delta_o G(AY_1Y_1...Y_N)$ has an identical defintion. The $\Delta_1 G$ terms are defined by

$$\Delta_1 G(X_{i_1}) = \Delta_o G(A...Y_{i_1-1}X_{i_1}Y_{i_1+1}...Y_N) - \Delta_o G(AY_1Y_2...Y_N) \qquad (3\text{-}1)$$

$$\Delta_1 G(X_{i_1}X_{i_2}) = \Delta_o G(A...Y_{i_1-1}X_{i_1}Y_{i_1+1}...Y_{i_2-1}X_{i_2}Y_{i_2+1}...Y_N) - \Delta_o G(AY_1AY_2...Y_N)$$

Generally

$$\Delta_1 G(X_{i_1}X_{i_2}...X_{i_L}) = \Delta_o G(A...X_{i_1}...X_{i_2}...X_{i_L}) - \Delta_o G(AY_1Y_2...Y_N)$$

where $1 \leq L \leq N$. The $\Delta_2 G$ terms are given by

$$\Delta_2 G(X_{i_1}X_{i_2}...X_{i_L}) = \Delta_1 G(X_{i_1}X_{i_2}...X_{i_L}) - \sum_{\underset{\sim}{A_1}} \Delta_1 G(X_{i_{j_1}})$$

where $2 \leq L \leq N$. The summation over the set $\underset{\sim}{A_1}$ on the right hand side is a sum over the contributions of all possible single groups selected from the given set of L groups. More explicitly

$$\sum_{\underset{\sim}{A_1}} = \sum_{j_1=1}^{L}$$

The $\Delta_3 G$ terms are defined by

$$\Delta_3 G(X_{i_1}X_{i_2}...X_{i_L}) = \Delta_2 G(X_{i_1}X_{i_2}...X_{i_L}) - \sum_{\underset{\sim}{A_2}} \Delta_2 G(X_{i_{j_1}}X_{i_{j_2}})$$

where $3 \leq L \leq N$. The summation over the $\underset{\sim}{A_2}$ on the right hand side is a sum over the contributions of all possible combinations of two groups selected from the given set of L groups. More explicitly

$$\underset{\sim}{A_2} = \sum_{j_1=1}^{L-1} \sum_{j_2=j_1+1}^{L}$$

Similarly

$$\Delta_4 G(X_{i_1} X_{i_2} \ldots X_{i_L}) = \Delta_3 G(X_{i_1} X_{i_2} \ldots X_{i_L}) - \sum_{\underset{\sim}{A_3}} \Delta_3 G(X_{i_{j_1}} X_{i_{j_2}} X_{i_{j_3}})$$

where $4 \leq L \leq N$. The summation over the set $\underset{\sim}{A_3}$ in the right hand side is a sum over the contributions of all possible combinations of three groups selected from the given set of L groups. More explicitly

$$\underset{\sim}{A_3} = \sum_{j_1=1}^{L-2} \sum_{j_2=j_1+1}^{L-1} \sum_{j_3=j_2+1}^{L}$$

The general terms is given by

$$\Delta_{k+1} G(X_{i_1} X_{i_2} \ldots X_{i_L}) = \Delta_k G(X_{i_1} X_{i_2} \ldots X_{i_L}) - \sum_{\underset{\sim}{A_k}} \Delta_k G(X_{i_{j_1}} X_{i_{j_2}} \ldots X_{i_{j_k}}) \quad (3\text{-}2)$$

where $k+1 \leq L \leq N$. The sum over the set $\underset{\sim}{A_k}$ on the right hand side is a sum over the contributions of all possible combinations of k groups selected from the given set of L groups. More explicitly

$$\underset{\sim}{A_k} = \sum_{j_1=1}^{L-k+1} \sum_{j_2=j_1+1}^{L-k+2} \cdots\cdots \sum_{j_s=j_{s-1}+1}^{L-k+s} \cdots \sum_{j_{k-1}=j_{k-2}+1}^{L-1} \sum_{j_k=j_{k-1}+1}^{L} \quad (3\text{-}3)$$

This sum has $\frac{L!}{(L-k)!k!}$ terms in it. This is true because the k subscrips $j_1, \ldots\ldots, j_k$ in each term of the sum are different, and only one combination of a specific set of k integers occurs in the sum (the k! permutations of the subscripts correspond to the same term) thus the total number of terms in the sum corresponds to the total number of ways of selecting k subscripts from a total of L.

An alternative way of expressing the sum over the set $\underset{\sim}{A}_k$ is by overcounting each term k! times and then correcting for this overcounting by dividing by k! as such

$$\sum_{\underset{\sim}{A}_k} = \frac{1}{k!} \sum_{j_1=1}^{L} \sum_{j_2=1}^{L} \cdots \sum_{j_k=1}^{L}$$

$j_i \neq j_k$ for all (i,j) pairs.

The last method for expressing $\underset{\sim}{A}_k$ may be useful in certain cases.

Definition 5: The k^{th} perturbation term is designated by $\Delta_k G(X_{i_1} X_{i_2} \ldots X_{i_k})$. Such term is defined by equation 3-2 by setting L= k+1, then replacing k by k-1. The k^{th} order perturbation sum is defined by

$$\sum_{\underset{\sim}{B}_k} \Delta_k G(X_{i_j} X_{i_2} \ldots X_{i_k}) \tag{3-4}$$

where the sum over the set $\underset{\sim}{B}_k$ is a sum over the contributions of all possible combinations of k groups selected from a given set of N groups. More explicitly

$$\sum_{\underset{\sim}{B}_k} = \sum_{j_1=1}^{N-k+1} \sum_{i_2=i_1+1}^{N-k+2} \cdots\cdots \sum_{i_s=i_{s-1}+1}^{N-k+s} \cdots \sum_{i_k=i_{k-1}+1}^{N} \tag{3-4a}$$

The Free Energy Decomposition Theorem

The free energy of binding of compound $AX_1X_2 \ldots X_N$ can be decomposed into contributions from the binding of a reference compound corrected for by first, second .... N^{th} order perturbation terms according to

$$\Delta_o G(AX_1X_2\ldots X_N) = \Delta_o G(AY_1Y_2\ldots Y_N) + \underset{\sim}{\sum_{B_1}} \Delta_1 G(X_{i_1}) + \underset{\sim}{\sum_{B_2}} \Delta_2 G(X_{i_1}X_{i_2})$$

$$+ \underset{\sim}{\sum_{B_3}} \Delta_3 G(X_{i_1}X_{i_2}X_{i_3}) + \ldots\ldots \underset{\sim}{\sum_{B_k}} \Delta_k G(X_{i_1}X_{i_2}\ldots X_{i_k})$$

$$+ \ldots\ldots\ldots\ldots\ldots + \Delta_N G(X_{i_1}X_{i_2}\ldots X_{i_N}) \qquad (3\text{-}5)$$

or more compactly

$$\Delta_o G(AX_1X_2\ldots X_N) = \Delta_o G(AY_1Y_2\ldots Y_N) + \sum_{k=1}^{N} \underset{\sim}{\sum_{B_k}} \Delta_k G(X_{i_1}X_{i_2}\ldots X_{i_k}) \qquad (3\text{-}6)$$

Proof:

The theorem is proved by induction. Setting k=N-1 and L=N in equation 3-2 gives

$$\Delta_N G(X_{i_1}\ldots X_{i_N}) = \Delta_{N-1} G(X_{i_1}\ldots X_{i_N}) - \underset{\sim}{\sum_{A_{N-1}}} \Delta_{N-1} G(X_{i_{j_1}}\ldots X_{i_{j_{(N-1)}}}) \qquad (3\text{-}7)$$

where the sum over $\underset{\sim}{A}_{N-1}$ is a sum over the contributions of all possible combinations of N-1 groups from the given set of N groups. Equation (3-6) can be rewritten as

$$\Delta_o G(AX_1\ldots X_N) = \Delta_o G(AY_1\ldots Y_N) + \sum_{k=1}^{N-2} \underset{\sim}{\sum_{B_k}} \Delta_k G(X_{i_1}\ldots X_{i_k})$$

$$+ \underset{\sim}{\sum_{B_{N-1}}} \Delta_{N-1} G(X_{i_1}\ldots X_{i_{N-1}}) + \Delta_N G(X_{i_1}\ldots X_{i_k}) \qquad (3\text{-}8)$$

Substituting equation 3-7 in equation 3-8 and noting that the sums over $\underset{\sim}{B}_{N-1}$ and $\underset{\sim}{A}_{N-1}$ cancel identically (see their respective definitions) gives

$$\Delta_o G(AX_1 \ldots X_N) = \Delta_o G(AY_1 \ldots Y_N) + \sum_{k=1}^{N-2} \sum_{B_k} \Delta_k G(X_{i_1} \ldots X_{i_k})$$

$$+ \Delta_{N-1} G(X_{i_1} \ldots X_{i_N}) \qquad (3\text{-}9)$$

Setting k=N-2 and L=N in equation 3-2 gives

$$\Delta_{N-1} G(X_{i_1} \ldots X_{i_N}) = \Delta_{N-2} G(X_{i_1} \ldots X_{i_N}) - \sum_{A_{N-2}} \Delta_{N-2} G(X_{i_{j_1}} \ldots X_{i_{j_{N-2}}}) \qquad (3\text{-}10)$$

where the sum over the set A_{N-2} is a sum over the contributions of all possible combinations of N-2 groups selected from the given set of N groups. Equation 3-9 can be rewritten as

$$\Delta_o G(AX_1 \ldots X_N) = \Delta_o G(AY_1 \ldots Y_N) + \sum_{k=1}^{N-3} \sum_{B_k} \Delta_k G(X_{i_1} \ldots X_{i_k})$$

$$+ \sum_{B_{N-2}} \Delta_{N-2} G(X_{i_1} \ldots X_{i_{N-2}}) + \Delta_{N-1} G(X_{i_1} \ldots X_{i_{N-1}}) \qquad (3\text{-}11)$$

Substituting equation 3-10 in 3-11 and noting that the sums over B_{N-2} and A_{N-2} cancel identically (see their respective definitions) gives

$$\Delta_o G(AX_1 \ldots X_N) = \Delta_o G(AY_1 \ldots Y_N) + \sum_{k=1}^{N-3} \sum_{B_k} \Delta_k G(X_{i_1} \ldots X_{i_k})$$

$$+ \Delta_{N-2} G(X_{i_1} \ldots X_{i_N}) \qquad (3\text{-}12)$$

Similarly, setting k=N-3 and L=N in equation 3-2 gives an expression for $\Delta_{N-2}G(X_{i_1}\ldots X_{i_N})$ which when substituted in equation 3-12 leads to a cancellation of all terms of the form $\Delta_{N-3}G(X_{i_1}\ldots X_{i_{N-3}})$ leaving

$$\Delta_o G(AX_1\ldots X_N) = \Delta_o G(AY_1\ldots Y_N) + \sum_{k=1}^{N-4} \sum_{B_k\sim} \Delta_k G(X_{i_1}\ldots X_{i_k}) + \Delta_{N-3}G(X_{i_1}\ldots X_{i_N}) \quad (3\text{-}13)$$

Proceeding in this manner one can show in general that when the upper limit on the sum over k in the right hand side of equation 3-13 is N-s, the general form of equation 3-13 is

$$\Delta_o G(AX_1\ldots X_N) = \Delta_o G(AY_1\ldots Y_N) + \sum_{k=1}^{N-s} \sum_{B_k\sim} \Delta_k G(X_{i_1}\ldots X_{i_k}) + \Delta_{N-s+1}G(X_{i_1}\ldots X_{i_N}) \quad (3\text{-}14)$$

Setting s=N-1 in equation 3-14 gives

$$\Delta_o G(AX_1\ldots X_N) = \Delta_o G(AY_1\ldots Y_N) + \sum_{B_1\sim} \Delta_1 G(X_{i_1}) + \Delta_2 G(X_{i_1}\ldots X_{i_N}) \quad (3\text{-}15)$$

Setting k=1 and L=N in equation 3-2 gives an equation for $\Delta_2 G(X_{i_1}\ldots X_{i_N})$ which when substituted into (3-15) gives

$$\Delta_o G(AX_1\ldots X_N) = \Delta_o G(AY_1\ldots Y_N) + \Delta_1 G(X_{i_1}\ldots X_{i_N}) \quad (3\text{-}16)$$

Substituting equation 3-1 into 3-16 completes the proof.

Lemma 1

There are precisely $\frac{N!}{(N-k)!k!}$ terms contributing to the k^{th} order perturbation sum (eqn. 3-4).

Proof:

The k^{th} order perturbation sum is a sum over all possible combinations of k groups selected from a total of N groups. The total number of combinations of k objects selected from N objects is

$$M_k = \frac{N!}{(N-k)!k!} \qquad \text{QED} \qquad (3\text{-}17)$$

Lemma 2

There are precisely 2^N perturbation terms on the right hand side of equation 3-5 and 3-6.

Proof:

There are M_k terms in the k^{th} order perturbation sum (lemma 1). If M is the total number of perturbation terms on the right hand side of equation 3-5 and 3-6 then

$$M = \sum_{k=0}^{N} M_k \qquad (3\text{-}18)$$

Substituting equation 3-17 in 3-18 gives

$$M = \sum_{k=0}^{N} \frac{N!}{(N-k)!k!} = \sum_{k=0}^{N} \frac{N!}{(N-k)!k!} N_1\, 1^{N-k}$$

Using the binomial theorem, M becomes

$$M = (1 + 1)^N$$

$$M = 2^N$$

QED

Lemma 3:

The 2^N terms in the Free Energy Decomposition Theorem are linearly independent.

Proof:

Let the total number of $\Delta_o G(AX_1X_2...X_N)$ terms (including $\Delta_o G(AY_1Y_2...Y_N)$) be M ($M=2^N$ by Lemma 2). These terms are linearly independent because they correspond to M independent measurements. Specifically $\Delta_o G(AY_1Y_2...Y_N)$ is independent of all other $\Delta_o G$ terms. Each of the $\Delta_1 G$ terms generated by the transformation

$$\Delta_1 G(AX_{i_1}X_{i_2}...X_{i_L}) = \Delta_o G(AX_{i_1}X_{i_2}...X_{i_L}) - \Delta_o G(AY_1Y_2...Y_N)$$

is linearly independent because each such term is generated by a linear combination of a unique $\Delta_o G(AX_{i_1}X_{i_2}...X_{i_L})$ term with $\Delta_o G(AY_1Y_2...Y_N)$. Moreover, there are M-1 such $\Delta_1 G$ terms, only N of which are retained as first order perturbation terms in the Free Energy Decomposition Theorem (lemma 1). The remaining (M-N-1) terms are used in generating $\Delta_2 G$ terms. The $\Delta_2 G$ terms generated by the transformation

$$\Delta_2 G(AX_{i_1}X_{i_2}...X_{i_L}) = \Delta_1 G(X_{i_1}X_{i_2}...X_{i_L}) - \underset{\underset{\sim}{A_k}}{\Sigma} \Delta_1 G(X_{i_{j_1}})$$

are linearly independent because each such $\Delta_2 G$ term is generated from an independent $\Delta_1 G(AX_{i_1}X_{i_2}...X_{i_L})$ term. The total number of $\Delta_2 G$ terms is thus (M-1-N). (N)(N-1)/2 of these $\Delta_2 G$ terms are retained as second order perturbation terms in the Free Energy Decomposition Theorem. The remaining $(M - \sum_{j=0}^{2} \frac{N!}{(N-j)!j!})$ terms are used

in generating $\Delta_3 G$ terms. Proceeding as such, one can show that after generating all the linearly independent 0^{th}, 1^{st}...k^{th} order perturbation terms which appear in the Free Energy Decomposition Theorem, there will be $(M - \sum_{j=o}^{k} \frac{N!}{(N-j)!j!}$ linearly independent terms left, only $\frac{N!}{(N-k-1)!(k+1)!}$ of which are retained as $(k+1)^{th}$ order perturbation terms. Setting k=N in the above equation completes the proof.

Lemma 4:

It is necessary and sufficient to have 2^N independent $\Delta_o G$ terms to obtain all the perturbation terms in the Free Energy Decomposition Theorem.

Proof:

The proof follows from lemma 3 by setting $M=2^N$.

Lemma 5:

To obtain the perturbation terms in the Free Energy Decomposition Theorem, it is necessary and sufficient to measure the free energy of 2^N independent compounds. Thus the Free Energy Decomposition Theorem is a linear mapping from a set of 2^N linearly independent free energy terms to a set of 2^N linearly independent perturbation terms.

Proof:

The proof follows from lemma 4.

IV.- COMMONLY USED ASSUMPTION IN OBTAINING THE BINDING CONSTANTS AND FREE ENERGIES.

There are two commonly used assumptions in determining the equilibrium constants.

The first assumption deals with handling the activities and concentrations. The chemical equation which expresses the binding of a ligand (AH) to a single site on a macromolecule, say a protein (P) to form a protein-ligand complex (PAH) is

$$P + AH \rightarrow PAH \tag{3-19}$$

the stoichiometric coefficient being unity for all components. The equilibrium constant (K'_{AH}) for this reaction is

$$K'_{AH} = \frac{a_{PAH}}{a_P \; a_{AH}} \tag{3-20}$$

where a_{PAH}, a_P and a_{AH} are the activities of PAH, P and AH respectively. The activities are dimensionless numbers. Hence, the equilibrium constants are dimensionless numbers too. The activities are expressed in terms of activity coefficients and concentrations by

$$a_{PAH} = (PAH) \; \gamma_{PAH} \tag{3-21}$$

$$a_P = (P) \; \gamma_P \tag{3-22}$$

$$a_{AH} = (AH) \; \gamma_{AH} \tag{3-23}$$

where the terms in brackets are the concentrations and the corresponding terms are the activity coefficients (with the dimension inverse concentration). Thus K'_{AH} becomes

$$K'_{AH} = \frac{(PAH)}{(p) \; (AH)} \; \frac{\gamma_{PAH}}{\gamma_P \; \gamma_{AH}} \tag{3-24}$$

By setting

$$\frac{(PAH)}{(P)(AH)} = K_{AH}$$

and

$$\frac{\gamma_{PAH}}{\gamma_P \ \gamma_{AH}} = K_\gamma$$

the expression for K'_{AH} becomes

$$K'_{AH} = K_{AH} \cdot K_\gamma \tag{3-25}$$

The activity coefficients of proteins and most small molecules are not available. They can be determined theoretically only for the simplest cases. The comon practice is to arbitrarily set the activity coefficients equal to unity. Under this assumption $K_\gamma = 1$ and

$$K'_{AH} = K_{AH} \tag{3-26}$$

It is well known thermodynamically that at infinite dilution the solute-solvent interactions are significant where as the solute-solute interactions are negligible due to the large distances that separate solute molecules. Under these conditions the solute behaves ideally. Thus the activity coefficients approach unity and the activities approach the concentrations. The dilution required for a particular solute to behave ideally depends on a multitude of factors including the structure and chemical nature of the solute and the solvent, the ionic strength,

etc. . It is conceivable that in certain experiments, the solutions are dilute enough to behave ideally. It is doubtful that this is true in all cases.

The second assumption deals with the models used in the data analysis. In virtually every case a macromolecule has multiple binding sites which may be grouped into subsets of identical sites (each containing one or more site) with no two subsets containing the same type of sites. Again in virtually all cases the sites are not independent of each other, i.e. binding at one site perturbs the binding at all other sites. A rigorous analysis of experimental data requires determining the binding constants to all such sites. This is never possible in practice. There are two reasons for this. First, even in the simplest models where the sites are treated as independent, the equations for several classes of sites are extremely cumbersome to work with. The situation deteriorates more rapidly if one tries to parametrize cooperative behaviour. Secondly, statistically fitting non linear equations requires experimental data of superb accuracy. In most situations however, it is relatively easy to discern sites whose affinities to the ligand differ by factors greater than 10. It is also straightforward to get (at least) crude estimates of the affinity constants.

The standard free energy ($\Delta G^{\circ}(AH)$ for the reaction shown in equation (3-19) is given by

$$\Delta G^{\circ}(AH) = - RT \ln K'_{AH} \qquad (3\text{-}27)$$

The standard state in this case is a state in which all the components have unit activities at the temperature and pressure of the reaction. Since all the information available to us is K_{AH} and

and not K'_{AH}, the superscript (°) in $G°$ is dropped and K'_{AH} is replaced by K_{AH}. Moreover, since we will be using AH as a reference compound in the subsequent discussions, we introduce the subscript (o) on the ΔG term. Equation (3-27) becomes

$$\Delta_o G(AH) = -RT \ln K_{AH} \tag{3-28}$$

Finally, we note that the commonly used linear free energy type parameters (σ, Es, π etc.) correspond to the first order terms ($\Delta_1 G$, equation 3-1) of the free energy decomposition.

V- SIMPLIFIED VERSION OF THE THEORY

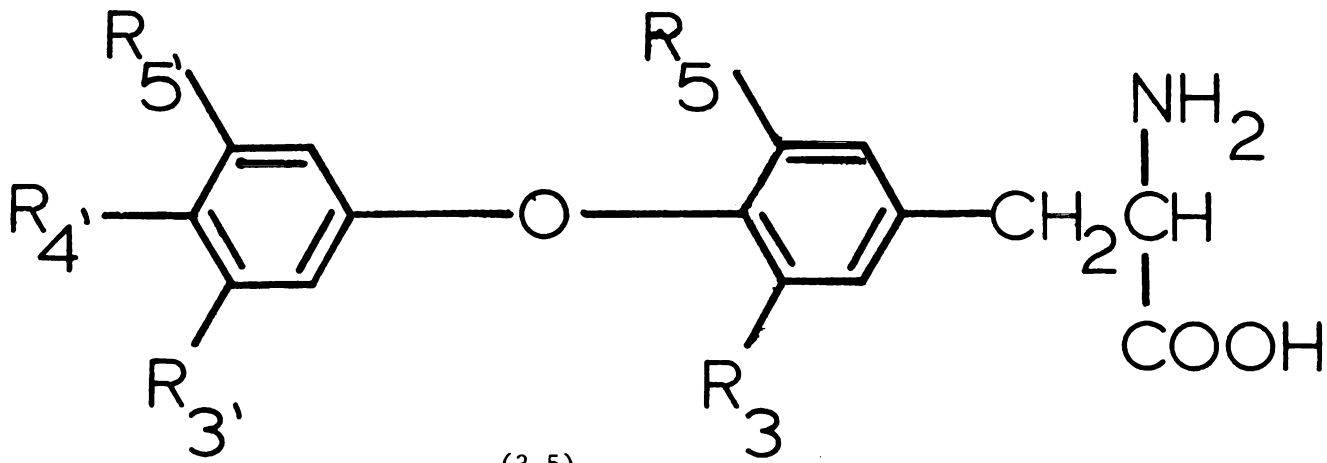
The theory of free energy decomposition presented in section III of this chapter is formal and general, two facts which may hide the simplicity of the ideas and hinder its useability. Its formulation as such was necessary to provide general definitions, ascertain their consistency and delineate the necessary conditions for its use. In this section we will give a much simpler formulation of the theory (albeit equally rigorous) that is easier to use in the more common situations. We will explicitly develop the free energy decomposition for one, two and three functional groups, show how the lemmas of section III can be used to give useful information, discuss the choice of a reference compound and finally give physical interpretations of the perturbation terms.

Suppose one has the set of eight thyroid hormone analogs shown in table 3-1 whose binding constants (K) to some specific site on a particular macromolecule are known. The free energy of binding of each compound $\Delta_o G$ can be determined by $\Delta_o G = -RT \ln K$. The eight values

Table 3-1. Thyroid Hormone Analogs Used in the Development of the Simplified Version of the Theory.[a]

Symbol	$R_{3'}$	$R_{4'}$	$R_{5'}$
AHHH	H	H	H
AXHH	X	H	H
AHYH	H	Y	H
AHHZ	H	H	Z
AXYH	X	Y	H
AXHZ	X	H	Z
AHYZ	H	Y	Z
AXYZ	X	Y	Z

[a]Substituents refer to the general structure (3-5) with R_3=R_5=I and Z=0.



(3-5)

General structure of thyroid hormone analogs.

which are obtained are: $\Delta_o G(AHHH)$, $\Delta_o G(AXHH)$, $\Delta_o G(AHYH)$, $\Delta_o G(AHHZ)$, $\Delta_o G(AXYH)$, $\Delta_o G(AXHZ)$, $\Delta_o G(AHYZ)$ and $\Delta_o G(AXYZ)$. Our aim is to obtain each of these values in terms of $\Delta_o G(AHHH)$ + first, second and third order perturbation terms.

The zeroth order perturbation term is

$$\Delta_o G(AHHH) \tag{3-29}$$

The first order perturbation terms ($\Delta_1 G$) are defined by

$$\Delta_1 G(X) = \Delta_o G(AXHH) - \Delta_o G(AHHH) \tag{3-30}$$

$$\Delta_1 G(Y) = \Delta_o (AHYH) - \Delta_o G(AHHH) \tag{3-31}$$

$$\Delta_1 G(Z) = \Delta_o (AHHZ) - \Delta_o G(AHHH) \tag{3-32}$$

$$\Delta_1 G(XY) = \Delta_o G(AXYH) - \Delta_o G(AHHH) \tag{3-33}$$

$$\Delta_1 G(XZ) = \Delta_o G(AXHZ) - \Delta_o G(AHHH) \tag{3-34}$$

$$\Delta_1 G(YZ) = \Delta_o G(AHYZ) - \Delta_o G(AHHH) \tag{3-35}$$

$$\Delta_1 G(XYZ) = \Delta_o G(AXYZ) - \Delta_o G(AHHH) \tag{3-36}$$

The second order perturbation terms ($\Delta_2 G$) are defined by

$$\Delta_2 G(XY) = \Delta_1 G(XY) - \{\Delta_1 G(X) + \Delta_1 G(Y)\} \tag{3-37}$$

$$\Delta_2 G(XZ) = \Delta_1 G(XZ) - \{\Delta_1 G(X) + \Delta_1 G(Z)\} \tag{3-38}$$

$$\Delta_2 G(YZ) = \Delta_1 G(YZ) - \{\Delta_1 G(Y) + \Delta_1 G(Z)\} \tag{3-39}$$

$$\Delta_2 G(XYZ) = \Delta_1 G(XYZ) - \{\Delta_1 G(X) + \Delta_1 G(Y) + \Delta_1 G(Z)\} \tag{3-40}$$

The third order perturbation term ($\Delta_3 G$) is defined by

$$\Delta_3 G(XYZ) = \Delta_2 G(XYZ) - \{\Delta_2 G(XY) + \Delta_2 G(XZ) + \Delta_2 G(YZ)\} \tag{3-41}$$

In terms of the above definitions, the $\Delta_o G$ of each of the initial eight compounds is given by

$$\Delta_o G(AHHH) = \Delta_o G(AHHH) \tag{3-42}$$

$$\Delta_o G(AXHH) = \Delta_o G(AHHH) + \Delta_1 G(X) \tag{3-43}$$

$$\Delta_o G(AHYH) = \Delta_o G(AHHH) + \Delta_1 G(Y) \tag{3-44}$$

$$\Delta_o G(AHHZ) = \Delta_o G(AHHH) + \Delta_1 G(Z) \tag{3-45}$$

$$\Delta_o G(AXYH) = \Delta_o G(AHHH) + \Delta_1 G(X) + \Delta_1 G(Y) + \Delta_2 G(XY) \tag{3-46}$$

$$\Delta_o G(AXHZ) = \Delta_o G(AHHH) + \Delta_1 G(X) + \Delta_1 G(Z) + \Delta_2 G(XZ) \tag{3-47}$$

$$\Delta_o G(AHYZ) = \Delta_o G(AHHH) + \Delta_1 G(Y) + \Delta_1 G(Z) + \Delta_2 G(YZ) \tag{3-48}$$

$$\begin{aligned}\Delta_o G(AXYZ) = {} & \Delta_o G(AHHH) + \Delta_1 G(X) + \Delta_1 G(Y) + \Delta_1 G(Z) \\ & + \Delta_2 G(XY) + \Delta_2 G(XZ) + \Delta_2 G(YZ) \\ & + \Delta_3 G(XYZ)\end{aligned} \tag{3-49}$$

The correctness of equations (3-42)-(3-49) was precisely the content of the proof of the Free Energy Decomposition Theorem.

There are several important features to be noted in equations (3-42)-(3-49). (i) Of all the eight experimentally determined $\Delta_o G$ terms and twelve theoretically determined $\Delta_1 G$, $\Delta_2 G$ and $\Delta_3 G$ terms (equations 3-30 through 3-41) there are only eight terms that appear explicitly on the right hand sides of equations (3-42)-(3-49). These are: $\Delta_o G(AHHH)$, $\Delta_1 G(X)$, $\Delta_1 G(Y)$, $\Delta_1 G(Z)$, $\Delta_2 G(XY)$, $\Delta_2 G(XZ)$, $\Delta_2 G(YZ)$, and $\Delta_3 G(XYZ)$. This is a consequence of the fact that we started with eight independent measured numbers, performed a linear transformation on them and ended with eight theoretically determined linearly independent numbers. The independence of the eight theoretical numbers is proved in lemma 3. (ii) In the set of eight linearly independent theoretically determined numbers there is one $\Delta_o G$ term, three $\Delta_1 G$ terms, three $\Delta_2 G$ terms and one $\Delta_3 G$ term. This distribution of terms is not coincidental. That

this distribution is correct is predicted and proved in lemma 1. This lemma specifies that there are $\frac{N!}{(N-k)!k!}$ terms of the form $\Delta_k G$ that will appear in the free energy decomposition. Setting N=3 (there are three groups X, Y and Z) and k = 0,1,2 and 3 gives a distribution of

$$\frac{3!}{3!0!} : \frac{3!}{2!1!} : \frac{3!}{1!2!} : \frac{3!}{0!3!} = 1 : 3 : 3 : 1$$

which is precisely the distribution that was obtained. (iii) The number of terms on the right hand sides of equations (3-42)-(3-49) are: one in (3-42), two in (3-43)-(3-45), four in (3-46)-3-48) and eight in (3-49). This is not coincidental either. Its correctness is predicted and proved in lemma 2. This lemma specifies that there are 2^N terms in a perturbation expansion of a compound which has N functional groups. Thus in (3-42) N = 0, (3-43)-(3-45) N = 1, (3-46) - (3-48) N = 2, and (3-49) N = 3 generating 2^0, 2^1, 2^2, and 2^3 = 1, 2, 4 and 8 terms respectively. (iv) Finally, according to lemma 5, if one needs to determine the free energy decomposition of N groups, 2^N compounds must be measured experimentally. Thus, to determine the decomposition of 0,1,2 and 3 groups one needs to measure 1,2,4 and 8 compounds respectively.

The choice of the reference compound (AHHH) is arbitrary. Any compound with any set of groups may serve as a reference compound. A reasonable choice of a reference compound would be one which has as many hydrogens as possible but this is not necessary. Once a reference compound has been chosen however, it must be used throughout. Thus, in order to determine the contribution of a 4'-hydroxyl relative to a reference group Y, say Y=H, to the binding of thyroid hormone analogs

to a certain protein one can choose any pair of analogs which are identical in every respect except that one of them has a 4'-hydrogen and the other has a 4'-hydroxyl. The contribution of the 4'-hydroxyl in different pairs of compounds is most likely not equal. This difference is due to the different perturbing effects exerted by the remaining groups in the different pairs of compounds.

Examining the $\Delta_1 G$ terms defined by equations (3-30), (3-31) and (3-32) shows that $\Delta_1 G(X)$ is the contribution of group X (whose position in the molecule is implicit in the symbol X) to the free energy of binding relative to hydrogen at the same position when the remainder of the molecule has the structure (A-HH). Similar interpretations can be given for the terms for groups Y and Z. Examining equation (3-37) shows that if the contribution of groups X and Y when present in the same molecule is the same as the sum of their contributions when present in different molecules then $\Delta_2 G(XY) = 0$ and $\Delta_1 G(XY) = \Delta_1 G(X) + \Delta_1 G(Y)$. In all cases that we know of however, $\Delta_2 G(XY) \neq 0$ indicating that when groups X and Y are present in the same molecule they mutually perturb the binding of each other by an amount equal to $\Delta_2 G(XY)$. Finally substituting equation (3-40) into (3-41) gives

$$\Delta_3 G(XYZ) = \Delta_1 G(XYZ) - \{\Delta_2 G(XY) + \Delta_2 G(XZ) + \Delta_2 G(YZ) + \Delta_1 G(X) + \Delta_1 G(Y) + \Delta_1 G(Z)\} \qquad (3\text{-}50)$$

which shows that $\Delta_3 G$ is a three body term which corrects for the three body perturbations of each other above and beyond that obtained from one and two body perturbations. In general, a $\Delta_k G$ term corrects for k body perturbations of each other above and beyond that obtained from 1, 2 ..... (k-1) body perturbations.

VI- EXAMPLES OF THE APPLICATION OF THE THEORY IN EXPERIMENTAL SITUATIONS

The simplified version of the theory has already been used by Jorgensen, et al. (27) to analyze the data of Koerner, et al. (26,28) on the binding of thyroid hormones and analogs to intact rat liver nuclei and this will be presented again in this section. It has also been used by Bolger and Jorgensen (20,21) to analyze the binding of thyroid hormones and analogs to solubilized nuclear "receptors", and this work appears in detail in reference 45. We will also present in this section an analysis of the data of Snyder et al. (29) on the binding of these hormones and analogs to thyroxine binding globulin. Finally, the theory is used in chapter 5 of this dissertation to analyze the data on the binding of thyroid hormones and analogs to prealbumin.

1-Binding of Thyroid Hormones and Analogs to Intact Rat Liver Nuclei:

Koerner, et al. (26,28) have measured the affinities of thyroid hormones and their analogs to the high affinity, low capacity binding sites of rat liver cell nuclei in vitro. The apparent association constant of L-T3 in this system was $6.1 \times 10^8\ M^{-1}$ (26), the corresponding $\Delta_o G(T3) = -12.5$ kcal/mole. Table 3-2 shows the ratios of the association constants of T3 analogs to that of T3 and the values for the change in free energy of binding for each analog relative to T3. Table 3-3 shows the calculated contributions ($\Delta_1 G$) of various substituents on the thyronine nucleus to the binding free energy.

3'-Substituents:

Table 3-3 shows that the 3'-alkyl substituent contributes from -0.95 to -3.60 kcal/mol to the binding free energy. The contribution increases as the alkyl group increases in size and hydrophobic character,

Table 3-2. Relative Binding Affinities of Thyroid Hormones and Analogs to Intact Rat Liver Nuclei[a,b]

No.	Compound	DL	R_3	R_5	$R_{3'}$	Z	$R_{4'}$	$R_{5'}$	$\frac{K(AX)}{K(T_3)} \times 100$	$\Delta_0 G^c$
1	T_3	L	I	I	I	O	OH	H	100	0.00
2	T_2	L	I	I	H	O	OH	H	0.3	+3.58
3	T_2Me	L	I	I	Me	O	OH	H	13.5	+1.23
4	T_2Et	DL	I	I	Et	O	OH	H	21	+0.96
5	T_2iPr	L	I	I	iPr	O	OH	H	104	-0.02
6.	T_2tBu	L	I	I	tBu	O	OH	H	38.5	+0.59
7	T_2iBu	L	I	I	iBu	O	OH	H	20	+0.99
8	T_2Phe	DL	I	I	Phe	O	OH	H	2	+2.41
9	T_2cHex	DL	I	I	cHex	O	OH	H	1.4	+2.63
10	3,3',5'-T_3	DL	I	H	I	O	OH	I	0.1	+4.25
11	3,3'-T_2	L	I	H	I	O	OH	H	0.5	+3.26
12	T_4	L	I	I	I	O	OH	I	12.5	+1.28
13	4'-H-T_3	DL	I	I	I	O	H	H	0.4	+3.40
14	4'-H-T_2Me	DL	I	I	Me	O	H	H	0.2	+3.82

Table 3-2. Continued

15	T_2Cl	L	I	I	Cl	O	OH	H	6.2	+1.71
16	T_2Cl_2	L	I	I	Cl	O	OH	Cl	4.5	+1.91
17	T_2Me_2	L	I	I	Me	O	OH	Me	6.2	+1.71
18	T_2iPr	DL	I	I	iPr	O	OH	H	100	0.00
19	T_2iPr_2	DL	I	I	iPr	O	OH	iPr	1.4	+2.63
20	TBr_2iPr	L	Br	Br	iPr	O	OH	H	36	+0.63
21	$TiPr_2I$	DL	iPr	iPr	I	O	OH	H	0.2	+3.83
22	TMe_3	L	Me	Me	Me	O	OH	H	0.1	+4.25
23	TMe_4	L	Me	Me	Me	O	OH	Me	0.1	+4.25
24	TMe_2iPr	L	Me	Me	iPr	O	OH	H	0.7	+3.06
25	MBT_3	DL	I	I	I	CH_2	OH	H	250	-0.56
26	MBT_4	DL	I	I	I	CH_2	OH	H	2.6	+2.25
27	ST_2	L	I	I	H	S	OH	H	1.3	+2.68
28	ST_3	L	I	I	I	S	OH	H	100	0.00
29	H_2NT_3	L	I	I	I	O	NH_2	H	0.76	+3.01

[a]Substituents refer to the general structure (3-5).

[b]References 26 and 28.

[c]Kcal/mole.

Table 3-3. First Order Perturbation Terms of Various Groups in the Binding of Thyroid Hormones and their Analogs to Intact Rat Liver Nuclei.[a,b]

Substituent X	Substituted Analog (AX)[c]	Reference Analog (AH)[c]	$\Delta_1 G(X)$[d]
3'-Substituent			
Me	3	2	-2.35
Et	4	2	-2.62[e]
iPr	5	2	-3.60
tBu	6	2	-2.99
iBu	7	2	-2.59
Phe	8	2	-1.17[e]
cHex	9	2	-0.95[e]
3,5, & 5'-Iodines			
3'-I	1	2	-3.58
5-I	1	11	-3.26
	12	10	-2.97[e]
5'-I	10	11	+0.99[e]
	12	1	+1.28
4'-Substitution			
4'-OH	1	13	-3.40[e]
4'-OH	3	14	-2.59[e]
4'-NH_2	29	13	-0.39[e]

Table 3-3 continued

5'-Substitution			
5'-Cl	16	15	+0.20
5'-Me	17	3	+0.48
5'-iPr	19	18	+2.63[e]

[a]Reference 27.

[b]Substituents refer to the general structure (3-5).

[c]The number in this column correspond to the number of compound (column 1) in table 3-2.

[d]Calculated using equation (3-30). Units are kcal/mole.

[e]AX,AH or both are DL mixture. In all other entries both AX and AH are L enantiomers.

hydrophobic character, reaching a maximum at isopropyl, and then diminishing as the 3' substituent further increases in size. This data suggests involvement of the 3'-alkyl group in a size-related hydrophobic association.

5,3, and 5' Iodine Atoms:

Table 3-3 shows that the contribution of the 3'-I is favorable by -3.58 kcal/mol, a value which falls between that of 3'-Et and 3'-iPr. As a measure of lipophilic character, the Hansch π values (3) of 3'-Et, 3'-I and 3'-iPr (0.97, 1.15 and 1.30, respectively) are in the same relative order as their contributions to the binding free energies. This suggests the involvement of the 3'-I in hydrophobic interactions. Table 3-3 further shows that adding the 5-I to either 3,3', 5'-T3 or to 3,3'-T2 increases binding by about 3 kcal/mol. It is likely that the contribution of the 5-I is due both to its hydrophobic interaction, and in concert with the 3-substituent, to its exerting steric constraints which favor a mutually perpendicular minimal energy conformation for the two aromatic rings. Thus, the apparent strong binding contribution of the 5-I would be due to an enhancement of the total hydrophobic binding of the very lipophilic and conformationally constrained ortho-diiododiphenyl ether structure relative to a poorly constrained and less lipophilic ortho-monoidodiphenyl ether such as 3,3'-T2.

4'-Substituents:

Addition of a 4'-hydroxyl groups to the 4'-deoxy analogs, 4'-H-T2Me and 4'-H-T3, increases the binding by 2.59 and 3.40 kcal/mol, respectively. This suggests an involvement of the 4'-hydroxy in hydrogen bonding with an acceptor on the protein. The greater binding effect for a phenolic

hydroxyl group ortho to an iodine atom indicates an interaction between substituents, in which the halogen atom enhances the hydrogen bond, possibly by its inductive effect. A 4'-amino group also favors the binding although not as strongly as the 4'-hydroxyl.

5'-Substituent:

Addition of a 5'-substituent to any of four 3,5,3'-trisubstituted compounds has an unfavorable effect on binding. The 5'-substituents size is in the order: Cl < CH_3 < I < iPr. This is the same order as their effect in decreasing binding. These results may be interpreted as a deleterious steric effect, either acting directly on the receptor surface, or indirectly by interfering with hydrogen bond formation between the adjacent 4'-hydroxyl group and an amino acid residue on the receptor.

Second Order Perturbation between the 5 and 5' Iodine Atoms:

It is possible to calculate a second order perturbation term for the 5 and 5' iodine atoms. Using equations (3-30),(3-31),(3-33) and (3-37) with X=5-I, Y=5'-I, AHHH = 3,3'-T2, AXHH = 3,5,3'-T3, AHYH = 3,3',5'-T3 and AXYH = 3,5,3',5'-T4 gives

$\Delta_1G(5\text{-}1) = \Delta_oG(3,5,3'\text{-T3}) - \Delta_o(3,3'\text{-T2}) = -3.28$

$\Delta_1G(5\text{-I}) = \Delta_oG(3,3',5'\text{-T3}) - \Delta_oG(3,3'\text{-T2}) = +0.99$

$\Delta_1G(5\text{-I},\ 5'\text{-I}) = \Delta_oG(3,5,3',5'\text{-T4}) - \Delta_oG(3,3'\text{-T2}) = -1.98$

$\Delta_2G(5\text{-I},5'\text{-I}) = \Delta_1G(5\text{-I},5'\text{-I}) - \{\Delta_1G(5\text{-I}) + \Delta_1G(5'\text{-I})\} = +0.29.$

A Δ_2G value of + 0.29 kcal/mol is small relative to the Δ_oG and Δ_1G terms. Moreover, this value is based on a measurement of 3,3',5'-T3 as a DL mixture (and all others being L enantiomers) and hence would be smaller if the Δ_oG value for the 3,3',5'-L-T3 was used. Thus one can conclude that the 5 and 5' iodine atoms do not significantly perturb the contribution of each other.

The Side Chain;

Koerner, et al(26) have shown that the acetic acid side chain analog of T3 (triac) binds at least as firmly as T_3 to isolated rat liver nuclei. Thus the carboxylate ion in the side chain of T_3 or triac is the primary contributor to side chain binding, presumably, by ion pair formation with a cationic residue of the protein.

A Generalized View of the Nuclear Receptor:

The thyroid hormone receptor shown schematically in figure 3-1 contains a positively charged site which forms an ion-pair with the carboxylate ion of the alanine side chain. The receptor contains a second polar region, a hydrogen bonding site, which associates with the 4'-phenolic hydroxyl or functionally equivalent groups. The intervening space between the two polar binding sites consists of a hydrophobic region, with highly specific steric requirements for maximal summation of dispersion forces and hydrophobic associative interactions with the aromatic rings and their 3,5, and 3'-substituents. The distal 3'-substituent forms an increasingly effective association in the order of size and hydrophobicity: H < Cl < Me < Br < Et < I < iPr. Groups larger than isopropyl in the distal 3'-position are less active in the order of increasing size: tBu > iBu > Phe > cyclohexyl, probably due to decreased fit to the limited dimensions of the hydrophobic pocket. A second substituent (5') ortho to the phenolic hydroxyl group produces a decrease in activity related to its size. Most interestingly, the summation of multiple weak contributions to receptor binding produces a strongly associated hormone-receptor complex.

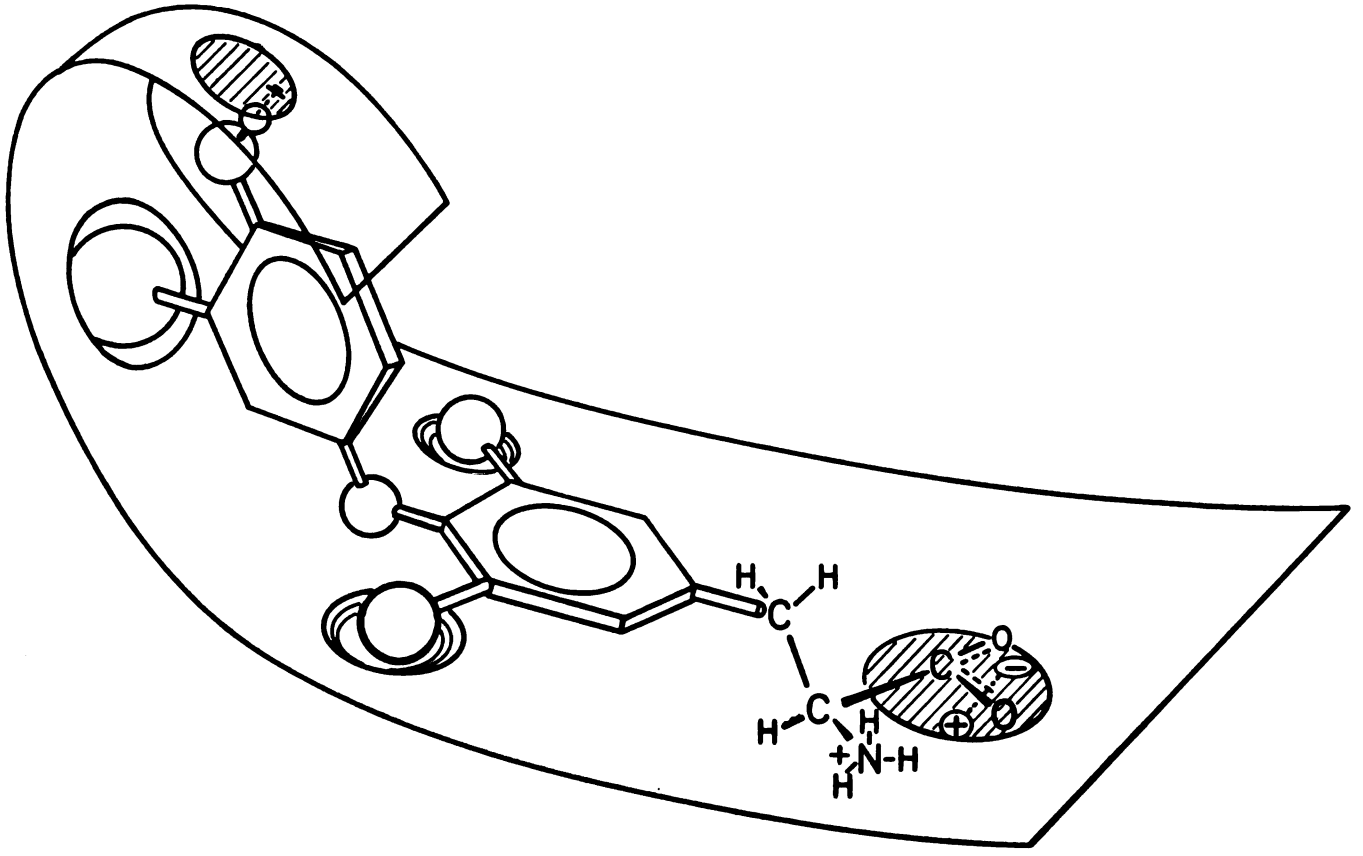


Figure 3-1. Schematic drawing of the thyroid hormone receptor.

2-Binding of Thyroid Hormones and Their Analogs to Thyroxine Binding Globulin:

Snyder, et al.(29) measured the affinities of thyroid hormones and their analogs to thyroxine binding globulin. The apparent association constant for L-T_4 is 2.5 x 10^9 M^{-1} at 25°C corresponding to a binding free energy of -12.81 kcal/mol. Table 3-4 shows the ratios of the affinity constants of thyroid hormones and analogs to that of L-T_4 and the values for the change in the free energy of binding of each analog relative to L-T_4. Table 3-5 shows the first order perturbation terms for different substituents calculated by equation (3-30). Table (3-6) shows the second order perturbation terms calculated for various pairs of groups by using equations (3-30), (3-31), (3-33) and (3-37).

Table (3-5) shows that the 3,5,3' iodine atoms contribute favorably to the binding. The 5'-iodine however which was unfavorable in the nuclear receptor, enhances the binding in thyroxine binding globulin. The second order perturbation terms for the pairs (3'-I, 5-I) and (3-I,5'-I) show that the members of each pair make each other's contribution more effective. The reverse is true however, for the (5-I,5'-I) pair. The origin of these effects is not obvious. It is equally interesting to mention here that the members of each of these three pairs of groups weaken each other's contribution to binding in the case of prealbumin.

Table 3-4. Relative Binding Affinities of Thyroid Hormones and Analogs to Thyroxine Binding Globulin.[a,b]

Number	Compound	DL	R_3	R_5	$R_{3'}$	$R_{4'}$	$R_{5'}$	$\frac{K(A)}{K(T_4)}$ x 100	G[c]
1	T_4	L	I	I	I	OH	I	100	0.00
2	T_4	D	I	I	I	OH	I	54	+0.38
3	T_3	L	I	I	I	OH	H	9.0	+1.48
4	$RevT_3$	L	I	H	I	OH	I	38	+0.60
5	$3,5\text{-}T_2$	L	I	I	H	OH	H	0.07	+4.47
6	$3,3'\text{-}T_2$	L	I	H	I	OH	H	1.3	+2.68
7	$3,5'\text{-}T_2$	L	I	H	I	OH	H	0.1	+4.25
8.	$3\text{-}T_1$	L	I	H	H	OH	H	0.05	+4.68
9	$3'\text{-}T_1$	L	H	H	I	OH	H	0.023	+5.16
10	$4'\text{-}OMeT_3$	L	I	I	I	OMe	H	3.5	+2.06
11	$4'\text{-}H\text{-}T_3$	DL	I	I	I	H	H	1.9	+2.44
12	$4'\text{-}NH_2T_3$	L	I	I	I	NH_2	H	2.4	+2.30

[a]Substituents refer to the general structure (3-5) with Z=0.

[b]Reference 29.

[c]Kcal/mole.

Table 3-4. Relative Binding Affinities of Thyroid Hormones and Analogs to Thyroxine Binding Globulin.[a,b]

Number	Compound	DL	R_3	R_5	$R_{3'}$	$R_{4'}$	$R_{5'}$	$\frac{K(A)}{K(T_4)}$ x 100	G[c]
1	T_4	L	I	I	I	OH	I	100	0.00
2	T_4	D	I	I	I	OH	I	54	+0.38
3	T_3	L	I	I	I	OH	H	9.0	+1.48
4	$RevT_3$	L	I	H	I	OH	I	38	+0.60
5	$3,5\text{-}T_2$	L	I	I	H	OH	H	0.07	+4.47
6	$3,3'\text{-}T_2$	L	I	H	I	OH	H	1.3	+2.68
7	$3,5'\text{-}T_2$	L	I	H	I	OH	H	0.1	+4.25
8.	$3\text{-}T_1$	L	I	H	H	OH	H	0.05	+4.68
9	$3'\text{-}T_1$	L	H	H	I	OH	H	0.023	+5.16
10	$4'\text{-}OMeT_3$	L	I	I	I	OMe	H	3.5	+2.06
11	$4'\text{-}H\text{-}T_3$	DL	I	I	I	H	H	1.9	+2.44
12	$4'\text{-}NH_2T_3$	L	I	I	I	NH_2	H	2.4	+2.30

[a]Substituents refer to the general structure (3-5) with Z=O.

[b]Reference 29.

[c]Kcal/mole.

Table 3-5. First Order Perturbation Terms of Various Groups in the Binding of Thyroid Hormones and Their Analogs to Thyroxine Binding Globulin.

Substituent (X)[a]	(AX)[b]	(AH)[b]	$\Delta_1 G(X)$[c]
Iodine Substitution			
3-I	4	7	-3.65[d]
	6	9	-2.48
5-I	1	4	-0.60
	3	6	-1.20
	5	8	-0.21
3'-I	3	5	-2.99
	6	8	-2.00
5'-I	1	3	-1.48
	4	6	-2.08
	7	9	-0.91[d]

Table 3-5 Continued

4'-Substitution

4'-OH	3	11	-0.96[d]
4'-OMe	10	11	-0.38[d]
4'-NH_2	12	11	-0.14[d]

[a]Substituents refer to structure (3-5).

[b]The numbers in this column correspond to the number of compound (column 1) in table 3-6.

[c]Calculated using equation (3-30). Units are kcal/mole.

[d]AX, AH or both are D enantiomers or DL mixtures. In all other entries AX and AH are L enantiomers.

Table 3-6. Second Order Perturbation Terms for Various Pairs of Groups in the Binding of Thyroid Hormones and their Analogs to Thyroxine Binding Globulin.

Substituent XY	AXYH	AXHH	AHYH	AHHH	$\Delta_2 G(XY)$
(3'-I,5-I)	3,5,3'-T_3	3,3'-T_2	3,5-T_2	3-T_1	-1.00
(3-I,5'-I)	3,3',5'-T_3	3,3'-T_2	3',5'-T_2	3'-T_1	-1.17
(5-I,5'-I)	3,5,3',5'-T_4	3,5,3'-T_3	3,3',5'-T_3	3,3'-T_2	+0.60

[a]Calculated using equations (3-30), (3-31), (3-33) and (3-37). Units are kcal/mole.

Chapter 4

Conformational Analysis of Diphenyl Ethers

I- THE HYPOTHESIS

Thyroid hormone analogs which have only one substituent ortho to the 4'-hydroxyl group exert their action in a conformation in which the substituent ortho to the 4'-hydroxyl is held in the distal 3'-position rather than the proximal 5'-position (figure 4-1) (30). In addition the two rings are almost mutually perpendicular.

The development of this hypothesis can be outlined as follows:

1- Substitution of the 3'-iodine atoms of T_3 by an alkyl group led to full retention of thyromimetic activity (1,2,30). Thus analogs having a methyl group instead of iodine in the outer ring were used to test the hypothesis.

2- 3'-methyl-3,5-diiodo-DL-thyronine (figure 4-2a) showed 50% the activity of L-thyroxine (31). The 2'-methyl,2',5'-dimethyl, and 2',3'-dimethyl derivatives of 3,5-diiodo-DL-thyronine (figures 4-2b, 4-2c and 4-2d respectively) showed 0.5, 0.5 and 50% the activity of L-thyroxine (32).

3- The 6' protons of the compounds in figures 4-2b, 4-2c and 4-2d had an anomalously high upfield chemical shift which was compatible with the concept that the 2'-methyl group in each of these compounds locks the two rings in a conformation in which the 2'-methyl group and the 3'-position are oriented distally with respect to the inner ring.

4- The observation that the activity of the 3'-methyl analog (figure 4-2a) is matched only by the 2',3'-dimethyl analog (figure 4-2d) (distal 3'-methyl) and not by the 2',5'-dimethyl analog (figure 4-2c) (proximal 5'-methyl) naturally led to the hypothesis.

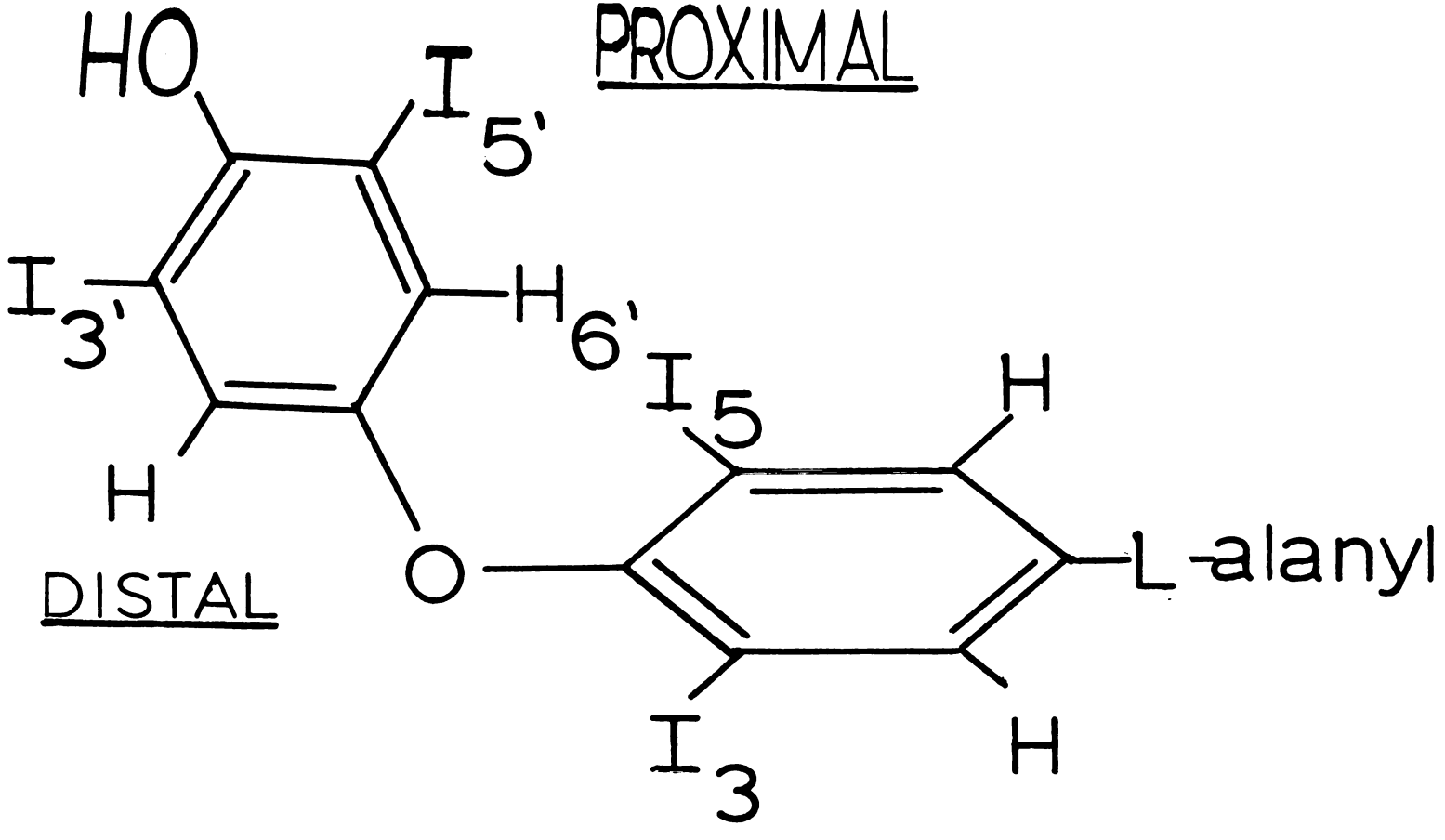


Figure 4-1. Proximal and distal positions of the outer ring substituents of thyroid hormones and analogs.

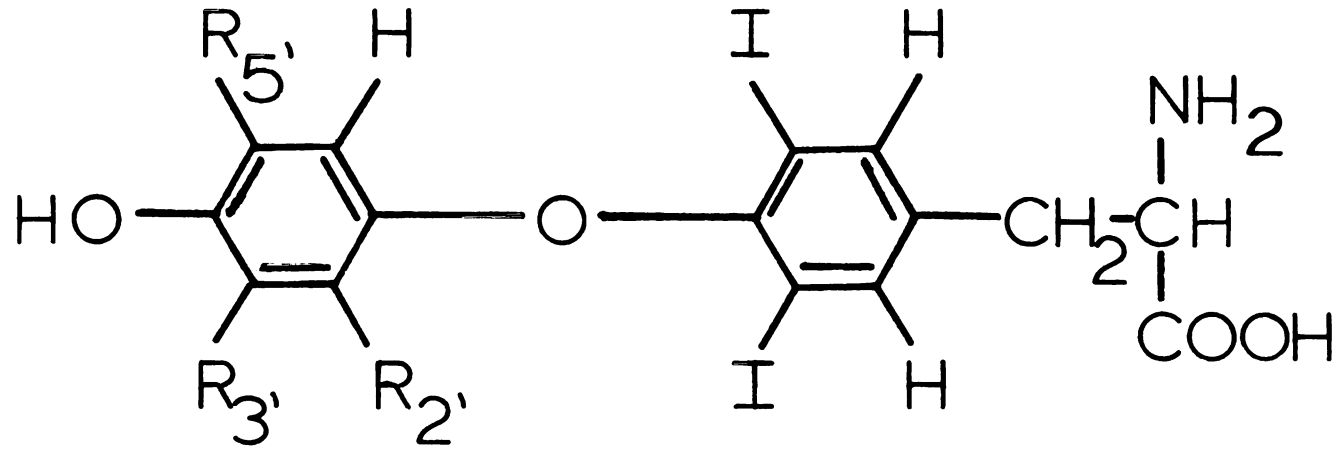


Figure 4-2.

a- R_2' = H , R_3' = CH_3 , R_5' = H

b- R_2' = CH_3 , R_3' = H , R_5' = H

c- R_2' = CH_3 , R_3' = H , R_5' = CH_3

d- R_2' = CH_3 , R_3' = CH_3 , R_5' = H

This hypothesis has clearly defined one of the variables that control thyromimetic activity and hence provided a logical basis for the explanation of the variation of thyromimetic activity of a certain class of compounds with their structure. Thus, it constitutes the starting point for the studies presented in this chapter.

II- TESTING THE HYPOTHESIS

There is compelling theoretical and experimental evidence (see Section VI) that for compounds which have no bulky 2' groups (i) the population of the proximal and distal conformations are almost equal, and (ii) the barrier for interconversion between the two conformers (for analogs with 3,5-iodine substituents) is of the order of 10 kcal/mol. Thus the rate of interconversion between the conformers is reasonably high at room temperature. This implies that if the distal population is depleted, it can be reconstituted from the remaining proximal. Given these two pieces of data alone, it is clear that there is no physical restriction on the validity of the hypothesis.

As is the case with many other hypotheses, there is no direct proof of it. There is some supportive evidence. Its ultimate acceptance is based on its ability to predict and interpret experimental results. Its validity may be questioned only if one can demonstrate that its prediction contradict experimental observations, such as, finding a pair of distal-proximal isomers in which the activity of the proximal is greater than that of the distal. Any test short of this can be easily rejected on logical grounds. Any attempt to disprove it (or prove it) based on the structure of a hormone or an analog in the solid state, solution,

or by conformational energy calculations on isolated molecules is meaningless.

We sought to test the consistency of one of the implications of the above hypothesis with available experimental data. In the original publication (30) it is not explicitly mentioned that the rings have to be in a mutually perpendicular conformation although this was implied. Later, theoretical and X-ray crystallographic studies (33,34) showed that the analogs used by Jorgensen (30) had an almost mutually perpendicular ring conformation. Thus we set out to test the following premise: if an analog is required to exert its biological activity in a conformation in which the two rings are mutually perpendicular then compounds for which it is "easier" to lock the rings into mutual perpendicularity should have higher biological activities than ones in which it is "more difficult" to lock the rings into mutual perpendicularity. We did not attempt to check the consistency of the proximal versus distal argument with available experimental data because the populations of these two isomers are almost equal for compounds with no 2'-substituent and hence their differentiation is a function of the "receptor(s)".

Presently, there are no experimental means by which the above premise can be tested. A thermodynamic function which measures the "difficulty" of locking an analog into a certain conformation is the free energy change attending the process in which every molecule in the population of molecules is locked into the given conformation. We have termed this measure ΔG_{lock}. The higher this free energy change, the more "difficult" it is to lock the compound into the given conformation. This measure will be derived in the next section.

III- STATISTICAL MECHANICAL DERIVATION OF ΔG_{lock}

The canonical ensemble partition function (Q) for N independent and indistinguishable molecules which obey Boltzmann statistics (the energy level density is much greater than the number of molecules) is given by (35a)

$$Q = \frac{q^N}{N!} \tag{4-1}$$

where q is the single molecule partition function. The set of degrees of freedom of each molecule may be partitioned into two subsets, one of which (typically the translations and rotations) can be treated classically and the second (the vibrations and the electronic states) has to be treated quantum mechanically (35b, 36). Thus

$$q = q_{class}\ q_{q.m.} \tag{4-2}$$

Let each molecule have a total of n classical and m quantum mechanical degrees of freedom. In this case

$$q_{class} = \frac{1}{h^n} \int dq_1 \ldots dq_n dp_1 \ldots dp_n\ e^{-\beta H_o} \tag{4-3}$$

where the q's and p's under the integral sign are the generalized coordinates and their conjugate momenta, respectively. $H_o(q_1 \ldots q_n p_1 \ldots p_n)$ is the hamiltonian of the classical degrees of freedom. Substituting equations (4-2) and (4-3) in (4-1) gives

$$Q = \frac{1}{N!} \left[\frac{q_{q.m.}}{h^n} \int dq_1 \ldots dq_n dp_1 \ldots dp_n\ e^{-\beta H_o} \right]^N \tag{4-4}$$

The conformation of diphenyl ethers is described by the dihedral angles Φ_1 and Φ_2 (figure 4-3). We introduce here two assumptions: (i) The motion along Φ_1 and Φ_2 can be treated classically, and (ii) the motion along these two coordinates is independent of the remaining $(n-2)$ classical coordinates. These assumptions imply that

$$H_o(q_1 \cdots q_n p_1 \cdots p_n) = H(\Phi_1, \Phi_2, P_{\Phi_1}, P_{\Phi_2}) + H'(q_3 \cdots q_n p_3 \cdots p_n) \quad (4\text{-}5)$$

Moreover

$$H(\Phi_1, \Phi_2, P_{\Phi_1}, P_{\Phi_2}) = K(P_{\Phi_1}, P_{\Phi_2}) + U(\Phi_1, \Phi_2) \quad (4\text{-}6)$$

where K and U are the kinetic and potential energy, respectively for motion along these two degrees of freedom. Substituting equations (4—5) and (4-6) in (4-4) and collecting terms gives

$$Q = \left(\int d\Phi_1 \, d\Phi_2 \; e^{-\beta U} \right)^N \times \frac{1}{N!} \left[\frac{q_{q.m.}}{h^n} \left(\int dP_{\Phi_1} \, dP_{\Phi_2} \; e^{-\beta K} \right) \times \left(\int dq_3 \cdots dq_n dp_3 \cdots dp_n \; e^{-\beta H'} \right) \right]^N \quad (4\text{-}7)$$

Setting the last term on the right hand side of equation (4-7) equal to C gives

$$Q = \left[\iint d\Phi_1 \, d\Phi_2 \; e^{-\beta U} \right]^N \times C \quad (4\text{-}8)$$

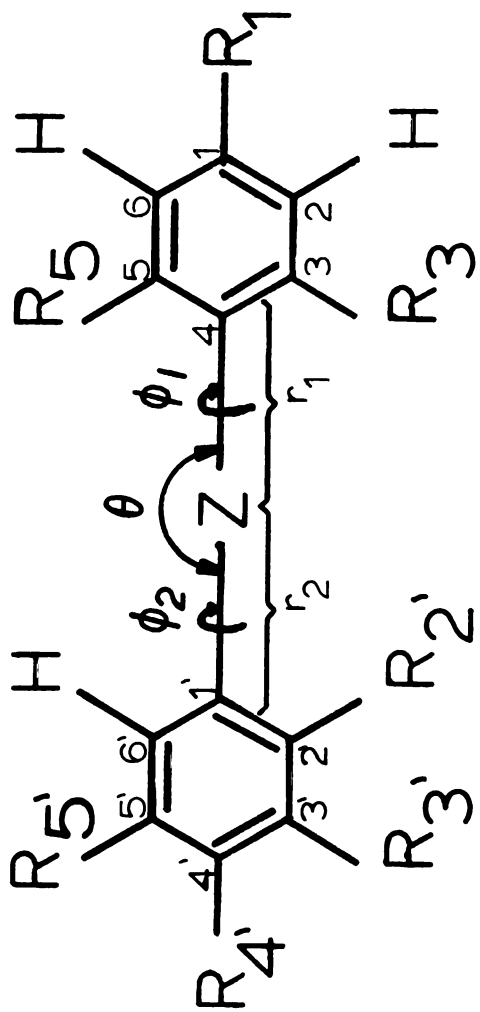


Figure 4-3. General structure of diphenyl ether analogs.

$\Phi_1 = \Phi_{34Z1'}$ and $\Phi_2 = \Phi_{4Z1'6'}$ where Φ_{ABCD} implies a clockwise rotation of BCD plane relative to ABC plane looking down the CB bond from C towards B.

If the N molecules are distributed over all (Φ_1,Φ_2) space the corresponding partition function (Q_T) is given by

$$Q_T = \left[\int_0^{2\pi}\int d\Phi_1\, d\Phi_2\, e^{-\beta U}\right]^N \times C \qquad (4\text{-}9)$$

In order to determine the corresponding partition function (Q_P) for the conformationally locked population, one needs a definition of a mutually perpendicular geometry. It is not obvious from the original formulation of the hypothesis what ranges of Φ_1 and Φ_2 precisely define the distal, proximal and mutually perpendicular conformation. Here we introduce a general definition of these conformations by:

Distal, mutually perpendicular: $90-\delta\Phi_1 \leq \Phi_1 \leq 90+\delta\Phi_1$

$$0-\delta\Phi_2 \leq \Phi_2 \leq 0+\delta\Phi_2 \qquad (4\text{-}10)$$

Proximal, mutually perpendicular: $90-\delta\Phi_1 \leq \Phi_1 \leq 90+\delta\Phi_1$

$$180-\delta\Phi_2 \leq \Phi_2 \leq 180+\delta\Phi_2 \qquad (4\text{-}11)$$

where $\delta\Phi_1$ and $\delta\Phi_2$ are small variation in these angles (in the grid that we will use later $\delta\Phi_1$ and $\delta\Phi_2$ = 15°). We also need to point out that there are four geometries in which the rings are mutually perpendicular. These are (Φ_1,Φ_2) = (90, 0), (90, 180), (270, 0) and (270, 180). The first and second pairs are related by a reflection in the plane of the inner ring to the third and fourth pairs, respectively. Thus each of the two pairs will generate identical contributions to the partition function. Hence we will only consider the first and second pairs, each multiplied by a factor of 2. Therefore

$$Q_p = 2^N\left[\int_{90-\delta\Phi_1}^{90+\delta\Phi_1} d\Phi_1 \left(\int_{0-\delta\Phi_2}^{0+\delta\Phi_2} d\Phi_2\, e^{-\beta U} + \int_{180-\delta\Phi_2}^{180+\delta\Phi_2} d\Phi_2\, e^{-\beta U}\right)\right]^N \times C \qquad (4\text{-}12)$$

The Helmholtz free energy for each of the two states is given by

$$A_T = -k_B T \ln Q_T \tag{4-13}$$

$$A_P = -k_B T \ln Q_P \tag{4-14}$$

The quantity

$$\Delta A = A_P - A_T \tag{4-15}$$

is the change in the Helmhotz free energy when the entire population of molecules having an equilibrium distribution over all possible energy states is locked into the subregions of phase space defined by equations (4-10) and (4-11). We also make the assumption that there is no change in the pressure and volume when such a locking process occurs. Thus, the energy and enthalpy are equal, so are the Gibbs and Helmholtz free energies. This assumption was necessary because we want to use the final result of this derivation to compare it with experimentally determined Gibbs free energies. It follows from this assumption and equations (4-13) to (4-15) that

$$\Delta G_{lock} = \Delta A = -k_B T \ln \frac{Q_P}{Q_T} \tag{4-16}$$

Comparing equations (4-12) and (4-9) shows that Q_P and Q_T are identical in every respect except that upper and lower limits of integration on the Φ_1 and Φ_2 coordinates. Dividing equation (4-12) by (4-9) gives after cancellation of C

$$\frac{Q_P}{Q_T} = \frac{2^N \left(\int_{90-\delta\Phi_1}^{90+\delta\Phi_1} d\Phi_1 \left[\int_{0-\delta\Phi_2}^{0+\delta\Phi_2} d\Phi_2 \, e^{-\beta U} + \int_{180-\delta\Phi_2}^{180+\delta\Phi_2} d\Phi_2 \, e^{-\beta U} \right] \right)^N}{\left(\int_0^{2\pi} \int d\Phi_1 \, d\Phi_2 \, e^{-\beta U} \right)^N} \qquad (4\text{-}17)$$

Equations (4-16) and (4-17) are our operational expressions. Since the analytical form of the function of U is not known, this function was determined numerically as shown in the next section.

The integrals in equation (4-17) were also evaluated numerically. This was achieved by multiplying the value of the probability density at each point in the (Φ_1, Φ_2) grid by the element of area and summing over the entire grid according to

$$\frac{Q_P}{Q_T} = \frac{2 \left(e^{-\beta U(90,0)} + e^{-\beta U(90,180)} \right) \Delta\Phi_1 \, \Delta\Phi_2}{\sum_{m=0}^{L1} \sum_{n=0}^{L2} e^{-\beta U(m\Delta\Phi 1, n\Delta\Phi 2)} \Delta\Phi_1 \, \Delta\Phi_2} \qquad (4\text{-}18)$$

where

$$L1 = (360/\Delta\Phi_1) - 1, \text{ and } L2 = (360/\Delta\Phi_2) - 1 \qquad (4\text{-}19)$$

$\Delta\Phi_1$ and $\Delta\Phi_2$ determine the fineness of the grid. Under these conditions, it is obvious that

$$\delta\Phi_1 = \Delta\Phi_1/2 \quad \text{and} \quad \delta\Phi_2 = \Delta\Phi_2/2 \qquad (4\text{-}20)$$

The calculation was done for $\Delta\Phi_1 = \Delta\Phi_2 = 10°$ for two compounds and compared with the results of $\Delta\Phi_1 = \Delta\Phi_2 = 30°$.

In the above derivations we have implicitly made the assumption that the only changes in the energy and entropy upon locking are those arising from the loss of the vibrational degrees of freedom Φ_1 and Φ_2. In other words we have neglected the changes in the rotational entropy upon locking inspite of the fact that the components of the inertia tensor (and hence the principal moments of inertia) are functions of Φ_1 and Φ_2.

We like to note here that similar methods have been used by Richards, et al.(37,38) in the analysis of conformational maps of physiologically active amines.

IV- COMPUTATIONAL DETAILS

In order to determine the potential energy surface, we needed a method that is inexpensive yet reliable enough to reflect the quantum mechanical effects like resonance and exchange repulsion. Thus the CNDO/2 method was used (39,40,41). Standard atomic parameters were employed for all atoms except for the halogens. The halogen parameters employed were those previously used by Kollman, et al. (33). Standard geometrical parameters (42,43) were used except where noted below.

The alanyl side chain was replaced by a hydrogen atom. This is justified on the basis of the calculation of Kollman, et al. (44). They (i) evaluated the energy for six selected points on the (Φ_1,Φ_2) potential energy surface (ranging from 0 to 16 kcal/mol in relative energy) for 3,5,3'-triiodo-4'-hydroxy diphenyl ether with and without the alanyl side

chain, and (ii) compared the energies for the two different amino acid side chain conformations discussed by Cody, et al.(45). They found that the relative energies of the amino acid containing structures and the structures in which the amino acid side chain is replaced by a hydrogen atom differed by less than 0.1 kcal/mol.

The minimum energy values of the geometrical parameters r_1,r_2 and θ (figure 4-3) were determined for X=O, CH_2 and S. The calculated and experimental values are in reasonable agreement (table 4-1), except for Z=S. Thus Z=O and CH_2, the calculated values of these geometrical parameters were used in the calculation of the surface; for Z=S the experimental geometries were employed.

The justification for using a classical instead of a quantum mechanical partition function is established on the basis that $kT > 20\ \Delta U$ where k is the Boltzmann constant, T=310°K and U is the separation between the vibrational energy levels (based on the force constant obtained from the curvature of the potential energy well).

The energy was calculated at 30° intervals of Φ_1 and Φ_2. To demonstrate the feasibility of using such a crude grid, the potential energies of 3,5,3'-triiodo-4'-hydroxy diphenyl ether and of its 3,5-dichloro analog were calculated at 10° intervals also. Table 4-2 shows that the minimum energy conformations, rotational barriers and ΔG_{lock} are in very good agreement in the two grids.

V- REVIEW AND COMMENTARY ON PREVIOUS WORK

Previous studies on the conformation of thyroid hormones and related compounds include structure determination in the solid state by X-ray

Table 4-1 Calculated and Observed Values of r_1, r_2, and θ for Various Inter-ring Linkages in Diphenyl Ethers and Related Compounds.[a]

	Calculated			Observed			References[c]
Z[a]	r_1[b]	r_2[b]	θ[b]	r_1[b]	r_2[b]	θ[b]	
O[d]	1.38	1.38	109	1.32-1.43	1.35-1.43	117-126	2-12b
O[e]	1.40	1.40	122				
CH_2[d]	1.48	1.48	114	1.514	1.522	114.3	13
S[d]	1.55	1.55	104	1.785	1.759	102.9	15

[a] Figure 4-3: $R_1=R_2,=R_5,=H$, $R_3,=R_3=R_5=I$, $R_4,=OH$.

[b] r_1 and r_2 in angstroms, θ in degrees.

[c] The numbers in this column refer to compounds in table 4-3.

[d] $\Phi_1=90$, $\Phi_2=0$. The calculated r_1, r_2 and θ were used in determining the Φ_1, Φ_2 potential energy surface except for Z=S where the Φ_1, Φ_2 potential energy surface was determined using both the calculated and observed values of r_1, r_2 and θ.

[e] $\Phi_1 = \Phi_2 = 90$

Table 4-2 Comparison of Minimum Energy Conformations, Rotational Barriers and ΔG_{lock} in the 10° and 30° grids of 3,5-Diiodo and 3,5-Dichloro Diphenyl Ether.[a]

		10° Grid					30° Grid				
		Minimum Energy Conformation		Rotational Barrier Around[b]			Minimum Energy Conformation		Rotational Barriers Around[b]		
R_3	R_5	Φ_1	Φ_2	C_4-Z bond	C_1'-Z bond	ΔG_{lock}[b]	Φ_1	Φ_2	C_4-Z bond	C_1'-Z bond	ΔG_{lock}[b]
I	I	90	0	54.41	10.64	4.64	90	0	54.41	10.64	4.72
		270	0				270	0			
Cl	Cl	90	0	14.56	2.47	5.30	90	0	14.56	2.47	5.33
		270	0				270	0			

[a]Figure 4-3; R_1=R_2,=R_5,=H, R_3,=I, R_4,=OH, Z=O.

[b]Kcal/mole.

crystallography, NMR studies in solution, and molecular orbital studies on isolated molecules.

The results obtained from crystal structures are summarized in table 4-3. The main features of these data are:

1. Compound 2: This compound has the smallest inner ring substituent (R_3=R_5=H) in the entire table. It also has the smallest value of Φ_1 and the largest value of Φ_2.

2. Compounds 3 to 9: These compounds have the features that R_3=R_5=I and Z=0. The observed ranges in dihedral angles in this set is: Φ_1=(72-116), Φ_2(-34)-(+33).

3. The pairs of compounds (10a,10b), (11a,11b) and (12a,12b): The members of each pair occur in the same crystal. The values of r_1 and r_2 and θ vary by less than 5% among the members of each pair. The values of Φ_1 and Φ_2 however, show significant variations. This is clear evidence that the minimum energy conformation in the solid state is strongly modulated by the crystal field. Hence, making any inferences about biologically active conformations on the basis of conformations in the solid state should be done with extreme caution. This is particularly important in cases where the conformational energy minima are shallow so that large regions of conformation space are populated at the temperature of interest.

4. Compounds 10a, 10b, 11a, 11b, 13 to 18: All these compounds have large 2' substituents. In all these compounds (except 14, 16 and 17) the observed conformation is distal. Compounds 14, 16 and 17 have inter-ring linkages, CO, SO and SO_2 respectively. In addition they all have a nitro group in the 2' position. Our interpretation of this apparent anomaly is that the repulsion (steric and/or electrostatic)

Table 4-3. Crystallographically Determined Geometrical Parameters of Thyroid Hormones and Analogs[a,b]

Number	R_3	R_5	$R_{2'}$	$R_{3'}$	$R_{4'}$	Z	r_1[b]	r_2[b]	θ[b]	Φ_1[b]	Φ_2[b]	P/D[b]	Ref
1[c]	I	I	H	H	MeO	NH	1.434	1.416	119.3	89	27	-	46
2[d]	H	H	H	H	OH	O	1.37	1.42	117	-37	-67	-	47
3[e]	I	I	H	H	OH	O	1.374	1.405	119	87[m]	21[m]	-	48
4[f]	I	I	H	I	OH	O	1.37	1.43	121	116	-21	D	49
5[g]	I	I	H	I	OH	O	1.32	1.36	121	90	-13	P	50
6[h]	I	I	H	I	OH	O	1.38	1.39	117	72[n]	33[n]	D	51
7[i]	I	I	H	iPr	OH	O	1.38	1.40	119	±88	±10	P	52
8[g]	I	I	H	iPr	OH	O	1.37	1.43	120	97	-26	P	53
9[j]	I	I	H	I	OH	O	-	-	-	101	-34	-	50
10a[k]	Cl	Cl	COOMe	H	NO2	O	1.387	1.354	117.9	76	8.8	D	54
10b[k]	Cl	Cl	COOMe	H	NO2	O	1.381	1.381	119.3	89.7	4.6	D	54
11a[L]	NO2	NO2	tBU	H	H	O	1.364	1.412	120.2	70.1	6.6	D	55
11b[L]	NO2	NO2	tBu	H	H	O	1.366	1.405	119.7	78.6	3.2	D	55
12a[L]	iPr	iPr	H	Me	NO2	O	1.404	1.370	119.7	77.2	5.7	D	56
12b[L]	iPr	iPr	H	Me	NO2	O	1.413	1.358	120.0	79.5	2.1	D	56

Table 4-3. Continued

13[c]	Me	Me	NO2	H	COOMe	CH_2	1.514	1.522	114.3	87.6	2.2	D	57
14[c]	Me	Me	NO2	H	COOMe	CO	1.503	1.502	116.5	51.5	40.2	P	58
15[c]	Me	Me	NO2	H	COOMe	S	1.785	1.759	102.9	86.8	4.2	D	59a
16[c]	Me	Me	NO2	H	COOMe	SO	1.798	1.819	101.4	51.6	48.1	P	59b
17[c]	Me	Me	NO2	H	COOMe	SO2	1.774	1.806	106.0	60.0	38.2	P	60
18[c]	Me	Me	NO2	H	COOMe	O	1.413	1.360	118.5	79	8.7	D	61

[a]Only references containing complete crystal data are included.

[b]Figure 4-3. Units of r_1 and r_2 are angstroms. Units of Φ_1, Φ_2 and θ are degrees. P=Proximal, D=distal.

[c]R_1 = Me, R_5, = H.

[d]R_1 = L-$CH_2CH(NH_3^+ Cl^-)COOEt$, R_5,=H.

[e]R_1 = L-$CH_2CH(NH_2)COOH:CH_3CONHCH_3$, R_5,=H.

[f]R_1 = L-$CH_2CH(NH_2)COOH$,R_5,=H.

[g]R_1 = L-$CH_2CH(NH_3^+ Cl^-)COOH.\ 3H_2O$, R_5,=H.

[h]R_1 = L-$CH_2CH(NH_2)COOMe$, R_5,=H.

[i]R_1 = CH_2CH_2COOEt, R_5,=H.

[j]R_1 = L-$CH_2CH(NH_3^+ Cl^-)COOH.H_2O$ R_5,=H.

[k]R_1 = Cl, R_5,=H.

[l]R_1 = H, R_5,=H.

Table 4-3. Continued

[m]In reference 48 the convention used in defining Φ_1 and Φ_2 was not clear to us. The values quoted here are those quoted by Cody in reference 51 where a clear definition of the conventions was made.

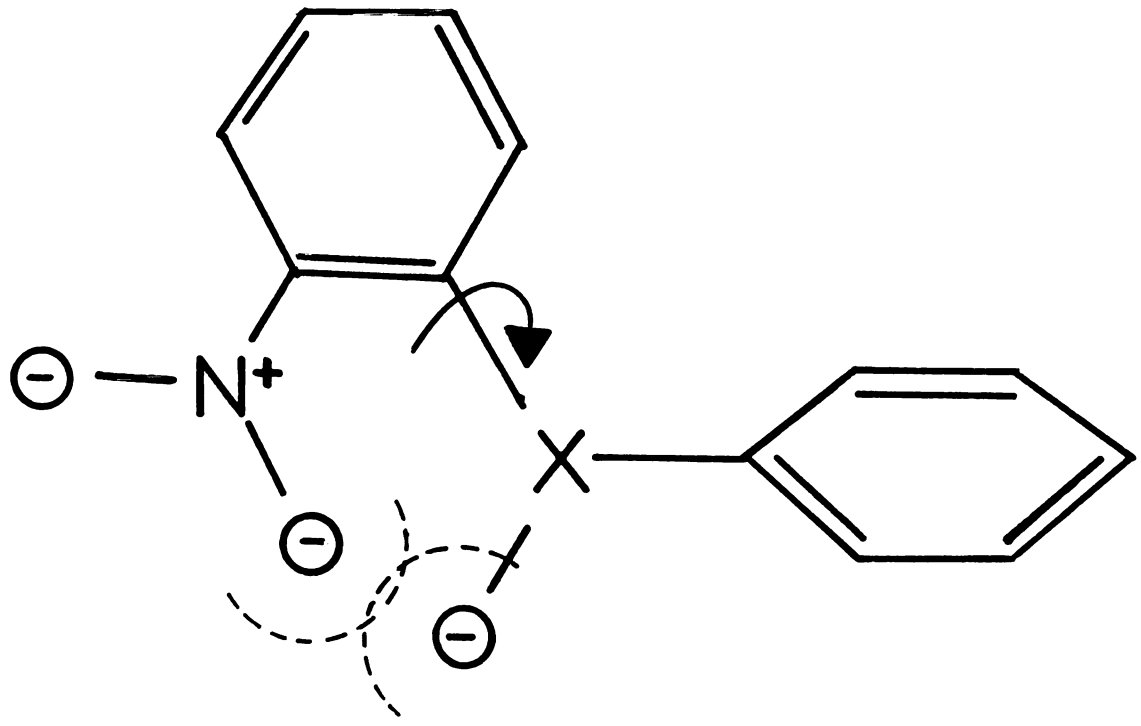
[n]Values quoted in the corresponding reference was $(\Phi_1, \Phi_2) = (-108, -33)$. In our convention these values become (72,33).

between the 2'-nitro and the oxygen "appendages" of the inter-ring linkage forces the 2'-nitro into the proximal position (see I).

The NMR data available can be summarized as follows:

1. Lehman and Jorgensen (62) were the first to provide support for the concept that the 2'-methyl group is 3,5-diiodo and 3,5-dinitro compounds locks the rings such that the 2'-methyl group is distal. This demonstration was achieved by showing that the NMR chemical shift of the 6' proton in 2'-methyl substituted thyroid hormone analogs was shifted upfield by about 1 ppm relative to the corresponding 2'-H compound. This upfield shift was interpreted as being due to the positioning of the 6' proton over the inner ring by the introduction of the 2'-methyl group.

2. Bergman et al. (63), Benjamins, et al. (64), and Emmett, et al. (65) estimated the rotational barrier for various diphenyl ether analogs. Their results are summarized in table 4-4. The main features of these rotational barriers are: (i) All compounds that have no bulky 2'-substituent (compounds 2-4 and 12-16) have rotational barriers less than 11 kcal/mol with the exception of compound 4 which has t-butyl groups in the 3 and 5 positions. (ii) All compounds with bulky 2'-substituents (compounds 1, 5-11) have rotational barriers greater than 17 kcal/mol. (iii) The barrier is higher in compound 16 than in 15. This can be explained on the basis that the lone pair orbital on the inter-ring oxygen in 16 can delocalize onto the outer ring carboxylate ester group to a greater extent than the corresponding lone pair in 15. Hence, the double bond character of the $C_{1'}$-O bond is greater in 16 than in 15 thus the barrier is higher in 16.



I. X = C, S, SO. Dashed semi-cricles represent Van Der Waal's radii. O^----O^- electrostatic and/or steric repulsion forces the nitro group into a proximal position. The figure is not drawn to scale.

Table 4-4. Rotational Barriers of Diphenyl Ethers Determined by NMR Spectroscopy[a]

Number	R_1	R_3	R_5	$R_{2'}$	$R_{3'}$	$R_{4'}$	$R_{5'}$	ΔG^b	Reference
1	H	iPr	iPr	NO_2	H	NO_2	H	17.8	63
2	H	iPr	iPr	H	H	NO_2	H	10.1-10.6	63
3	H	iPr	iPr	H	Me	NO_2	H	10.7	63
4	tBu	tBu	tBu	H	H	Br	H	16.5	63
5	H	iPr	iPr	COOMe	H	NO_2	H	20.0	64
6	H	iPr	iPr	COOMe	H	NH_2	H	17.5	64
7	H	iPr	iPr	COOMe	H	I	H	18.3	64
8	H	iPr	iPr	COOMe	H	H	H	18.6	64
9	H	iPr	iPr	NO_2	H	COOMe	H	18.1	64
10	H	iPr	iPr	NH_2	H	COOMe	H	17.3	64
11	H	iPr	iPr	I	H	COOMe	H	21	64
12	H	iPr	iPr	H	H	COOMe	H	<10	64
13	CH_3	I	I	H	I	OH	I	8.5±.2	65
14	CH_3	I	I	H	I	OH	H	7.9±.3	65
15	CH_3	I	I	H	H	OH	H	<7.7	65
16	CH_2CH (NHAC)COOEt	I	I	H	H	COOEt	H	9.3±.2	65

a- Figure 4-3 Z=0.
b- Rotational free energy barrier in kcal/mole.

The molecular orbital results can be summarized as follows:

1. The Camermans (66), using Extended Hückel Molecular Orbital Theory, found that the proximal geometry is more stable than the distal by 132 kcal/mol.
2. Kier and Hoyland (67) calculated the rotational energy barriers for 3,5,3'-triiodo, tribromo, and trichloro diphenyl ethers and found that the barriers are related to the size of the halogen atoms.
3. Kollman, et al. (33) found that the minimum energy geometries for 3,5- H_2, Me_2, F_2, Cl_2, Br_2 and I_2 are (90,90), (30,90), (60,30), (90,0) and (90,0) respectively. They also found the barriers to internal rotation to be of the order of 10-15 kcal/mol for compounds having a 2'-hydrogen. They calculated that the 2'-methyl distal conformation is more stable than the proximal by 37 kcal/mol.

VI- RESULTS AND DISCUSSIONS

Examining the structures of the compounds in table 4-3 as well as the structures of the large number of thyroid hormone analogs that have been synthesized and tested suggests that at least two effects might play a role in determining the ring conformations. These are (a) The mesomeric effect: The lone pair orbitals on the inter-ring oxygen prefer to conjugate with the p orbitals on the adjacent ring carbon atom (C_1, and C_4) and facilitate the delocalization of the π electrons of the two rings. This effect favors a coplanar conformation of the rings. (b) The steric effect: The steric interaction of the 3,5 substituents with the outer ring and its substituents (particularly the 2' and 6' substituents) favors a conformation in which the two rings are mutually perpendicular. Compounds that are identical in all respects but differ in the type of inter-ring

linkage (O, CH_2, CO, NH, S, SO, SO_2, etc.) are useful in studying the resonance effect; compounds with oxygen in the inter-ring linkage but differing in the 3,5 substituents (I_2, Br_2, Cl_2, F_2, H_2, IH, Me_2, iPr_2, $(NO_2)_2$, etc.) can be used to study the steric effect.

The potential energy maps obtained for various diphenyl ether analogs are shown in figure 4-4. At the temperature of the biological measurement (310°K) 95% of the population was found to be within the 2 kcal/mol contours. The maps show several interesting features:

(a) Comparison of the maps shows that in all cases (except for R_3=I, R_5=H; figure 4-4h) the two regions Φ_1 = 0-180, Φ_2 = 180-360 are identical. This is to be expected due to the equivalence of the upper and lower surfaces of the inner ring.

(b) Comparing the maps of Z=O, R_3=R_5=I, Br, Cl, F and H (figures 4-4c, d, e, f and g) shows that as the size of the 3,5 substituent decrease, the allowed areas of the maps (less than 2 kcal/mol in energy) increase. The most conspicuous change is seen in going from 3,5-dichloro to 3,5-difluoro and dihydrogen where large areas that were completely forbidden in the former become populated in the latter two compounds.

(c) Comparing the maps for Z=O, CH_2 and S (figures 4-4c, b and a) shows that for the latter two cases the area within the 1 kcal/mol contour is broader (larger range in Φ_2) than for the oxygen bridged case. This can be explained on the basis that the lone pair orbitals on the inter-ring oxygen atom can be conjugated with the p orbitals of the adjacent outer ring carbon atom C_1, to a greater extent than the hyperconjugation of the C-H bonds or the conjugation of the sulfur orbitals ($r_2(S) > r_2(O)$) with the result of a greater rotational freedom around the Z-C_1, bond for

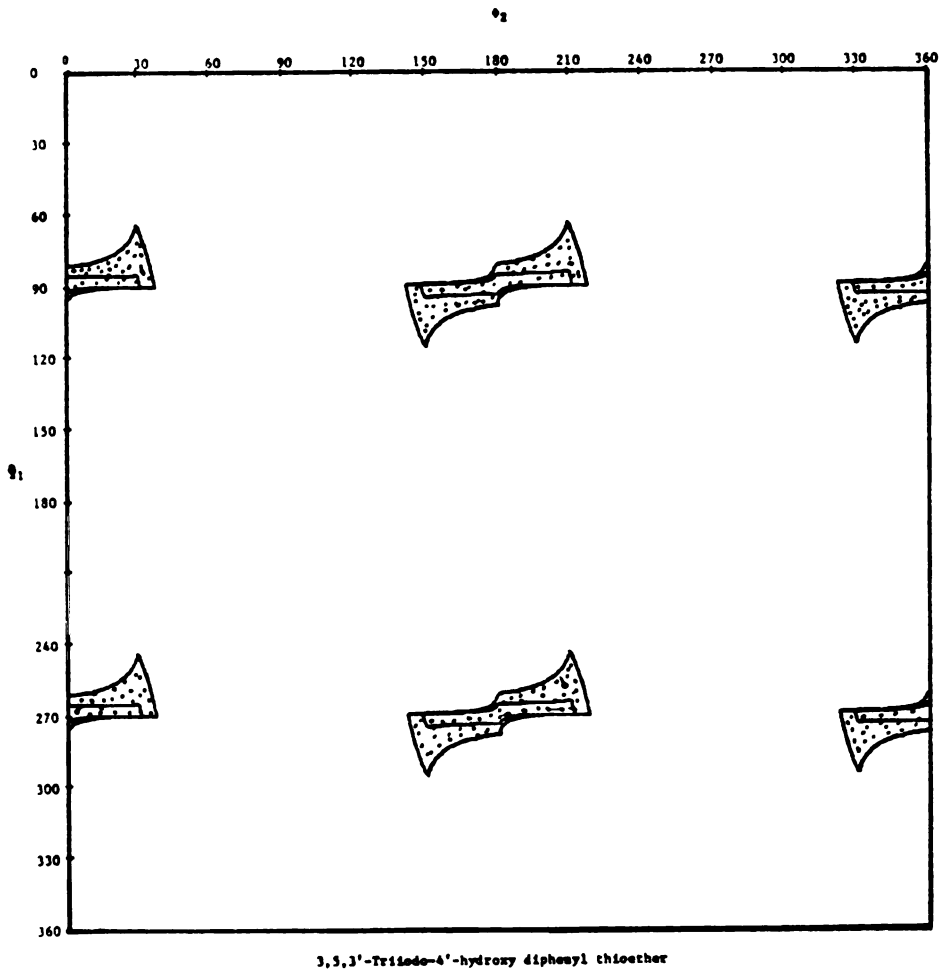

φ2
0
30
60
90
120
150
180
210
240
270
300
330
360
φ1
3,5,3'-Triiodo-4'-hydroxy diphenyl thioether

Figure 4-4a.

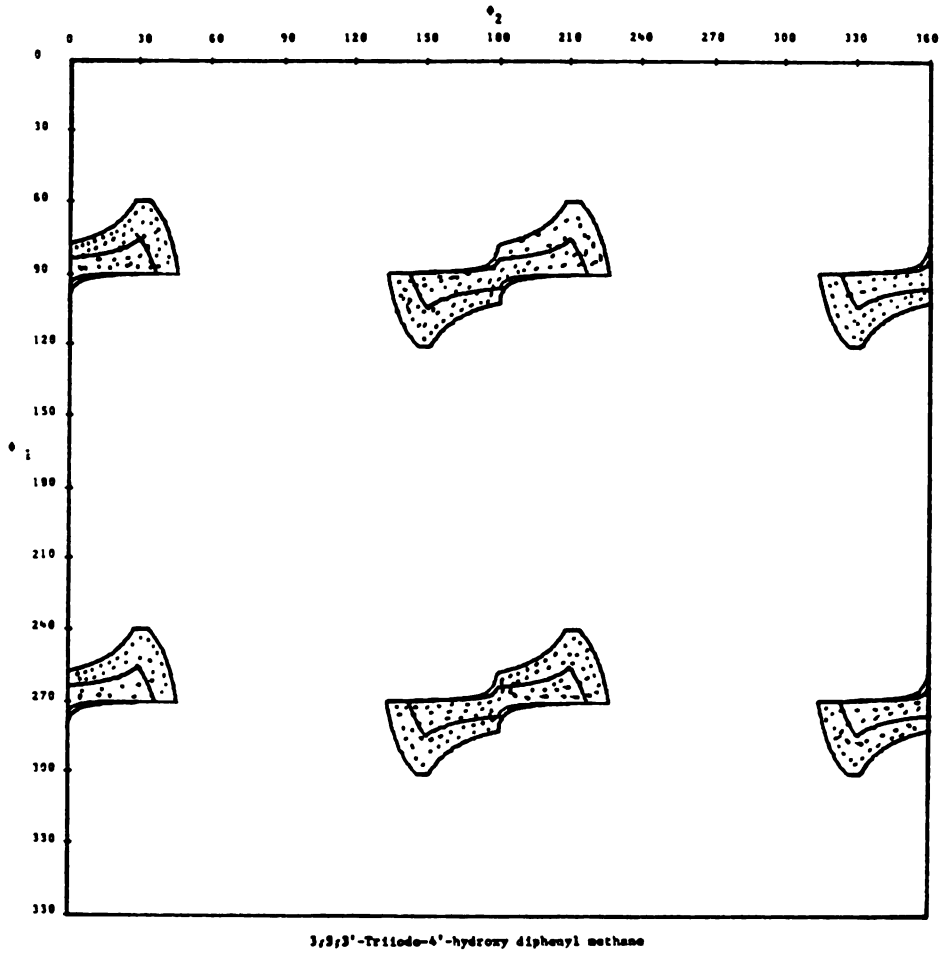

ϕ_2
0
30
60
90
120
150
180
210
240
270
300
330
360
ϕ_1
3,3,3'-Triiodo-4'-hydroxy diphenyl methane

Figure 4-4b.

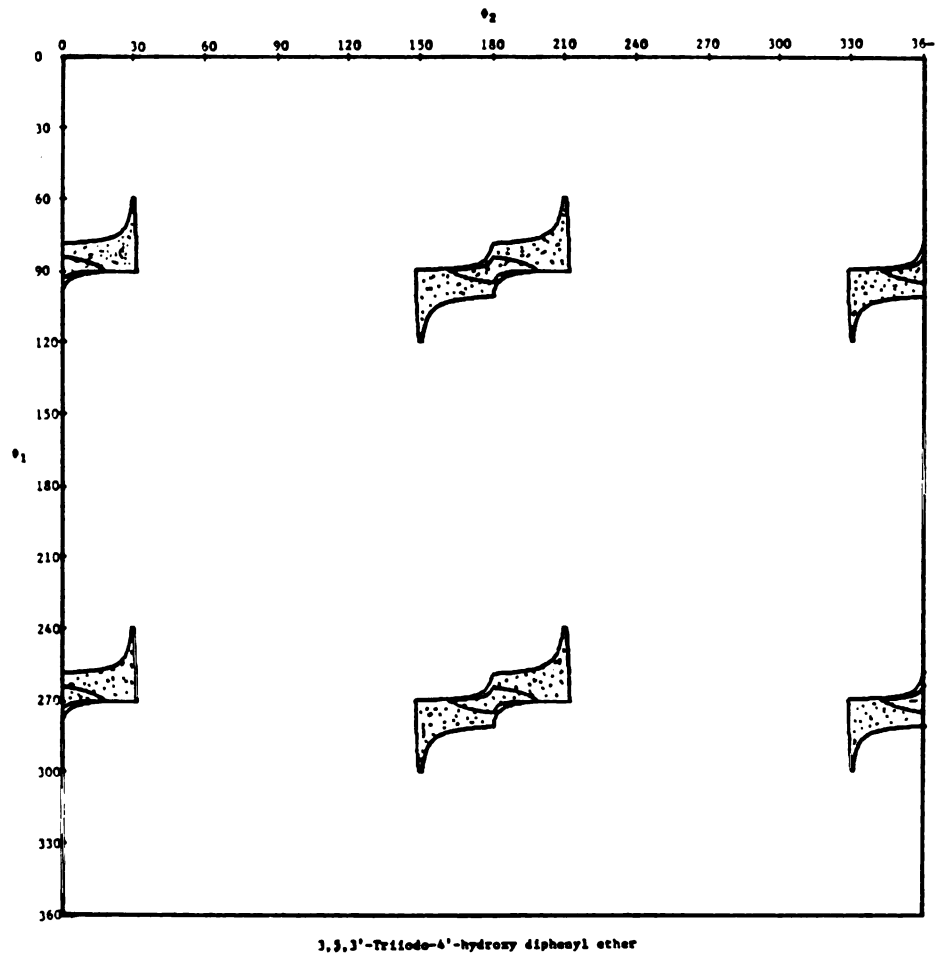

ϕ_2
0
30
60
90
120
150
180
210
240
270
300
330
360
ϕ_1
3,5,3'-Triiodo-4'-hydroxy diphenyl ether

Figure 4-4c.

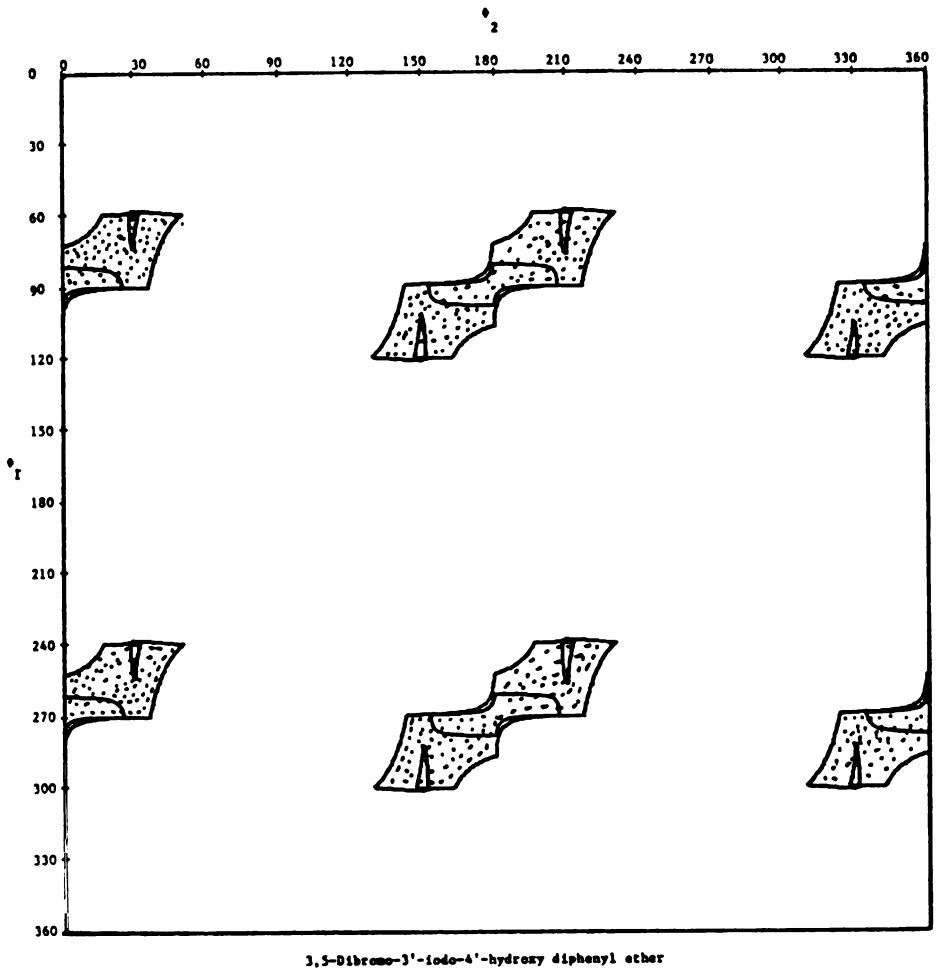

φ2
0
30
60
90
120
150
180
210
240
270
300
330
360
φ1
3,5-Dibromo-3'-iodo-4'-hydroxy diphenyl ether

Figure 4-4d.

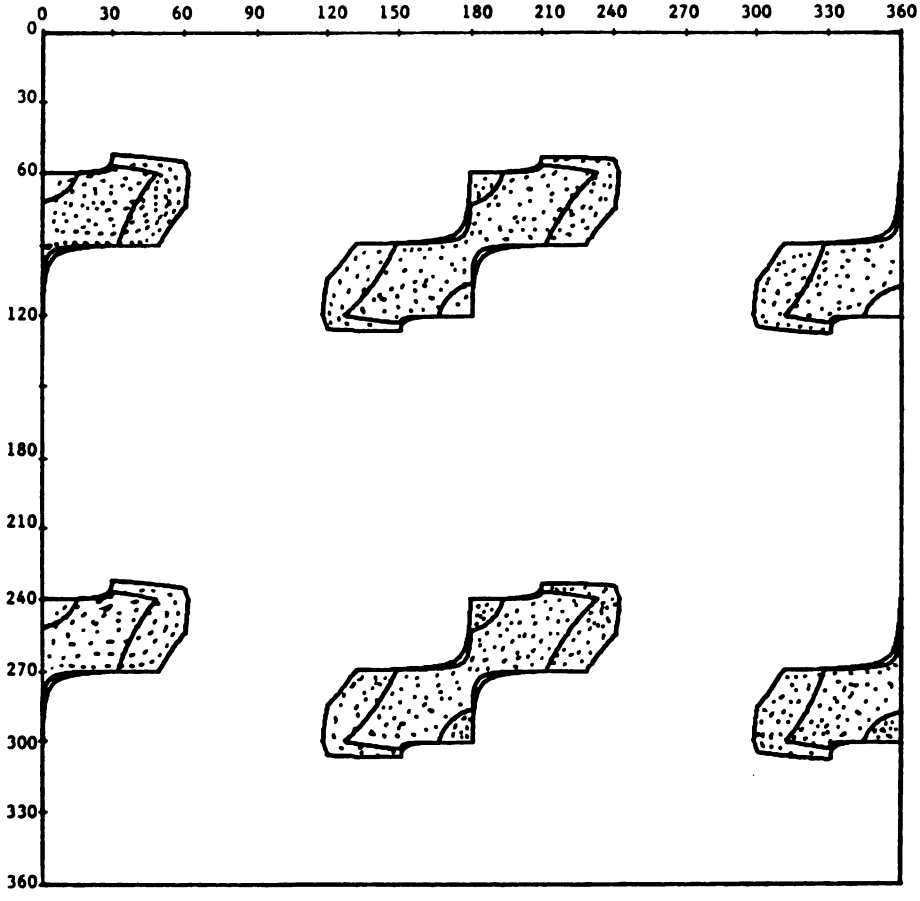

0
30
60
90
120
150
180
210
240
270
300
330
360
3,5-Dichloro-3'-iodo-4'-hydroxy diphenyl ether

Figure 4-4e.

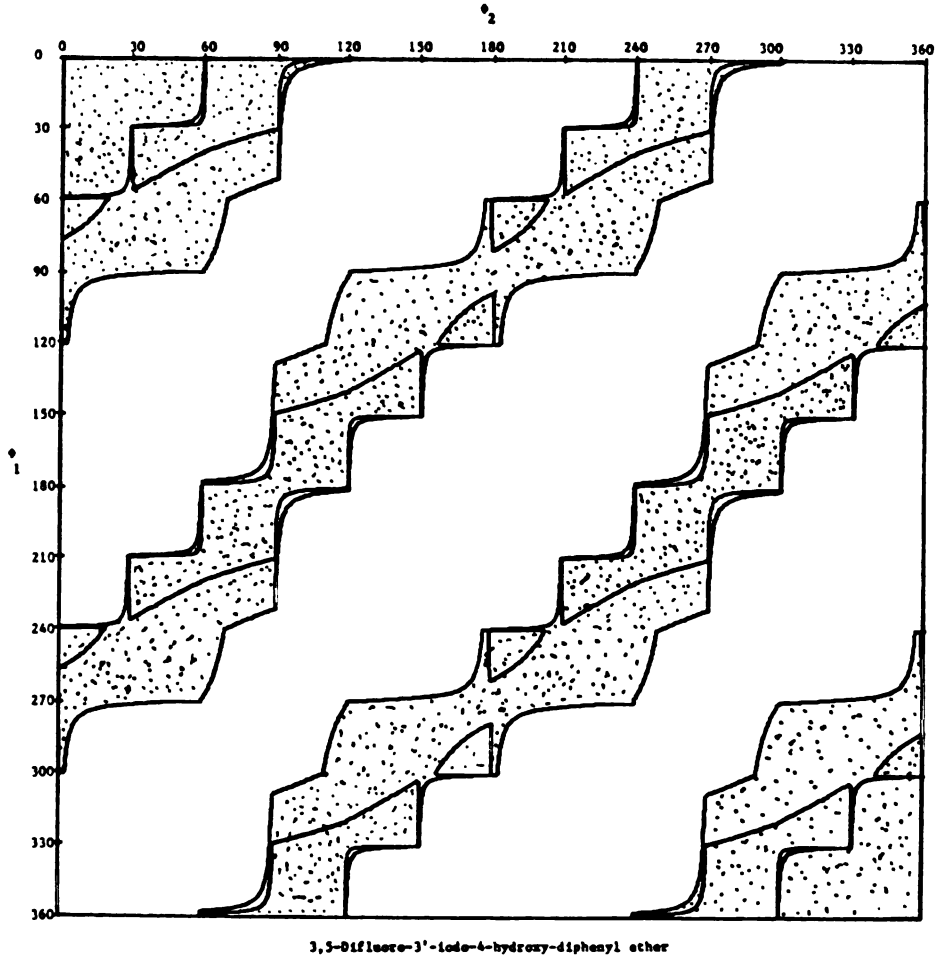

φ2
0
30
60
90
120
150
180
210
240
270
300
330
360
φ1
3,5-Difluoro-3'-iodo-4-hydroxy-diphenyl ether

Figure 4-4f.

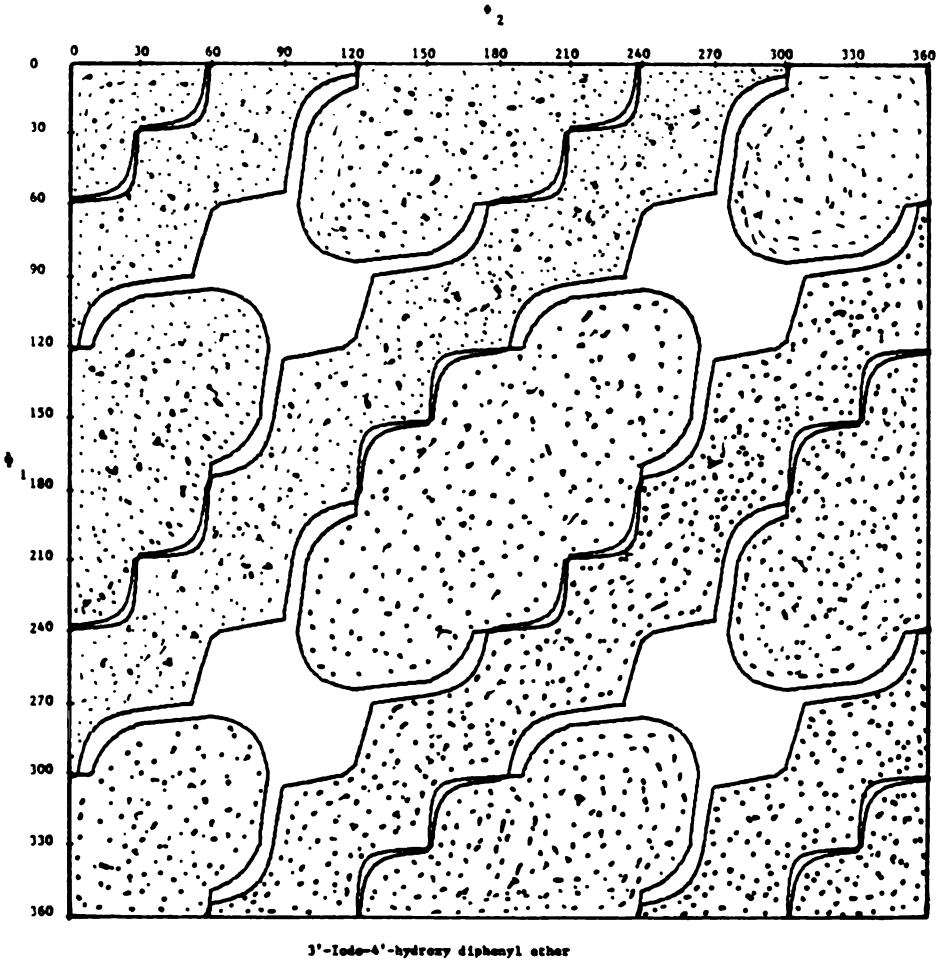

φ2
0
30
60
90
120
150
180
210
240
270
300
330
360
φ1
3'-Iodo-4'-hydroxy diphenyl ether

Figure 4-4g.

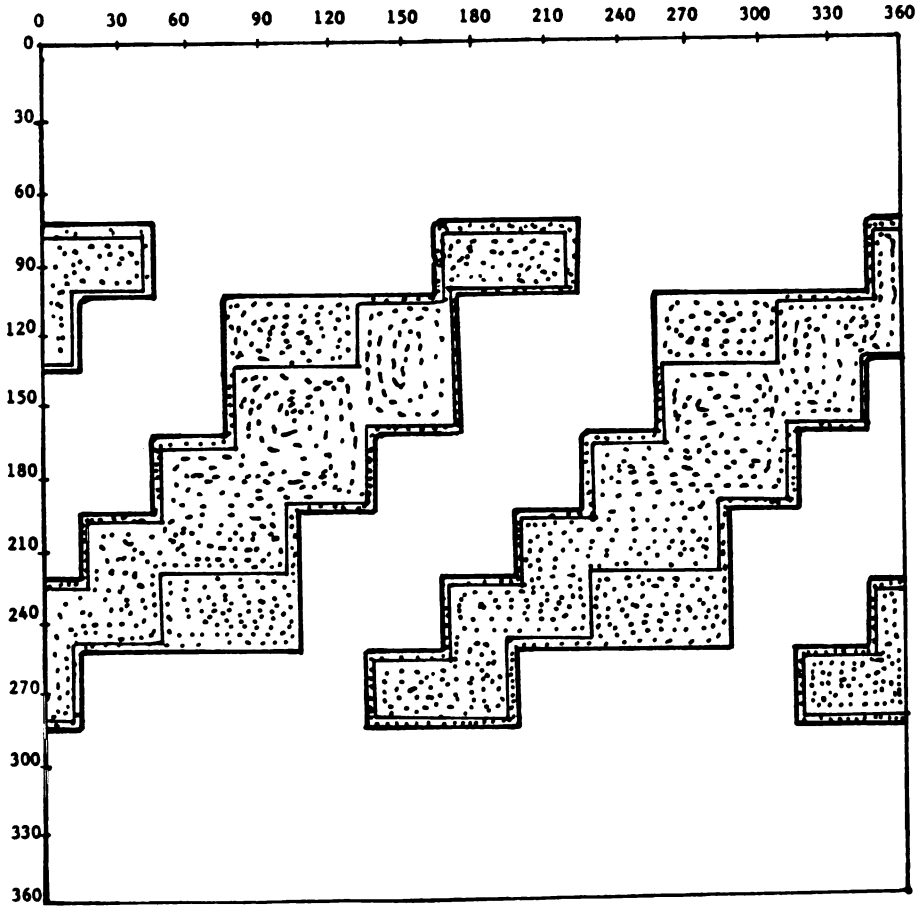

0
30
60
90
120
150
180
210
240
270
300
330
360
3,3'-Diiodo-4'-hydroxy diphenyl ether

Figure 4-4h.

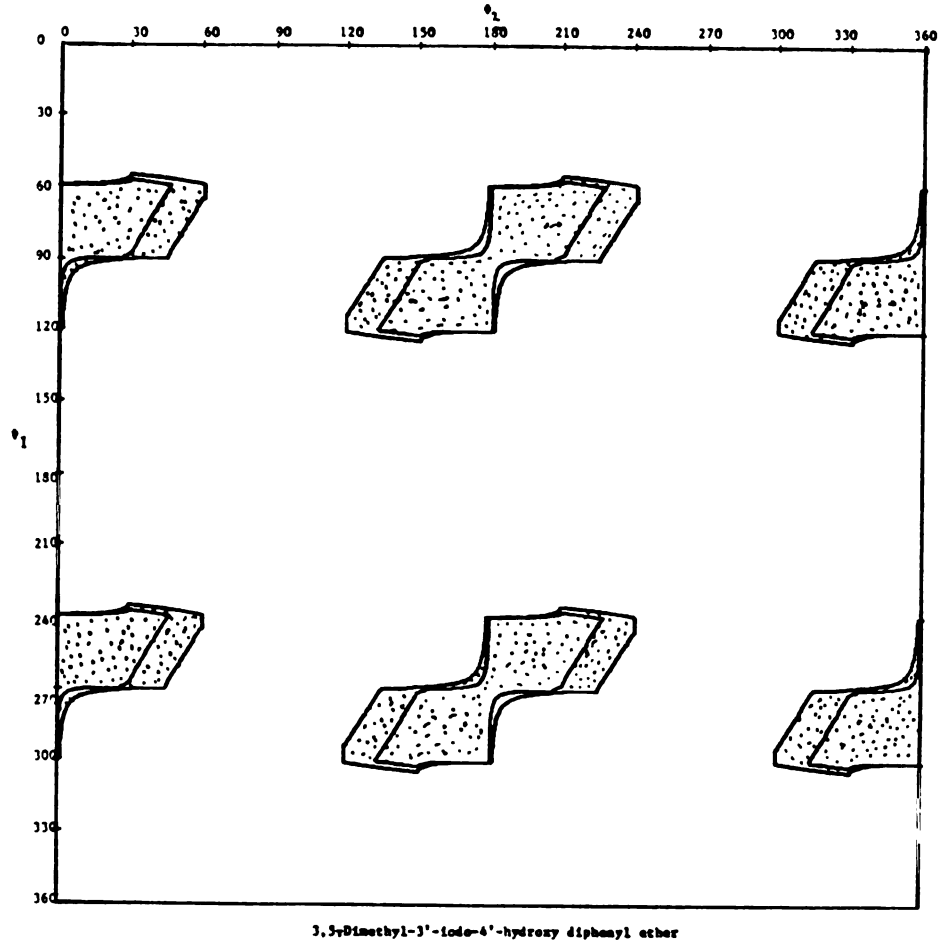

ϕ_2
0
30
60
90
120
150
180
210
240
270
300
330
360
ϕ_1
3,5-Dimethyl-3'-iodo-4'-hydroxy diphenyl ether

Figure 4-4i.

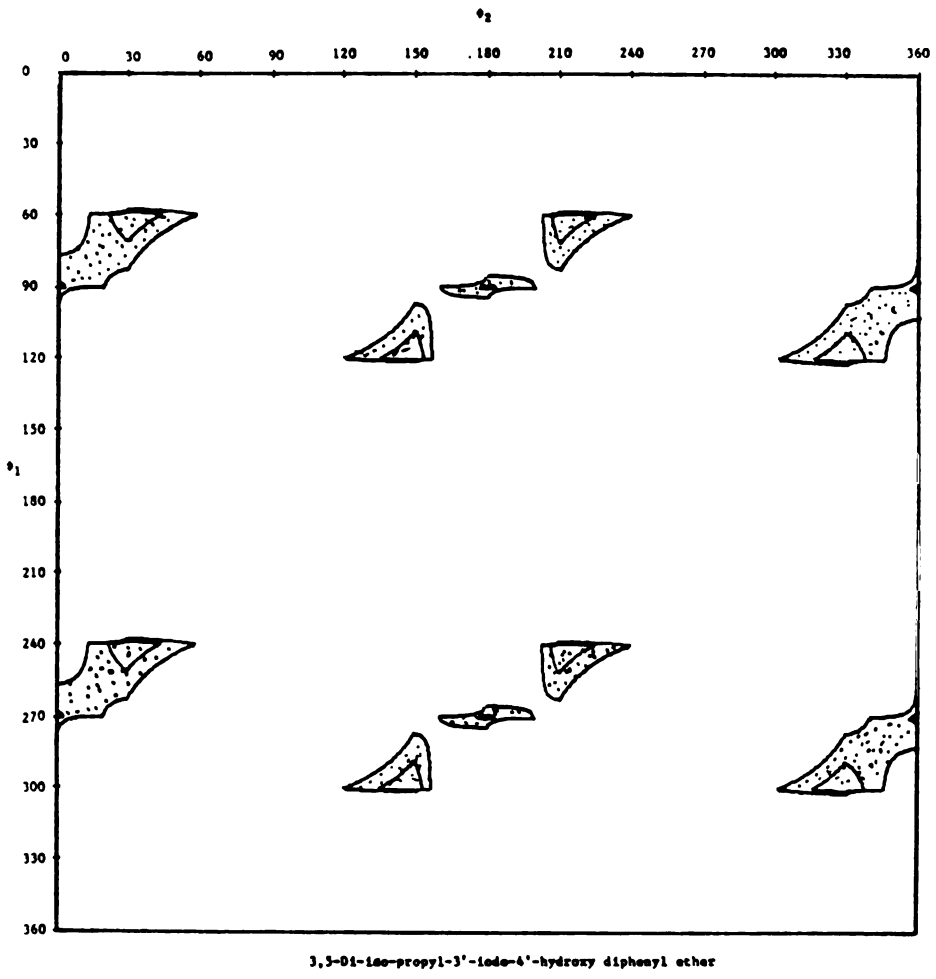

ϕ_2
0
30
60
90
120
150
180
210
240
270
300
330
360
ϕ_1
3,5-Di-iso-propyl-3'-iodo-4'-hydroxy diphenyl ether

Figure 4-4j.

Figure 4-4.

Φ_1,Φ_2 Potential energy maps for diphenyl ether analogs (figure 4-3: $R_1=R_2,=R_5,=H$, $R_3,=I$, $R_4,=OH$). Energy contours are 1 and 2 kcal/mol and the dotted areas represent energies less than 2 kcal/mol above the minimum energy. The substituents in the 3 and 5 positions are:

(a) $R_3 = R_5 = I$; $Z = S$.

(b) $R_3 = R_5 = I$; $Z = CH_2$.

(c) $R_3 = R_5 = I$; $Z = O$.

(d) $R_3 = R_5 = Br$; $Z = O$.

(e) $R_3 = R_5 = Cl$; $Z = O$.

(f) $R_3 = R_5 = F$; $Z = O$.

(g) $R_3 = R_5 = H$; $Z = O$.

(h) $R_3 = I$; $R_5 = H$; $Z = O$, $\Phi_1 = 180$ places the outer ring nearer $R_5 = H$ than $R_3 = I$.

(i) $R_3 = R_5 = CH_3$; $Z = O$.

(j) $R_3 = R_5 = iPr$; $Z = O$.

$Z=CH_2$ and S than for Z=O. It is also interesting to note here that the allowed ranges of Φ_1 are similar for the three cases. This is in agreement with the idea that steric interaction between the outer ring and the 3,5-iodine atoms is stronger than the resonance of the bridging atom with the inner ring.

(d) For Z=O and large 3,5 substituents (I_2, Br_2, and Cl_2, figures 4-4c, d and e, respectively; table 4-5) there are four global minima at positions in which the phenyl rings are mutually perpendicular, whereas for smaller groups (Me_2, F_2 and H_2 figures 4-2i, f and g, respectively; table 4-5) there are four global minima positioned on both sides of the mutually perpendicular rings conformation. The reason that the 3,5-diisopropyl compound (figure 4-4j; table 4-5) show four global minima inspite of the large size of these groups is that each isopropyl group can rotate about the bond connecting it to the inner ring in a concerted manner with the rotation of the outer ring (an effect which we investigated) keeping its methine hydrogen rather than its bulky methyl groups pointed "towards" the outer ring. Thus, the outer ring "feels" more or less the same repulsion from the 3,5-diisopropyl groups as from the 3,5-dimethyl groups.

(e) Finally, the maps and table 4-5 show that for every global minimum, there is a local minimum (whose calculated energy differs by not more than 0.1 kcal/mol from the energy of the global minimum) obtained by changing Φ_2 by 180°. The global and local minima correspond to the 3'-iodine held proximally or distally.

To further examine the relative roles of the resonance and steric effects, the minimum energy geometries were determined for analogs

Table 4-5 Calculated Minimum Energy Geometries, and Calculated and Observed Rotational Energy Barriers for Various Diphenyl Ethers and Related Compounds.[a]

No.	R_4'	Z	R_3	R_5	Calcd. Minimum Energy Geometry: Global Φ_1	Global Φ_2	Local Φ_1	Local Φ_2	Rotational Energy Barrier[b]: Calculated ($\Delta E^{\ddagger}$) Path 1	Calculated ($\Delta E^{\ddagger}$) Path 2	Observed ($\Delta G^{\ddagger}$)
1	OH	CH_2	I	I	90	0	90	180	4.74	58.34	--
					270	0	270	180			
2	OH	CO	I	I	90	10	-	-	2.73	--	--
3	OH	S	I	I	90	180	90	0	2.04	--	--
					270	180	270	0			
4	OH	SO	I	I	90	10	-	-	0.89	--	--
5	OH	NH	I	I	90	0	-	-	13.41	--	--
6	OH	O	I	I	90	0	90	180	10.64	54.41	7.9[c]
					270	0	270	180			
7	CO_2Et	O	I	I	-	-	-	-	11.15	--	9.3[d]
8	OH	O	Br	Br	90	180	90	0	5.78	32.72	--
					270	180	270	0			
9	OH	O	Cl	Cl	90	0	90	180	2.47	14.55	--
					270	0	270	180			

Table 4-5 Continued

10	OH	O	F	F	30	30	30	210	2.29	2.29	--
					150	330	150	150			
					210	30	210	210			
					330	330	330	150			
11	OH	O	NO_2	NO_2	50	60			18.43	--	--
12	OH	O	iC_3H_7	iC_3H_7	60	210	60	30	6.68	41.79	$<10^e$
					120	150	120	330			
					240	210	240	30			
					300	150	300	330			
13	OH	O	CH_3	CH_3	60	210	60	30	6.06	41.89	--
					120	150	120	330			
					240	210	240	30			
					300	150	300	330			
14	OH	O	H	H	30	210	30	30	1.19	1.19	--
					150	150	150	330			
					210	210	210	30			
					330	150	330	330			
15	OH	O	I	H	180	90	none	none	2.94	--	--
					180	270					

Table 4-5. Continued

[a]Figure 4-3: $R_1 = R_2' = R_5' = H$, $R_3' = I$, $R_4' = OH$.

[b]Kcal/mole.

[c]$R_1 = CH_3$, $R_3' = I$, $R_5' = H$ (Reference 65).

[d]$R_1 = CH_2CH(NHAC)COOEt$ (Reference 65).

[e]$R_1 = R_3' = R_5' = H$, $R_4' = CO_2CH_3$ (Reference 64).

of 3,5,3'-triiodo-4'-hydroxy diphenyl ether in which Z=CO, SO and NH. These three compounds showed minimum energy geometries of (Φ_1,Φ_2) = (90,10), (90,10) and (90,0) respectively (table 4-5), indicating that the steric effect of the bulky inner ring iodine atoms is the main determinant of conformation. Finally, replacing the 3,5-iodine atoms by 3,5-nitro groups (Z=0) causes the minimum energy geometry to change from (Φ_1,Φ_2)=(90,0) to (50,60) (table 4-5). This change can be accounted for by: (a) The smaller steric effects of the nitro groups in a direction perpendicular to the inner ring. This is due to the coplanarity of the nitro groups with the inner ring and the small Van der Waals radii of nitrogen and oxygen compared to iodine. (b) The delocalization of the π electron system onto the nitro groups enhances the conjugation of the lone pair orbital of the inter-ring oxygen with the inner ring π system, and tends to orient the two rings in a coplanar conformation.

Table 4-6 shows a comparison between the X-ray crystallographically observed geometries and theoretically determined minimum energy conformations. The agreement is as good as can be expected considering the fact that the calculations were done on isolated molecules whereas in the crystal each molecule experiences the force field generated by all other molecules in an essentially infinite lattice.

Another feature that can be elucidated from the potential energy surfaces is the path of internal rotation. The path of internal rotation is defined as the set of points in (Φ_1,Φ_2) space which define the route of minimum energy between the various local and global minima on the surface. As was mentioned above, 3,5,3'-triiodo-4'-hydroxy diphenyl ether has two local and two global minima. The path of internal rotation

Table 4-6. Calculated and Observed Minimum Energy Geometries of Various Diphenyl Ethers.[a]

				Minimum Energy Geometry			
				Calculated[a]		Observed[b]	
No.	R_3	R_5	Z	ϕ_1	ϕ_2	ϕ_1	ϕ_2
1	I	I	CH_2	90	0	--	--
2	I	I	CO	90	10	--	--
3	I	I	S	90	0	--	--
4	I	I	SO	90	10	--	--
5	I	I	NH	90	0	89	27
6	I	I	O	90	0	64-90	4-34
7	Br	Br	O	90	0	--	--
8	Cl	Cl	O	90	0	--	--
9	F	F	O	30	30	--	--
10	NO_2	NO_2	O	50	60	--	--
11	iPr	iPr	O	60	30	77.2 79.5	5.7 2.1
12	Me	Me	O	60	30	--	--
13	H	H	O	30	30	37	67

Table 4-6. Continued

[a]Figure 4-3: $R_1=R_2,=R_5=H$, $R_3,=I$, $R_4,=OH$.

[b]observed values are obtained from table 4-3.

for this compound is depicted in figure 4-5. This figure shows that there are two paths of rotation (a) Path 1: In this path interconversion occurs between the distal and proximal conformations on the same surface of the inner ring. Compounds 3 to 9 (table 4-3) have the features that $R_3 = R_5 = I$ and $Z = 0$. The crystallographically observed (Φ_1, Φ_2) values of these seven compounds may be qualitatively interpreted as points that lie on the path of internal rotation. These seven points are also depicted in figure 4-5. The only compound that seems to be misplaced is 4. (b) Path 2: In this path, the distal (proximal) conformation on one surface of the inner ring is converted to a proximal (distal) conformation on the opposite surface of the inner ring.

The energy barrier to internal rotation is defined as the energy difference between the global (or local minimum) and the energy at the highest energy point along the path of rotation. Table 4-5 lists the calculated barriers and compares them to experimentally available numbers. The calculated barriers shows some interesting features:
(a) The highest path 1 barrier is 18.34 kcal/mol for 3,5-dinitro-3'-iodo-4'-hydroxy diphenyl ether (compound 11). (b) Replacing the 3,5-diiodo atoms (Z=0) by dibromo, dichloro, difluoro and dihydrogen (compounds 6, 8, 9, 10 and 14) causes a progressive decrease in the energy barrier of path 1. This can be explained on the basis of the progressive decrease in the size of the 3,5-substituents which leads to less steric constraint on rotation. (c) Replacing Z=0 by CH_2, CO, S and SO (compounds 5, 1, 2, 3 and 4) lowers the energy barrier of path 1. There are two factors which might contribute to this (i) r_1 and r_2 are larger for Z = CH_2, CO, S and SO than for Z = 0,

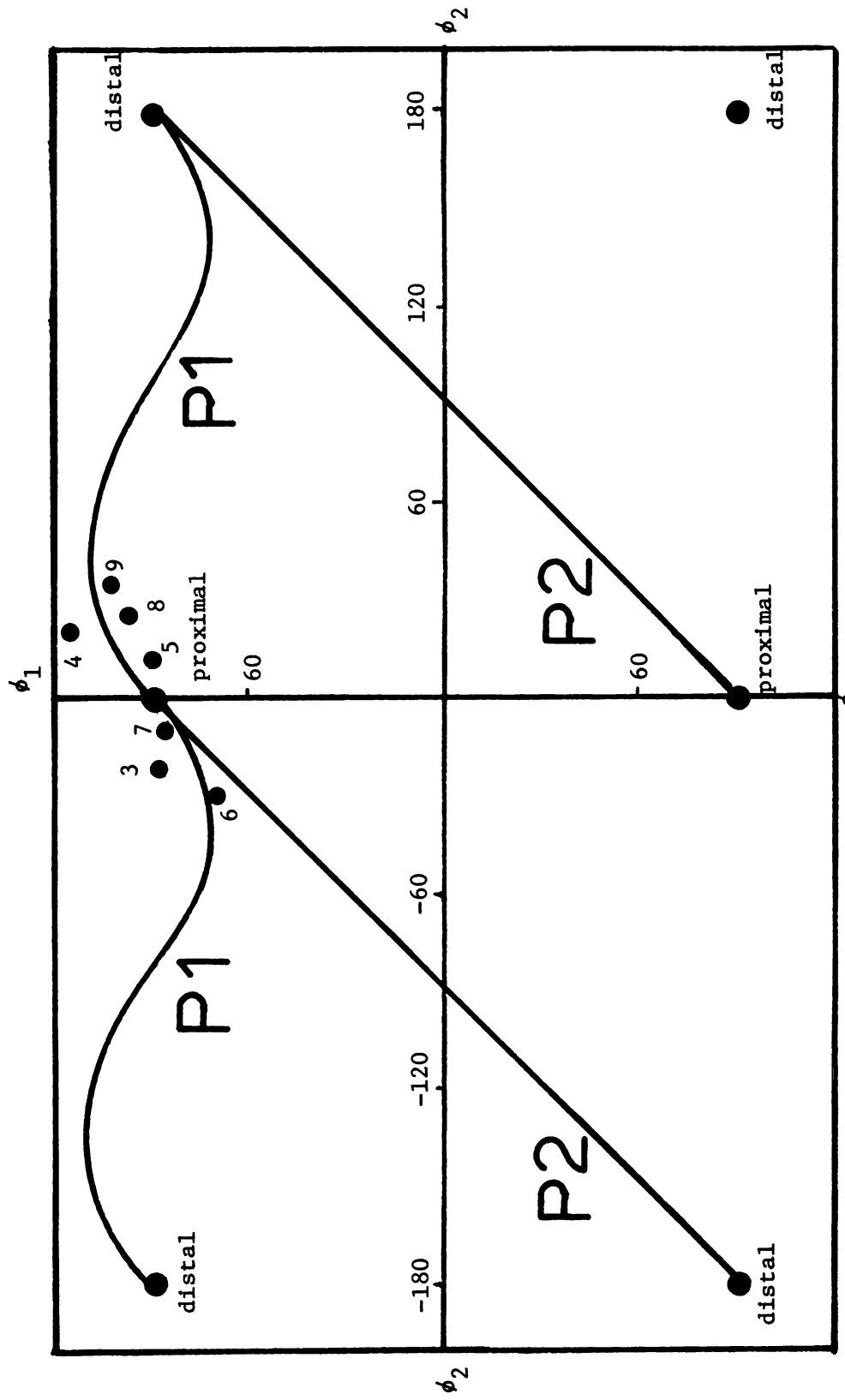


Figure 4-5. Rotation paths of 3,5-diiodo diphenyl ethers. P1 = path 1 (allowed), P2 = path 2 (forbidden). The numbered points give the experimentally observed geometries. The numbers correspond to entries in table 4-3.

thus the steric constraint to rotation arising from the 3,5 substituents is less in the first four cases than in the last one, and (ii) the overlap of the lone pair orbitals on the oxygen atom (Z=O) with the π electrons of the outer ring is larger in magnitude than in the case of $Z=CH_2$ (no lone pair orbitals available) and S, CO, S and SO (larger r_1 and r_2) and hence bonds connecting the Z atom to the outer ring have more double bond character in the first case than in the last four. (d) The barriers of path 1 and path 2 in 3,5-iPr_2 (compound 12) are almost the same as those of the 3,5-Me_2 (compound 13) in spite of the much larger bulk of the former. This can be explained on the basis that each isopropyl group can rotate about the bond connecting it to the inner ring in a concerted manner with the rotation of the outer ring (an effect which we investigated) keeping its methine hydrogen atom rather than the bulky methyl groups pointed "towards" the outer ring. Thus, the outer ring "feels" more or less the same repulsion from the 3,5-iPr_2 groups as from the 3,5-Me_2 groups. (e) Finally, at the temperature of the biological system, rotation along path 2 for Z=O, 3,5-diiodo, dibromo, dimethyl and diisopropyl compounds is highly unlikely due to their high energy. On the other hand such rotation is expected to occur frequently in the 3,5-dichloro, difluoro and dihydrogen compounds.

The validity of the "distal active conformation" has been questioned by the Camermans (66, 68, 69) partly on the basis of the observation that in their crystallizing medium, all thyroid hormone analogs that were studied crystallized in the 5'-proximal conformation and partly on their finding that Extended Hückel molecular orbital method predicts the proximal conformation to be more stable than the distal by 132 kcal/mol. Our calculations using CNDO/2 method (33 and this study) indicate that

the "proximal" and the "distal" conformations differ by not more than 0.1 kcal/mol in all cases studied. Based on this small energy difference, the ratio of proximal and distal conformer populations is about 1.2. Cody, et al. (49, 51, 70) found that in their crystallzing media , all thyroid hormones and analogs studied crystallized in the "distal" conformation. This fact, taken in conjunction with Camerman's observation of the "proximal" conformation (53) supports our observation that the two conformations are close in energy so that small modifications of the crystallizing medium favors one conformation or another. Recently, the low temperature NMR finding of Emmett and Pepper (65) that the population of the "distal" conformer is 0.5-0.6 (and hence, the ratio of the populations of the proximal/distal is 0.7-1.0) for 1-methyl-3,5,3'-triiodo-4'-hydroxy diphenyl ether again attests to the almost equal energies of the two conformers.

The values of ΔG_{lock} for several compounds are listed in table 4-7. The features of the data can be summarized as follows (a) Comparing compounds 1, 2 and 3 shows that for $R_3 = R_5 = I$, it is easier to lock the oxygen bridged analog than the sulfur and methylene bridged analogs. (b) For Z=0, as the size of the 3,5-halogen increases (F, Cl, Br, I) it becomes easier to lock the compound into a mutually perpendicular conformation. (c) ΔG_{lock} for the 3,5-dimethyl and 3,5-diisopropyl analogs are almost equal. We like to note here that since the ΔE_{lock} is negative in all cases, the large positive ΔG_{lock} is strictly due to a large negative ΔS_{lock}. The negative entropy arises from the fact that when the entire population of molecules is locked into the mutually perpendicular conformation, a large vibrational entropy for the vibration

Table 4-7 Calculated ΔG_{lock} Values for Various Thyroid Hormone Analogs[a]

No.	R_3	R_5	Z	Calculated ΔG_{lock}[b]
1	I	I	CH_2	4.77
2	I	I	S	4.71
3	I	I	O	4.29
4	Br	Br	O	4.61
5	Cl	Cl	O	4.90
6	F	F	O	6.57
7	iPr	iPr	O	5.48
8	Me	Me	O	5.50
9	H	H	O	5.96
10	I	H	O	5.91

[a]Figure 4-3: R_1=R_2,=R_5,=H, R_3,=I, R_4,= OH.

[b]Kcal/mole.

along the Φ_1 and Φ_2 coordinates has to be sacrificed. In order to use these ΔG_{lock} values we have to elucidate these contributions of the various functional groups ($\Delta_1 G_{lock}$). This is achieved by using compound 3 in table 4-7 as a reference compound and substracting its ΔG_{lock} value from all other values in the table, thus, generating the contribution of $Z=CH_2$ and S relative to O and of various 3,5-substituents relative to 3,5-iodines. These results are shown in the third column of table 4-8. They are compared with experimental $\Delta_1 G(X)$ (determined by using equation 3-30, 3-33 and the binding data in intact rat liver nuclei as shown in table 3-2). The last column in table 4-8 is the contribution of these functional groups to the logarithm of the biological activity (1,2) as defined by:

$$\Delta \ln BA(X) = \ln BA(AX) - \ln BA(AH) \qquad (4\text{-}21)$$

where AX and AH are two compounds that are identical in every respect except that one of them has group X in a certain position and the other has a hydrogen atom in the same position. Examining table 4-8 shows that there is a fair correlation between $\Delta_1 G_{lock}$ and Δ ln BA. The data of columns 3 and 4 in table 4-8 were submitted to linear regression analysis and were found to obey the equation:

$$\Delta_1 G(X) = 2.79 \; \Delta_1 G_{lock}(X) - 0.62$$

$$r = .89, \quad n = 7$$

Table 4-8 Calculated Values of $\Delta_1 G_{lock}$ (X) and Observed Values of $\Delta_1 G(X)$ and Δ ln BA(X) for Various X Groups Relative to 3,5-Diiodo and Inter-ring Oxygen.[a]

No.	X	$\Delta_1 G_{lock}X)$[b]	$\Delta_1 G(X)$[b]	Δ ln BA(X)[b]
1	$Z=CH_2$	+0.48	-0.56	(-1.63)-(-4.61)
			+0.97	
2	Z=S	+0.41	-0.90	(-0.91)-(-1.21)
			0.00	
3	Z=O	0.00	0.00	0.00
4	$3,5-Br_2$	+0.31	+0.65	(-1.15)-(-2.64)
5	$3,5-Cl_2$	+0.61	----	(-4.61)-(-1.90)
6	$3,5-F_2$	+2.28	----	----
7	$3,5-(iPr)_2$	+1.19	+3.83	----
8	$3,5-(Me)_2$	+1.21	+3.08	(-3.22)-(-5.30)
			+3.02	
			+2.54	
9	$3,5-H_2$	+1.67	+3.26	<-11.2
10	3-I-5-H	+1.61	+2.97	<-11.2

a- Figure 4-3.
b- For determination of this value see text.

VII- COMMENTARY ON AVAILABLE METHODS IN DETERMINING BIOLOGICALLY ACTIVE CONFORMATIONS

A medicinal chemist, attempting to define the biologically active conformation would typically (i) synthesize a series of analogs locked in various conformations, (ii) screen them for biological activity, and (iii) infer the biologically active conformation from the conformation of the most active analog. Currently there are several experimental and theoretical methods for studying conformations of biologically active small molecules. Of these, the most widely used are X-ray crystallography, NMR spectroscopy, and molecular orbital theory. Other methods, such as dipole moment measurement and conformational energy calculations using empirical potential energy functions have also been used. We will give a brief commentary on each of these approaches.

Each molecule has three classes of degrees of freedom: translations, rotations (of the molecule as a whole) and vibrations. The subclass of vibrations known as internal rotations are usually the ones that are most drastically affected by conformation locking. Thus the tantamount effect of conformation locking is to confine one or more of the internal rotational degrees of freedom to limited regions of conformation space. In almost all cases, such conformation locking is accomplished either (i) by cyclization of an otherwise freely rotating moiety of a molecule, or (ii) by introduction of a large functional group which sterically constrains the molecule into a smaller region of conformation space. Thus, locking a molecule into a certain conformation can only be accomplished by the introduction of an extraneous feature. Such features by themselves most likely influence the biological activity directly

in addition to influencing it via conformation locking. There is absolutely no guarantee that the inferred biologically active conformation would be the same in the absence of the extraneous feature. In spite of this shortcoming, conformation locking is the only presently available direct method of probing biologically active conformation. Thus, it is important to accept this approach with the above mentioned precaution in mind.

A structure determined by X-ray crystallography corresponds to only one point in conformation space. Including the thermal ellipsoids increases the region of conformation space defined by X-ray crystallography. Thus, the X-ray structure may be a meaningful estimate of biologically active geometries for compounds having very restricted internal rotations (such as steroids that have no "loose" side chains). This technique however, has serious pitfalls for molecules that have internal rotational degrees of freedom whose minima are shallow so that large regions of conformation space are populated at the temperature of the biological system. Inferring a biologically active conformation from the crystal structure of such compounds may be incorrect particularly since forces exerted by neighboring molecules in the crystal can play significant roles in determining the conformation of segments that have shallow minima. Typical cases in point are the alanyl side chains and diphenyl ether conformation of thyroid hormone, the ethyl amine side chain of histamine, etc. Under similar circumstances NMR spectroscopy can pick up contributions from all of conformation space. Thus NMR spectroscopy may be useful in defining the relative probabilities of different regions of conformation space. It is not capable, however, of defining the probability of a single geometry. Finally, theoretical

methods have the advantage of giving the energy (and hence, the probability) of each point in conformation space. Although the actual numerical value of the energies are not necessarily exact, the trends have frequently been shown to be correct. The most serious weakness of theoretical methods is the neglect of perturbations by the environment (solvent, other solutes, the adjacent groups on the receptor, etc.) which most certainly would modulate and distort the conformational profiles calculated for isolated molecule.

To summarize and conclude: Conformation locking, X-ray crystallography, NMR spectroscopy and molecular orbital calculations are among the most used tools in studying biologically active conformations. Each of these techniques has some advantages as well as shortcomings.

Chapter 5

Binding of Thyroid Hormones and Analogs to Prealbumin

I- INTRODUCTION

There are three proteins in human blood whose interaction with thyroid hormones and their analogs have been studied. These are thyroxine binding globulin (TBG), thyroxine binding prealbumin (TBPA), and albumin. The affinity constants of these proteins to thyroxine are in the order: TBG (10^9) > TBPA (10^7) > albumin (10^5). Their abundance in human blood is in the reverse order. All three proteins have been isolated and purified.

Recently, prealbumin has been crystallized and its three dimensional structure determined in the solid state (71,72). The highlights of the structure can be summarized as follows:

1- Prealbumin is a tetramer with four identical subunits.

2- The four subunits can be oriented in cartesian space so that each of the three cartesian axes is a C_2 symmetry axis. Thus prealbumin molecule belongs to the D_2 point group.

3- A central channel runs through the molecule and defines two identical binding sites for thyroxine.

4- The amino acid residues extending into the binding channel can be classified as polar-un-ionized (serine and threonine), polar-ionized (lysine, glutamate, histidine and arginine) and non polar (alanine, valine, leucine and methionine).

5- The positions of the four iodine atoms of bound thyroxine have been determined with some uncertainty.

These studies (and similar ones that have recently emerged on other small molecule-macromolecule systems) provided the opportunity to understand the binding of biologically active ligands to macromolecules

in terms of topographical complimentarity between the two molecules. Thus they provided us with an incentive to study the binding affinities of thyroid hormone analogs whose structures are systematically varied and an opportunity to interpret the variation in these affinities in terms of the physical chemical nature of the interaction between the ligand and its complementary surface.

The binding of thyroid hormones and a few of their analogs to prealbumin has already been investigated by some authors. The structure-binding affinity picture is far from complete. We will refer to these studies in a later section, confining our attention to experimental data that have a sound physical chemical basis.

II- EXPERIMENTAL SECTION

a- Materials

Prealbumin was obtained from Behringwerke AG, Marburg, Germany with an immunological purity of 98% and was used without further purification. Thyroid hormones and analogs were obtained from the collection of Dr. Jorgensen. The catalog numbers for each hormone and analog is shown in table 5-1. The buffer components: monobasic sodium phosphate, sodium phosphate, tris, sodium chloride and EDTA were purchased from Baker. High specific activity ^{125}I-T4 was purchased from Abbott and New England Nuclear. The latter was used in most experiments due to its higher purity (73). The dialysis tubing was purchased from Thomas. Iobeads were purchased from Technicon.

b- The Buffers: Composition and pH Adjustment

The components, pH, and ionic strengths of the buffers employed are shown in table 5-2. The buffers were prepared and their pH adjusted as

Table 5-1 Sources of the Thyroid Hormones and Analogs Studied

Compound	Catalog Number[a]	Source and Lot
L-T_4		Smith Kline and Frence (BS-1171)
D-T_4		National Biochemical Corp.
3,5,3'-L-T_3		National Biochemical Corp.
3,5,3'-D-T_3		Sigma/103C-2810
3,3',5'-L-T_3		Hennig-Bulers
3,5-L-T_2	133L	Sigma/103C-0190
3,3'-L-T_2	107L	PB-2019b
3',5'-DL-T_2	111DL	EJ-II-30a
3-L-T_1	106L	PB-12726
3'-DL-T_1		I-135
L-thyronine		Sigma, #1626
triac	157	SKF #5013
tetraform	162	W1489 (lot 125)
tetrac	164	Cyclo(#13740) Lot-H-3491
tetraprop	166	W1524
tetrabut	167	W1620
thyroxamine	192	RPR-77-1

[a]Dr. Jorgensen's collection.

Table 5-2. Buffers Used in Studying the Binding of Thyroid Hormones and Analogs to Prealbumin.[a]

Buffer Concentrations	$NaH_2PO_4.H_2O$[b]	Na_2HPO_4[b]	Tris[b]	NaCl[b]	pH[c]	Ionic Strength[d]
0.02	3.80×10^{-3}	1.62×10^{-2}		0.15	7.40	0.194
0.05	9.50×10^{-3}	4.05×10^{-2}		0.10	7.40	0.211
0.10	1.90×10^{-2}	8.10×10^{-2}		0.10	7.40	0.322
0.05			.05	0.15	7.40	0.150
0.1125			.1125	.071	7.40	0.150
0.05	6.90×10^{-3}	4.31×10^{-2}		.139	8.00	0.275
0.10	1.38×10^{-2}	8.62×10^{-2}		.003	8.00	0.275
0.05			.05	.256	8.00	0.275
0.10			.10	.238	8.00	0.275
0.10			.10	.113	8.00	0.150

[a]All buffers contained 10^{-3} moles/liter EDTA.

[b]All concentrations are in moles/liter.

[c]All pH values are adjusted at 37°C.

[d]Calculated using $I = \sum C_i Z_i^2$ where C_i is the concentration of the ith species and Z_i is its charge. The concentrations of the different ionic species of phosphate and tris were calculated from the dissociation constants reported by N. E. Good, et al., Biochemistry, 5, 467 (1966).

follows: The required components were weighed and dissolved in the required amount of glass distilled water. The solution was brought to the temperature of the experiment and the pH was adjusted to the required value at that temperature by addition of either NaOH or HCl. This precaution was necessary since the pH could fluctuate significantly with temperature.

c- The Dialysis Units

The dialysis units were designed by Drs. Ralph C. Cavalieri and Ira D. Goldfine of the Veterans Administration Hospital, San Francisco, and were manufactured by Mr. Michelson (Research and Development Laboratory) of the same institution. Figure 5-1 shows the units and their dimensions. Each unit is made of two plexiglass blocks: F(free side) and B (bound side) each with the dimensions (15 cm x 7.5 cm x 2.5 cm). Six grooves are carved into each block. The dimensions of the grooves are: B grooves (4.5 cm x .6 cm x .3 cm = .8 cm^3) and F grooves (4.5 cm x 1.1 cm x .6 cm = 3 cm^3 volume). The grooves are carved in such a way that each groove on one side matches and complements a groove on the other side as accurately as possible. Thus, each pair of matching grooves forms a dialysis cell when the two blocks are brought in contact and separated by a dialysis membrane. Each (B and F) pair of blocks forms a unit of six dialysis cells. Each block also has a set of six holes through which the solutions are introduced into the cells. Each hole is provided with a teflon screw cap. Each dialysis unit is supplied with nuts and bolts to hold the two sides firmly together during the course of dialysis.

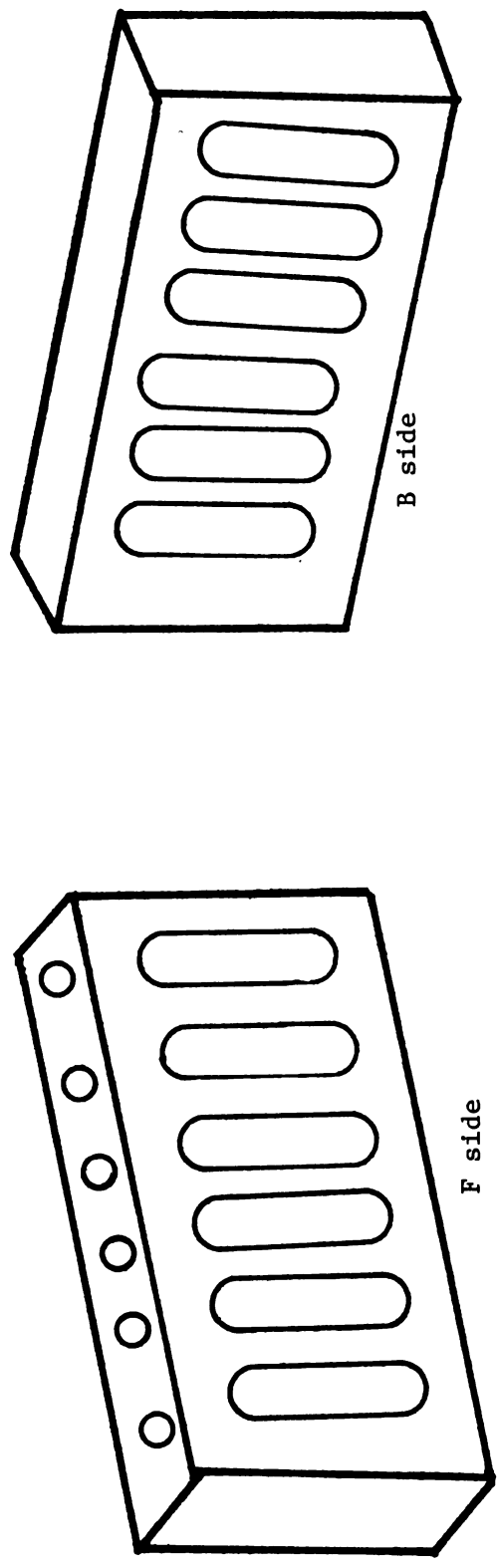


Figure 5-1. Dialysis unit used in equilibrium dialysis experiments.

d- Preparation and Cleaning of the Dialysis Units

Previously cleaned dialysis units are prepared for an experiment according to the following procedure:

1- Dialysis tubing is cut into 5.0-5.5 cm long pieces. Each piece is trimmed to a width of 1.2 - 1.5 cm by cutting along each of the two edges. The pieces are then soaked in buffer for 30 minutes.

2- Wearing vinyl gloves, the two halves of each piece are separated by placing the piece between the thumb and index finger and applying pressure in opposite directions. Each piece thus separates into two identical halves.

3- Each half is positioned on a groove on side B in such a way as to cover the groove entirely. When all six grooves of side B are covered, side F is placed in position and fastened with nuts.

4- In order to fill the cells, the unit is held upside down at 30° angle and 0.8 ml of solution B (to be defined in section e - "Experimental Design") pipetted into the B side. When the B sides of all cells in the unit are filled, the unit is turned right side up and 2.60 ml of pure buffer are pipetted into each side F of each cell of the unit. The twelve holes in each unit are sealed with the teflon screw caps and the unit is incubated at the required temperature for the required amount of time until equilibrium (determined in section f below) is reached.

5- When the required incubation time has elapsed, the screw caps are removed. 0.50 ml is withdrawn from each of the B and F sides and counted. Withdrawing solution from the B side is done with the cells in an upright position, and from the F side with the cells in an almost horizontal position.

At the end of each experiment, the dialysis units are cleaned as follows:

1- A simple apparatus which consists of a pasteur pipette connected to the house vacuum via two intervening traps is assembled.

2- The solution remaining in the B and F sides of each cell is removed (by the action of vacuum) by inserting the pipette of the apparatus above into the hole on the B side then simply puncturing the dialysis membrane to introduce the pipette into the F side. The solution collects in the first trap.

3- The liquid radioactive waste is disposed of and the traps and pipette rinsed with water.

4- The dialysis units are disassembled. The membranes are carefully removed (with the aid of Intrex applicators supplied by Gentec). The spent membranes and applicators are discarded.

5- The blocks and teflon screw caps are rinsed at least twice with tap water, once with 20% aqueous solution of NaOH, followed by several thorough rinsings with tap water and finally once with glass distilled water.

6- The blocks are set aside to dry.

e- Experimental Design

Two distinct experimental designs are required. In the first type of experiment, the Scatchard plots, affinity constants and number of binding sites of T4 in different buffer systems are determined. In the second type, the affinity constants of the analogs relative to T4 are determined.

For the first type of experiment, the following solutions are prepared:

1- Solution S1: 40 microliters of the stock solution of ^{125}I-T4 (New England Nuclear) is dissolved in 5.2 mls of buffer, 0.35 gm iobeads are mixed in and the suspension is allowed to stand for 30 minutes at room temperature. 4.0 mls of the supernate are removed and labelled S1.

2- Solution P : 0.9 - 1.0 mg of prealbumin in a polyethylene culture tube is dissolved in an exact volume of buffer to obtain a concentration of 5.00×10^{-7} mole/liter (using a molecular weight of 55000 for prealbumin). This dilution will give a prealbumin concentration of 5.00×10^{-8} mole/liter in the final B solution.

3- Solutions D1, D2, D3, and D4: These correspond to different dilutions of unlabelled T4 in buffer. They contain 10^{-11}, 10^{-10}, 10^{-9}, and 10^{-8} mole/ml respectively. These dilutions correspond to 1000 fold range in the concentration. It is not necessary to use all of them in a single experiment. One might desire to explore a narrower concentration range in a particular experiment.

4- Solution B: This solution contains buffer, unlabled T4, labelled T4 and prealbumin. The volumes used for each solution are shown in table 5-3. The total volume of each B solution is 1.00 ml. The concentration of T4 in each B solution is calculated from the concentration of the D1, D2, D3 and D4 solutions and the volumes used of each.

In the second type of experiment solution S1 and P are prepared as above. In addition, the following solutions are prepared:

Table 5-3 Components of Solution B Used in the Binding of L-Thyroxine to Prealbumin.

Buffer[a]	D_1[a]	D_2[a]	D_3[a]	D_4[a]	S_1[a]	P[a]
0.60	0.20				0.10	0.10
0.30	0.50				0.10	0.10
0.00	0.80				0.10	0.10
0.60		0.20			0.10	0.10
0.30		0.50			0.10	0.10
0.00		0.80			0.10	0.10
0.60			0.20		0.10	0.10
0.30			0.50		0.10	0.10
0.00			0.80		0.10	0.10
0.60				0.20	0.10	0.10
0.30				0.50	0.10	0.10
0.00				0.80	0.10	0.10

[a]Volume in ml.

1- Solution S2 : This is a solution of T4 in buffer. It contains 2.00×10^{-10} mole T4 per ml.

2- Solution A : This is a solution of the analog to be tested in buffer. Its concentration is so chosen as to cause an observable displacement of bound T4.

3- Solution B : This solution contains buffer, analog, unlabelled T4 labelled T4 and prealbumin. The volumes used of each is shown in table 5-4. The total volume of each B solution is 1.00 ml. The concentration of analog in each B solution is calculated from the concentration and volume of solution A used.

f- Equilibrium Time

This experiment was done to determine the time required for the system to reach equilibrium (no further flux of T4 across the membrane) at 37°C. The experimental design was identical to an experiment of the first type (see "Experimental Design") with the only difference that all the B solutions contained identical amounts of T4. The B and F solution were sampled at different time points. The results are shown in figure 5-2. It is clear that the system reaches equilibrium by 24 hours. Thus all experiments were incubated for 24 hours.

We note however that, rigorously this equilibrium time holds only for T4. We have assumed that the equilibrium time in the presence of other analogs is the same.

Table 5-4 Components of Solution B Used in Studying the Binding of Thyroid Hormone Analogs to Prealbumin.

Buffer[a]	A[a]	S_2[a]	S_1[a]	P[a]
0.60	0.10	0.10	0.10	0.10
0.50	0.20	0.10	0.10	0.10
0.40	0.30	0.10	0.10	0.10
0.30	0.40	0.10	0.10	0.10
0.20	0.50	0.10	0.10	0.10
0.10	0.60	0.10	0.10	0.10

[a]Volume in ml.

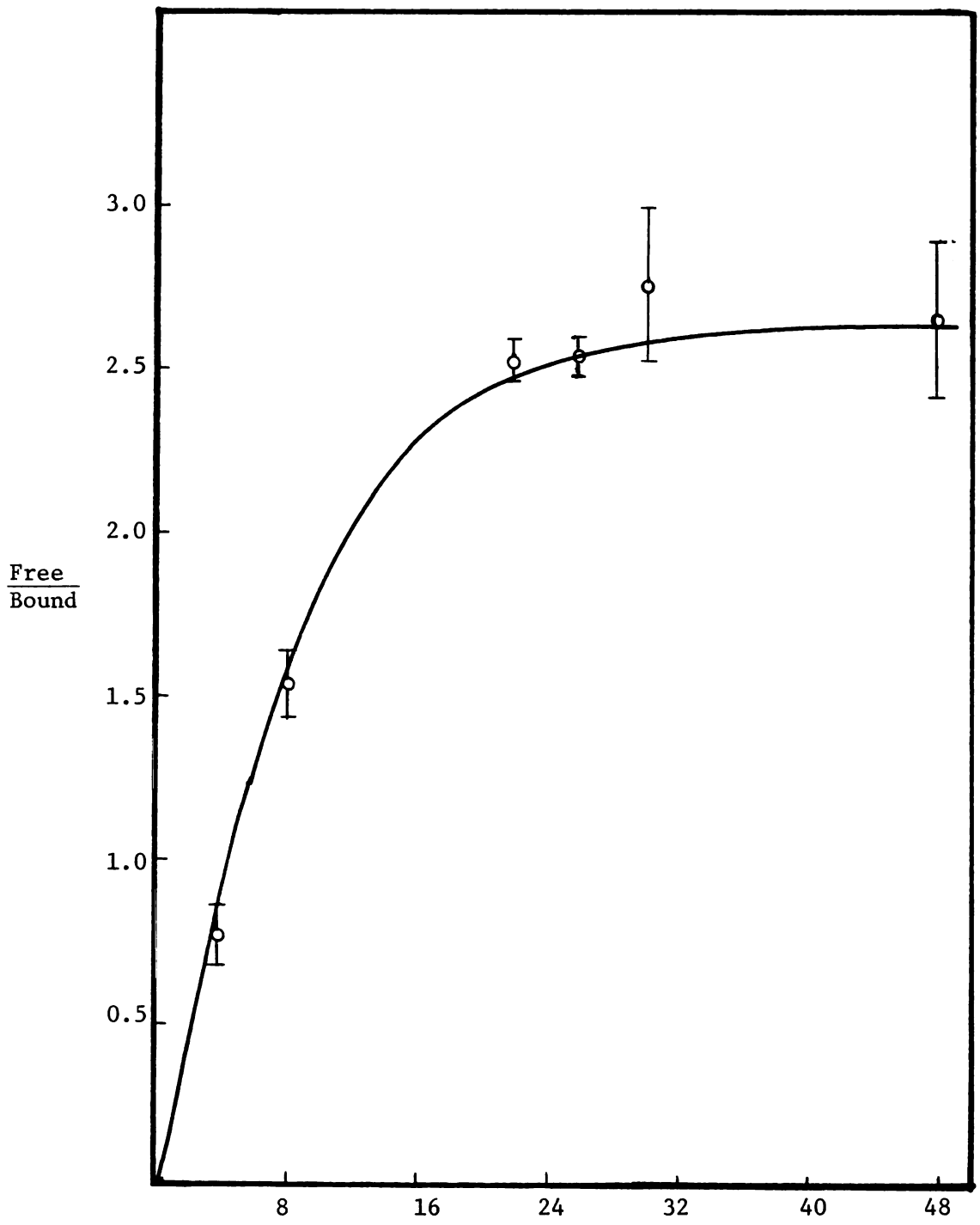


Figure 5-2. Equilibrium time determination for L-T_4-prealbumin binding at 37(±1)°C.

III- MODELS AND PROCEDURES USED IN DATA ANALYSIS

a- Binding of L-Thyroxine

1- Theoretical Model

The data were analyzed according to Scatchard's model for two independent sites (74). This choice of model is discussed in section c below.

In this model

$$\frac{\nu}{TF} = \frac{n_1 K_1}{1 + K_1(TF)} + \frac{n_2 K_2}{1 + K_2(TF)} \qquad (5\text{-}1)$$

where ν is the average number of T4 molecules bound per prealbumin molecule, TF is the concentration (moles/liter) of free T4, K_1 and K_2 are the affinity constants (liter/mole) of the high and low affinity sites, respectively, n_1 and n_2 are the number of high and low affinity sites per prealbumin molecule. Equation (5-1) can be rearranged into

$$\frac{\nu}{TF} = (n_1K_1+n_2K_2) - (K_1 + K_2)\,\nu + (n_1 + n_2)K_1K_2(TF)$$

$$- K_1K_2(\nu TF) \qquad (5\text{-}2)$$

Setting

$$n_1K_1 + n_2K_2 = A \qquad (5\text{-}3)$$

$$K_1 + K_2 = B \qquad (5\text{-}4)$$

$$(n_1 + n_2)\,(K_1K_2) = C \qquad (5\text{-}5)$$

$$K_1K_2 = D \qquad (5\text{-}6)$$

equation (5-2) becomes

$$\frac{\nu}{TF} = A - B\nu + C(TF) - D(\nu TF) \qquad (5\text{-}7)$$

In order to obtain the binding parameters (K_1, K_2, n_1 and n_2) we note that if the values of A, B, C and D are known, equations (5-3)-(5-6) are four simultaneous equations in the four unknown binding parameters. We also note that (ν/TF), ν, (TF) and (νTF) are linearly independent variables. Thus the parameters A, B, C and D can be determined by statistically fitting the observed values of (ν/TF) to the values of ν, (TF), and (νTF) according to equation (5-7). In order to solve equations (5-3)-(5-6) for the binding parameters, equation (5-5) is divided by (5-6) and rearranged to give

$$n_2 = \frac{C}{D} - n_1 \tag{5-8}$$

Rearranging (5-4) gives

$$K_2 = B - K_1 \tag{5-9}$$

Which upon substitution into (5-6) and rearranging gives

$$K_1^2 - B K_1 + D = 0 \tag{5-10}$$

Equation (5-10) is a quadratic in K_1 whose two roots are given by

$$K_1 = \frac{B \pm (B^2 - 4D)^{1/2}}{2} \tag{5-11}$$

If the root with the positive sign is taken to be K_1, then the root with the negative sign must be K_2. This is easily shown by substituting equation (5-11) with the positive sign into (5-9) to get

$$K_2 = \frac{B - (B^2 - 4D)^{1/2}}{2} \tag{5-12}$$

In order to determine n_1 and n_2 equation (5-8) is substituted into (5-3) and rearranged to give

$$n_1 = \frac{A - K_2(C/D)}{(K_1 - K_2)} \qquad (5\text{-}13)$$

n_2 is determined by substituting (5-13) in (5-8)

2- Calculation of the Parameters of Equation (5-7) from Experimental Data

In this section we will show how a set of experimentally observed values at each concentration of T_4 is transformed into a set of parameters suitable for Scatchard analysis. First, we recall that there are 2.60 mls on the F side and 0.80 mls on the B side so that the total volume of solution in the cell is 3.40 mls. The experimental data available are: T (moles of T_4/ml solution B x 1000 x (0.8/3.4)), C_{B+F} (counts per minute/0.5 ml of the solution on side B at equilibrium), C_F (counts per minute/0.5 ml of the solution on side F at equilibrium). The parameters required for the Scatchard analysis are: (the average number of molecules of T_4 bound per molecule of prealbumin) and TF (moles per liter free T_4 at equilibrium).

We define the following additional symbols:

C_B - Counts per minute/0.5 ml of bound hormone at equilibrium.

C_T - Total counts per minute per cell.

SA - Specific activity (cpm/mole T_4)

PT - Concentration (mole/liter) of bound T_4 at equilibrium.

P - Total concentration (mole/liter) of prealbumin in solution B.

The experimental data are treated as follows:

$C_B = C_{B+F} - C_F$

$C_T = (C_{B+F}) \times 1.6 + C_F \times 5.2$

$SA = (C_T \times 1000)/(T \times 3.4)$

$PT = (C_B \times 2000) / SA$

$TF = (C_F \times 2000) / SA$

$\nu = PT / P$

Note that

$$T = TF + \frac{PT}{4.25}$$

where the factor of 4.25 in the above expression for T arises from the fact that AF is distributed in a volume (3.40 mls) which is 4.25 times as large as the volume (0.80 mls) in which PA is distributed. We should note here that the definition of T given in the first paragraph in this section implies that if the B and F sides are expanded proportionately so that the total volume of the cell becomes one liter, (T) is the total number of moles of T_4 in this one liter.

b- Binding of Thyroid Hormone Analogs

1- Theoretical Model

The analysis of the competition of T_4 by an analog at two sites is a very complicated process. If one can manipulate the experimental conditions so that the major part of the competition occurs at the level of a single site, substantial simplication can be achieved. Our experiments were designed with this in mind. The adequacy of this choice is discussed in section c below.

If we focus our attention on competition at a single site, we can derive a very simple equation. We first define some relevant symbols.

A - Concentration (mole/liter) of analog in solution B at the start of the experiment.

PT - Concentration (mole/liter) of bound T_4 at equilibrium.

TF - Concentration (mole/liter) of free T_4 at equilibrium.

PA - Concentration (mole/liter) of bound analog at equilibrium.

AF - Concentration (mole/liter) of free analog at equilibrium.

PF - Concentration (mole/liter) of free sites at equilibrium.

K_T - Binding constant (liter/mole) of T_4.

K_A - Binding constant (liter/mole) of the analog.

Application of mass action laws gives

$$K_T = \frac{PT}{(PF)(TF)} \tag{5-14}$$

$$K_A = \frac{PA}{(PF)(AF)} \tag{5-15}$$

Equations (5-14) and (5-15) are rearranged into

$$PF = \frac{PT}{(K_T)(TF)} \tag{5-16}$$

and

$$PF = \frac{PA}{(K_A)(AF)} \tag{5-17}$$

respectively. Equating the right hand sides of equations (5-16) and (5-17) and rearranging gives

$$\frac{PA}{AF} = \frac{K_A}{K_T} \times \frac{PT}{TF} \tag{5-18}$$

Equation (5-18) represents a straight line with ordinate and abscissa intercepts of zero, and a slope equal to the ratio of the affinity

constants of the analog to that of T_4.

2- Calculation of the Parameters of Equation (5-18) from Experimental Data

The parameters of equation (5-18) that must be determined are PT, TF, PA and AF. We define (A) by

$$A = (\text{moles of analog per ml solution B} \times 1000)\ (0.8/3.4) \qquad (5\text{-}19a)$$

Thus if the B and F sides of each cell are expanded proportionately so that the volume of the cell becomes one liter, (A) is the total number of moles of in this one liter. The only justification for defining such an obscure parameter is that it simplifies the calculations. Therefore

$$A = AF + \frac{PA}{4.25} \qquad (5\text{-}19b)$$

The factor of 4.25 in equation (5-19b) arises from the fact that AF is distributed in a volume (3.40 ml) which is 4.25 times as large as the volume (0.8 ml) in which PA is distributed.

From the conservation of total number of sites we get

$$P = PF + PA + PT \qquad (5\text{-}20)$$

where P again is the total concentration (mole/liter) of prealbumin. Substituting equation (5-16) into (5-20) and rearranging gives

$$PA = P - PT - \frac{PT}{(K_T)(TF)} \qquad (5\text{-}21)$$

PT and TF on the right hand side of equation (5-21) are determined as in section (a) above. The value of AF is determined by substituting the value of A and PA in equation (5-19b) and solving for AF.

c- Discussion of the Assumptions Used in Each of the Models

1- Scatchard Model of T4 Binding

In all our experiments (see "Results, Discussions and Conclusions") and those from other authors (75,76), the Scatchard plots for T4-prealbumin binding intercept the abscissa at a value close to 2 indicating the presence of two binding sites in prealbumin. In the crystal, prealbumin has been shown to have two indistinguishable thyroxine binding sites within the central channel. It is reasonable to expect that the sites are indistinguishable in solution too.

If the two sites are independent in addition to being indistinguishable, a Scatchard plot of the data would be linear according to

$$\frac{\nu}{TF} = nK_T - K_T \nu$$

where n=2 and K_T is the affinity constant. The experimental curves obtained by us (see "Results, Discussions and Conclusions") and other authors (75,76) however, were nonlinear in a manner which implies that the binding at the two sites is anti-cooperative i.e. binding at one site decreases the affinity of the second site.

On the other hand, there is no guarantee that the second site observed in solution is the second site in the central channel. It is conceivable that thyroxine could bind on the surface of the protein with any one or more of the amino acid residues extending out from the protein. In this case, the two sites observed in solution are distinguishable and most likely not independent.

The relevance of these discussions becomes apparent when one is trying to choose a model to analyze the binding data. Should one

use a model for two "distinguishable and independent" sites? two "distinguishable and interacting sites"? or two "indistinguishable and interacting" sites? If interactive models are used what sort of interaction parameters should be introduced and what is their physical origin? Moreover, it is apropos to note here that the actual mode of binding cannot be delineated on the basis of goodness of fit of the data to the equations of these models, because, all such models can be made to fit the same observed set of data by appropriate adjustment of parameters. For example, Ferguson, et al.(75) were able to obtain excellent fits of the same set of data using equations for two diametrically opposed models: the "distinguishable-independent sites" model and an "indistinguishable-interactive sites" model. The values of the binding constants obtained from the two models were significantly different.

Since there is no apriori physical reason for favoring any particular model, we chose to use the one that we find the simplest and most convenient to work with, namely the "distinguishable-independent sites" model.

2- Equations (5-18) and (5-21) for Analog-T4 Competition

In deriving equations (5-18) and (5-21) for analog binding, we have assumed that we can manipulate the experimental conditions so that the significant competition is occurring at the level of the high affinity site. In order to examine thoroughly the implications of this assumption, we need to write out explicitly the complete equations and point out what we have neglected in order to obtain equations (5-18) and (5-21).

Prealbumin has two binding sites which we will assume to be distinguishable, independent and that the second site cannot be occupied unless the first is occupied. This allows us to write the reaction equations as follows"

$$P + T \xrightarrow{KT1} PT \qquad (5\text{-}22)$$

$$PT + T \xrightarrow{KT2} PT2 \qquad (5\text{-}23)$$

$$P + A \xrightarrow{KA1} PA \qquad (5\text{-}24)$$

$$PA + A \xrightarrow{KA2} PA2 \qquad (5\text{-}25)$$

$$PT + A \xrightarrow{KA2} PAT \qquad (5\text{-}26)$$

$$PA + T \xrightarrow{KT2} PAT \qquad (5\text{-}27)$$

where PA2, PT2 and PAT corresponds to a protein molecule which is doubly occupied by two analog molecules, two thyroxine molecules and one analog-one thyroxine molecule, respectively. From equations (5-22) - (5-27) one obtains

$$KT1 = \frac{PT}{(PF)(TF)} \qquad (5\text{-}28)$$

$$KT2 = \frac{PT_2}{(PT)(TF)} \qquad (5\text{-}29)$$

$$KA1 = \frac{PA}{(PF)(AF)} \qquad (5\text{-}30)$$

$$KA2 = \frac{PA2}{(PA)(AF)} \qquad (5\text{-}31)$$

$$KT2 = \frac{PAT}{(PA)(TF)} \qquad (5\text{-}32)$$

$$KA2 = \frac{PAT}{(PT)(AF)} \tag{5-33}$$

From equation (5-28) and (5-30) one gets

$$PF = \frac{PT}{(KT1)(TF)} \tag{5-34}$$

and

$$PF = \frac{PA}{(KA1)(AF)} \tag{5-35}$$

respectively. Equating the right hand sides of equation (5-34) and (5-35) and rearranging gives

$$\frac{PA}{AF} = \frac{KA1}{KT1} \times \frac{PT}{TF} \tag{5-36}$$

Equations (5-36) and (5-18) are identical. Thus equation (5-18) holds whether competition is occurring at a single site only or at two sites. The crux of the assumption is not the validity of equation (5-18) per se but the validity of being able to actually determine (or even get a good estimate of) the values of PA, PF, PT and AF for every experimental point. The conservation of mass requires that

$$A = AF + \frac{PA + 2PA2 + PAT}{4.25} \tag{5-37}$$

$$T = TF + \frac{PT + 2PT2 + PAT}{4.25} \tag{5-38}$$

$$P = PF + PA + PT + PA2 + PT2 + PAT \tag{5-39}$$

When one examines closely the derivations and discussions in sections a and b above (particularly equations 5-19, the equations in section a-2 above and equation 5-20) it becomes apparent that we have set

$$A = AF + \frac{PA}{4.25} \tag{5-40}$$

$$T = TF + \frac{PT}{4.25} \tag{5-41}$$

$$P = PF + PA + PT \tag{5-42}$$

Comparison of equations (5-37), (5-38) and (5-39) with (5-40), (5-41) and (5-42) respectively reveals that we have ignored the concentrations of PA2, PT2 and PAT but not PF, PA, or PT. In other words, we have assumed that the protein is either unoccupied by ligand or else it is singly occupied by an analog molecule or a thyroxine molecule. That is, in our treatment the concentration of any doubly occupied protein was ignored.

How justified are we in doing this? If we can rigorously show the concentration of doubly occupied protein is small compared to the concentration of the singly occupied protein, then we are justified in our assumption. Comparing equations (5-37 with 5-40), (5-38 with 5-41) and (5-39 with 5-42) indicates specifically that it is necessary and sufficient to demonstrate that

$$PA >>> 2PA2 + PAT \tag{5-43}$$

and

$$PT >>> 2PT2 + PAT \tag{5-44}$$

in order to justify the validity of our assumption. (Note that it is

sufficient to demonstrate conditions 5-43 and 5-44 to satisfy the condition PA + PT >>> PA2 + PT2 + PAT which is deduced from comparing equations 5-39 with 5-42)

Using equation 5-29 and 5-32 with the experimental observation that TF is about 10^{-9} and KT2 is about 3×10^{6} it is easy to demonstrate that

$$PT >>> 2PT2 \qquad (5\text{-}45)$$

and

$$PA >>> PAT \qquad (5\text{-}46)$$

This however, is a far cry from the stringent requirements of conditions (5-43) and (5-44). We simply have no rigorous proof that condition (5-43) and (5-44) hold in our experiments.

On the other hand, we have measured KT1 and KT2 and found that $KT1/KT2 \tilde{=} 10$. If $KA1/KA2 \tilde{=} 10$ also then we can expect PA to be of the order of 10 times PA2. If we use only experimental points in which the analog is showing small displacements of T4, then the chances of having negligible concentrations of PA2 and PAT becomes better. We will not belabor this point any further.

There is a second source of error that must be examined. In the next section we report an anomalous behaviour in the Scatchard plots in the region where the average number of ligand molecules bound per protein molecule is < 0.3. In order to avoid this region we have accepted only experimental points for which

$$\frac{PT + PA}{P} > 0.3$$

where $P = 5.00 \times 10^{-8}$ moles/liter in all our competition experiments.

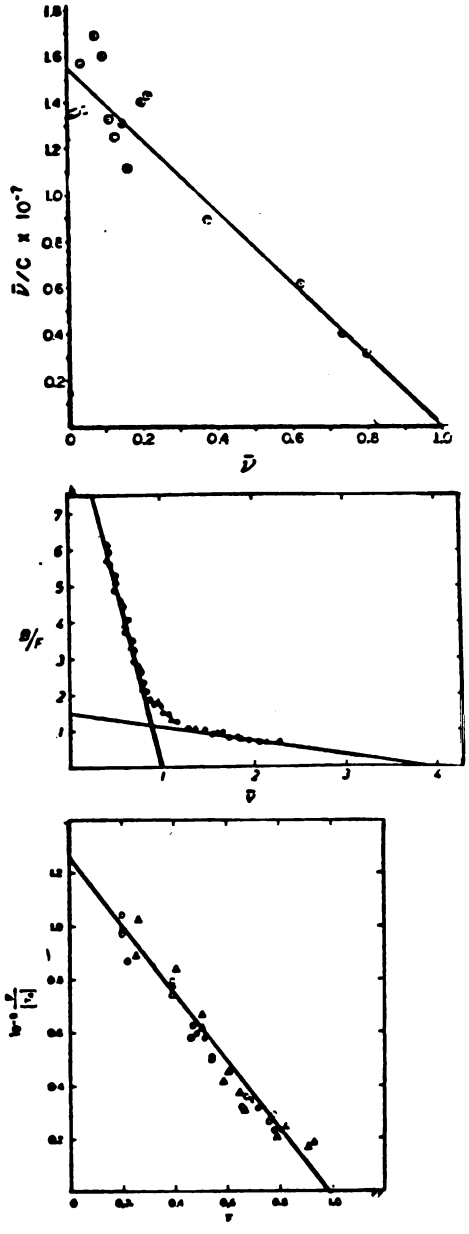
Finally, a last check consists of checking the consistency of the data with the type of plot that obeys equation (5-18). Specifically, the plot of equation (5-18) should be linear with abscissa and ordinate intercepts of zero. Moreover, if the analog used is T_4, the plots obtained should have a slope of unity in addition to the (0,0) intercept. These again are necessary condition for the validity of the assumptions. We do not know if they are sufficient.

IV- RESULTS, DISCUSSIONS AND CONCLUSIONS

a- Review of Past Work

The binding affinities of thyroxine as well as the conditions and types of experiments used by different investigators are summarized in table 5-5. Figure 5-3 is a compilation of the corresponding Scatchard plots of T_4 binding. The main features to be noted in table 5-5 are: (i) The binding constants of thyroxine are comparable in tris and phosphate buffers. This was specifically noted by Nilsson and Petersen (35). (ii) The high affinity site has an affinity constant of 10^7 - 10^8 M^{-1} for L-thyroxine. (iii) The number of high affinity sites is about one. The total number of sites (and hence the number of low affinity sites) shows larger variation. Examining figure 5-3 shows that the observation of one high affinity size and no low affinity sites in figures 5-3a and 5-3c is a consequence of termination of measurements at low T_4 concentrations. Figures 5-3b, 5-3d and 5-3e show about 4, 2 and 1.3 total sites respectively.

Table 5-6 summarizes the affinities of other thyroid hormones and analogs as well as the conditions under which they were determined. It includes only analogs that have been simultaneously measured by ourselves.

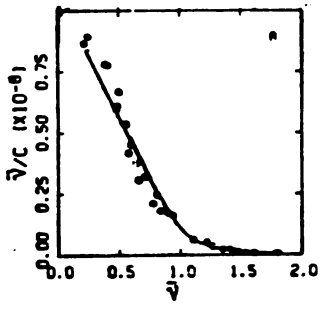


(a) Reference 77

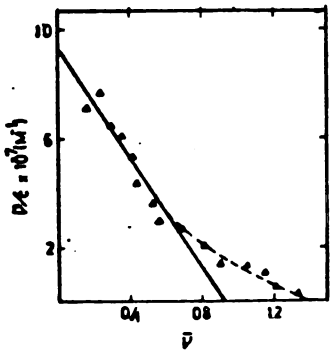
(b) Reference 78

(c) Reference 79

Figure 5-3. Scatchard plots of L-T_4-prealbumin binding as determined by various authors.



(d) Reference 75



(e) Reference 76

Figure 5-3 continued.

Table 5-5 Prealbumin-L-Thyroxine Binding Parameters as Determined by Various Investigators.[a]

Buffer[b]	NaCl[b]	pH	Temperature °C	K_1[c]	K_2[c]	N_1[c]	NT[c]	Reference
.06P	0.00	7.4	24	1.5×10^7		0.96		77
.01P	0.14	7.4	24	1.6×10^7		1.00		77
.01P	0.14	7.4	37	1.3×10^7		.84		77
.02T	0.20	7.4	4	2.2×10^7		1.00	4	78
.02P	0.15	7.4	37	7.8×10^6				78
.02T	0.20	8.0	4	2.2×10^7				78
.02P	0.15	8.0	37	7.8×10^6				78
.05P	0.10	7.4	25	1.3×10^8		1.00		79
.05P	0.10	7.4	25	1.1×10^8	9.6×10^5		2.0	75
.05P	0.15	7.5		9.3×10^7		0.95	1.3	76

[a]Only equilibrium dialysis data are included.

[b]Concentration in moles/liter. P = Phosphate. T = Tris.

[c]K_1, K_2, N_1, NT are the affinity constant of the high affinity site, affinity constant of the low affinity site, the number of high affinity sites, and total number of sites respectively.

Table 5-6. Binding Affinities of Some Thyroid Hormones and Analogs to Prealbumin at pH 7.4 (±.1), 25°C as Determined by Various Investigators.

Analogs	K[a]	Method[a]	Reference
3,5,3'-L-T_3	1.3×10^6	F & ED	78[b]
	9.0×10^6	ED	76
	1.2×10^7	ED	79
	2.0×10^7	F & ED	80
3,5-L-T_2	6.2×10^4	F	78[b]
N-acetyl-L-T_4	1.2×10^7	F & ED	80
Tetrac	2.5×10^8	F	80
Tetraprop	3.98×10^8	F	80
	$2.5 \times 10^{8}_{7}$	ED	
Tetrabut	5.01×10^7	F	80

[a] K = Affinity constant. F = Flourescence assay. ED = Equilibrium dialysis.

[b] The authors specify that they used 3,3',5'-L-T_3 and 3',5'-T_2. Most likely however, they meant 3,5,3'-L-T_3 and 3,5-T_2.

b- The Binding Parameters of L-Thyroxine

The binding of L-thyroxine to prealbumin was measured in phosphate and tris buffers at pH 7.4 and 8.0. Two phosphate buffers were tested at pH 7.4: 0.05 M and 0.10 M phosphate with 0.1 M NaCl and 10^{-3}m EDTA. Figure 5-4 and 5-5 show that data points obtained from several experiments as well as the regression curve. Table 5-7 shows the binding parameters which characterize the regression curve. Figure 5-6 shows that the regression curve in 0.1 M phosphate lies below the curve in 0.05 M phosphate. This indicates that for any value of ν the free ligand concentration is higher in 0.1 M than in 0.05 M phosphate. This cannot be interpreted on the basis of affinity constants because both affinity constants have higher values in the 0.1 M buffer (table 5-7). It can be interpreted however, on the basis of the lower number of both classes of sites in the 0.1 M buffer. This suggests that the phosphate is blocking some of the thyroxine binding sites on prealbumin.

Two phosphate buffers were tested at pH 8: 0.05 M phosphate-0.14 M NaCl and 0.1 M phosphate-0.003 M NaCl,both buffers contained 10^{-3} M EDTA. The results are shown in figure 5-7. Here again the 0.10 M curve lies below the 0.05 M curve possibly due to a similar inhibition of thyroxine binding by phosphate.

Two tris buffers were used at pH 7.4: 0.05 M tris-.15 M NaCl and .1125 M tris-.071 M NaCl, each buffer contained EDTA. Figure 5-8 is a Scatchard plot of the data. The experimental points obtained in the two buffers are essentially indistinguishable. Moreover, the curves are linear (!!!) in the range tested with an average binding constant of about 8.8×10^{6} and about 2.5 sites per prealbumin molecule. The reason for the linearity is not clear.

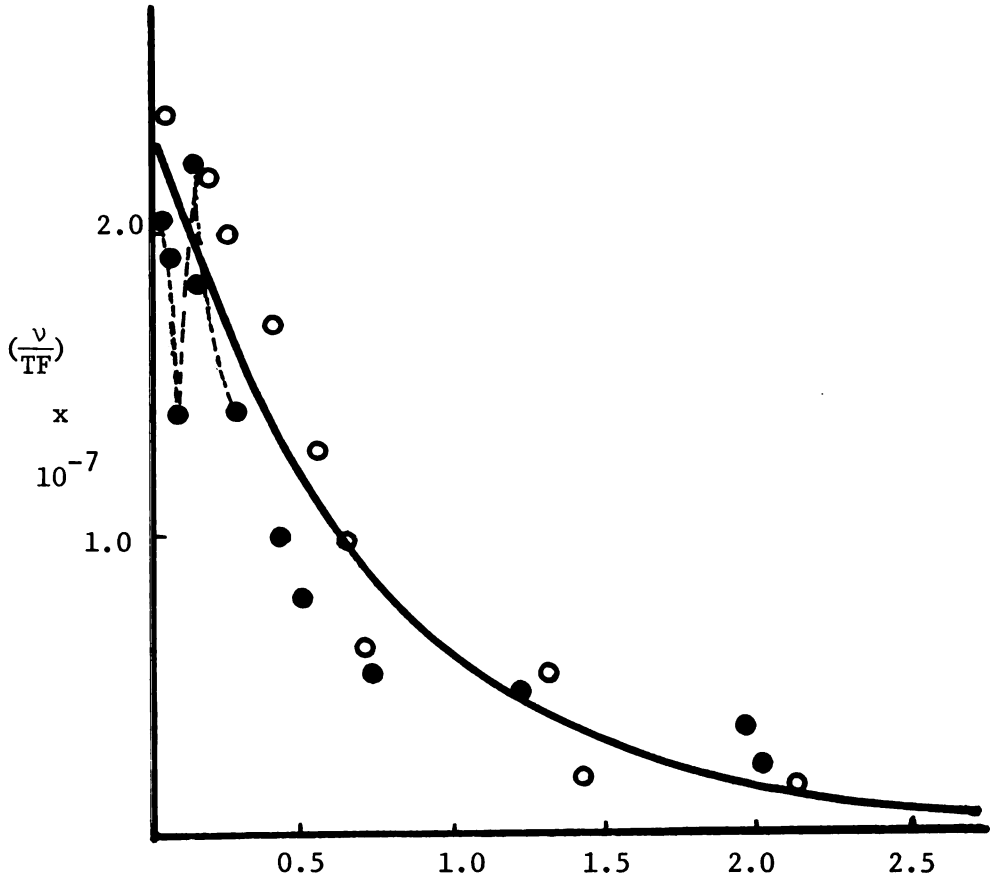


Figure 5-4. Scatchard plot of L-T_4-prealbumin binding in 0.05 M phosphate buffer containing 0.1M NaCl and 1 mM EDTA, at pH 7.4 and 37 ($\pm$1)°C. Different symbols correspond to different experiments. The solid curve is calculated from the binding parameters in table 5-7. The dashed curve connects the experimental points for one of the experiments in the domain $\nu<0.3$.

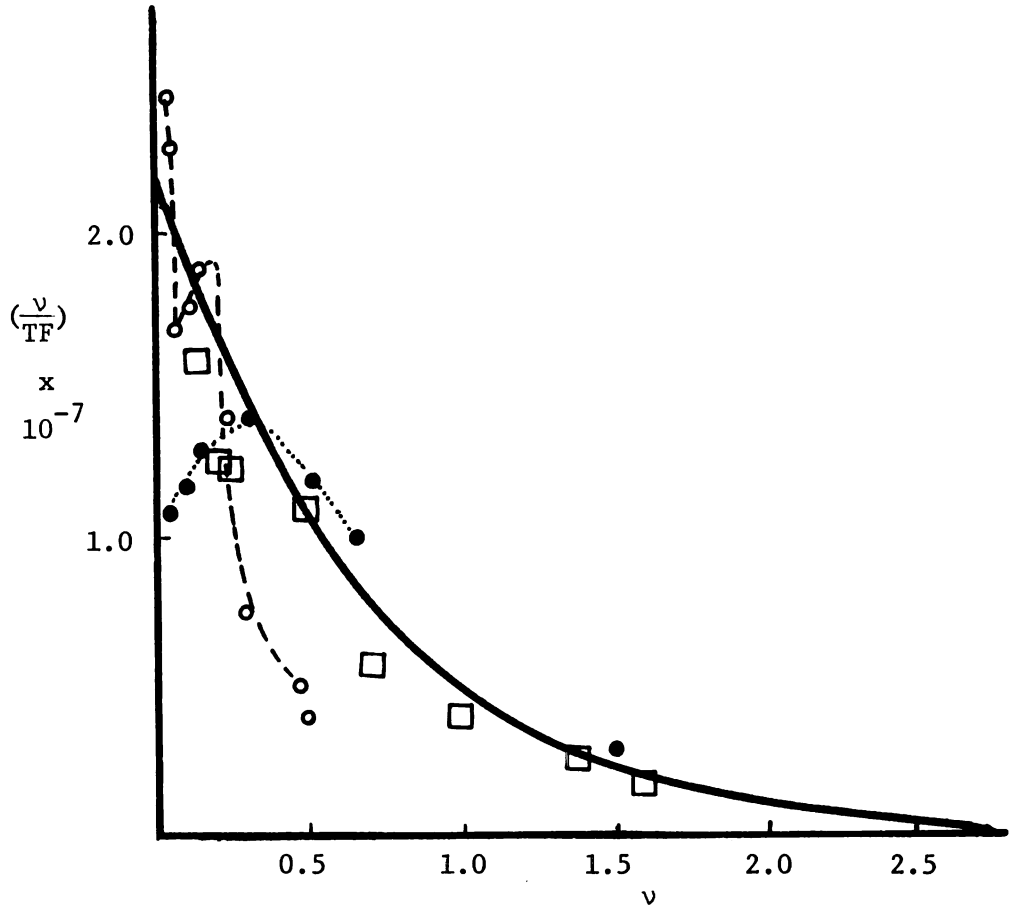


Figure 5-5. Scatchard plot of L-T_4-prealbumin binding in 0.1M phosphate, containing 0.1M NaCl and 1 mM EDTA, at pH 7.4 and 37 ($\pm$1)°C. Different symbols correspond to different experiments. The solid curve is calculated from the binding parameters in table 5-7. The dotted and dashed curves connect the points of individual experiments in the domain $\nu<0.3$.

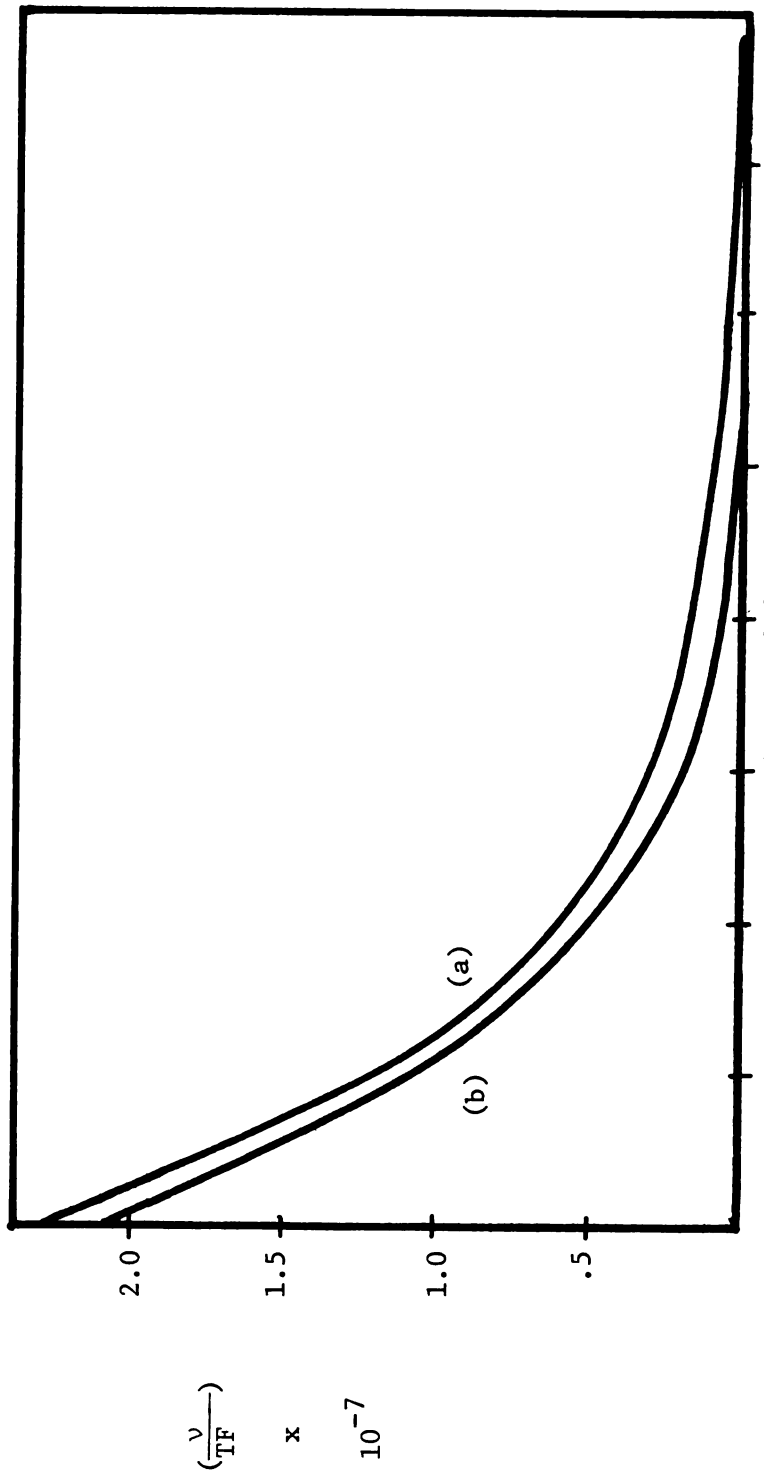


Figure 5-6. The best fit curves of figures (a) 5-4 (0.05M phosphate, pH 7.4 and 37°C), and (b) 5-5 (0.1M phosphate, pH 7.4 and 37°C).

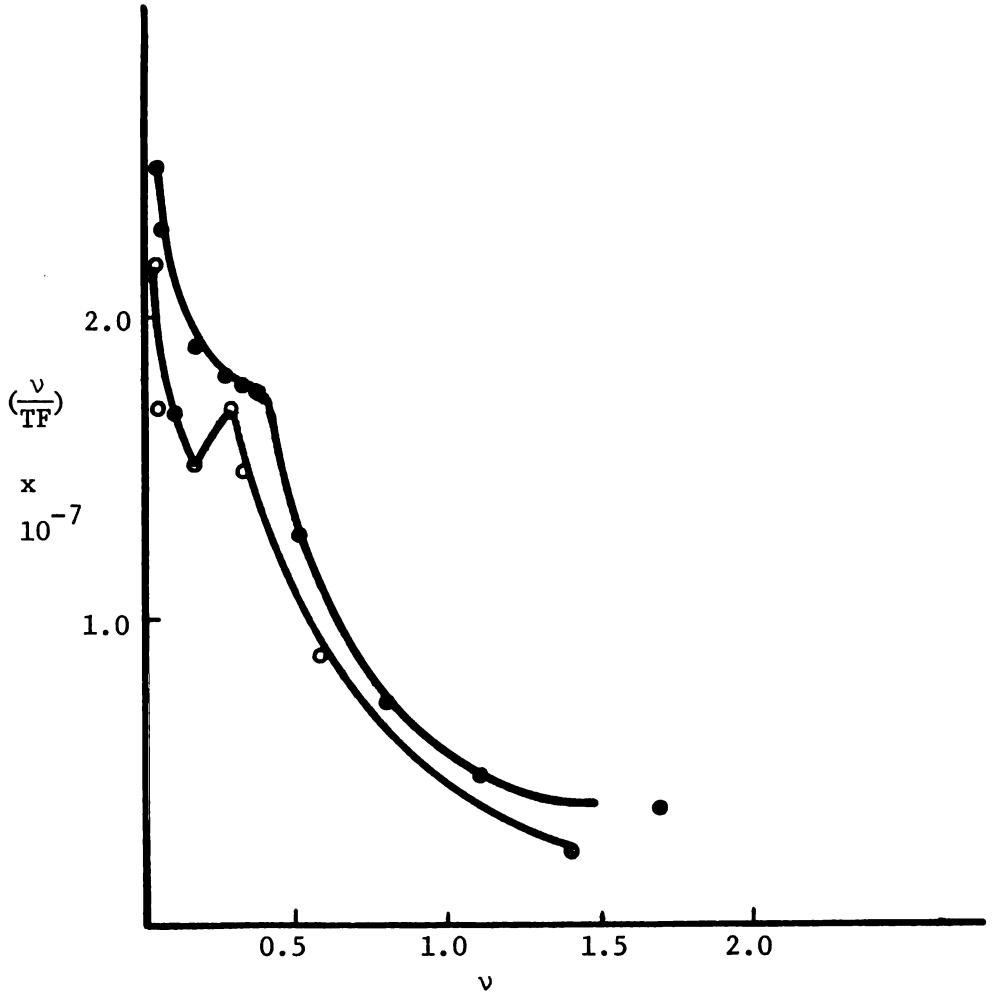


Figure 5-7. Scatchard plots of L-T_4-prealbumin binding in 0.05M phosphate buffer (●) containing 0.139m NaCl and 1 mM EDTA, and 0.1M phosphate buffer (o) containing 0.003M NaCl and 1 mM EDTA. Both sets of data were taken at pH 8.0 and 37 (±1)°C.

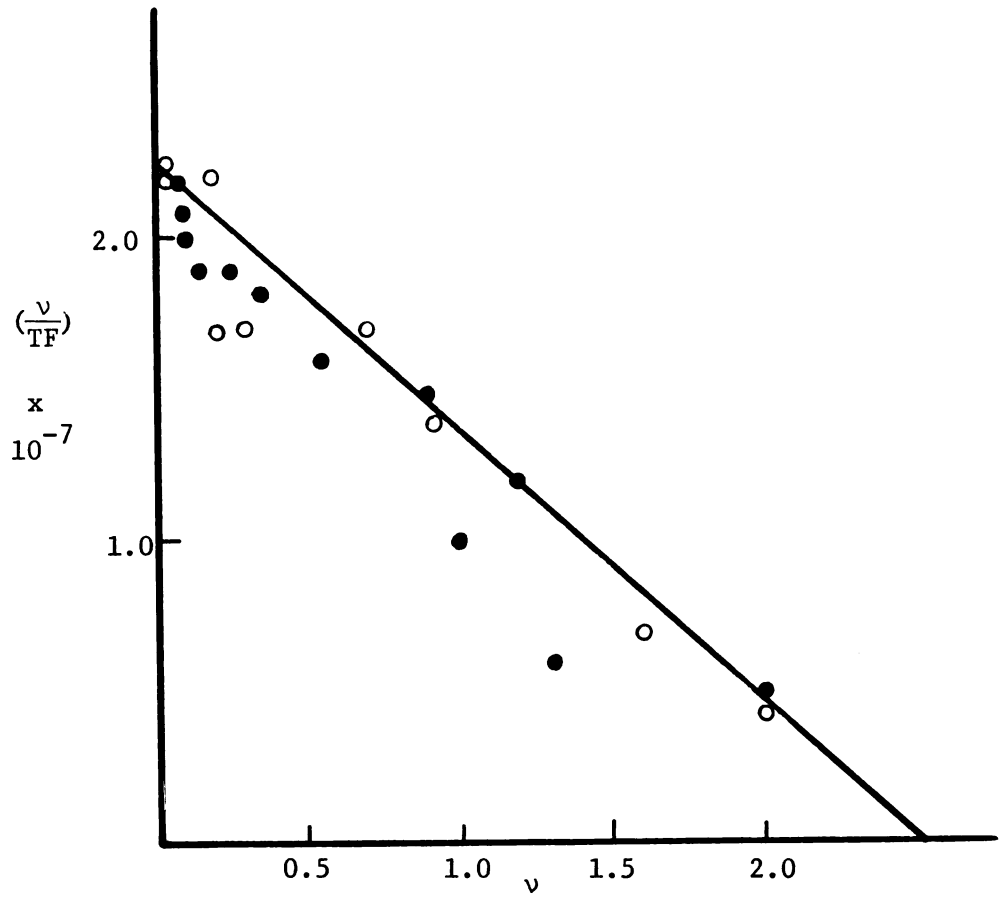


Figure 5-8. Scatchard plots of L-T_4-prealbumin binding in 0.05M tris buffer (o) containing 0.15M NaCl, and 0.1125M tris buffer (●) containing 0.071M NaCl. Both buffers contained 1 mM EDTA. Both sets of data were taken at pH 7.4 and 37 (±1)°C.

Table 5-7. Calculated Values of L-Thyroxine-Prealbumin Binding Parameters at 37 (± 1)°C.

Buffer	NaCl[a]	pH	K_1[b]	K_2[b]	N1[b]	N2[c]
0.05 M Phosphate	0.10	7.40	2.43×10^7	7.34×10^5	0.86	2.85
0.10 M Phosphate	0.10	7.40	3.94×10^7	1.64×10^6	0.47	2.22
0.05 and 0.112M Tris	0.275	8.00	4.91×10^7	3.20×10^6	0.75	1.28

[a]Concentration in moles/liter.

[b]See Footnote c table 5-1.

[c]N2 is the number of low affinity sites.

Two tris buffers were tested at pH 8: 0.05 M tris - .256 M NaCl and 0.1 M tris - .238 M NaCl, both buffers contained 10^{-3} M EDTA. Figure 5-9 shows a Scatchard plot of the data as well as the regression curve. Table 5-7 shows the binding parameters that characterize the regression curve. The data in the two buffers were identical for $\nu > 0.3$, and hence both sets of data were used in determining the regression curve. Comparing tables 5-5 and 5-7 shows that our observed binding parameters (K_1, K_2, n_1, n_2) are compatible with those obtained by other authors.

c- Anomalous Behaviour of the Scatchard Plots

Scatchard's equation for two independent sites has the qualitative behaviour shown in figure 5-10a where the value of ν/TF decreases monotonically as ν increase. In this normal situation, an increase in the ligand concentration leads to an increase in the average number of ligand molecules bound per protein molecule (ν). The concentration of free ligand (TF) simultaneously increases. TF however, increases faster than ν. Thus as more ligand is added, ν and TF increase but ν/TF decrease. The slope of the ν/TF vs. ν plots is always negative.

Examining the Scatchard plots for individual experiments in various buffers (figures 5-4, 5-5, 5-7, 5-8 and 5-9), it is noted that (except for figure 5-8) the plots do not resemble fig 5-10a but rather behave anomalously in the domain $0 < \nu < 0.3$. In this domain, the curves do not decrease monotonically as predicted by the Scatchard model for independent sites. This anomaly can be characterized graphically as follows: in most cases, in the domain $0 < \nu < 0.3$, there are subdomains where the slope of the (ν/TF vs ν) curves is positive, and there is at least one point where the slope is zero. Figure 5-10b qualitatively summarizes the various observed modes of behaviour in the domain of anomaly. In

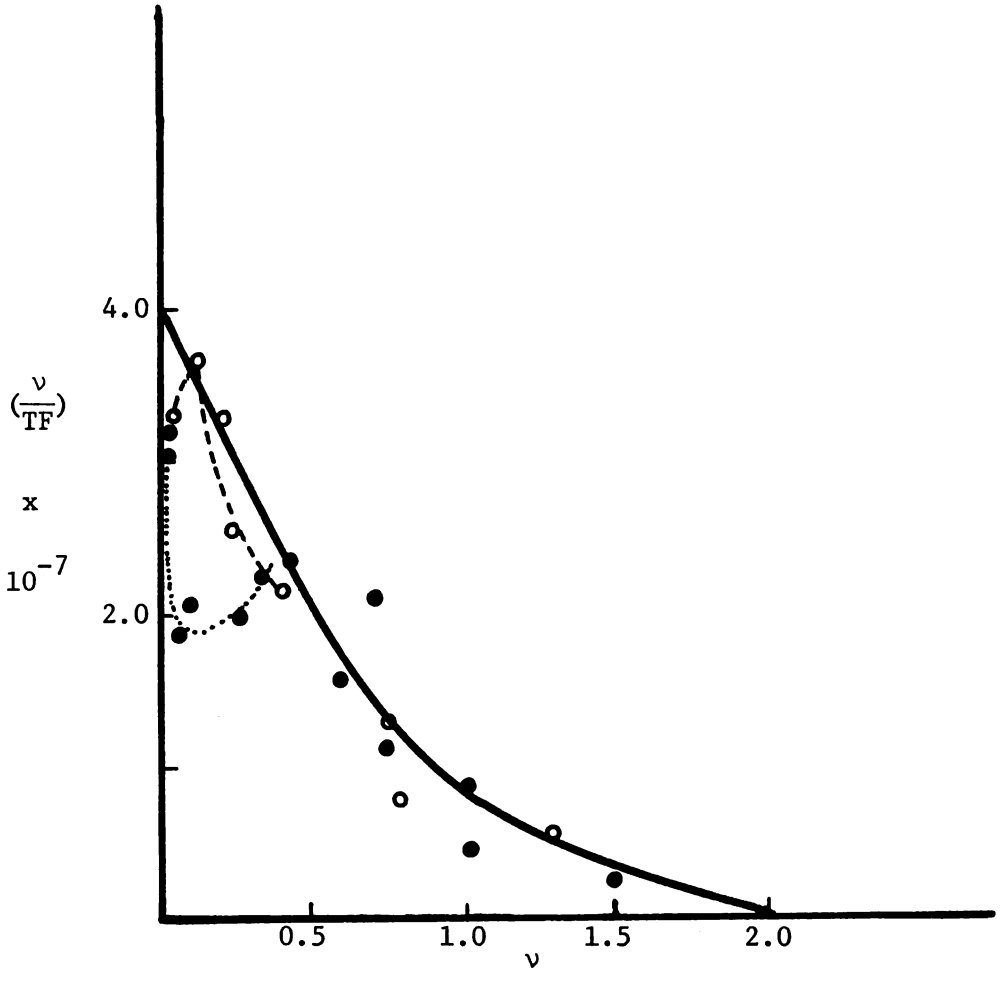


Figure 5-9. Scatchard plots of L-T_4-prealbumin binding in 0.05M tris buffer (●) containing 0.256M NaCl, and 0.1M tris buffer (o) containing 0.238M NaCl. Both buffers contained 1 mM EDTA. Both sets of data were taken at pH 8.0 and 37 ($\pm$1)°C. The solid curve is calculated from the binding parameters of table 5-7. The dotted curve connects the experimental points for ν<0.3 in 0.05M tris. The dashed curve connects the experimental points for ν<0.3 in 0.1M tris.

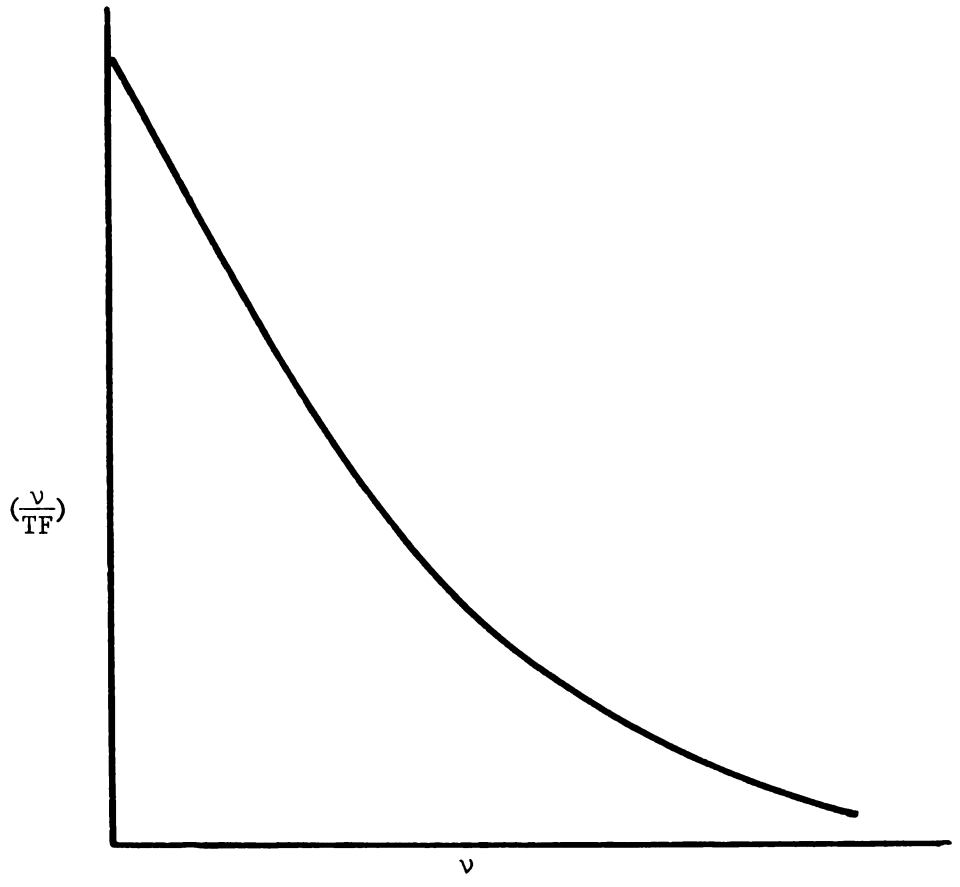


Figure 5-10. (a) Normal Scatchard type behaviour: ν/TF is a monotonically decreasing function of ν.

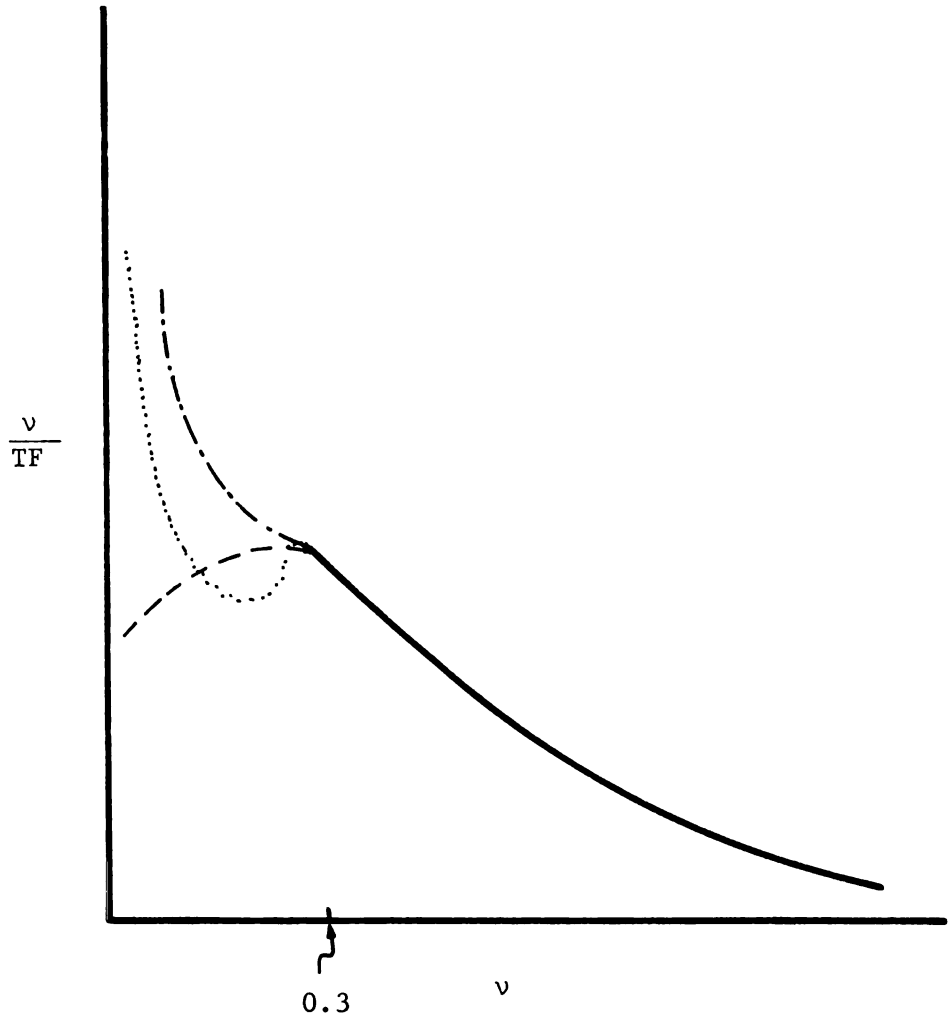


Figure 5-10. (b) Anomalous behaviour of L-T_4-prealbumin Scatchard plots in the domain $\nu<0.3$.

qualitative terms this means that in the domain of anomaly, as more ligand is added and increases, TF either decreases, does not change or increases at a rate slower than that of ν. In our experiments, the third situation was the only observed mode of behaviour, namely, TF increases at a slower rate than ν. Presently, we do not have an interpretation of these observations. Neither do we claim that these modes of behaviour in the region of anomaly are unambiguously defined. One can speculate however, that (i) prealbumin is a mixture of monomers, dimers, trimers and tetramers, and (ii) the equilibrium constant for the formation of the tetramer (which is the only species that binds the hormone with high affinity) is a sharply increasing function of the concentration of T_4 so that addition of T_4 causes a sharp shift in equilibrium towards the tetramer. This speculation would explain the region with positive slope.

At this point, it is interesting to find where other investigators stand on this point. Figure 5-3 is a compilation of Scatchard plots obtained by different authros for T_4-prealbumin binding. The only plot which shows experimental points in the domain $0 < \nu < 0.3$ is figure 5-3a which clearly shows the anomalous behaviour that we are discussing. In figures (5-3b) to (5-3e) there are virtually no data points in this domain. The corresponding authors must have either (i) not explored that region altogether, (ii) discarded the data ascribing irregular behaviour to experimental error, or (iii) explored the region and observed the anomalous behaviour but the data points were inadvertently not included in the graphs. Moreover, none of these authors mention any abnormal behaviour in their respective discussions.

What is the possibility that this anomaly is ascribable to experimental error? We cannot completely rule out this possibility, particularly

that the entire analysis depends on the value of TF at each experimental point. Any error in the measurement of TF would certainly lead to an error in the position of the corresponding point on the graph. On the other hand, it is highly unlikely that this error consistently recurs in the observed domain of anomaly.

d- Binding of other Thyroid Hormones and Analogs

The competition of thyroxine by analogs was studied at pH 8.0 in 0.1 M tris containing 0.1 M NaCl and 10^{-3}M EDTA. Figure 5-11 is a graph of PT/TF vs. log C (C = 4.25 x A where A is defined in equation 5-19a). Figure 5-12 shows plots of equation (5-18) for various analogs. The slopes and intercepts obtained from linear regression analysis of the data in the plots of figure 5-12 are shown in table 5-8. The binding affinities and binding free energies of the analogs studied are shown in table 5-9. It can be seen in figure 5-11 that the affinities of thyroid hormones and analogs are in the same order as in table 5-9. The contribution of individual functional groups to binding as well as the perturbative effects that groups have on each other are best expressed in terms of the free energy decomposition scheme of chapter 3. Specifically, we will use equations (3-30), (3-31), (3-33) and (3-37).

1- Contribution of the Iodine Atoms:

What is the relative significance of the inner and outer ring iodine atoms? In order to answer this question, the scheme presented in figure (5-13) was devised. In this scheme, all the possible combinations of 0,1, 2,3, and 4 iodine atoms in the 3,5,3' and 5' positions are arranged in a hierarchy according to the number of iodine atoms. Examining the figure, leads one to conclue that: for any compound, losing (or gaining) an outer ring iodine results in a greater loss (or gain) in the binding free energy

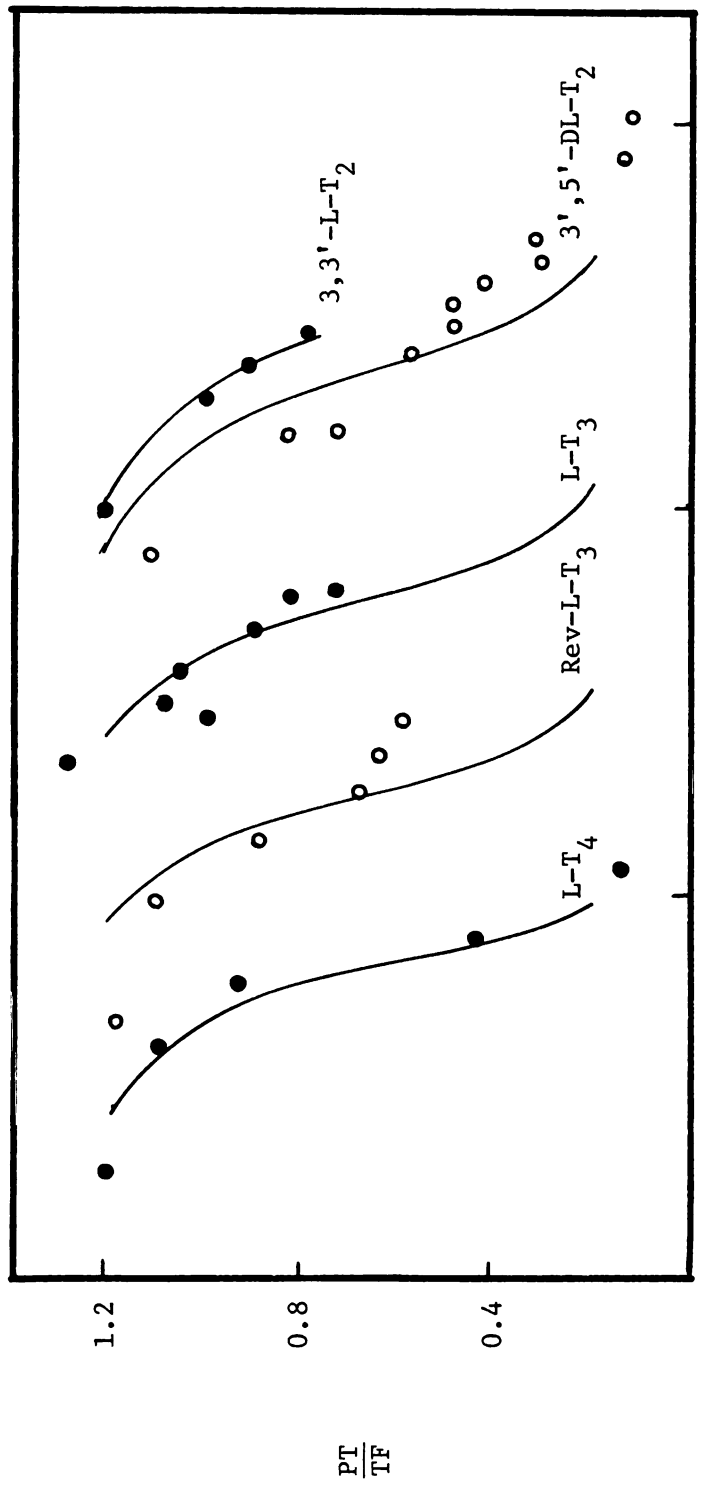
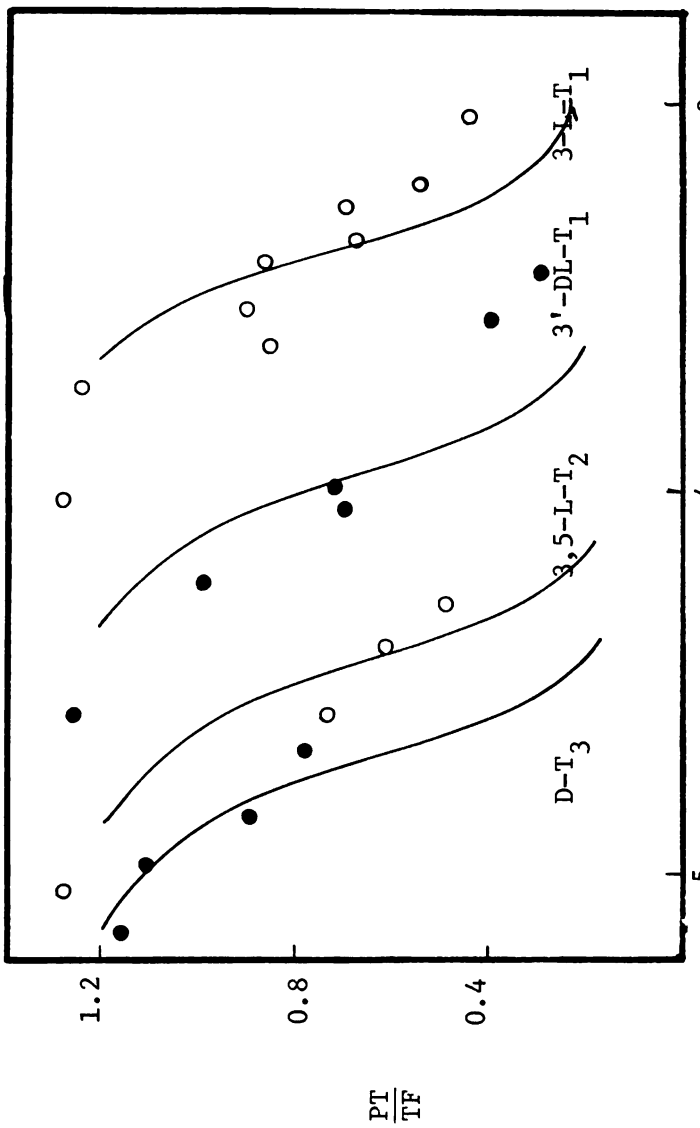


Figure 5-11. Displacement curves for the binding of thyroid hormones and their analogs to prealbumin. (C = 4.25 x A where A is defined by equation 5-19a).



(b)

Figure 5-11 continued.

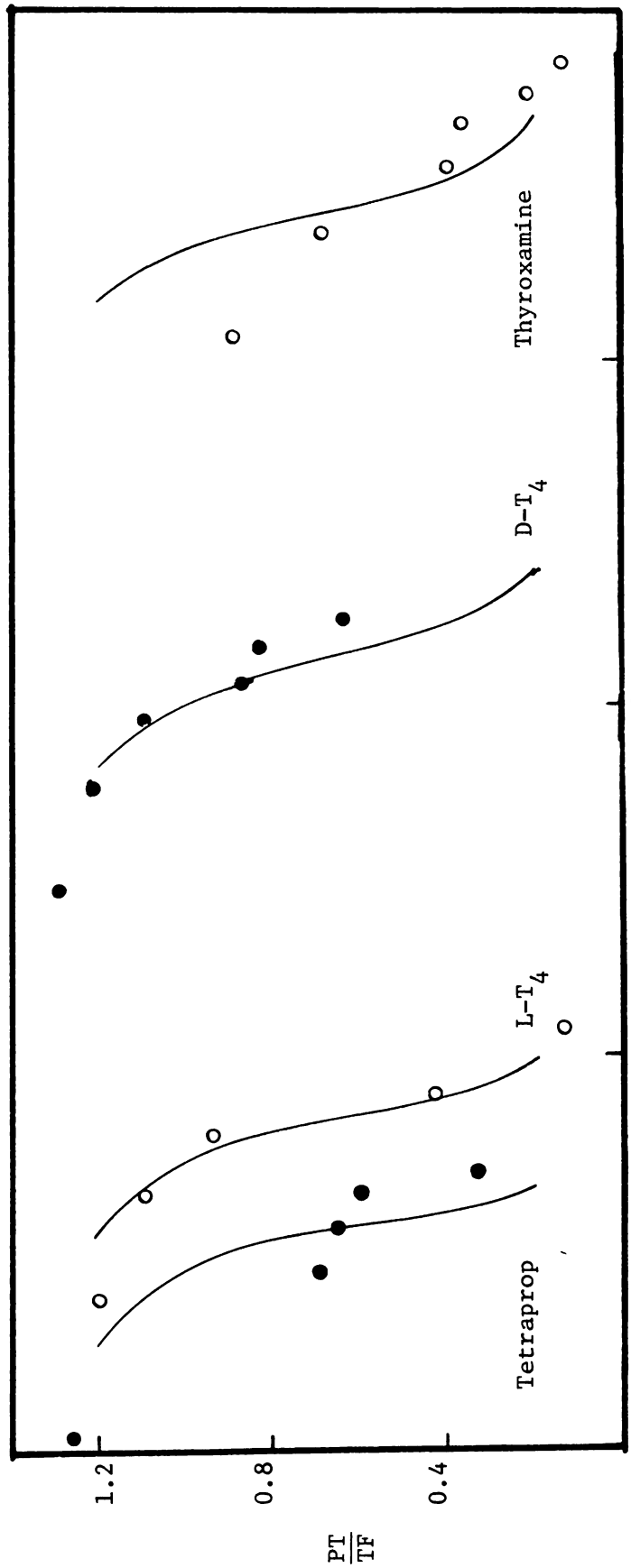


Figure 5-11 continued.

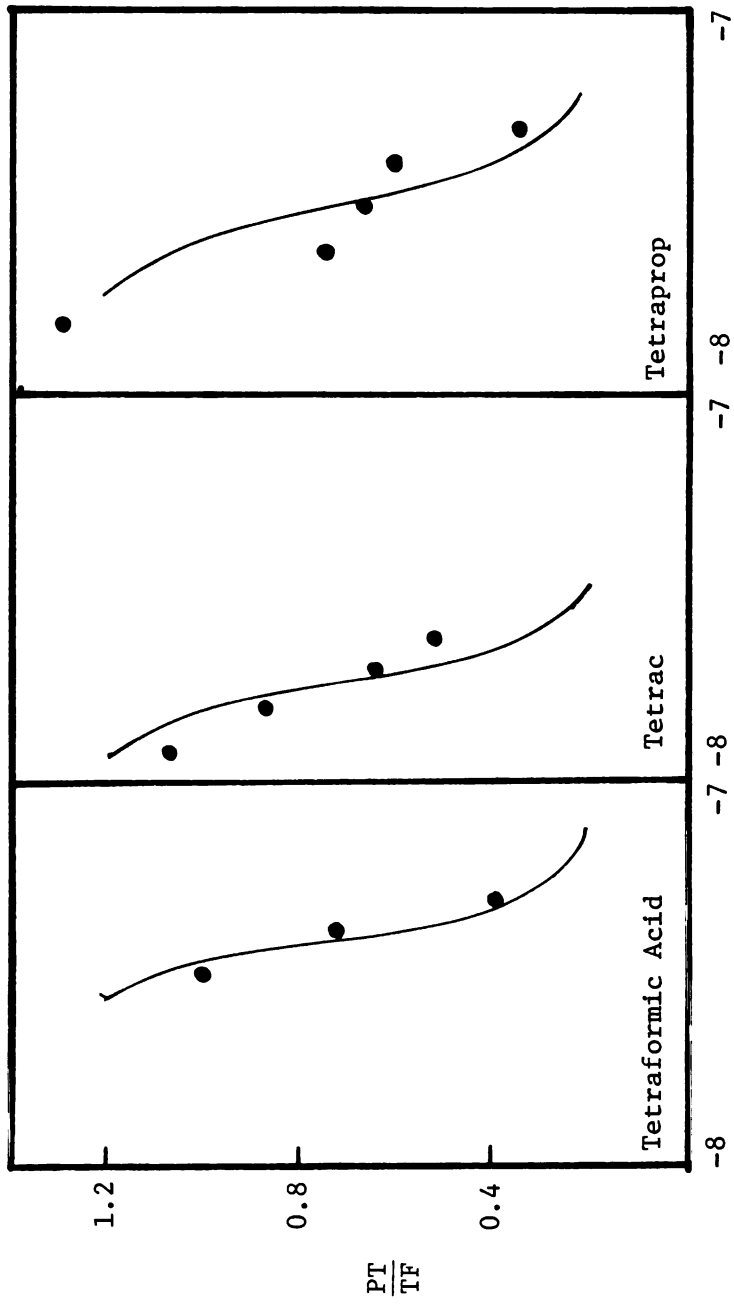


Figure 5-11 continued.

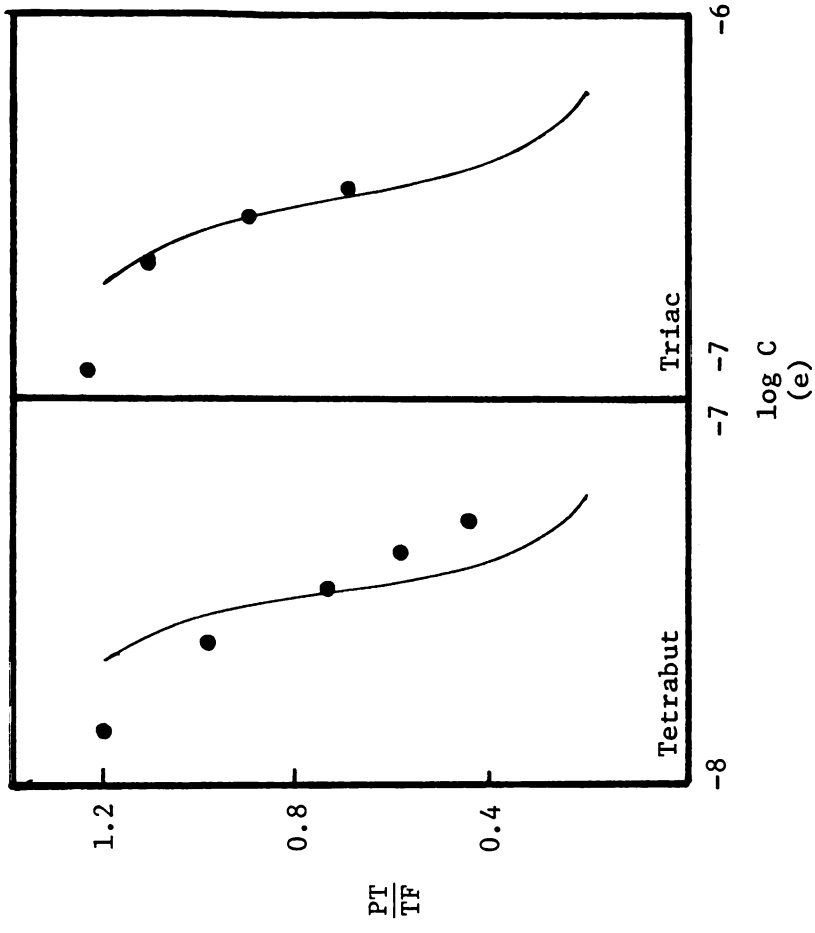


Figure 5-11 continued.

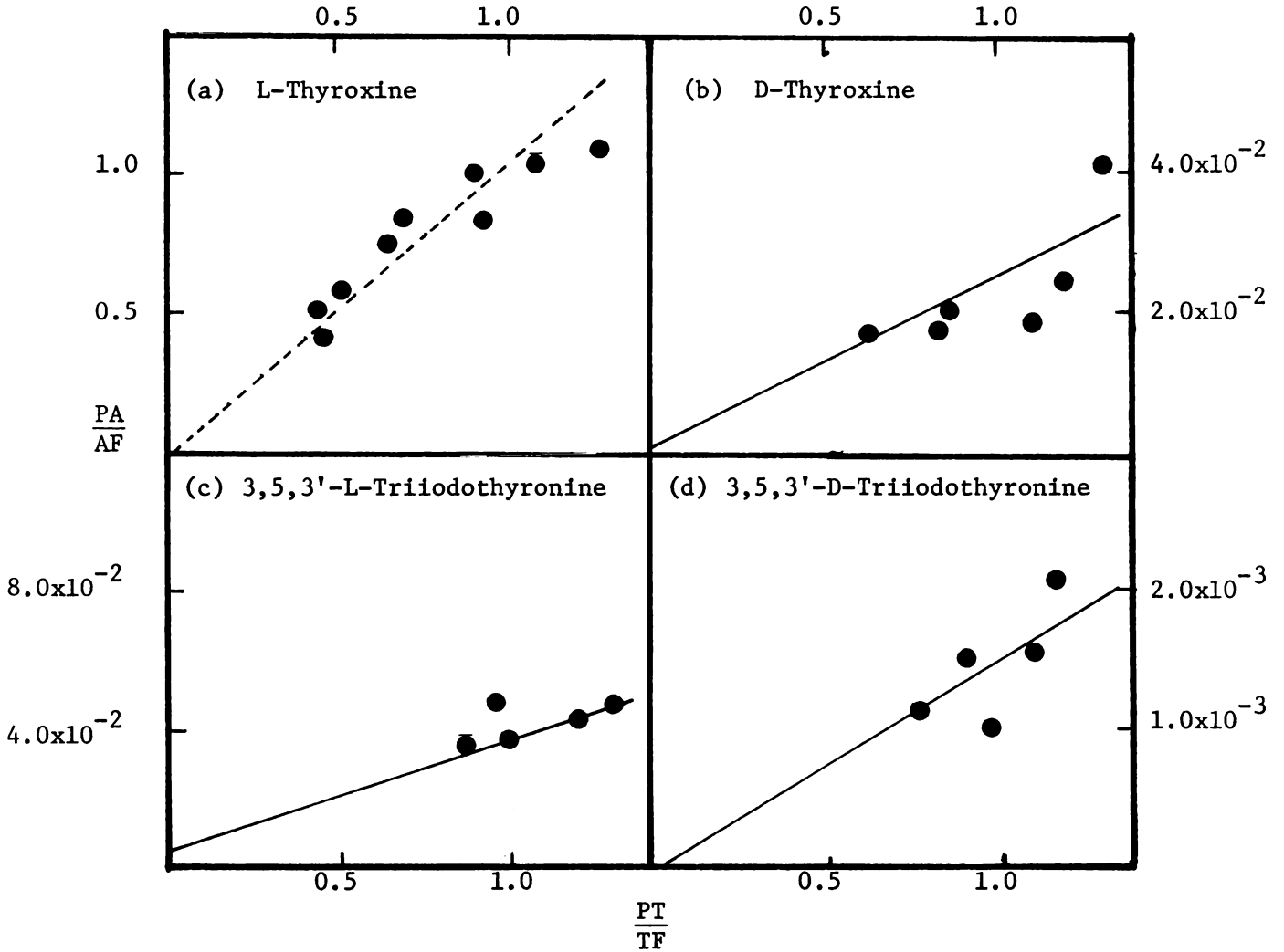


Figure 5-12. Plots of equation 5-18 for thyroid hormones and various analogs. The ordinates and abscissas of all graphs are PA/AF and PT/TF respectively.

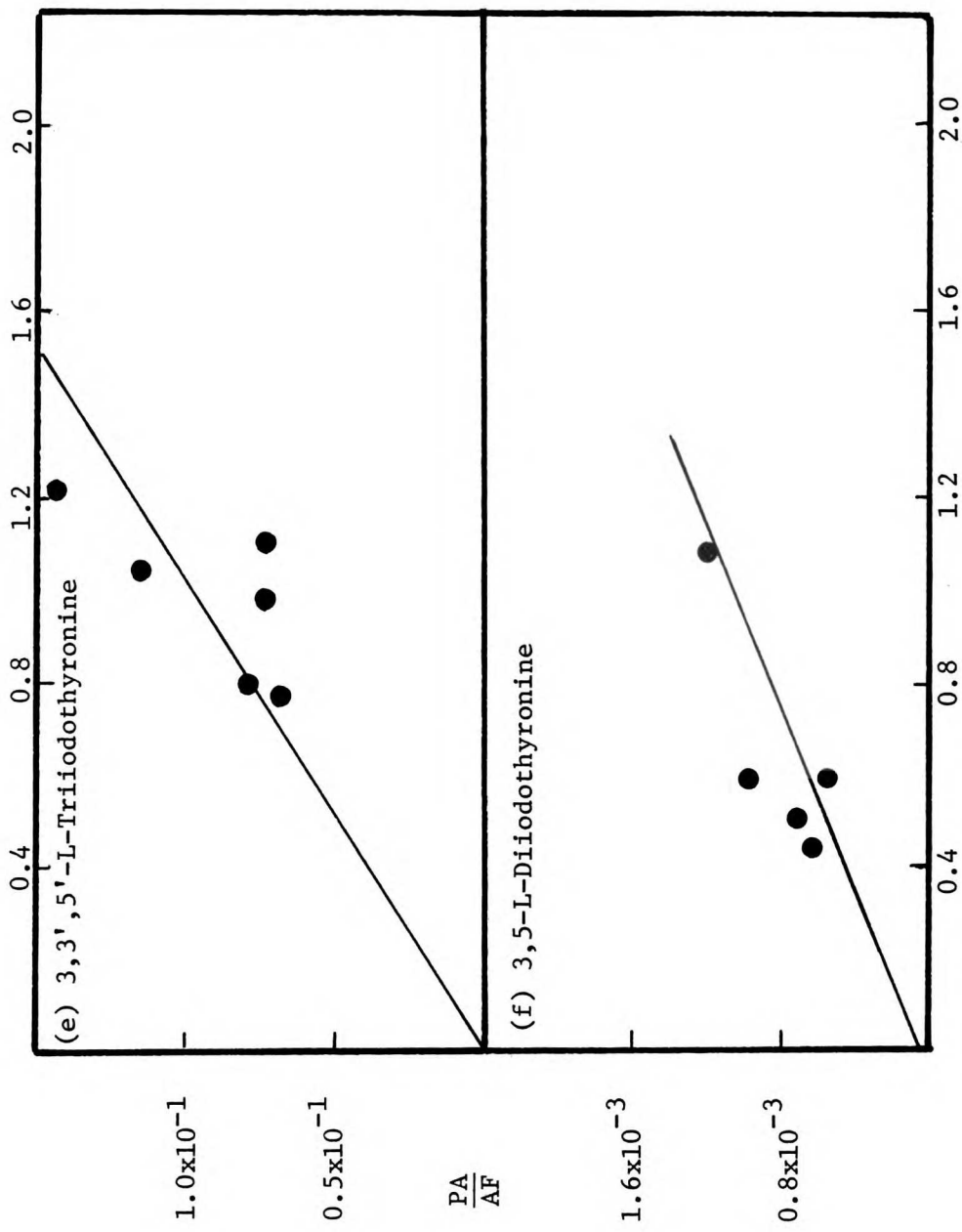


Figure 5-12 continued.

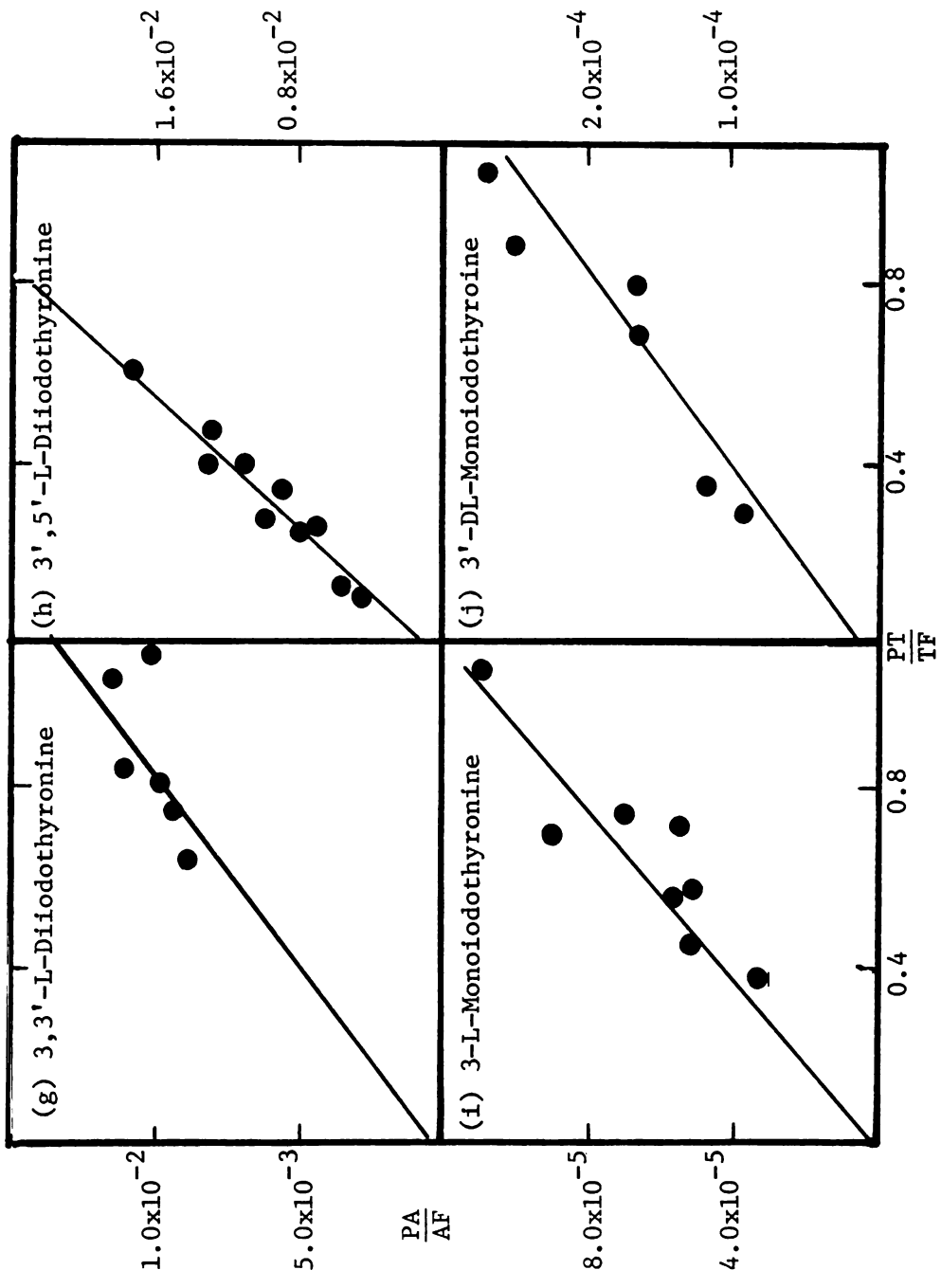


Figure 5-12 continued.

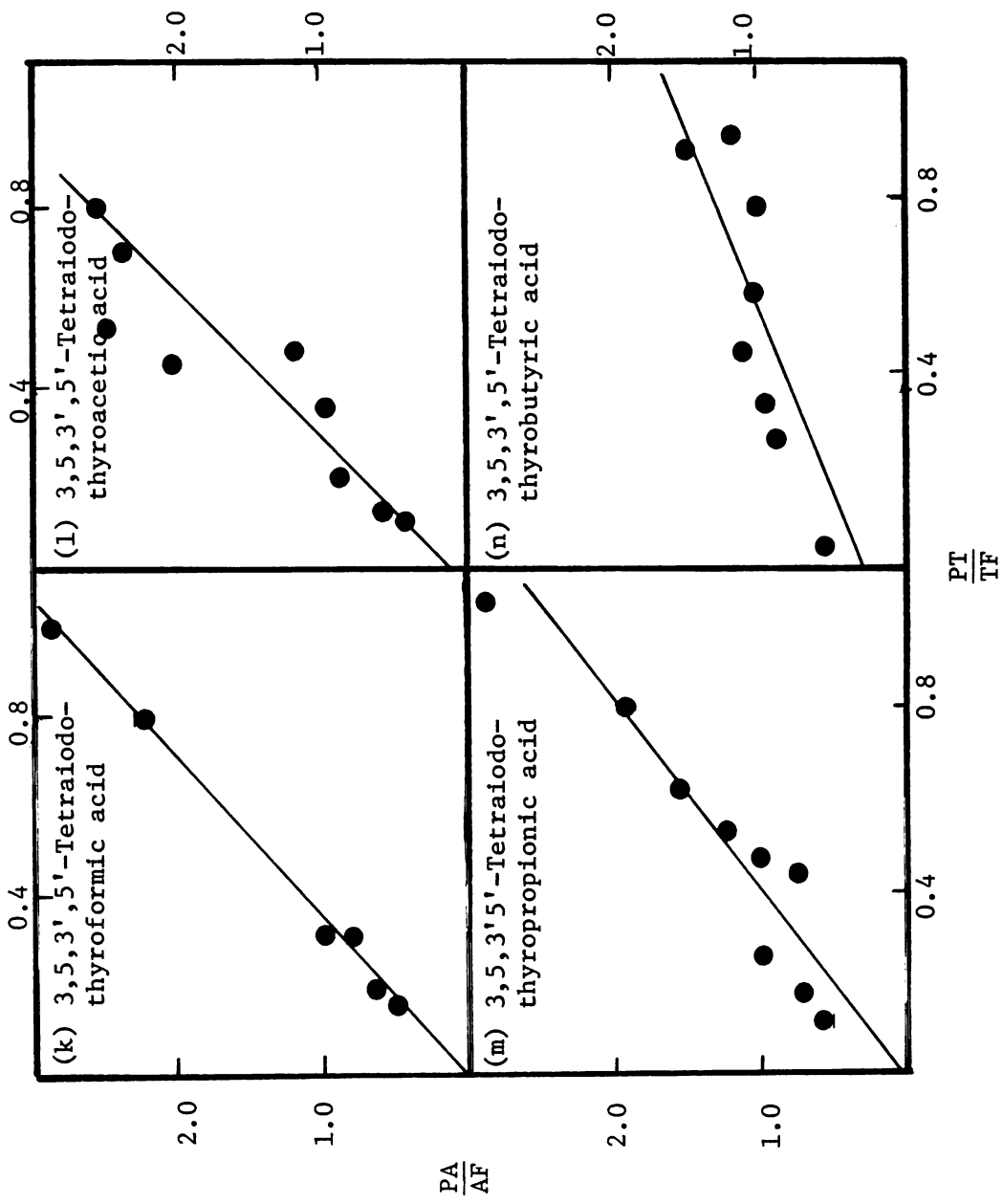


Figure 5-12 continued.

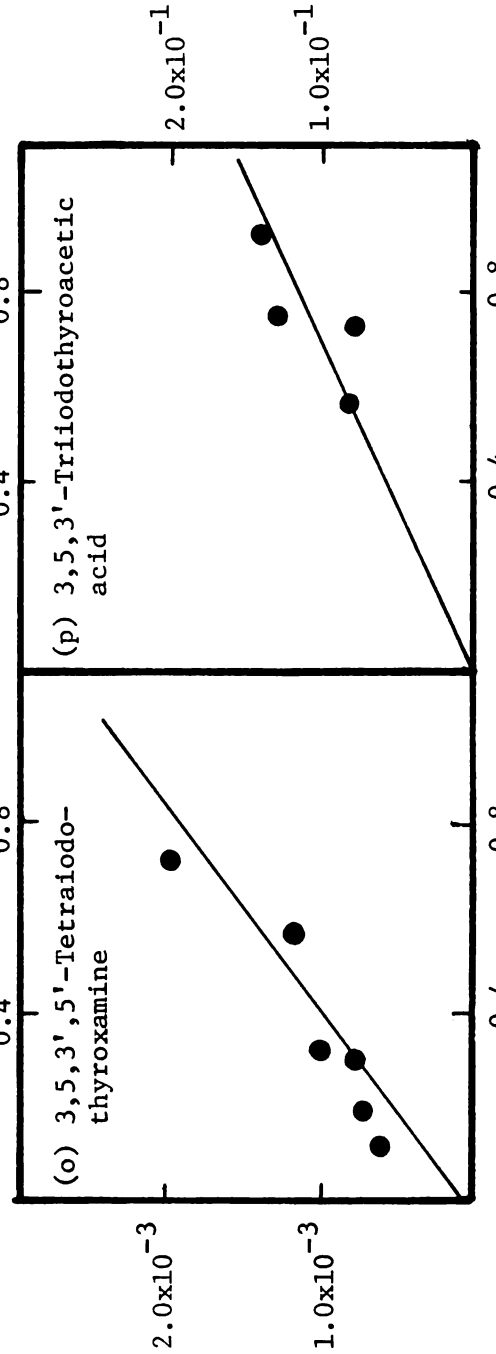


Figure 5-12 continued.

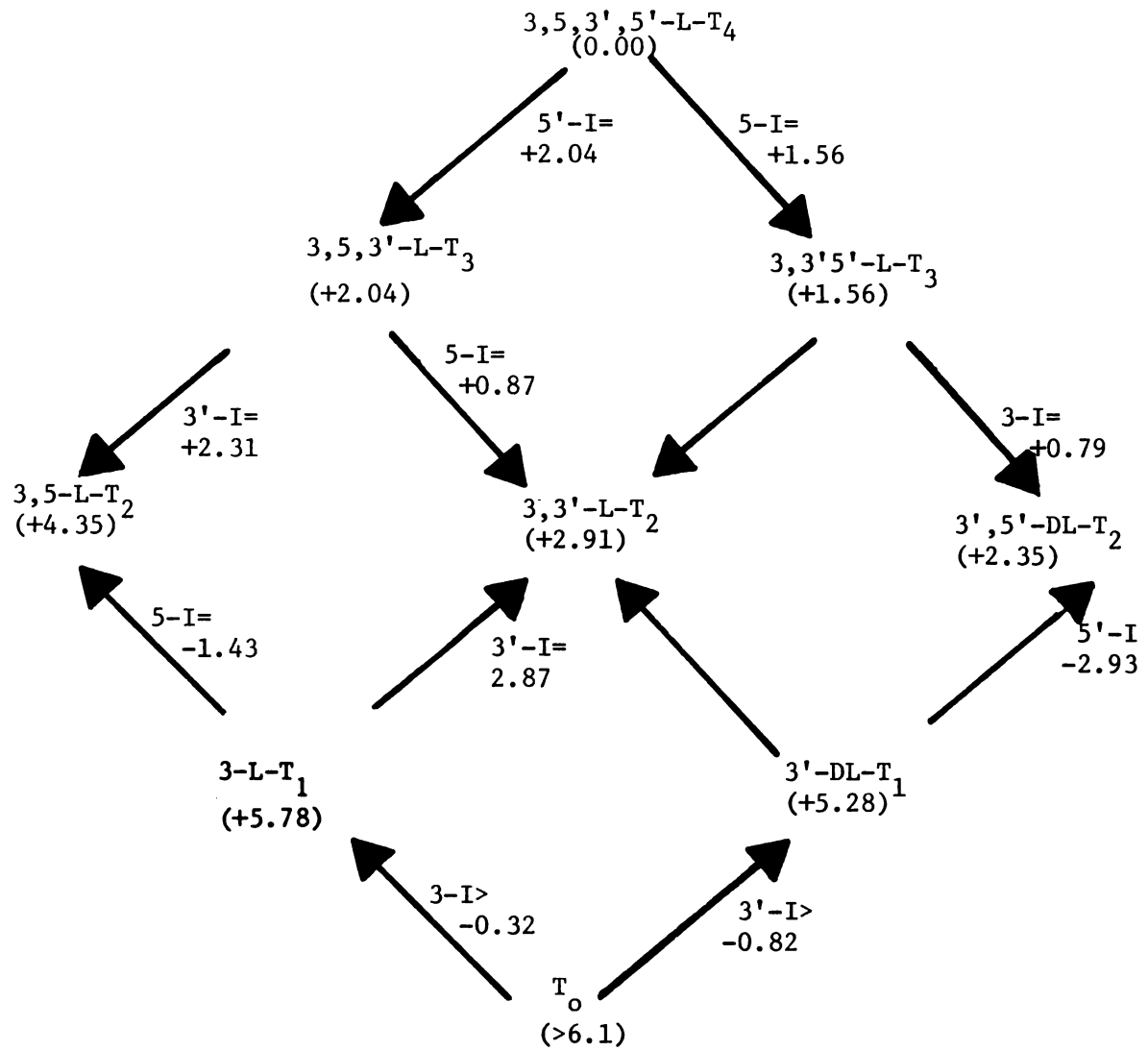


Figure 5-13. Diagramatic representation of the effect of sequential addition or removal of iodines on the free energy of binding. The numbers in brackets are the observed binding free energies ($\Delta_o G$) terms. The number of each arrow corresponds to (i). In the upper half of the diagram: change in binding free energy when the iodine atom indicated on the arrow is replaced by hydrogen. (ii) In the lower half of the diagram: change in binding free energy when the iodine atom indicated on the arrow replaces hydrogen.

Table 5-8. Linear Regression Analysis of Equation 5-18 for the Binding of Thyroid Hormones and Analogs to Prealbumin.

Analog	Slope	Intercept
$L\text{-}T_4$		
$D\text{-}T_4$	$2.41\ (\pm\ .58) \times 10^{-2}$	-7.77×10^{-4}
thyroxamine	$1.95\ (\pm\ .26) \times 10^{-3}$	1.55×10^{-4}
$3,5,3'\text{-}L\text{-}T_3$	$3.67\ (\pm\ .76) \times 10^{-2}$	7.83×10^{-3}
$3,5,3'\text{-}D\text{-}T_3$	$1.46\ (\pm\ .24) \times 10^{-3}$	-1.99×10^{-5}
$3,3',5'\text{-}L\text{-}T_3$	$7.95\ (\pm\ .21) \times 10^{-2}$	-3.16×10^{-3}
$3,5\text{-}L\text{-}T_2$	$8.57\ (\pm\ 2.3) \times 10^{-4}$	1.12×10^{-4}
$3,3'\text{-}L\text{-}T_2$	$8.85\ (\pm\ 1.48) \times 10^{-3}$	8.27×10^{-4}
$3',5'\text{-}DL\text{-}T_2$	$2.22\ (\pm\ .15) \times 10^{-2}$	1.11×10^{-3}
$3\text{-}L\text{-}T_1$	$8.39\ (\pm\ 1.05) \times 10^{-5}$	-2.80×10^{-6}
$3'\text{-}DL\text{-}T_1$	$1.89\ (\pm\ .24) \times 10^{-4}$	1.85×10^{-5}
tetraform	2.29 (± .09)	.058
tetrac	2.54 (± .38)	.14
tetraprop	1.94 (± .23)	.088
tetrabut	1.21 (± .25)	.25
triac	.119 (± .02)	-1.11×10^{-3}

Table 5-9 Affinities and Free Energies of Binding of Thyroid Hormones and Analogs to Prealbumin.

Compound	K_A/K_{T_4}	Binding Free Energy relative to L-T_4
L-T_4	1.00	0.00
3,5,3'-L-T_3	3.67×10^{-2}	+2.04
3,3',5'-L-T_3	7.95×10^{-2}	+1.56
3,5-L-T_2	8.57×10^{-4}	+4.35
3,3'-L-T_2	8.85×10^{-3}	+2.91
3',5'-DL-T_2	2.22×10^{-2}	+2.35
3-L-T_1	8.39×10^{-5}	+5.78
3'-DL-T_1	1.89×10^{-4}	+5.28
T_o	$< 5 \times 10^{-5}$	>6.1
tetraform	2.29	-0.51
tetrac	2.54	-0.57
tetraprop	1.94	-0.41
tetrabut	1.21	-0.12
D-T_4	2.41×10^{-2}	+2.29
thyroxamine	1.95×10^{-3}	+3.84
3,5,3'-D-T_3	1.46×10^{-3}	+4.02
triac	1.19×10^{-1}	+1.31

[a]Kcal/mole relative to L-T_4.

than losing (or gaining) an inner ring iodine. The only compounds in the figure for which the validity of this statement may be questioned are 3',5'-T_2 and 3'-T_1, because they were measured as DL mixtures whereas all other compounds were measured as the L enantiomers. This however, does not affect the generality of the above statement because, an L enantiomer has a higher binding affinity than the corresponding D enantiomer (see the section the side chain contributions). Thus, if the binding free energies of the L enantiomers of these two compounds are used instead of the binding free energies of the DL mixtures, the numbers on the arrows (in figure 5-13) leading to and away from these two compounds would change in a manner consistent with the above statement. The discussion presented in this paragraph as well as figure 5-13 were developed entirely by Dr. Jorgensen. They are included here because they form an integral part of the development of the subject.

The free energy contributions (as calculated by equation 3-30) of each iodine atom are also presented in table 5-10. Examining the table leads one to conclude that: each of the iodine atoms in the 3,5,3' and 5' positions contributes (-1) to (-3) kcal/mole to the free energy of binding. One may speculate on the physical chemical origin of these contributions as follows: A prealbumin molecule which is not bound to a hormone or analog molecule has its central channel occupied by water whose entropy is probably lower than bulk water. A hormone or analog molecule is surrounded by water whose entropy is also lower than bulk water. When the binding event occurs, some of the water molecules in the central channel as well as those surrounding the hormone or analog molecule are released to the bulk water with a concomitant increase in the entropy of the system.

Table 5-10. First Order Perturbation Terms for Various Groups in the Binding of Thyroid Hormones and their Analogs to Prealbumin

Iodine Atom	AX	AH	$\Delta_1 G(X)^{b,c}$
3-I	3-L-T_1	L-T_o	<-0.32
	3,3'-L-T_2	3'-DL-T_1	-2.37[a]
	3,3',5'-L-T_3	3'- 5'-DL-T_2	-0.79[a]
5-I	3,5-L-T_2	3-L-T_1	-1.43
	3,5,3'-L-T_3	3,3'-L-T_2	-0.87
	L-T_4	3,3',5'-L-T_3	-1.56
3'-I	3'-DL-T_1	L-T_o	<-0.82[a]
	3,3'-L-T_2	3-L-T_1	-2.87
	3,5,3'-L-T_3	3,5-L-T_2	-2.31
5'-I	3',5'-DL-T_2	3'-DL-T_1	-2.93[a]
	3,3',5'-L-T_3	3,3'-L-T_2	-1.35
	L-T_4	3,5,3'-L-T_3	-2.04

[a]These $\Delta_1 G(X)$ cannot be directly compared with other values obtained for the iodine in the same position because in this case either AX, AH or both is (are DL.

[b]Units are Kcal/mole.

[c]Calculated using equation (3-30).

Replacement of a hydrogen atom on an analog by iodine causes an incremental increase in size which in turn leads to an incremental increase in the amount of "low entropy water" surrounding the analog. Thus when the iodo analog is bound to prealbumin, the amount of "low entropy water" released to the bulk solvent is greater than in the case of the hydrogen analog. There is hardly anything novel in these ideas. They are essentially an expression of the hydrophobic effect. The observation that the contribution of each iodine is (-1) to (-3) kcal/mole is consistent with the observation that the contribution of an iodine atom to the free energy of transfer of a compound from water to an organic solvent is about -1.5 kcal/mole (as calculated from the π value of iodine). It is also conceivable that there are some enthalpic differences in the binding of iodo and hydrogen analogs. The observation that the 3' and 5' iodine atoms contribute favorably to the binding (table 5-10) may also be interpreted on the basis that prealbumin prefers to bind the negatively ionized 4' hydroxyl rather than its unionized counterpart. This discussion suggests that further interpretations can only be made when the entropy and enthalpy of binding of the various analogs have been determined.

Finally one can demonstrate some interesting perturbative effects among the iodine atoms. Specifically, using equation 3-37, one can obtain the second order perturbation ($\Delta_2 G$) between the two members of each of the following pairs: (3-I, 5'-I), (3'-I, 5-I) and (5-I, 5'-I). The results are shown in table (5-11). The positively signed value of $\Delta_2 G$(3'-I, 5-I) indicates that the total contribution of these two iodine atoms to the strength of binding is less when they are present in the same molecule (3,5,3'-T_3) than the sum of their contributions when present in different molecules (3,3'-T_2 and 3,5-T_2). Similar analysis may be

Table 5-11. Second Order Perturbation Terms for Various Pairs of Groups in the Binding of Thyroid Hormones and Their Analogs to Prealbumin.[a]

Substituent XY	AXYH	AXHH	AHYH	AHHH	$\Delta_2G(XY)$
(3'-I,5-I)	3,5,3'-T_3	3,3'-T_2	3,5-T_2	3-T_1	+0.56
(3-I,5'-I)	3,3',5'-T_3	3,3'-T_2	3',5'-T_2	3'-T_1	+1.58[b]
(5-I,5'-I)	3,5,3',5'-T_4	3,5,3'-T_3	3,3'-5'-T_3	3,3'-T_2	-0.69

[a]Calculated using equations (3-30), (3-31), (3-33), and (3-37). Units are kcal/mole.

[b]One may question the validity of the number (+1.58) for Δ_2G(3-I,5'-I) on the basis that 3',5'-T_2 and 3'-T_1 were measured as DL mixtures. The DL effect however, almost cancels in equation (3-31) for AHYH = 3'5'-DL-T_2 and AHHH = 3'-DL-T_1 because both compounds are measured as DL mixtures. Moreover, when the Δ_1G terms from equations (3-31) and (3-33) are substituted into equation (3-37), the Δ_oG(3'-T_1) cancels regardless of whether 3'-T_1 was measured as D, L or DL mixture. Thus we feel that a value of (+1.56) for Δ_2G(3-I, 5'-I) is reasonably accurate.

done for the 3 and 5' iodine atoms. The physical origin of these perturbative effects is not known. It is intriguing to note here that in chapter 3 section VI we demonstrated that these second order perturbation terms are negatively signed (the groups reinforce each other's contribution) in thyroxine binding globulin whereas $\Delta_2 G$(5-I, 5'-I) is almost zero in the case of nuclear binding.

2- The Contributions of the Side Chain

By far, the most interesting observations are in the binding of the side chain analogs. The binding affinities and binding free energies are shown in table 5-9.

The binding affinity increases in the order: thyroxamine < D-T_4 < L-T_4 < tetraprop. There are four charged amino acid residues extending from each monomer into the binding channel in the immediate vicinity of the location of the hormone. At the pH of the experiment, two of these (Glu 7 and Glu 54) are negatively charged and two (Lys 9 and Lys 15) are positively charged. Cheng, et al. (81) found that Lys 15 is exclusively alkylated in affinity labelling experiments. In later reports (82) they found that Lys 9 is alkylated with about eight times the frequency of Lys 15 and that Gly 1 (which resides at the entrance of the channel) is also alkylated to a certain extent. Molecular models of prealbumin show that in the region between Lys 15 and Lys 9 there are four positively charged and two negatively charged groups (each monomer contributes Lys 9, Lys 15 and Glu 54). Our observation of the above mentioned order of binding affinities can be interpreted as follows: (i) The carboxylate anion of tetraprop is experiencing electrostatic repulsion from two negative charges and attraction from four positive charges and hence it has a net of two attractive interactions. (ii) Addition of a positively charged ammonium group to

tetraprop to form L-thyroxine leads to a decrease in binding affinity because the extra ammonium group experience repulsion from four positively charged groups and attraction from two negatively charged groups and hence adds two net repulsive interactions. (iii) A change of configuration from L to D leaves the carboxylate its most favourable interaction site (say, the energy well around the region of highest electrostatic potential) but alters the position of the thyroxine ammonium groups in such a way to decrease its attraction with the negative glutamate and/or increase its repulsion with the lysines. This results in the observed decrease in affinity of D-T_4 relative to L-T_4. (iv) Removal of the carboxylate anion of D-T_4 to form thyroxamine, leads to a loss of two net attractive interactions and a concomitant decrease in binding affinity. To summarize: the side chain of tetraprop, L-T_4, D-T_4 and thyroxamine have a net two attractive, zero, zero and two repulsive interactions with groups in their immediate vicinity.

The observation that the L enantiomer binds with higher affinity than the D is duplicated in the case of T_3. Moreover, we note that the difference in the binding free energy between D and L-T_4 (-2.29 kcal/mole in favour of the L enantiomer) is almost identical with the difference between D and L-T_3 (-1.98 kcal/mole also in favour of the L enantiomer).

With regard to the homologous series of analogs having one carboxylate anion in the side chain, the affinities are in the order: tetraform < tetrac > tetraprop > tetrabut. This observation may be interpreted as follows: The diphenyl ether moiety of the analogs is anchored deep in the binding channel. Both tetraform and tetrac experience attractions mainly from the Lys 15's. Tetrac however, due to its additional torsional

degree of freedom can place its carboxylate out of the plane of the inner ring and can approach the positive ammonium of the lysines more closely than in tetraform, hence exhibit higher affinity. Addition of one and two methylenes to form tetraprop and tetrabut causes the carboxylate to extend into a region where it effectively "feels" the repulsions from the glutamate and hence ellicit lower binding affinities.

It should be stressed however that all these interpretations are to be regarded as tentative. If they are valid, the following conditions should be satisfied: (i) We have implicitly assumed that the variation of the binding affinity in all these side chain analogs can be explained on the basis of interaction energy. Thus, if our interpretations are correct, experimental determination of the binding enthalpy and entropy should reveal that all these analogs have almost equal binding entropies but differ in the binding enthalpy. (ii) The observed trends should be theoretically reproducible by the use of potential energy functions which retain exchange repulsions, coulombic interactions and hydrogen bonding interaction. These tests would only provide supportive evidence for the validity of the interpretations. By no means do they constitute a proof.

e- Implications of the Symmetry Properties of Biological Proteins

The L enantiomer has 25-50 times the binding affinity of the corresponding D enantiomer. The lack of reflective symmetry (mirror plane or improper axis) precludes the equality of the binding affinity of the D and L enantiomers. This can be understood by simply noting that there is not a single binding configuration in which the interaction of one enantiomer with the field of this protein is the same as the interaction of the other enantiomer with the same field. This observation has the following implications:

1- Any protein composed of all L or all D amino acids lacks reflective symmetry. The proof of this statement is almost trivial: any reflection operation changes a D enantiomer into an L enantiomer and vice versa, thus a protein composed of only one type of enantiomers cannot have reflective symmetry. Most (if not all) known biological proteins are exclusively composed of L amino acids.

2- The binding affinity of the D and L enantiomers to any protein that lacks reflective symmetry are unequal. Their difference however, may not be large enough to measure experimentally.

3- A compound having two asymmetric centers has four diastereomers which can be grouped into two enantiomeric pairs: a(DD - LL) pair and a (DL - LD) pair. Since the two members of each pair are enantiomeric, they have different binding affinities to a protein which lacks reflective symmetry. Moreover, the affinity of either member of one pair to a protein which lacks reflective symmetry is not equal to the affinity of either member of the other pair to the same protein.

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