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Molecular interactions between thyroid hormone analogs and the solubilized rat liver nuclear receptor

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MOLECULAR INTERACTIONS BETWEEN THYROID HORMONE ANALOGS AND THE SOLUBILIZED RAT LIVER NUCLEAR RECEPTOR

by

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DISSERTATION

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[redacted]

ABSTRACT

MOLECULAR INTERACTIONS BETWEEN THYROID HORMONE ANALOGS AND THE SOLUBILIZED RAT LIVER NUCLEAR RECEPTOR

Michael Bruce Bolger

Ph.D. Dissertation

Department of Pharmaceutical Chemistry

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San Francisco

Chromatin-localized non-histone receptors, which may mediate the action of thyroid hormones, can be solubilized from rat liver nuclei. A study of the binding affinities of L-[3'-^{125}I]3,5-triiodothyronine and a series of thyroid hormone analogs to the solubilized nuclear receptor has provided information about the nature and magnitude of structural and stereochemical interactions in the hormone-receptor complex.

Five new thyroid hormone analogs were synthesized and tested for thyromimetic activity in the rat antigoiter bioassay and/or in binding to the solubilized rat liver nuclear receptor. The receptor binding affinities of 55 thyroid hormone analogs were determined in a competitive binding assay with L-[3'-^{125}I]3,5-triiodothyronine. The results of the binding assay for these analogs were used to: (1) develop a quantitative correlation between their measured *in vitro* binding affinity and their known *in vivo* antigoiter biological activity and (2) calculate for each analog the free energy of binding (ΔG^{o}) in order to determine, by first and second order partitioning of free energies, the nature and magnitude

of specific substituent interactions with the receptor.

The first analysis of the enthalpic and entropic contributions to the free energy change for L-[3'-^{125}I]3,5-triiodothyronine binding to the solubilized nuclear receptor was conducted by examining the temperature dependence of the equilibrium association constant and the kinetic rate constants.

A computergraphic study, utilizing the x-ray coordinates of the plasma transport protein, human plasma prealbumin, was undertaken in order to generate stereo-views of a theoretical binding interaction between L-3,5, 3'-triiodothyronine and prealbumin.

From these studies it has been determined that <u>in vivo</u> antigoiter activities correlate well with <u>in vitro</u> binding to the solubilized rat liver nuclear receptor when adjustments are made for <u>in vivo</u> metabolism of 4'-deoxy and 4'-methoxyl analogs. This result further supports the physiological relevance of the nuclear receptor as an initial mediator of <u>in vivo</u> hormonal activity.

The specific molecular interactions between 3,5,3'-triiodothryonine substituents and the receptor ($\Delta G^{o}_{T_3}$ = -12.4(±0.2) kcal/mol) can be summarized as follows: (1) The 4'-hydroxyl participates in a donor hydrogen bond oriented toward the 5'-side of the outer ring and adds -1.2(±0.2) kcal/mol of binding free energy. (2) The 3'-substituent participates in hydrophobic and van der Waals bonding with a size limit at isopropyl, and adds to the strength of the 4'-hydroxyl interaction. The contribution to the binding free energy of a 3'-iodine is -4.1(±1.2) kcal/mol. (3) The optimal 3,5-substituents are iodine atoms which can each contribute an average of -2.6(±0.7) kcal/mol. (4) The alanine side chain probably participates in an electrostatic attraction between the carboxylate anion and a positively charged amino acid residue in the receptor and adds -1.9(±0.8) kcal/mol to the binding free energy.

From the temperature dependence study of the association and rate constants, it has been shown that the predominant driving force for complex formation is contributed by the enthalpy change ($\Delta H^{o}_{T_3}$ = -11.0 (±0.8) kcal/mol) which represents a decrease in the binding free energy due to formation of specific electrostatic bonds, hydrogen bonds, and dispersion force interactions. It is suggested that a trade-off between favorable hydrophobic interactions and restriction of the translational and rotational degrees of freedom of L-3,5,3'-triiodothyronine results in a very small contribution of entropy to the binding free energy.

The postulated model of prealbumin binding, based on computergraphic orientation of L-3,5,3'-triiodothyronine in between two subunits of the tetrameric prealbumin, illustrates the first specific molecular interactions between substituents on a hormone and the amino acid side chains of a biologically important protein with which it associates in a structurally specific manner.

ACKNOWLEDGEMENTS

I would like to express my sincere appreciation to the following persons who helped me to write this dissertation:

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To my parents, Bruce and Marie, for providing continual encouragement, love, and the ability to accomplish a goal.

To Penny, who provided the reserve energy, love, and encouragement that made me enjoy the last two years.

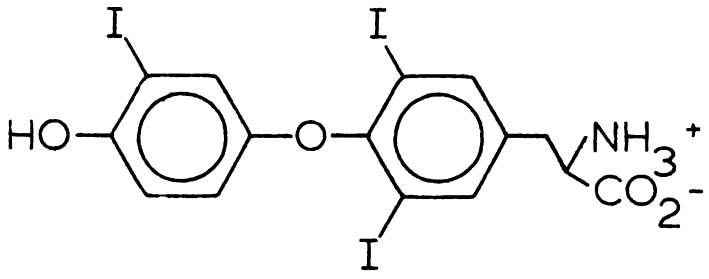
TABLE OF CONTENTS

Chapter One: Introduction

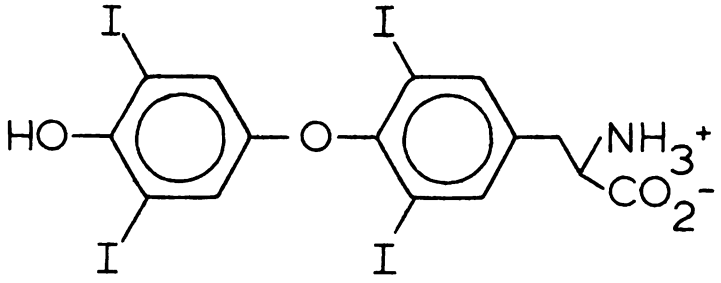
Many drug induced biological responses are recognized to be mediated by the initial formation of a reversible complex between the drug and its cellular receptor. The structural and stereospecificity of the ligand-macromolecular complex formation results in the diversity of response observed for the interaction of living systems and organic molecules. These receptors must be able to translate complex formation into a specific biological response. Investigation of ligand-macromolecule complex formation and determination of the magnitude and nature of the molecular interactions between drugs and their cellular receptors are the first steps toward understanding the physical basis for the relative efficacy of drug molecules. This dissertation presents a study of the changes in free energy, enthalpy, and entropy which accompany the complex formation and are used to determine the strength of the molecular interactions between thyroid hormone and the

solubilized rat liver nuclear receptor.

Two of the endogenous thyroid hormones, 3,5,3'-triiodo-L-thyronine (T_3) and 3,5,3',5'-tetraiodo-L-thyronine (T_4 or thyroxine), are seen in Figure 1-1.



3,5,3'-Triiodo-L-thyronine (T_3)



3,5,3',5'-Tetraiodo-L-thyronine (T_4)

These hormones, produced in the thyroid gland, show a wide variety of biological activity. They have been shown to induce metamorphosis in amphibia[1] and jellyfish.[2] In man, for example, the lack of thyroid hormones can result in severe developmental deficiencies in the central nervous,[3] respiratory, and skeletal systems.[4] Thyroid hormones also have marked influences on metabolism and thermogenesis in the adult.[5]

The apparent diversity in thyroid responsive tissues has contributed to the slow progress in defining the biochemical basis of thyroid hormone action. There are no well defined "target tissues" as in the case of many steroid hormones which have been used as probes for studying the regulation of gene expression, differentiation, and development.

Thyroid hormone has been shown to affect many tissues including the anterior pituitary, the liver, and the heart which are the most responsive.

On the cellular level, there are two major organelles which have been studied as the initial site of action of the thyroid hormones. Groups studying the action of the hormone on the molecular level are divided into those who suggest that thyroid hormones interact directly with mitochondria and those who suggest that thyroid hormones exert their primary effects by stimulating transcription in the nucleus. An excellent review of the literature on mitochondrial and nuclear thyroid hormone effects has been prepared by Oppenheimer and Surks.[6] Based in part on the work of Ismail-Beigi and Edelman,[7,8,9] which showed that upon hormone administration the increase in oxygen consumption followed an initial stimulation of an energy requiring system, Oppenheimer and Surks concluded that the in vitro and in vivo effects on mitochondria are not the primary effect of thyroid hormone administration, but a secondary phenomena following the stimulation of specific energy-requiring systems such as the sodium pump.

The early work of Tata and associates resulted in the first proposal that thyroid hormones initiate their action primarily by stimulation of transcription of genetic information at the nuclear level.[10] These workers demonstrated that the initial events following a pulse injection of T_3 into thyroidectomized rats are related to nuclear activity. The sequential events were shown to be as follows: an increase in the rate of incorporation of ^{14}C-orotic acid into nuclear RNA, augmentation of the activity of DNA-dependent RNA polymerase, and finally, an increase in the total production of nuclear RNA. These early changes

were followed subsequently by an enhanced rate of amino acid incorporation into microsomal proteins, an increase in mitochondrial enzyme activity (cytochrome C reductase), and an increase in mitochondrial Q_{O_2} and total body oxygen consumption.[11] The investigators concluded that the augmentation of mitochondrial respiration was a relatively late effect which appeared to be entirely dependent on new protein synthesis stimulated at the nuclear level. Despite the evidence which suggested that thyroid hormone interacted directly with the nucleus, no one had been able to isolate or detect a specific binding site for the hormone in the nucleus.

The presence of limited-capacity binding sites in the nuclei of both rat liver and kidney was first reported by Oppenheimer and coworkers in 1972.[12] As discussed in Chapter Three, these non-histone protein binding sites have been isolated, purified, and characterized and have generally become recognized as the physiologic receptors for the thyroid hormones.

At the present time, the T_3 receptor is being studied on the molecular level by a number of groups in an attempt to determine the mechanism of the thyroidal regulation of transcription. In light of the strong evidence for regulation of specific mRNA levels, Baxter and associates have investigated the effects of thyroid hormone administration on the synthesis of growth hormone by an isolated pituitary tumor cell culture system. They have found that the stimulation of cellular growth hormone production in response to thyroid hormone is associated with a similar increase in growth hormone mRNA relative to the total RNA.[13]

Utilizing formaldehyde fixation studies, Charles and associates have shown that the receptor is closely associated with the slowly sedimenting fractions of rat liver chromatin which contain most of the template

capacity for RNA synthesis.[14] In addition, MacLeod and Baxter have shown that the solubilized receptors bind to DNA and have suggested that this reaction may be the mechanism for receptor localization in chromatin.[15] These studies suggest a role for the T_3 receptor which would be somewhat analogous to the repressor or derepressor concept introduced by Jacob and Monod for the lactose operon.[16]

Another theory which has not been so actively investigated is based on the observation that T_3 stimulates an increase in the degree of phosphorylation of non-histone proteins.[17] Kleinsmith and Allfrey reported that calf thymus and rat liver nuclei contain phosphoprotein which accounts for four percent and nine percent of the dry weight of the nucleus, respectively. These values are much higher than would be expected for an enzyme in catalytic amounts and point to the conclusion that phosphoproteins are a major structural component of the cell nucleus.[18]

These workers also showed that the isolated non-histone protein contains endogenous kinase activity but no phosphorylase activity since there is a very rapid turnover of labeled ^{32}P-phosphoprotein only when it is added back to a suspension of calf thymus nuclei.[19] Since the only labeled product obtained was ^{32}P-inorganic phosphate, they concluded that the protein is probably not involved in the metabolic transfer of high energy phosphate groups from one molecule to another. They propose the hypothesis that the phosphorylation and dephosphorylation serve the purpose of modifying the structural and functional characteristics of the protein similar to the action of phosphorylase and phosphorylase kinase in regulating glycogen synthesis and breakdown. Their final theory is that the stimulated protein kinase activity, induced

by T_3 administration, may result in an increase in non-histone protein phosphorylation, which could lead in turn to enhanced template activity of chromatin and hence to the well-established increase in liver RNA biosynthesis.

Figure 1-1 shows a schematic diagram of the proposed sequence of nuclear events following thyroid hormone administration. As discussed, the exact nature of the interaction of the receptor with chromatin is not known. Previously, many groups have measured the binding affinity and biological activity of many thyroid hormone analogs. However, there have been no studies on the strength and nature of the molecular interactions between thyroid hormone analogs and nuclear receptor.

This dissertation will present the synthesis of five new thyroid hormone analogs, the results of a study of the binding affinity of thyroid hormone analogs to the solubilized rat liver nuclear receptor, and a computer-graphic study of a theoretical binding interaction between triiodothyronine and the plasma transport protein, human plasma prealbumin.

Chapter Two presents the rationale, schemes, and experimental details for the synthesis of five new thyroid hormone analogs.

Chapter Three discusses: (1) the history of the isolation and characterization of the high affinity, limited capacity binding sites for the thyroid hormones found in rat liver nuclei, (2) the ammonium sulfate solubilization of protein from rat liver nuclei, and (3) the partial purification of the solubilized receptor preparation by removal of histone proteins and some DNA.

Chapter Four describes the procedure used in the thyroid hormone analog binding assay and the methods for aquisition of data for kinetic,

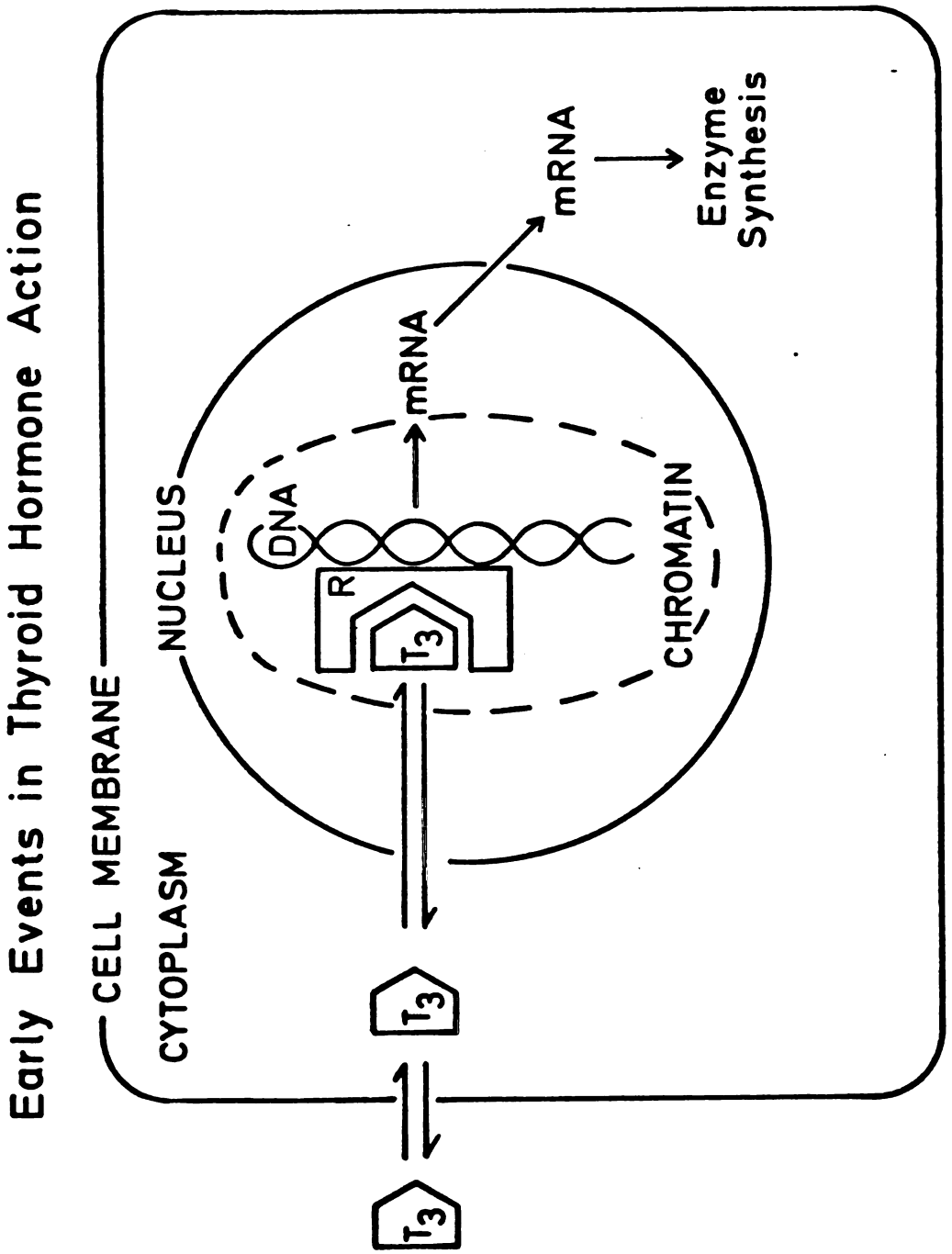

Early Events in Thyroid Hormone Action
CELL MEMBRANE
CYTOPLASM
NUCLEUS
CHROMATIN
DNA
R
T3
mRNA
mRNA
Enzyme Synthesis
T3
T3

Figure 1-2.

Scatchard, and equilibrium analog competition binding analysis. The results of the binding affinity of 50 analogs is presented as a percentage of L-T_3, and a quantitative correlation between *in vivo* rat antigoiter activity and *in vitro* binding to the soluble nuclear receptor is developed.

In Chapter Five, the binding affinities for the thyroid hormone analogs that were presented in Chapter Four are recalculated to represent a change in binding free energy. These binding free energies are partitioned into substituent group contributions in order to determine the strength of the binding interaction between the receptor and specific substituents on the T_3 molecule. Finally, second order partitioning of the binding free energies of 3'-substituted-4'-(hydroxy and deoxy)-3,5-diiodothyronines is used to determine the interactive effect of 3'-substituents on the strength of the interaction between the 4'-hydroxyl and the receptor.

Chapter Six presents the first analysis of the enthalpic and entropic contributions to the free energy change for T_3 binding to the solubilized nuclear receptor by examining the temperature dependence of the equilibrium association constant and the kinetic rate constants. A further analysis of the entropy term is made by calculation of the loss of entropy due to restriction of translational and rotational degrees of freedom in the T_3 molecule when bound to the receptor. The activation parameters $\Delta G^{\ddagger}$, $\Delta H^{\ddagger}$, and $\Delta S^{\ddagger}$ are calculated and used to obtain insights into the nature of the transition state for T_3-receptor complex formation.

Chapter Seven contains the methods and results of a computer-graphic study of a theoretical binding interaction between T_3 and the plasma transport protein, human plasma prealbumin. Utilizing the X-ray coor-

dinates of Blake and the PROPHET computer, stereo-views of a theoretical binding interaction between T_3 and human plasma prealbumin were generated. The stereo-views are described with respect to specific molecular interactions between the amino acid side chains of prealbumin and substituents on the T_3 molecule. This binding reaction is compared and contrasted with the previous description of nuclear receptor binding.

Finally, Chapter Eight presents the concluding remarks and suggestions for future research.

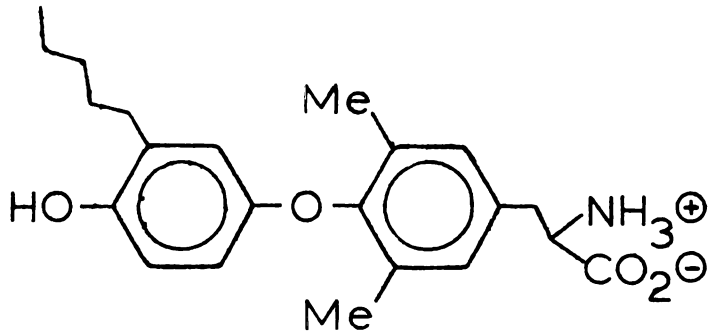
Chapter Two: Synthesis of Thyroid Hormone Analogs

SYNTHETIC RATIONALE

The synthetic projects undertaken fall into three main categories. First, intermediates were synthesized for a compound which would set the sum of the π-lipophilicity of the 3,5,3'-positions of L-thyronine to a value which should theoretically maximize biological activity for a completely halogen-free analog. Second, 3,5-diiodo-L-thyronine was brominated in the 3', and 3',5'-positions. These compounds, previously synthesized as DL-isomers, were prepared to test the anomalously low activities reported in the rat antigoiter assay.[20,21] Finally, three new 4'-deoxy-3'-halogen-3,5-diiodo-L-thyronine compounds were synthesized in order to study the strength of the 3'-halogen and 4'-hydroxy interaction with the receptor.

The rationale for the synthesis of 3,5-dimethyl-3'-n-pentyl-

L-thyronine (2-1) is clear if one looks at a plot of the logarithm of rat antigoiter activity versus the sum of the 3,5,3'-position π-lipophilicities presented in figure 2-1.[22] For a series of 3'-substituted-3,5-diiodo-L-thyronines there is a skewed parabolic relationship that shows maximal activity at a sum of π(3,5,3') ≃ 3.60. A similar trend is seen in a limited series of analogs that contain 3,5-dimethyl substitution. We proposed to test whether or not lipophilic character could be added to the 3'-position to make up for the loss due to the reduced lipophilicity of 3,5-dimethyl substitution. If such were the case, maximal activity should be achieved in non-halogenated analogs similar to that seen with 3,5-diiodothyronines. In order to achieve a Σπ near 3.6 in the halogen-free series, we proposed to synthesize the (3'-n-pentyl, 3'-n-propyl, and 3'-s-butyl)-3,5-dimethyl-L-thyronine analogs, which should have the activity indicated by the open circles in figure 2-1.



2-1

The 3'-n-propyl[34] and 3'-s-butyl[35] analogs were synthesized and tested with the results shown in closed squares. These early results showed that activity was not related to Σπ 3,5,3'. It was therefore decided to suspend the 3'-n-pentyl project. Nevertheless the scheme and experimental results for intermediate synthesis are discussed in

Table 2-1. Data for Graph (Fig 2-1)

Antigoiter Activity Versus Sum of π-3,5,3'

Substituted Thyronine DL	3,5	3'	Antigoiter Activity (% L-T_3)[a]	Sum π-3,5,3'[b]
L	I	OH	0.27	1.81
L	I	H	0.81	2.30
L	I	Me	14.47	2.81
L	I	Et	93.50	3.27
L	I	iPr	142.1	3.60
L	I	nPr	39.5	3.73
L	I	secBu[d]	79.9	3.96[d]
L	I	tBu	21.7	3.98
L	I	iBu[e]	7.74	4.10[e]
DL	I	Ph	3.50[c]	4.19
L	Me	Me	0.54	1.53
L	Me	I	0.90	2.17
L	Me	iPr	3.60	2.32
L	Me	nPr	2.36	2.45
L	Me	secBu[d]	2.91	2.67[d]
L	Me	nPentyl[f]	--	3.42[f]

a. Antigoiter data corrected to molar basis.[36]

b. From the phenoxyacetic acid system of Hansch et al.,[37] unless otherwise noted.

c. Corrected to molar basis assuming L = DL/0.58.

d. Calculated ($\pi_{3'\text{-}\underline{s}Bu}$ = 1.66) from the 4-sec-butyl-phenoxyacetic acid by multiplying by the ratio $\pi_{3\text{-}I}/\pi_{4\text{-}I}$ = 0.913.

e. Estimated $\pi_{3'\text{-}\underline{i}Bu}$ = 1.81.[36]

f. Estimated $\pi_{3'\text{-}\underline{n}Pentyl}$ = 2.40.

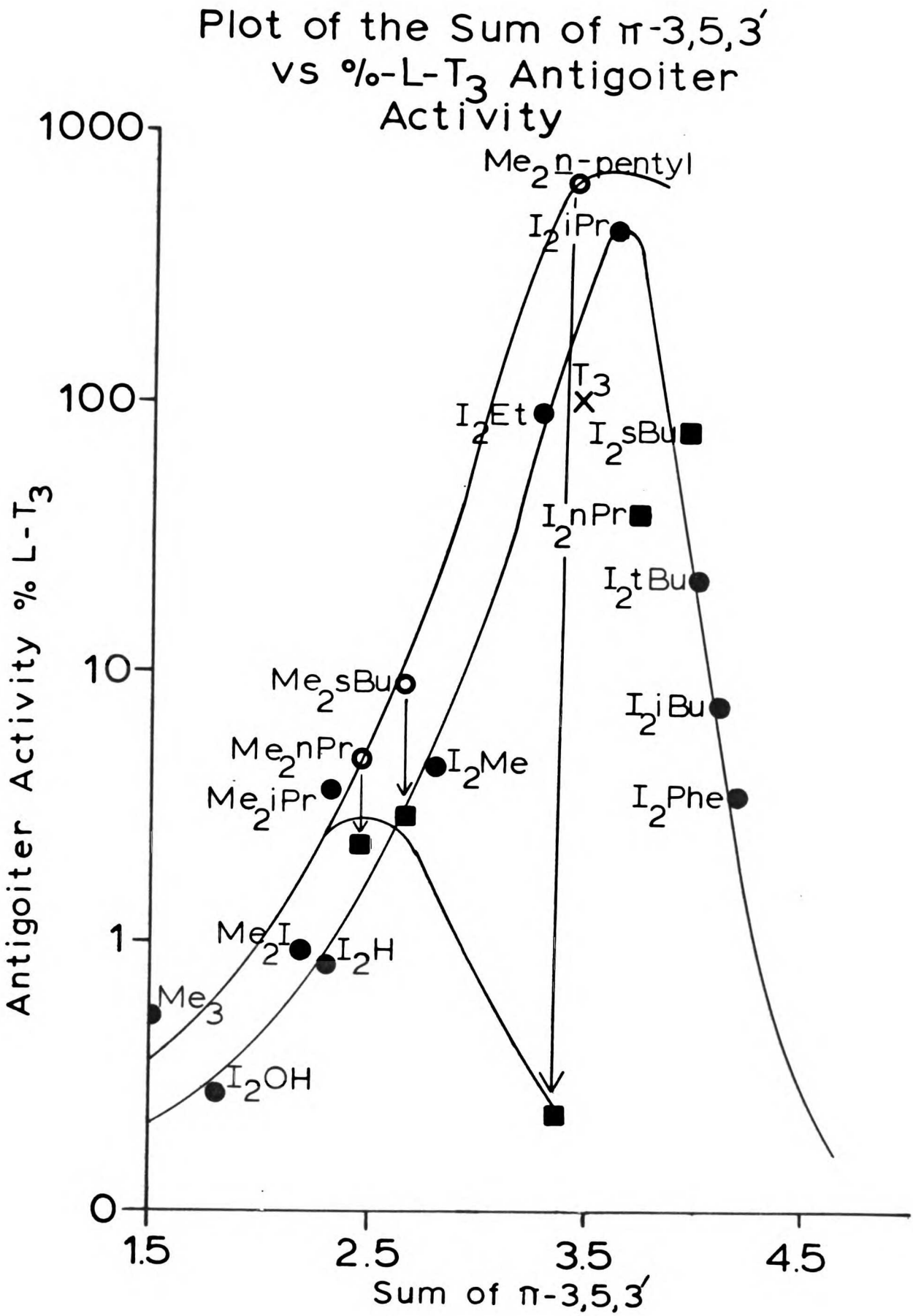
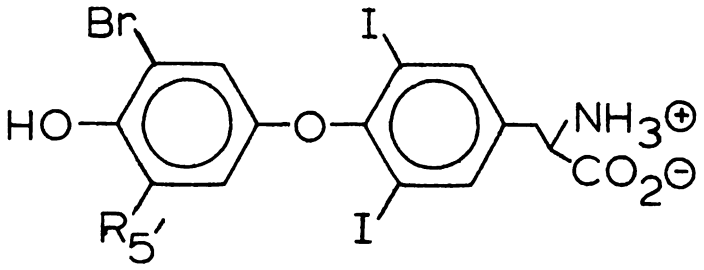

Plot of the Sum of π-3,5,3′ vs %-L-T3 Antigoiter Activity
Antigoiter Activity % L-T3
1000
100
10
1
0
1.5
2.5
3.5
4.5
Sum of π-3,5,3′
Me2 n-pentyl
I2iPr
T3
I2Et
I2sBu
I2nPr
I2tBu
Me2sBu
I2iBu
Me2nPr
I2Me
Me2iPr
I2Phe
Me2I
I2H
Me3
I2OH

Figure 2-1.

the following sections of this chapter. The results obtained with the 3'-n-propyl and 3'-s-butyl analogs indicate that there is probably a strict steric requirement for the 3'-substituent in the mechanism of antigoiter activity. This will be discussed in more detail in Chapter Five.

The synthesis and biological testing of 3,5-diiodo-3'-bromo-DL-thyronine and 3,5-diiodo-3',5'-dibromo-DL-thyronine (2-2) had been reported by Musset and Pitt-Rivers.[20,21]



2-2

$R_{5'}$ = H

$R_{5'}$ = Br

These investigators were the first to notice that for a given halogen the 3'-monosubstituted compound had higher biological activity than the 3',5'-disubstituted compound.

Table 2-2. Antigoiter Activity (%DL-T_3) of 3'-Mono and 3',5'-Disubstituted-3,5-Diiodo-DL-Thyronines[20,21]

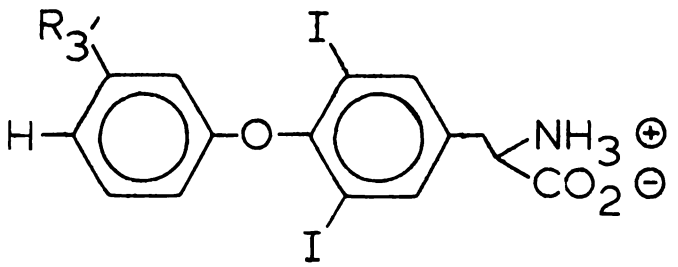
3'subs.	% Activity	3',5'-subs.	% Activity	Ratio di/mono
I	100	I	20	0.20
Br	27.6	Br	1.4	0.05
Cl[23]	4.8	Cl[23]	3.8	0.79
F	2.14	F	0.7	0.33

As shown in Table 2-2, the ratio of activities for the disubstituted/monosubstituted compounds was particularly low for the bromine analogs. One would expect the order of increasing activities for the disubstituted compounds would be the same as for the monosubstituted compounds, increasing as halogen size and lipophilicity increases from fluorine to iodine. Since the 3,5-diiodo-3',5'-dibromo-DL-thyronine is out of line with the rest of the analogs, it was decided to prepare the new compounds, 3,5-diiodo-3',5'-dibromo-L-thyronine and the 3'-bromo analog, for testing in binding to the solubilized rat liver nuclear receptor and in the rat antigoiter assay.

As will be described in Chapter Five, the development of the solubilized receptor binding assay provided the means for a direct study of molecular interactions between the hormone and the receptor binding site. One of the first tools, an idea of Tariq Andrea, was to partition the free energy of binding into specific group contributions.[24] For example, using the equilibrium association constant (K_a), and the relationship $\Delta G^o = -RT \ln K_a$: ($\Delta G_{T_3} = -12.38$ kcal/mol) and ($\Delta G^o_{T_4} = -11.23$ kcal/mol). Therefore $\Delta\Delta G_{5'-I} = +1.15$ kcal/mol. That is, +1.15 kcal of binding free energy is lost when the 5'-iodine is added to T_3 to form T_4. In order to determine directly the contribution of the 4'-hydroxyl to the free energy of binding, the 4'-H analogs of a series of 3'-substituted-3,5-diiodo-L-thyronines are compared to the corresponding 4'-hydroxy analogs. Accordingly, the 3,5-diiodo-4'-deoxy-3'-(fluoro, chloro, and bromo)-L-thyronines (2-4) were synthesized.

These compounds also allowed the calculation of the contribution of the 3'-substituent to the binding free energy by comparison of the 3'-substituted compound to the corresponding 3'-hydrogen compound. As will be discussed in Chapter Five, this type of analysis could be extended

to a development of the interactive term between 3' and 4' substituents, and led to a thorough investigation of the effect of the 3'-substituent on the strength of the 4'-OH interaction with the receptor.



2-4

$R_{3'}$ = F,Br,Cl

SYNTHETIC SCHEMES

The two general methods for synthesis of optically active analogs of 3,5,3'-L-triiodothyronine differ primarily in the method of formation of the diphenyl ether. If there is an o-R-phenol available, the shortest and most desirable method to introduce the alkyl group, R, into the 3'-position is to first methylate the phenol to form o-R-anisole. Two moles of the anisole are allowed to react with iodine-tris-(trifluoroacetate) to form the di(3-R-4-methoxyphenyl)-iodonium iodide.[25] This reactive intermediate is condensed with N-acetyl 3,5-diiodo-L-tyrosine ethyl ester to form the diiododiphenyl ether.[29]

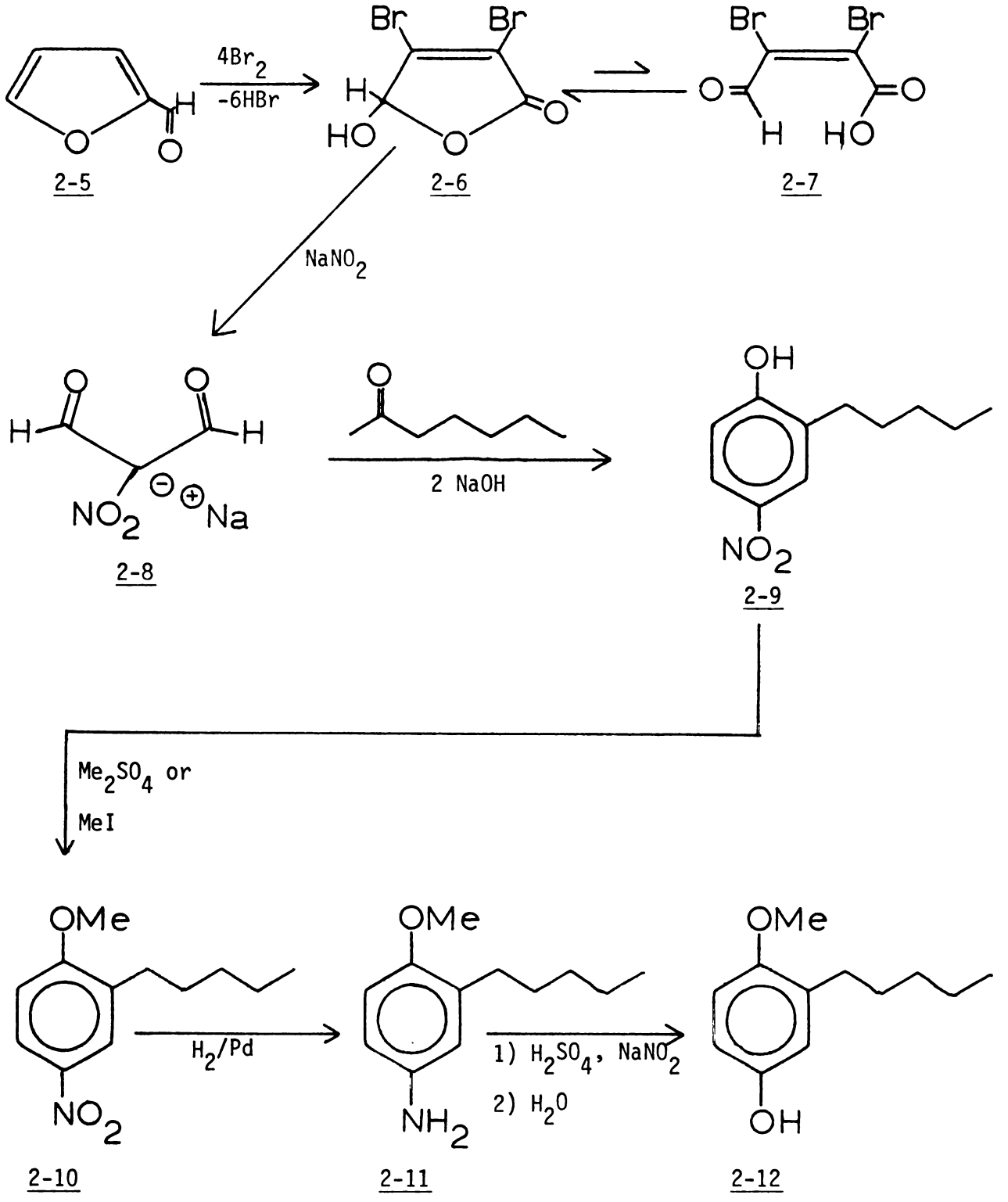
In order to prepare intermediates for the 3'-n-pentyl-3,5-dimethyl-L-thyronine in the absence of available o-n-pentylphenol, the following synthetic scheme was initiated. 2-Furfuraldehyde (2-5) was converted to mucobromic acid (2-7) with four moles of bromine in 89% yield. The reaction proceeded with loss of six moles of hydrogen bromide and one

mole of CO_2.[26] In most literature references mucobromic acid is drawn as the 2,3-dibromo-3-formyl acrylic acid (2-7). However, the IR spectrum indicates that the equilibrium lies in the direction of the α,β-unsaturated-γ-hydroxylactone (2-6). There is a broad OH stretching vibration at 3325 cm^{-1}, a sharp γ-lactone carbonyl stretching vibration at 1760 cm^{-1}, and a tetra-substituted alkene stretching vibration at 1625 cm^{-1}. This corresponds well with the literature absorbance values of Kuh and Shepard.[27] Sodium nitromalondialdehyde monohydrate (2-8) was prepared with sodium nitrite in water at 55°.[28] The yield of 35%, obtained in this synthesis, approached the literature yield of 36%.

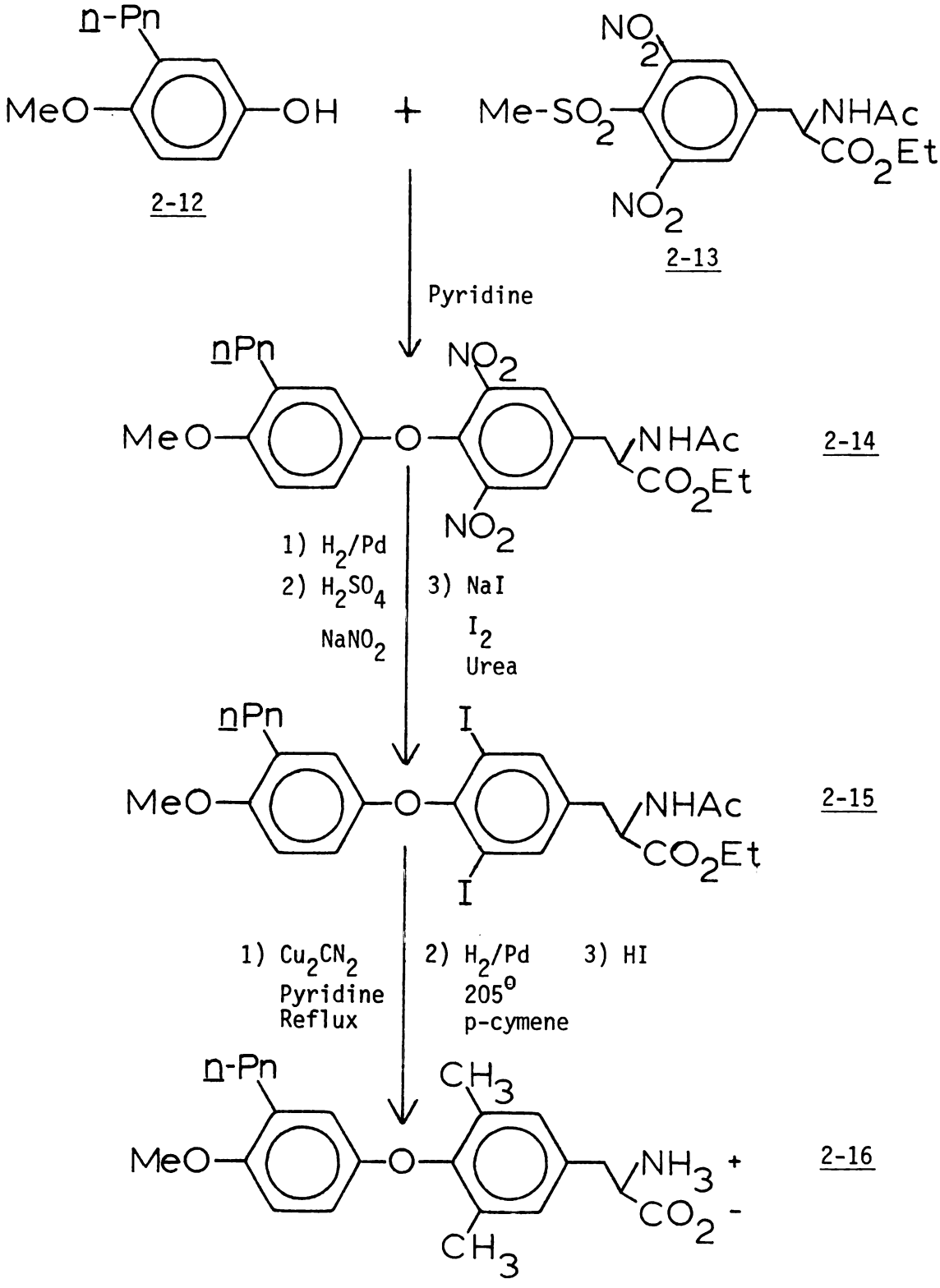
Finally, a condensation between sodium nitromalondialdehyde (2-8) and 2-octanone in the presence of a 1 M excess of 2 N NaOH gave the 2-n-pentyl-4-nitrophenol (2-9) in 50% yield.[29] We decided to abandon the synthesis at this point and conditions for preparing 2-n-pentyl-4-nitroanisole (2-10) were not worked out.

This rather complicated, unambiguous, low yield synthesis of the starting material 3-n-pentyl-4-methoxyphenol (2-11) avoids isomerization of the n-pentyl substituent which would be encountered in Friedel Crafts alkylation of p-nitrophenol. A limited study was made of a Friedel Crafts acylation followed by reduction of the 3-n-pentanoyl-4-methoxyphenol. However, the conditions were not worked out for the production of pure product. If this synthesis had been continued, 3-n-pentyl-4-methoxyphenol (2-12) would have been condensed with N-acetyl 4-methane sulfonyl-3,5-dinitro-L-tyrosine ethyl ester (2-13) in the presence of refluxing pyridine to give the protected 3,5-dinitro-3'-n-pentyl-L-thyronine (2-14). This would be reduced, diazotized, and iodinated to give the protected 3,5-diiodo-3'-n-pentyl-L-thyronine (2-15). Reaction with cuprous cyanide, reduction of the 3,5-cyano groups to methyl groups,

Synthetic pathway to the 3-n-pentyl-4-methoxyphenol



Synthetic pathway to 3,5-dimethyl-3'-n-pentyl-L-thyronine

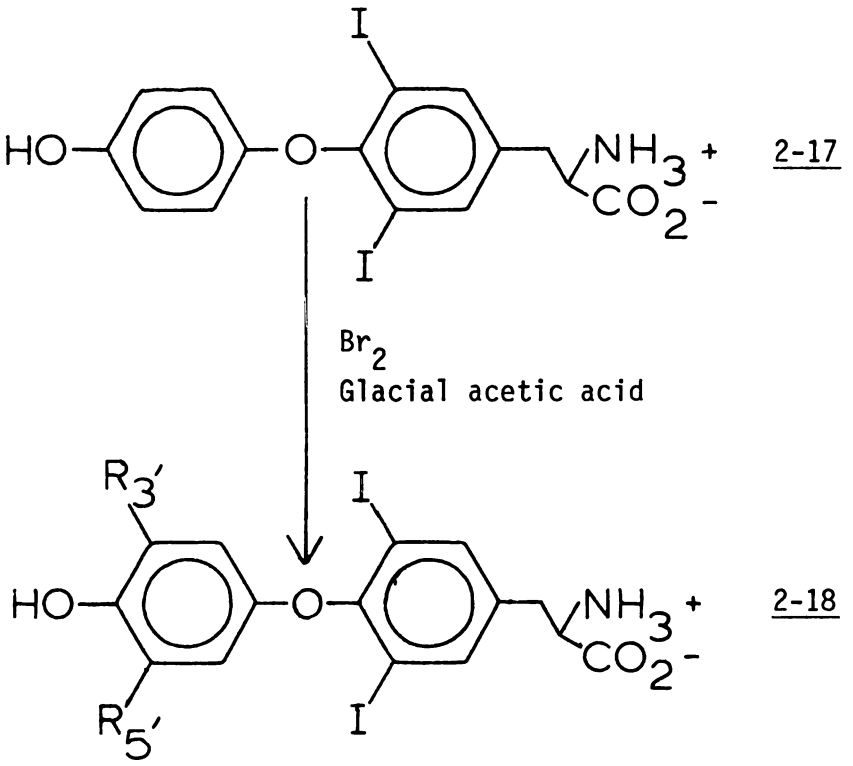


and hydrolytic removal of protecting groups would give the final 3,5-dimethyl-3'-n-pentyl-L-thyronine (2-16)

The next two new analogs, 3,5-diiodo-3'-bromo-L-thyronine (2-18) and 3,5-diiodo-3',5'-dibromo-L-thyronine (2-19) were synthesized from 3,5-diiodo-L-thyronine (2-17) by addition of a carefully controlled amount of bromine in glacial acetic acid at room temperature for 20 hours. Isoelectric precipitation of the amino acids gave 88% yield.[30]

The last three new analogs, 3,5-diiodo-4'-deoxy-3'-(fluoro, chloro, and bromo)-L-thyronines, were prepared by a synthetic route similar to that used for the formation of N-acetyl 3,5-diiodo-3'-n-pentyl-L-thyronine ethyl ester (2-15). Resublimed 3-(fluoro, chloro, and bromo)-phenols (2-20, 2-21, 2-22) were condensed with N-acetyl 4-methane sulfonyl-3,5-dinitro-L-tyrosine ethyl ester in refluxing pyridine to produce the N-acetyl 3,5-dinitro-4'-deoxy-3'-(fluoro, chloro, and bromo)-L-thyronine ethyl esters (2-23, 2-24, 2-25) in 60 to 70% yield.[31] These compounds were reduced, diazotized, and iodinated to yield the N-acetyl-3,5-diiodo-4'-deoxy-3'-substituted-L-thyronine ethyl esters (2-26, 2-27, 2-28).[29] Finally, the hydrolysis of protecting groups with refluxing concentrated hydrochloric acid and glacial acetic acid gave the desired 3,5-diiodo-4'-deoxy-3'-substituted-L-thyronines (2-29, 2-30, 2-31).[31] Reduction of the 3'-bromo compound required the use of iron, acetic acid, and hydrochloric acid, since catalytic reduction, following a procedure of J.H. Barnes et al.,[32] gave rise to a final amino acid which is slightly high (0.45%) in carbon and slightly low(0.77%) in iodine. No attempt was made to modify reaction conditions, or to furthur purify the product due to lack of time and the knowledge that inner ring mono or di-deiodination would give rise to only low-affinity, low-activity compounds,

Synthetic pathway to 3,5-diiodo-3',5'-dibromo-L-thyronine



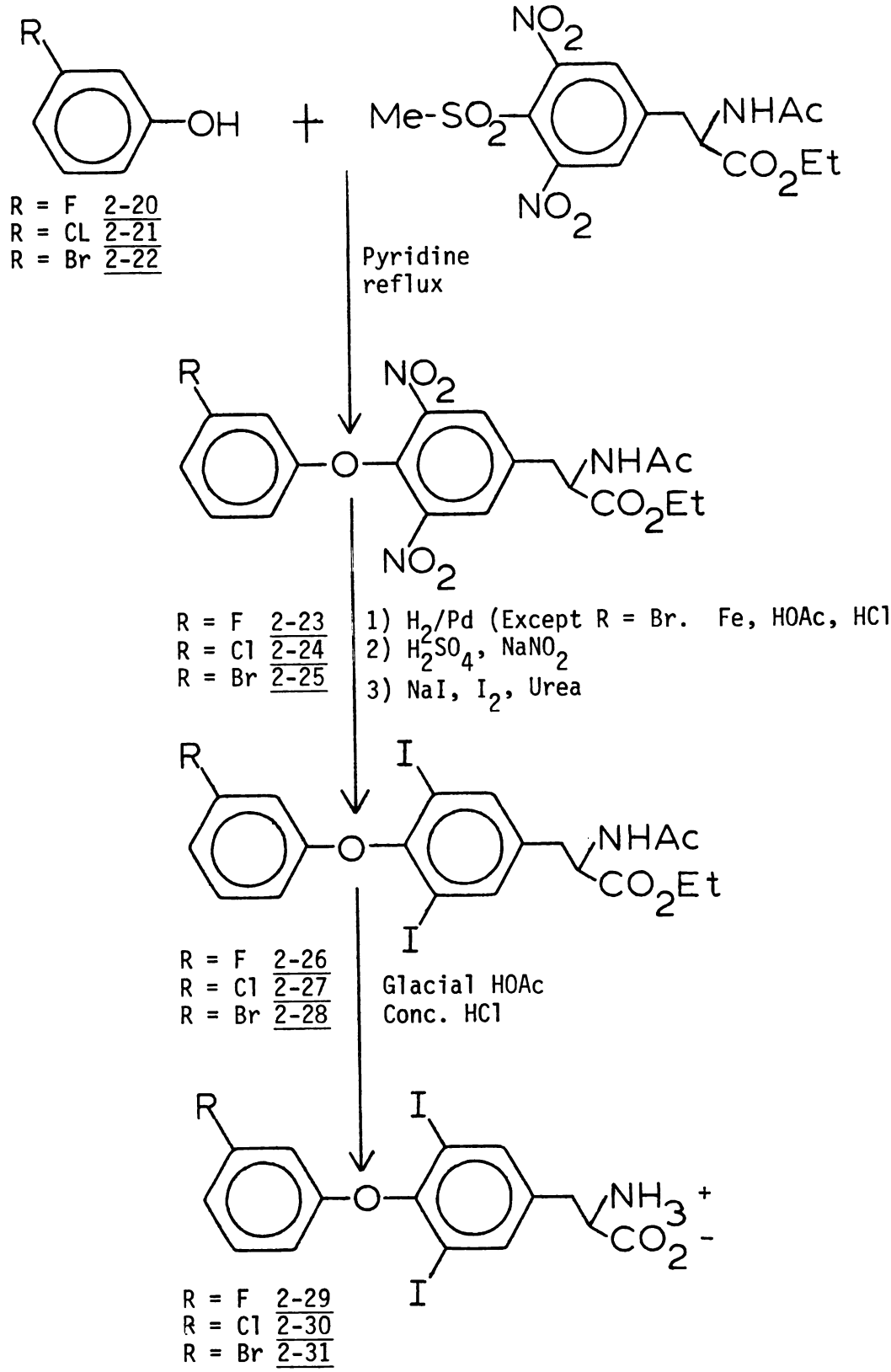
2-17

2-18

$R_{3'}$ = Br $R_{5'}$ = H 2-18

$R_{3'}$,$R_{5'}$ = Br 2-19

Synthetic pathway to 3,5-diiodo-4'-deoxy-3'-substitiuted-L-thyronine



which would produce minimal interference in biological studies in vivo and in vitro.

EXPERIMENTAL SECTION

Melting points, determined with a Thomas-Hoover Uni-Melt stirred oil capillary tube melting point apparatus, are uncorrected.

Proton magnetic resonance (PMR) spectra were determined at 60 MHz with a Varian Model A-60A PMR spectrometer. PMR chemical shift values are expressed in δ units (parts per million) relative to a TMS or sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) internal standard. For the presentation of the PMR spectra, the following abbreviations are used: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, comp m = complex multiplet. Infrared (IR) spectra were recorded with a Perkin-Elmer Model 337 grating infrared spectrometer (P.E.) and a Beckman Acculab 4 (Beck). Microanalyses were performed by the Microanalytical Laboratory, University of California, Berkeley, Calif. Optical rotations were measured with a Perkin-Elmer Model 141 Polarimeter (microcell: 10 cm path length, 1 ml cell volume).

Thin layer chromoatography (TLC) was routinely used to check the purity of samples, to follow the progress of reactions, and to analyze fractions obtained by column chromatography. For presentation of the TLC data, the following abbreviations are used for plate types: A = pre-coated silica gel sheets with fluorescent indicator (100 μ coating on flexible plastic sheets Eastman Kodak Company, #6060); B = pre-coated 4-channel silica gel plates with fluorescent indicator (250 μ coating on glass with pre-absorbent loading zone; Quantum Industries, Inc.,

#5052); C = precoated alumina sheets without fluorescent indicator (100 μ coating on flexible plastic sheets; Eastman Kodak Company, #6062).

2,3-Dibromo-3-formylacrylic acid (2-7).[26] To 200 ml (2.41 moles) of recently distilled 2-furfuraldehyde (2-5) in two liters of water, cooled to 0 to 5°, bromine liquid (1.82 Kg, 11.33 moles) was added dropwise for 5 hr with overhead stirring while the temperature was maintained at less than 5°. The dropping funnel was replaced with a reflux condenser and the mixture was heated with a heating mantle and allowed to reflux at 70° for 30 min while excess bromine vapor flowed from the reaction vessel. The resulting clear orange solution was distilled until the colorless distillate indicated no excess HBr. After cooling to room temperature, white crystals fell out and were filtered and washed with cold H_2O. Crystals were dried to give the product (481.1 g, 89.6%; lit.[26] 83%). Mp 119–123° (lit.[26] 124-125°). IR (mull, halocarbon oil) (PE), 3325 cm^{-1} (C–OH), 1760 cm^{-1} (α,β-unsaturated-γ-lactone), 1625 cm^{-1} (alkene).

Sodium nitromalonaldehyde monohydrate (2-8).[28] Sodium nitrite (258 g, 3.74 moles) was dissolved in 250 ml of water with slight heating. When the temperature reached 54°, mucobromic acid (2-7) (258 g, 1.0 mole) in 250 ml of warm 95% EtOH was added while maintaining the temperature at 54 ± 1°. After addition was complete, the temperature was maintained at 54 ± 1° for 10 min more. The reaction was then cooled to 0 to 5° and the yellow precipitate was collected on a previously chilled Buchner funnel. The moist cake was recrystallized from EtOH and H_2O after hot filtration to remove an insoluble yellow precipitate. Tan crystals were dried in air away from light. (CAUTION: The sodium salt of nitromalonaldehyde is impact sensitive and thermally unstable and should be handled as a

potentially explosive material.) Obtained (27.4 g, 35%; lit.[28] 36%). IR (KBr pellet) (P.E.), 1650 cm^{-1} (α,β-unsaturated, aliphatic aldehyde).

2-n-Pentyl-4-nitrophenol (2-9). This compound was prepared by the general method of Blank et al.[29] First, 2-octanone (Aldrich Chem. Co. 93%, bp 173 °C, n_D^{20} 1.4150) was redistilled. To an aqueous solution of sodium nitromalondialdehyde (2-8) (29.9 g, 0.190 moles) in 150 ml of H_2O was added a 10% solution of 2-octanone (25.6 g, 0.2 moles, d^{15} 0.819 g/ml, 31.3 ml) in 95% EtOH. This was followed by addition of a 1 M excess of 2 N NaOH (0.38 moles, 190 ml of 2 N NaOH). The reaction mixture was allowed to stir for 45 hr at room temperature. The EtOH was removed with a rotary evaporator under reduced pressure. The resulting yellow crystals were filtered and the mother liquor was cooled and filtered again. The combined product was recrystallized from ethyl-acetate after hot filtration with decolorizing carbon. The yellow,sodium·monohydrate was obtained (20 g, 40%). Mp 197-200° (Yellow·H_2O---Red non-hydrated). IR (KBr pellet) (Beck) 3450 cm^{-1} ($(CH_2)_n$ stretch), 1580 cm^{-1} and 1300 cm^{-1} (aromatic-NO_2). PMR (60 MHz) (D_2O) 1.05 (t, J = 5.0 Hz, 3H, n-pentyl-CH_3), 1.56 (comp m, 6H, n-pentyl-CH_2-Ar), 6.70 (d, J = 10 Hz, 1H, Ar-6H), 8.17 (m, 2H, Ar-3,5 H). Analysis: $C_{11}H_{16}NO_4K$ (K-salt from KOH wash)
Calculated C, 49.77; H, 6.03; N, 5.28
Found C, 50.4; H, 6.2; N, 5.4

3,5-Diiodo-3'-bromo-L-thyronine (2-18). 3,5-diiodo-L-thyronine (2-17) (Sigma lot 103C-0190) was brominated by the method of Dibbo et al.[30] In order to carefully control the bromination to obtain the maximum yield of mono brominated product, Br_2 (0.9720 g, 0.0061 mol) was weighed into 5 ml of glacial acetic acid in a 10 ml volumetric flask. The volume was brought to 10 ml with glacial acetic acid to make a 9.72%

solution. Next, 1.9 ml (1.16×10^{-3} mol) of this solution was added to a stirred solution of 3,5-diiodo-L-thyronine (2-17) (0.6 g, 1.14×10^{-3} mol) in 30 ml of glacial acetic acid plus one or two drops of concentrated HCl to help dissolve the amino acid. This mixture was stirred at room temperature for 20 hr. The reaction color changed from dark red to very pale yellow. The solvent was removed with a rotary evaporator under reduced pressure. The resulting amber glass was dissolved in 10 ml of EtOH with two drops of concentrated HCl. The pH was brought to 4.7 with 5% sodium acetate and the resulting precipitate was collected by centrifugation. The ppt was washed 3 times with H_2O to remove inorganic salts, and dried overnight in a vacuum oven at 65°. The dried product weighed 661 mg, 95% yield. TLC (UV) Rf (A: $HCCl_3$-MeOH-conc. NH_4OH/20:10:1) 0.38. A slight amount of the dibrominated product was seen at Rf 0.25. The crude product was dissolved in 10 ml of EtOH with two drops of conc. HCl, with decolorizing carbon and filtered hot to remove a small insoluble ppt. Five percent sodium acetate solution was added until cloudiness appeared. The product was allowed to crystallize two days in the refrigerator to produce (425 mg, 61% yield). Mp 245-249° (decomp.) (lit.[30] 247-249° for the D-isomer). $[\alpha]_D^{25}$ = +25.7° (c, 1.0, in HCl/EtOH/1:1) (lit.[30] for the D-isomer $[\alpha]_D^{20}$ = -25.6°). TLC (UV) Rf (A: $HCCl_3$-MeOH-conc. Me_2NH:OH/20:10:5) 0.60 shows no starting material T_2 (2-17) Rf - 0.64. Analysis: $C_{15}H_{12}NO_4BrI_2$

Calculated: C, 29.89: H, 1.99; Br, 13.23

Found: C, 29.79; H, 2.04; Br, 13.50

3,5-Diiodo-3',5'-dibromo-L-thyronine (2-19). Into a stirred solution of 3,5-diiodo-L-thyronine (2-17) (0.439 g, 8.36×10^{-4} mol) in 22 ml of glacial acetic acid with ten drops of concentrated HCl at roon temperature

was added to 4 ml (0.40 g, 2.51 x 10^{-3} mole) of a Br_2 solution (1.002 g Br_2 in 10 ml glacial acetic acid). This was allowed to react for 22 hr until a slight yellow ppt was seen. The solvent was removed with a rotary evaporator at 41° under reduced pressure. Absolute EtOH (20 ml) was added to dissolve the resulting glass. Five percent sodium acetate solution was used to bring the pH to 4.7 and the mixture was allowed to precipitate in a centrifuge tube. The ppt was collected by centrifugation and washed twice with H_2O. After drying overnight under reduced pressure at 65° an analytical sample was dried six hours under 0.1 mm pressure at 78° to give the desired product (0.50 g 87.5%; lit.[30] 94%). Mp 241-242° (decomp.) (lit.[30] 246° decomp for the D-isomer). $[\alpha]_D^{24}$ = +20.9° (c, 1.0, in HCl/EtOH/1:1) (lit.[30] for the D-isomer $[\alpha]_D^{20}$ = -21°). TLC (UV) Rf (B: $HCCl_3$-MeOH-NH_4OH/20:10:1) 0.25. The monobromination product (2-18) Rf = 0.38 was not present. Analysis: $C_{15}H_{11}NO_4Br_2I_2$

Calculated: C, 26.38; H, 1.63; N, 2.05; Br, 23.40

Found: C, 26.55; H, 1.77; N, 2.06; Br, 23.18

N-Acetyl 3,5-dinitro-4-(3'-fluorophenoxy)-L-phenylalanine Ethyl Ester (2-23). The condensation of 3-fluorophenol (2-20) and N-acetyl 3,5-dinitro-L-tyrosine ethyl ester (supply on hand $[\alpha]_D^{22}$ = -6.46 (c, 6.0, dioxane) lit.[31] $[\alpha]_D^{23}$ = -8.0°) was accomplished using the method of Jorgensen et al.[31] Into a 500 ml 3-neck round bottom flask fitted with a dropping funnel and reflux condenser was placed N-acetyl 3,5-dinitro-L-tyrosine ethyl ester (29.3 g, 0.086 mol) and 165 ml of dry pyridine. The mixture was stirred and brought to reflux. Methane sulfonyl chloride (7.2 ml, 0.093 mol) was added slowly through a septum with a syringe and the mixture was allowed to reflux for two min. After a slight cooling period, 119 g (0.169 mol) of 3-fluoro-

phenol (Aldrich 96%, redistilled, bp 178°) was added dropwise and the mixture was allowed to reflux for 20 min. The pyridine was removed under H_2O aspirator vacuum with a rotary evaporator and the residue was taken up in 300 ml of $CHCl_3$ and washed successively with: 300 ml of 2 N HCl, 150 ml of 2 N NaOH, 75 ml of 2 N NaOH, and 300 ml of saturated NaCl. The organic layer was dried over Mg_2SO_4. After filtration the $CHCl_3$ was removed under reduced pressure with a rotary evaporator. The residue was taken up in 55 ml of ethyl acetate and filtered hot. A brownish solid was deposited during overnight refrigeration. The solid was filtered and washed with abs EtOH to remove the brown color. The compound was air dried in the dark to give (24.2 g, 64.6%). Mp 125-126° $[\alpha]_D^{24}$ = +44.2° (c, 1.0 $CHCl_3$). TLC (UV) Rf (B: n-BuOH-t-BuOH-H_2O/ 5:5:1) 0.69, Rf (C: $CHCl_3$) 0.46. PMR (60 MHz) ($CDCl_3$) δ 1.30 (t, J = 7.0 Hz, 3H, CO_2Et-CH_3), 2.02 (s, 3H, N-Ac-CH_3), 3.25 (m, 2H, β-CH_2) 4.28 (q, J = 7 Hz, 2H CO_2Et-CH_2), 4.95 (m, 1H, α-CH), 6.52-7.45 (comp m, 5H, 2',4',5',6'-H and NAc-NH), 8.15 (s, 2H, 2,6-H). Analysis $C_{19}H_{18}N_3O_8F$

Calculated: C, 52.42; H, 4.16; N, 9.65

Found: C, 52.27; H, 4.25; N, 9.66

N-Acetyl 3,5-dinitro-4-(3'-chlorophenoxy)-L-phenylalanine Ethyl Ester (2-24). The procedure of Jorgensen et al. was used to synthesize (2-24) in a manner similar to the above (2-23).[31] Into a 500 ml, three neck round bottom flask was placed N-acetyl 3,5-dinitro-L-tyrosine ethyl ester (27.2 g, 0.079 mol) and 160 ml of pyridine (dried over KOH). After the stirred mixture was brought to reflux, 6.6 ml (0.085 mol) of methanesulfonyl chloride was added slowly through a septum with a syringe. The mixture was allowed to reflux for 2 min and then cooled slightly. m-Chlorophenol (2-21) (25 g) (Eastman) was sublimed at 0.01 mm, 40°. The

The PMR Spectrum of the resulting pure white crystals matched the Sadtler Index #232 exactly. The m-chlorophenol (2-21) (20 g, 0.155 mol) in 10 ml of pyridine was added to the reaction mixture and allowed to reflux for 20 min. The excess pyridine was removed by vacuum distillation under H_2O aspirator pressure at 27°. The resulting oily residue was taken up in 300 ml of $CHCl_3$ and washed successively with: 300ml of 2 N HCl, 150 ml of 2 N NaOH, 75 ml of 2 N NaOH, and 300 ml of saturated NaCl. The organic layer was dried over Mg_2SO_4 overnight. After filtration and solvent removal the product was recrystallized in 100 ml of hot ethyl acetate. The light yellow crystals that formed in the refrigerator overnight were filtered and dried in a house vacuum oven at room temperature to give (23 g, 64%). Mp 141-142°. $[\alpha]_D^{24}$ = +42.1° (c, 1.0 $CHCl_3$). TLC (UV) Rf (B: n-BuOH-t-BuOH-H_2O/5:5:2) 0.70, Rf (C:$CHCl_3$) 0.47. PMR (60 MHz) ($CDCl_3$) δ 1.32 (t, J = 7 Hz, 3H, CO_2Et-CH_3) 2.04 (s, 3H, N-Ac-CH_3), 3.32 (m, 2H, β-CH_2), 4.30 (q, J = 7 Hz, 2H, CO_2Et-CH_2-), 4.93 (m, 1H, α-CH), 6.46 (d, J = 7 Hz, 1H, N-Ac-H), 6.61-7.50 (comp m, 4H, 2',4',5',6'-H), 8.12 (s, 2H, 2,6-H). Analysis $C_{19}H_{18}O_8N_3Cl$

Calculated: C, 50.51; H, 4.01; N, 9.30; Cl, 7.85

Found: C, 50.57; H, 4.05; N, 9.25; Cl, 7.89

N-Acetyl 3,5-dinitro-4-(3'-bromophenoxy)-L-phenylalanine Ethyl Ester (2-25). 3-Bromophenol (2-22) (Eastman) was redistilled under 1 mm pressure at 90°. The PMR spectra of (2-22) compared exactly with the Sadtler Index #6296. To 20.1 g (0.059 mol) of N-acetyl 3,5-dinitro-L-tyrosine ethyl ester in 120 ml of pyridine (dried over KOH) was added 5 ml (0.064 mol) of methane sulfonyl chloride and the mixture was heated under reflux for two min. After slight cooling, 20 g (0.115 mol) of 3-bromophenol (2-22) was added dropwise and the mixture was heated under

reflux for 20 min. The pyridine was then removed under reduced pressure. The resulting oily black residue was taken up in 240 ml of $CHCl_3$ with slight heating on a steam cone and successively washed with: 240 ml of 2 N HCl, 120 ml of 2 N NaOH, 60 ml of 2 N NaOH, and 240 ml of saturated aqueous NaCl. The organic layer was dried over Mg_2SO_4. After filtration, solvent removal, and drying for 6 hr under 0.02 mm pressure at 78° a light yellow solid was obtained (20.2 g, 69%). Mp 142-144°. $[\alpha]_D^{24}$ = +34.6° (c, 2.0 dioxane). TLC (UV) Rf (B: n-BuOH-t-BuOH-H_2O/5:5:2) 0.69, Rf (C: $CHCl_3$), 0.49. PMR (60 MHz) ($CDCl_3$) δ 1.32 (t, J = 7 Hz, 3H, CO_2Et-CH_3), 2.05 (s, 3H, N-Ac-CH_3), 3.32 (m, 2H, β-CH_2), 4.30 (q, J = 7 Hz, 2H, CO_2Et-CH_2), 4.97 (m, 1H, α-CH), 6.36 (d, J = 7 Hz, N-Ac-NH), 6.56-7.45 (comp m, 4H, 2',4',5',6'-H), 8.09 (s, 2H, 2,6-H). Analysis: $C_{19}H_{18}O_8N_3Br$

Calculated: C, 45.98; H, 3.65; N, 8.47; Br, 16.10

Found: C, 45.90; H, 3.68; N, 8.47; Br, 16.03

N-Acetyl 3,5-diiodo-4-(3'-fluorophenoxy)-L-phenylalanine Ethyl Ester (2-26). This compound was prepared by the general method of Blank et al.[29] A nitrosyl sulfuric acid solution was prepared in a one liter, three neck round bottom flask fitted with thermometer, overhead stirrer, and addition funnel by slow addition of $NaNO_2$(4.5 g, 0.66 mole) to 91 ml of sulfuric acid and 36 ml of glacial acetic acid at 60 to 70°. At the same time 5 g (0.011 mol) of N-acetyl-3,5-dinitro-4-(3'-fluorophenoxy)-L-phenylalanine ethyl ester (2-23) was reduced in a Parr hydrogenation apparatus under 1.84 x 10^3 mm initial pressure with 1.1 g of 10% Pd/C catalyst. Hydrogen uptake was monitored carefully to avoid possible dehalogenation. At 19° the initial pressure of 1.84 x 10^3 mm (35.5 psi) fell to 1.55 x 10^3 mm (30 psi) (94% of theoretical H_2 uptake) in 13 min.

The Pd/C was removed by filtration through a fine sintered glass funnel. The amine solution was poured into a 125-ml addition funnel which had been flushed with nitrogen and covered with aluminum foil to prevent the formation of a dark colored amine solution. The amine was added to the stirred, cooled (0 to 5°) nitrosyl sulfuric acid solution so that the temperature never rose above 5°. After addition was complete the solution was stirred and cooled an additional hour. The resulting tetrazonium solution was added rapidly to a stirring mixture of: 9.9 g of sodium iodide, 12.6 g of iodine, 2.3 g of urea, 200 ml of water, and 200 ml of chloroform. The mixture was allowed to stir for one hour at room temperature. After the organic and aqueous layers were separated, the aqueous layer was extracted twice with $CHCl_3$. The combined organic fractions were washed with; H_2O (150 ml x 2), fresh 10% sodium bisulfite (100 ml x 2), 5% potassium bicarbonate (200 ml x 1), and H_2O (200 ml x 1). The orange colored organic layer was dried overnight with Mg_2SO_4. The $CHCl_3$ was removed and the oil which resulted spontaneously solidified on standing for one day. This solid was triturated on a cold watch glass with hexane to yield a dry light tan solid (6.4 g, 97%). This was taken up in 60 ml of hot benzene, and hexane was added until the solution started to become cloudy. The solution was cooled to room temperature and placed in the refrigerator overnight. The resulting white needles were filtered and dried to give (3.5 g, 52%). Mp 122-123°. $[\alpha]_D^{24}$ = +52.7° (c, 1.0 $CHCl_3$). TLC (UV) Rf (B: $CHCl_3$-MeOH-NH_4OH/20:10:1) 0.79, Rf (C: $CHCl_3$) 0.62. PMR (60 MHz) ($CDCl_3$) δ 1.30 (t, J = 7 Hz, 3H, CO_2Et-CH_3), 2.06 (s, 3H, N-Ac-CH_3), 3.06 (d, J = 6 Hz, 2H, β-CH_2), 4.23 (q, J = 7 Hz, 2H, CO_2Et-CH_2-), 4.83 (m, J = 6 Hz, 1H, α-CH), 6.35 (d, J = 6 Hz, 1 H, N-Ac-NH), 6.52-7.40 (comp m, 4H, 2',4',5',6'-H), 7.68 (s, 2H, 2,6-H).

Analysis: $C_{19}H_{18}NO_4FI_2$

Calculated: C, 38.22; H, 3.04; N, 2.34; I, 42.51

Found: C, 38.42; H, 3.05; N, 2.43; I, 42.68

N-Acetyl 3,5-diiodo-4-(3'-chlorophenoxy)-L-phenylalanine Ethyl Ester (2-27). The general method of Blank et al.[29] was followed as above for the reduction of (2-26). N-Acetyl 3,5-dinitro-4-(3'-chlorophenoxy)-L-phenylalanine ethyl ester (2-23) (5 g, 0.011 mol) in 110 ml of glacial acetic acid was reduced with 1.1 g of 11% Pd/C. The reduction reached 94% completion in 15 min. The amine was then diazotized and iodinated as above for (2-26) to produce an oil from the washed organic fractions. This oil was eluted with $CHCl_3$ from a 40 g alumina column and the fractions containing the desired product (TLC (UV) Rf (C: $CHCl_3$) 0.62) were combined. The solvent was removed from the combined fractions to produce an oil which yielded a tan colored solid when triturated with hexane and a small amount of 95% ethanol. This solid was recrystallized from 25 ml of hot benzene after enough hexane was added to cause a cloudiness to occur. The desired product crystallized overnight in the refrigerator to give (1.5 g, 22% yield). Mp 106-107°. $[\alpha]_D^{24}$ = +51.2° (c, 1.0, $CHCl_3$). TLC (UV) Rf (B: $CHCl_3$MeOH-NH_4OH/20:10:1) 0.79, Rf (C: $CHCl_3$) 0.62. N-acetyl-3,5-diiodo-L-thyronine ethyl ester Rf (C: $CHCl_3$) 0.63 was not detected in this product. Chemical Ionization M.S. (Source temp. 195°, reactant gas i-Bu, pressure ≃ 0.5 torr) indicated a molecular ion at M/E = 614 with 20% M/E = 579 which corresponds to a loss of Cl. Since the PMR and the analysis showed no dehalogenation, the dehalogenation must have occured in the M.S. probe. PMR (60 MHZ) ($CDCl_3$) δ 1.30 (t, J = 7 Hz, 2H, CO_2Et-CH_3), 2.05 (s, 3H, N-Ac-CH_3), 3.08 (d, J = 6 Hz, 2H, β-CH_2), 4.27 (q, J = 7 Hz, 2H, CO_2Et-CH_2-), 4.87 (m, 1H,

α-CH), 6.42 (d, J = 6 Hz, 1H, N-Ac-NH), 6.59-7.48 (comp m, 4H, 2',4', 5',6'-H), 7.75 (s, 2H, 2,6-H). Analysis: $C_{19}H_{18}NO_4ClI_2$

Calculated: C, 37.19; H, 2.95; N, 2.28; Cl, 5.78

Found: C, 37.29; H, 2.98; N, 2.35; Cl, 5.59

N-Acetyl 3,5-diiodo-4-(3'-bromophenoxy)-L-phenylalanine Ethyl Ester (2-28). Since this compound undergoes dehalogenation when reduced by catalytic hydrogenation, the method of Blank et al.[29] was modified for the initial reduction to be done with iron, acetic acid, and hydrochloric acid.[32] N-Acetyl 3,5-dinitro-4-(3'-bromophenoxy)-L-phenylanine ethyl ester (2-25) (5 g, 0.01 mole) was dissolved in 300 ml of warm glacial acetic acid and then cooled to 30°. Iron (10 g, 0.179 mol) (Baker, reduced with hydrogen), was added to the solution and the system was flushed with nitrogen. Concentrated HCl (60 ml) was added slowly from a dropping funnel for a period of one hour while trying to avoid a large increase in the temperature. After allowing the reaction to stir for one additional hour, the inorganic solids were filtered off and the amine solution was concentrated to 20 ml. A solid white precipitate developed which was dissolved in 200 ml of $CHCl_3$. A saturated aqueous solution of $NaHCO_3$ (200 g) was added slowly to the $CHCl_3$ solution to neutralize the HCl. The organic layer was separated and dried over Mg_2SO_4 for one hour. After filtration, the solvent was removed from the amine solution to produce a light yellow oil. This oil was dissolved in 75 ml of glacial acetic acid and the solution was slowly added to a nitrosyl sulfuric acid solution (prepared by adding 4.1 g of sodium nitrite to 91 ml H_2SO_4 and 36 ml of glacial acetic acid) so that the temperature stayed between 0 and 5°. A red color appeared in the reaction vessel which indicated the formation of the tetrazonium compound. After cooling and stirring

for one hour, the reaction mixture was added rapidly to a stirred mixture of: 9.0 g of sodium iodide, 9.0 g of iodine, 1.6 g of urea, 200 ml of H_2O, and 200 ml of $CHCl_3$. This iodination reaction was stirred for three hours, the organic and aqueous layers were separated, and the aqueous layer was washed four times with 50 ml of $CHCl_3$. The combined organic fractions were washed with: H_2O (100 ml x 3), 10% sodium bisulfite (100 ml x 2), 1 N NaOH (100 ml x 2), and H_2O (100 ml x 2). The $CHCl_3$ layer was then dried over Mg_2SO_4 overnight. After the solvent was removed, the resulting oil was eluted from a 40 g alumina column to produce another oil. It was found that trituration of the original oil with hexane and EtOH on a cold watch glass produces a higher yield (see preparation of 2-26). The tan solid obtained from trituration was recrystallized from hot benzene (15 ml/g solid) with enough hexane added to cause a slight cloudiness to occur in the mixture. The recrystallization mixture was cooled in the refrigerator overnight to give the desired product (900 mg, 14%). Mp 110-112°. $[\alpha]_D^{24}$ = +48.9° (c, 1.0 $CHCl_3$). TLC (UV) Rf (B: $CHCl_3$-MeOH-NH_4OH/20:10:1) 0.78, Rf (C: $CHCl_3$) 0.62. PMR (60 MHz) ($CDCl_3$) δ 1.30 (t, J = 7 Hz, 3H, CO_2Et-CH_3), 2.06 (s, 3H, N-Ac-CH_3), (d, J = 6 Hz, 2H, β-CH_2), 4.27 (q, J = 7 Hz, 2H, CO_2Et-CH_2-), 4.88 (m, J = 6 Hz, 1H, α-CH), 6.36 (d, J = 7 Hz, 1H, N-Ac-NH), 6.65-7.57 (comp m, 4H, 2',4',5',6'-H), 7.76 (s, 2H, 2,6-H). Analysis: $C_{19}H_{18}NO_4BrI_2$

Calculated: C, 34.68; H, 2.75; N, 2.13; Br, 12.14

Found: C, 35.27; H, 2.81; N, 2.59; Br, 12.44

3,5-Diiodo-3'-fluoro-4'-deoxy-L-thyronine (2-29). Hydrolysis of the N-acetyl and ethyl ester protecting groups was accomplished using the general procedure of Jorgensen et al.[31] N-Acetyl 3,5-diiodo-4-

(3'—fluorophenoxy)-L-phenylalanine ethyl ester (2-26) (2 g, 3.34×10^{-3} mol) in a 100-ml round bottom flask with reflux condenser was dissolved in 20 ml of glacial acetic acid and 20 ml of concentrated hydrochloric acid. The reaction mixture was heated at reflux for two hr and then added to 160 ml of cold water. The pH was adjusted to 5.0 with concentrated NH_4OH. The solution was allowed to precipitate for one hr and the resulting ppt was filtered and washed with water. The final white solid was dried overnight under reduced pressure at 40°. An analytical sample was dried for 10 hours at 78° under 0.03 mm. The total product obtained was (1.69 g, 96%). Mp 242-245° decomposition. $[\alpha]_D^{24}$ = +12.6° (c, 1.0, EtOH-1N HCl/3:1). TLC (UV) Rf (B: $CHCl_3$-MeOH-NH_4OH/20:10:1) 0.34 Rf (B: ethyl acetate-iPr-NH_4OH/27:18:10) 0.40. Analysis: $C_{15}H_{13}NO_3FI_2$

Calculated: C, 34.18; H, 2.29; N, 2.66; I, 48.15

Found: C, 33.9; H, 2.3; N, 2.6; I, 48.0

3,5-Diiodo-3'-chloro-4'-hydrogen-L-thyronine (2-30). N-Acetyl 3,5-diiodo-4-(3'-chlorophenoxy)-L-phenylalanine ethyl ester (2-27) (1.0 g, 1.62×10^{-3} mol) was hydrolysed in 10 ml of glacial acetic acid and 10 ml of concentrated hydrochloric acid at reflux for two hours. The reaction mixture was poured into four times the volume of cold H_2O. The product (881 mg, 91%) was obtained by isoelectric precipitation in a centrifuge tube with 5% sodium acetate. Mp 249-253° decomposition. $[\alpha]_D^{24}$ = +23.2° (c, 1.0, EtOH-1N HCl/3:1) TLC (UV) Rf (B: $CHCl_3$-MeOH-NH_4OH/20:10:1) 0.37, Rf (B: EtOAc-iPr-NH_4OH/27:18:10) 0.38. Analysis: $C_{15}H_{13}NO_3ClI_2$

Calculated: C, 33.15; H, 2.22; N, 2.58; Cl, 6.52; I, 46.70

Found: C, 32.0; H, 2.4; N, 2.6; Cl, 6.7; I, 46.4

3,5-Diiodo-3'-bromo-4'-hydrogen-L-thyronine (2-31). N-Acetyl 3,5-diiodo-4-(3'-bromophenoxy)-L-phenylalanine ethyl ester (2-28) (600 mg, 9.10×10^{-4} mol) was dissolved in a 100-ml round bottom flask fitted with reflux condenser, with 10 ml of concentrated HCl and 10 ml of glacial acetic acid. The mixture was refluxed for two hours, and then poured into four times the volume of cold H_2O (80 ml). While the mixture was stirred over an ice bath, the pH was carefully brought to 5.0 with NH_4OH. The mixture was filtered with a fine sintered glass funnel and washed with cold H_2O. The compound was dried under reduced pressure at 60^o for two days to give (530 mg, 98%). Mp 238-241° decomposition. $[\alpha]_D^{24} = +23.2^o$ (c, 1.0, EtOH-1N HCl/3:1). TLC (UV) Rf (B: $CHCl_3$-MeOH-NH_4OH/20:10:1) 0.40, Rf (B: EtOAc-iPr-NH_4OH/27:18:10) 0.36. Analysis: $C_{15}H_{13}NO_3BrI_2$

Calculated: C, 30.64; H, 2.05; N, 2.38; Br, 13.59, I, 43.17

Found: C, 31.09; H, 2.20; N, 2.64; Br, 13.36; I, 42.40

3,5-Diiodo-4-hydroxybenzaldehyde[33] (2-32). This was prepared from the method of Matsuura and Cahnman.[33] Iodine monochloride (25 ml) was redistilled at 97°. After collection of 10 ml of ICl liquid the distillation was stopped. Solid I_2 was left in the pot. A solution of 3.95 g (0.024 mol) of ICl in 5 ml of 20% HCl was added within four minutes to a stirred solution of 1.22 g (0.01 mol) of p-hydroxybenzaldehyde (mp 115-117°) in 14 ml of 20% HCl and 120 ml of H_2O. In one minute a light yellow precipitate formed. The reaction was stirred at room temperature for 2 hours. After 2 hours the ppt was filtered and washed with H_2O and then dried under reduced pressure at 40^o to give (3.4 g, 90% yield, Mp 185-195°). This was recrystallized from 70 ml of hot EtOH and 30 ml of hot H_2O. The long clear needles which formed overnight at room temperature were filtered and dried to give (2.6 g, 69%). Mp 198-200° (lit.[33] 199-

Chapter Three: Solubilization and Purification of the Rat Liver Nuclear Protein Receptor

BACKGROUND OF METHOD

The presence of limited-capacity binding sites for triiodothyronine in the nuclei of both rat liver and kidney was first reported by Oppenheimer and coworkers in 1972.[12] The limited-capacity sites were detected by determination of the amount of [^{125}I]T_3 (40 μCi/μg) in isolated nuclei 30 min after intravenous injection of tracer doses of [^{125}I]T_3 along with increasing quantities of nonradioactive hormone. These *in vivo* displacement studies had very low sensitivity because of the low specific activity of the [^{125}I]labeled hormone and the authors made no attempt to calculate an equilibrium constant or to determine binding capacity. However, they did notice that 3,5,3'-triiodothyronine was more potent than thyroxine in displacing [^{125}I]T_3 from the binding sites. In 1973 Oppenheimer made a study of the binding affinity

of a very limited number of thyroid hormone analogs and found that if differences in metabolism and distribution were taken into account there was a good qualitative correlation between the analog binding affinity and its biological potency.[38] In addition, these workers found limited-capacity binding sites in all tissues studied in the rat. However, the capacity in tissues generally considered non-responsive to thyroid hormone was very low compared to the capacity of thyroid responsive tissues such as anterior pituitary, liver and heart.[40] Thus, the evidence suggested that these nuclear binding sites were indeed the cellular receptors for the thyroid hormones.

Several groups undertook studies of these nuclear binding sites and attempted to isolate and characterize these putative "receptors". Samuels and Tsai were the first to study the action of thyroid hormone in cell culture.[41] They used a rat pituitary tumor cell line, GH-1, that showed a 3-fold increase in the rate of growth under the influence of triiodothyronine and thyroxine. They demonstrated high-affinity, low-capacity binding sites for these hormones in intact cells and in isolated nuclei, but not in isolated mitochondrial or cytosol fractions. Koerner, Surks and Oppenheimer also demonstrated *in vitro* the presence of specific T_3 binding sites in isolated rat liver nuclei.[39] In collaboration with Jorgensen, these workers used the binding to intact rat liver nuclei to study the affinity of an extensive series of thyroid hormone analogs.[42] This data provided information on the structural requirements for thyroid hormone *in vitro* binding and the correlation of this with *in vivo* activity, and further supported the physiological relevance of the nuclear sites.

High-affinity, limited capacity thyroid hormone binding sites have been also identified in the cytosol,[43-46], mitochondria,[47] and other cell

fractions.[44] The specific binding seen in these other cell fractions leads to two questions: Is there an analogy between the thyroid, and the steroid type mechanism where the hormone interacts with a cytoplasmic protein and is translocated to the nucleus? Are these other high affinity binding sites the physiologically important initiators of thyroid hormone action? Several studies have addressed the first question and all have concluded that the cytosol binding site is not required to localize the thyroid hormone binding sites in the nucleus.[46,48] As for the second question, Samuels showed that the cytosol binding proteins in cultured pituitary cells have a higher affinity for thyroxine than for triiodothyronine which is opposite to that seen for in vivo hormonal activity.[49] This suggests that these cytosol binding sites are not likely to be physiologic "receptors". The binding sites in other cell fractions have not been studied in as much detail as the nuclear sites and their roles, if any, in thyroid hormone action have not been determined. In summary, there is significant evidence to suggest that the triiodothyronine nuclear binding sites may be the hormone receptors but no equally convincing evidence has been presented with respect to other cellular fractions.

The next step in the isolation and characterization of the thyroid "receptor" was the solubilization of the rat liver, kidney, and GH-1 cell nuclear binding activity with 0.4 M KCl, by Samuels and coworkers.[50] They were able to prepare a nuclear extract which maintained thyroid hormone binding activity at 37°. This was in contrast to the earlier work of Surks et al., who found that the T_3-macromolecular complex was unstable at 25 to 37°. Since then several groups have isolated soluble nuclear receptors and some binding characteristics have been reported.[15,49,52-55] The binding activity is sensitive to proteolytic but not other hydrolytic

enzymes.[49,51,52] This nuclear protein has been shown to have a molecular weight between 55,000 and 65,000 by Sephadex G-100 column chromatography and sedimentation on glycerol gradients.[54,55] From the Stokes radius of 35 Å and the sedimentation coefficient of 3.5 S, a frictional ratio of 1.4 can be calculated which indicates that the protein is somewhat asymmetrical.[55] The binding protein has been shown to be an acidic non-histone protein by elution from a weak carboxylic acid column, extraction in 0.4 M KCl at pH 8.5 from whole nuclei, and partial purification by removal of histones with quaternary aminoethyl Sephadex chromatography.[49,51,55] The nuclear binding activity has been shown to be localized in the chromatin by formaldehyde fixation which can only occur when a protein is very closely associated with DNA.[14] MacLeod and Baxter have shown that the solubilized receptors bind to DNA and have suggested that this reaction may be the mechanism for receptor localization in chromatin.[15]

In order to more clearly define the nature of the molecular interactions between thyroid hormone and the nuclear receptor, a solubilized nuclear protein preparation was chosen for our studies to measure the binding affinity of an extensive set of thyroid hormone analogs. Previous work by Oppenheimer had measured binding affinity to intact nuclei but there was still some concern about the possible effect of the nuclear membrane in this binding reaction.[42] By utilizing the methods of Latham et al., a preparation of solubilized nuclear protein was obtained which could be stored for over six months at liquid N_2 temperature and retain high-affinity, saturable specific binding activity with [^{125}I]T_3. Most previous studies of in vivo and in vitro thyroid hormone activity have tried to determine the basic structural features of the thyroxine molecule

required for activity. The present analog binding studies were chosen to obtain information about the nature of the molecular interactions between the hormone and its receptor. These binding studies with the solubilized rat liver nuclear receptor have allowed us to determine: (a) the strength of the 4'-OH interaction with the receptor, (b) define the size limit of hydrophobic and dispersion force interaction of the 3'-substituent with the receptor, (c) define the probable type of electrostatic interaction between the alanine side chain and the receptor, and (d) perform the thermodynamic and kinetic studies of complex formation with the receptor. In addition, we have studied the quantitative correlation between *in vitro* solubilized receptor binding and *in vivo* rat antigoiter activity.[56]

AMMONIUM SULFATE SOLUBILIZATION OF PROTEIN FROM RAT LIVER NUCLEI

The following procedure describes the process of isolating a soluble receptor preparation from 80 g of frozen rat liver. The yield of nuclear extract is 40 ml which is enough for 800 points in a Scatchard, kinetic, or competition assay, assuming 50 µl per incubation. The procedure should be scaled up or down in multiples of 80 g liver.

Thirty male Sprague-Dawley rats (350 g/rat, assume 10 g tissue/liver) were sacrificed as supplied by the vendor (Simonsen). The animals were lightly anesthetized with chloroform before cervical dislocation, then the aorta was severed and blood was drained from the liver. Livers were manually removed and immediately stored in a liquid nitrogen cryogenic storage container (MVE, SX-34).

After preparation of all the appropriate buffers, 80 g of frozen liver was weighed into a mortar and quickly crushed with a pestle. The

chunks of frozen liver were poured into 160 ml of warm (35°) Liver Prep. Solution A (0.34 M sucrose, 15 mM $MgCl_2$, 0.24 mM Spermine·4 HCl) and stirred until thawed to give a final temperature of about 4°. The liver pieces were then drained using a coarse nylon mesh, and added to ice cold Homogenization Solution B (240 ml of 2.1 M sucrose, 1.44 ml of 1 M $MgCl_2 \cdot 6\ H_2O$, 0.25 ml of 0.1 M Spermine · 4 HCl). The mixture was then homogenized for three 1-minute periods, separated by one minute of cooling, using a Tekmar Dispex Homogenizer (Cincinnati, Ohio) at maximum speed. This preparation, when stained with 1% crystal violet, was observed by light microscopy to contain cell debris, intact nuclei and no whole cells. The mixture was then filtered through one layer of Miracloth (Calbiochem) with kneading to obtain a homogeneous mixture without large pieces of connective tissue. Six Sorvall GSA-rotor bottles were filled with about 150 ml each and then 125 ml of 2.1 M sucrose was added to the bottom of the bottles with a separatory funnel in order to float the liver mixture to the top. The nuclei were then pelleted through the 2.1 M sucrose by centrifugation (25,000 g, 90 min, 4°) in a Sorvall RC-2B with a GSA rotor (diameter 14.61 cm). After centrifugation the cell debris and sucrose solution was sucked off from the top to avoid contamination of the nuclear pellet. The nuclear pellets were each washed with gentle suspension in 50 ml of Nuclear Pellet Washing Buffer C (20 mM Tricine, 2 mM $CaCl_2 \cdot H_2O$, 1 mM $MgCl_2 \cdot 6\ H_2O$, pH 7.6) containing 0.5% Triton X-100. The suspensions were repelleted by centrifugation (500 g, 10 min, 5°) in an International Refrigerated Centrifuge. After decanting the supernatant the final nuclear pellets were each resuspended in 20 ml of ice cold Resuspension Buffer D (20 mM Tris (pH 8.0), 0.25 M sucrose, 1.0 mM EDTA, 0.1 mM dithiothreitol, 5% glycerol), and 1.05 ml of saturated (4 M) ammonium sulfate was added to make 0.2 M. This suspension was

then sonicated two times for 15 sec at 95 watts with a microtip, allowing for ice cooling between bursts. The sonicated mixture was finally centrifuged (48,000 g, 30 min, 4^{o}) in a Sorvall RC-2 and the supernatant medium (120 ml designated as nuclear extract) was stored in 2 ml fractions at liquid nitrogen temperature in sealed plastic test tubes (A/S NUNC, Cole Scientific, 38 x 12.5 mm, #1076, w/screw caps and teflon sealing rings). This extract did not lose binding activity on freezing or after prolonged storage (up to six months) in liquid nitrogen. Endogenous triiodothyronine in the nuclear extract was reported by Latham to be less than 1 pM as measured by radioimmunoassay.[55] Since the binding assays routinely used 0.1 nM hormone, 1 pM endogenous cold triiodothyronine would be insignificant. It was therefore considered unnecessary to thyroidectomize the rats to lower endogenous hormone levels.

SEPHADEX QAE A-50 ANION EXCHANGE PURIFICATION OF NUCLEAR EXTRACT

This procedure is the same as that described by Latham and associates without major modification.[55] The principle of this procedure is to titrate the nuclear extract to pH 9.6. This pH is near the pI of the basic histones that contain a high percentage of lysine and arginine residues. This causes the histone to precipitate and, in theory, to coprecipitate some of the DNA. By dialyzing this mixture against 100 volumes of a low salt solution, ammonium sulfate is removed from the nuclear extract. After centrifugation to remove the precipitated histone-DNA mixture, the supernatant is applied to a column of quaternary amine anion exchange medium in pH 9.6 buffer. This causes the ionized carboxylic acid-containing non-histone proteins to be retained by the cationic quaternary amine medium and the soluble histone fraction to

be eluted with the pH 9.6 buffer. To remove the acidic non-histone proteins from the column, the elution buffer is changed to pH 5.7 and the receptor protein-containing fractions are collected and combined. After precipitating with ammonium sulfate and redissolving in a smaller volume, the QAE purified nuclear extract is stored at liquid nitrogen temperature.

PARTIAL PURIFICATION PROCEDURE

All the following steps are carried out at 0 to 4^{o}. The frozen nuclear extract was thawed quickly with a 37^{o} bath but was placed on ice before the temperature rose above 4^{o}. To 50 ml of nuclear extract was added 50 ml of 1 M Tris base. The final pH was 9.6. (Note: It is more important to add all 50 ml of 1 M Tris base than to carefully titrate to pH 9.6.) This solution was dialyzed against 80 volumes of Dialysis Buffer E (0.1 M NaCl, 0.05 M Tris, 1.0 mM dithiothreitol, pH 9.6) for 1.5 hours at 5^{o}. Following dialysis the protein mixture was centrifuged (25,000 g, 30 min, 4^{o}) in a Sorvall RC-2 ultracentrifuge and the supernatant was loaded onto a Sephadex QAE A-50 column. The functional group, diethyl-(2-hydroxypropyl) aminoethyl, is a strongly basic anion exchanger. Sephadex QAE A-50 (10 g) was swelled 24 hours in Buffer E to give a bed volume of 1 ml/1 ml sample in a 3.5 x 15 cm column. The column was eluted with one bed volume of Buffer E and two bed volumes of Buffer F (0.2 M $(NH_4)_2SO_4$, 25 mM Na citrate, 50 mM monosodium phosphate, 1.0 mM dithiothreitol, pH 5.7.) Fractions of 4 ml each were collected and analyzed by measuring protein concentration (Appendix II) and binding activity. Fractions containing nuclear receptor were combined on the basis of high protein concentration and binding activity. The protein in the combined

fractions was precipitated by adding 0.3 g/ml solid $(NH_4)_2SO_4$ (Schwarz/Mann, enzyme grade) with stirring until dissolved, and then letting the solution stand for four hours. After four hours at 5^o the combined and precipitated fractions were centrifuged (5000 g, 10 min, 5^o) and the supernatant was discarded. The pellet was resuspended in Buffer G (10 mM Tris, 0.6 M NaCl, 10 mM dithiothreitol, pH 8.5, 1 ml for each 3 ml of starting nuclear extract). The resuspended mixture was centrifuged (25,000 g, 20 min, 4^o) and the resulting supernatant medium was stored at liquid nitrogen temperature in 2 ml aliquots.

The following graphs (figures 3-1 and 3-2) show the direct assay of nuclear extract and the elution profile of the QAE purified nuclear extract. The binding assay will be discussed in Chapter Four.

A direct assay is a plot of the amount of $[^{125}I]T_3$ bound versus an increasing concentration of added nuclear extract. The total protein concentration is determined by the method of Lowry,[57] and the amount bound is considered to be the total moles bound minus the amount of non-specifically bound hormone determined by a parallel incubation with 1000-fold excess of cold-T_3. The direct assay shows that the amount of T_3 bound increases linearly with the amount of protein added until protein aggregation and a low hormone to protein ratio causes a leveling off. All of the kinetic experiments were performed in the linear range.

The first protein peak in the elution profile of the QAE purified nuclear extract is mainly histone protein. It can be seen that very little binding activity is associated with the histones. However, the second peak elutes after changing to the pH 5.7 buffer and contains non-histone protein which shows very high binding activity. The peak corresponding to high binding activity is the one which is pooled and precipitated

Direct Assay of Nuclear Extract

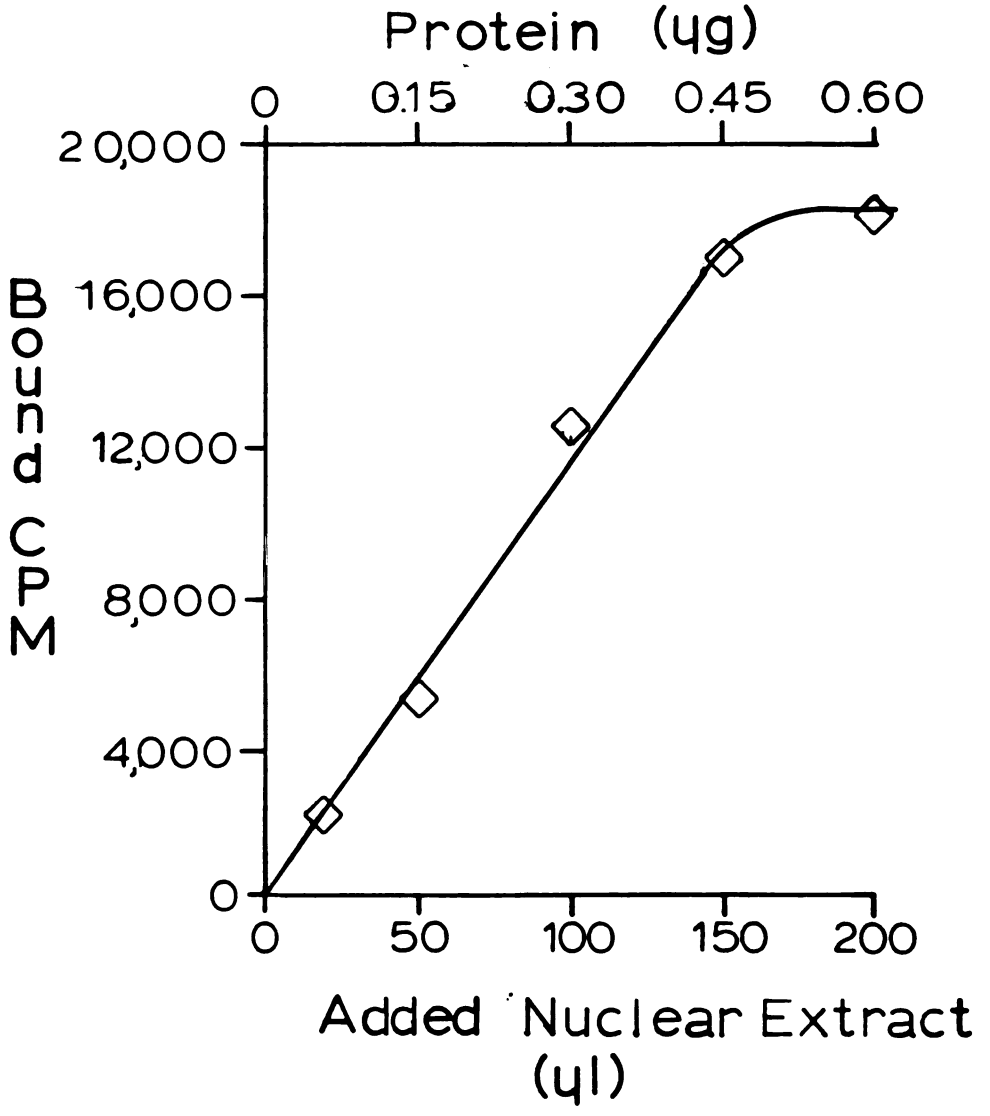


Figure 3-1.

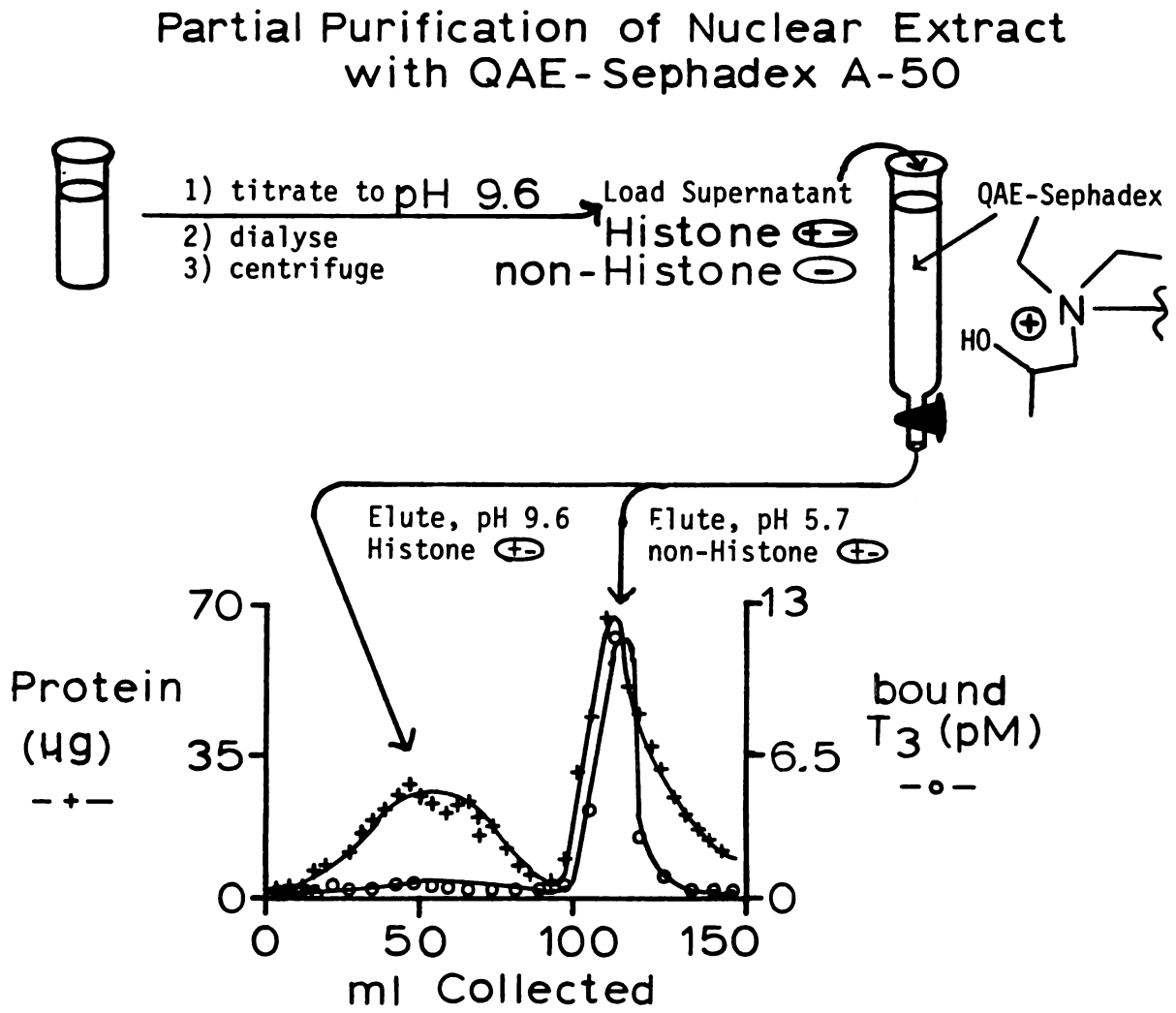

Partial Purification of Nuclear Extract
with QAE-Sephadex A-50
1) titrate to pH 9.6
2) dialyse
3) centrifuge
Load Supernatant
Histone ⊕⊖
non-Histone ⊖
QAE-Sephadex
HO
N
Elute, pH 9.6
Histone ⊕
Elute, pH 5.7
non-Histone ⊕
Protein
(µg)
-+-
bound
T3 (pM)
-o-
70
35
0
13
6.5
0
0
50
100
150
ml Collected

Figure 3-2.

Chapter Four: A Correlation Between Protein Binding and Production of a Cellular Response to Thyroid Hormone

In order to assign physiological relevance to a binding reaction involving a hormone and a high-affinity, limited-capacity binder, the interaction of the hormone and its analogs with the binder should conform to known steric and structural specificity. This chapter describes a study of the correlation between the known *in vivo* antigoiter activity and the recently measured *in vitro* thyroid hormone receptor binding affinities. The first section describes the detailed methods for measuring the *in vitro* binding affinity of thyroid hormone and its analogs. The next section presents the general principles of the *in vivo* antigoiter assay. Finally, the results of a quantitative correlation between *in vivo* antigoiter activity and *in vitro* binding to the solubilized rat liver nuclear receptor are presented.

IN VITRO BINDING TO SOLUBILIZED RAT LIVER NUCLEAR RECEPTOR

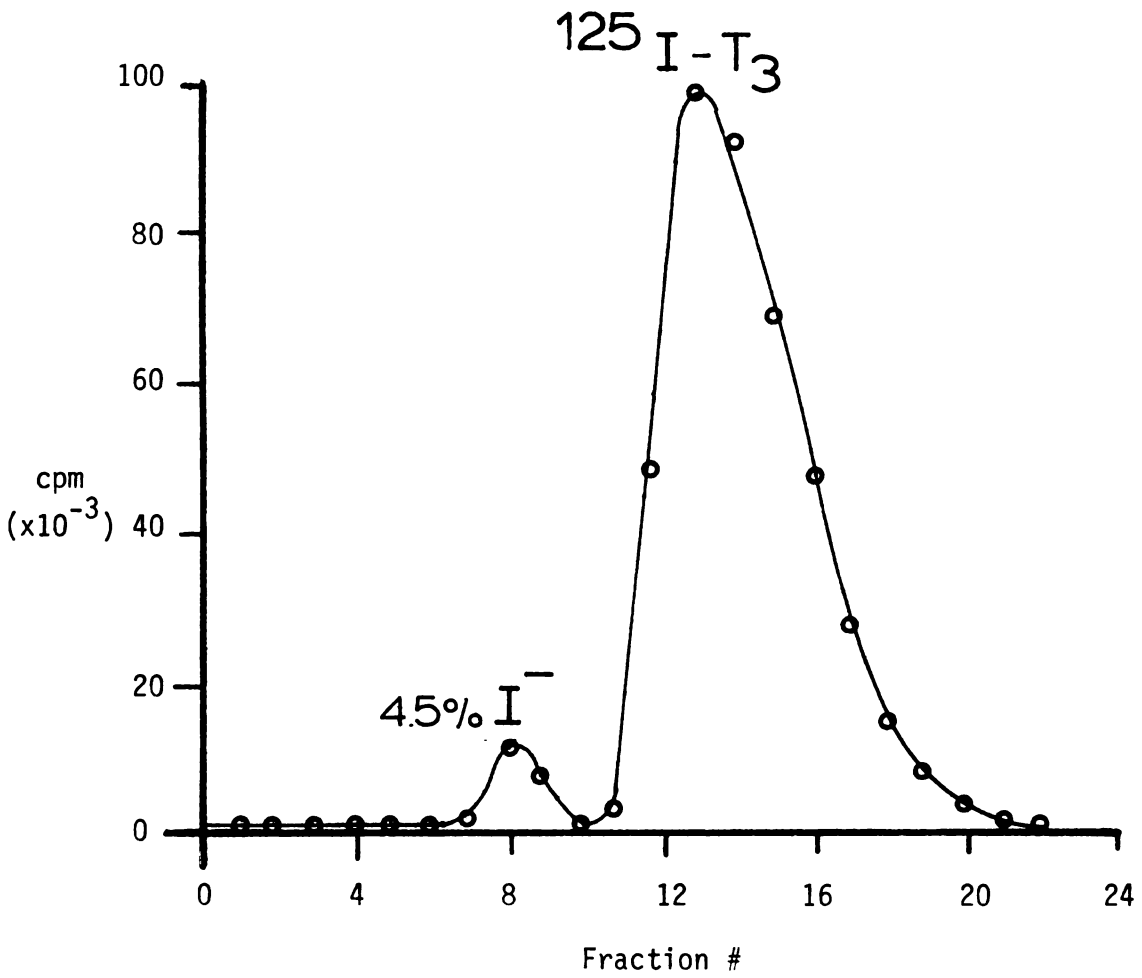
Most cell nuclei contain non-histone proteins strongly associated with the chromatin, which possess high-affinity, limited-capacity binding sites for the thyroid hormones and analogs.[14,42,55] These non-histone nuclear proteins can be solubilized with retention of binding affinity for thyroid hormones and analogs.[50,55] The techniques of Latham et al.[55] have been adopted for this study and the detailed methods of the binding assay are described. This section covers: (1) the acquisition and purity of the radioactively labeled 3,5,3'-triiodothyronine, (b) the detailed methods used in the binding assay, and (c) the general prodecure involved in the acquisition of data for kinetic, Scatchard, and equilibrium analog competition analysis.

The in vitro binding assay utilizes the solubilized nuclear extract which is prepared as described in Chapter Three. The binding assay involves incubation of the nuclear extract, or the QAE-purified nuclear extract, with 3,5,3'-triiodo-L-thyronine which has been radioactively labeled with ^{125}Iodine at the 3'-position. This labeled hormone, which shall be referred to as [^{125}I]T_3 can be obtained from New England Nuclear (N.E.N.) with a specific activity of approximately 914 μCi/μg (5.89 x 10^5 μCi/μ mol, Cat. #NEX-110 H). Typically 0.1 μCi ($80.00) would be purchased under radioisotope license #4248 with an allowance of one week for stockroom ordering and delivery. The hormone is delivered at a concentration of ∿5 x 10^{-7} M in 0.5 ml of 50% n-propanol/H_2O and usually contains 3% of unbound [^{125}I]iodide ion. Due to the loss of [^{125}I]iodide (1-3%/mo) and the short radioactive half-life of 60 days for ^{125}Iodine, each preparation should not be used for more than two months from the N.E.N. assay date.

The stock solution of [^{125}I]T_3 can be routinely assayed for [^{125}I]iodide, [^{125}I]T_3, and [^{125}I]T_4 using a method developed by Green[58] and modified by Latham et al.[59] as follows: A sample of [^{125}I]T_3 (0.1 ml of 2 x 10^{-8} M, ≃100,000 cpm) is diluted 1:1 with 0.03 N NaOH, 1.0 M NaCl and incubated at 37^{o} for 10 min. A 0.1 ml portion is loaded onto a Sephadex G-25 (fine) column (bed volume = 2.0 ± 0.05 ml, in a disposable Pasteur pipette with a cotton plug) equilibrated in 0.015 N NaOH, 0.5 M NaCl, and eluted at ambient room temperature in 0.25 ml fractions with column equilibration solution. Protein associated-[^{125}I], [^{125}I]iodide, and[^{125}I]triiodothyronine elute as sharp peaks at 1.0, 2.0, and 3.25 ml, respectively. [^{125}I]Thyroxine elutes as a broad peak at 5.25 ml (figure 4-1).

There are many names for the competitive binding assay such as: saturation analysis, isotope dilution, equilibrium analysis, radioligand assay, etc, but all of these techniques rely on (a) the partial saturation of a specific binding agent (receptor) by the substance undergoing testing (hormone analog), and (b) on the competition of the test substance with a radioactive, chemically similar or identical "indicator" substance (the labeled hormone) for the available binding sites on the binder.[70] The differences in the degree of saturation of the binder by the labeled and unlabeled ligands are the basis for the calculation of the equilibrium binding parameters. Saturation analysis experiments allow for determination of kinetic rate constants and measurement of the thermodynamic, entropic, and enthalpic contributions to the binding free energy. The binding assay used in this study is the method for determining the degree of saturation of the receptor by [^{125}I]triiodothyronine (bound counts).

Figure 4-1. Radiopurity of [^{125}I]Triidothyronine



SOLUBILIZED RECEPTOR BINDING ASSAY DESCRIPTION

Two parallel sets of incubation tubes (12 x 75 mm) are prepared to determine (a) the total amount of labeled hormone that is bound to the receptor and (b) the amount of "non-specifically" bound radioactivity. The non-specific component is defined as non-saturable binding of [^{125}I]T_3 to low-affinity, high-capacity binding sites and is detected as radioactivity which is bound even in the presence of a 1000-fold excess of unlabeled triiodothyronine.

A tracer dose of [^{125}I]T_3 is added to each tube. This concentration is approximately half of the equilibrium dissociation constant (K_d) for T_3 binding to the receptor, and should contain at least 100,000 counts per minute (cpm). To determine non-specific binding, a concentrated solution of unlabeled triiodothyronine (5×10^{-5} M) is added to half of the tubes so that the final T_3 concentration is approximately 10^3 times the concentration of [^{125}I]T_3. Incubation Buffer I (50 mM sodium phosphate (pH 7.6), 0.2 M ammonium sulfate, 1.0 mM dithiothreitol, 5% glycerol) is added to each tube so that the final incubation volume is 0.5 ml when the volume of added nuclear extract is taken into account. The radioactivity of the tubes is counted before the assay is started in order to have an accurate measure of the initial concentration of [^{125}I]T_3.

To start the assay, nuclear extract or QAE-purified nuclear extract is added to the incubation tubes. The tubes are then mixed with a vortex mixer and placed in an appropriate constant temperature bath (for analog competition T = 25 ± 1^{o}). The amount of nuclear extract to be added can be determined from a direct plot (figure 3-1). The incubation tubes should produce 10 to 20 x 10^3 cpm bound and still be in the linear range of the direct plot. In a typical nuclear extract preparation, 0.1 ml

of nuclear extract contains 20 to 200 pM of binding sites (∿0.30 mg protein, 15,000 bound cpm). After a two hr incubation at 25^{o} the tubes are quickly cooled to 4^{o} with an ice bath. This low temperature slows the dissociation rate constant (k_{-1}) and allows for separation of bound and free counts without significant loss of bound counts in the gel filtration medium. For 50 tubes, the bound and free hormone can be separated in 15 min. Since the dissociation rate constant (k_{-1}) at 4^{o} is 5.2×10^{-4} min^{-1}, there is no significant dissociation within 15 min. To separate the bound and free hormone populations, a small Sephadex G-25 (medium) column (bed volume = 2.0 ± 0.05 ml, equilibrated in Incubation Buffer I) is prepared for each incubation tube from a 5 inch disposable Pasteur pipette with a cotton plug (dental supply #3) at the narrow end. A sample of incubation mixture (0.4 ml) is layered on these minicolumns with a Pipetman^{R} digital pipette. Incubation Buffer I (0.4 ml) is added to the columns to start the elution of bound counts. The columns are then moved over on top of a new set of clean tubes and the macromolecular-bound counts are collected in the excluded peak by elution with an additional 0.8 ml of Buffer I. The amount of ^{125}I is then determined with a Searle Auto-gamma spectrometer (efficiency = 74%). Specific binding (ie. high-affinity, limited-capacity) is calculated by subtracting the cpm for non-specific binding from the cpm for total binding. Free hormone is calculated by subtracting the cpm for total binding from the cpm for total labeled hormone added.

A plot of radioactive cpm versus the number of ml collected from a typical Sephadex G-25 minicolumn is shown in figure 4-2. The triangles represent an incubation without the excess unlabeled triiodothyronine and the circles represent an incubation with a 1000-fold excess of unlabeled triiodothyronine. The first peak to elute from the column is

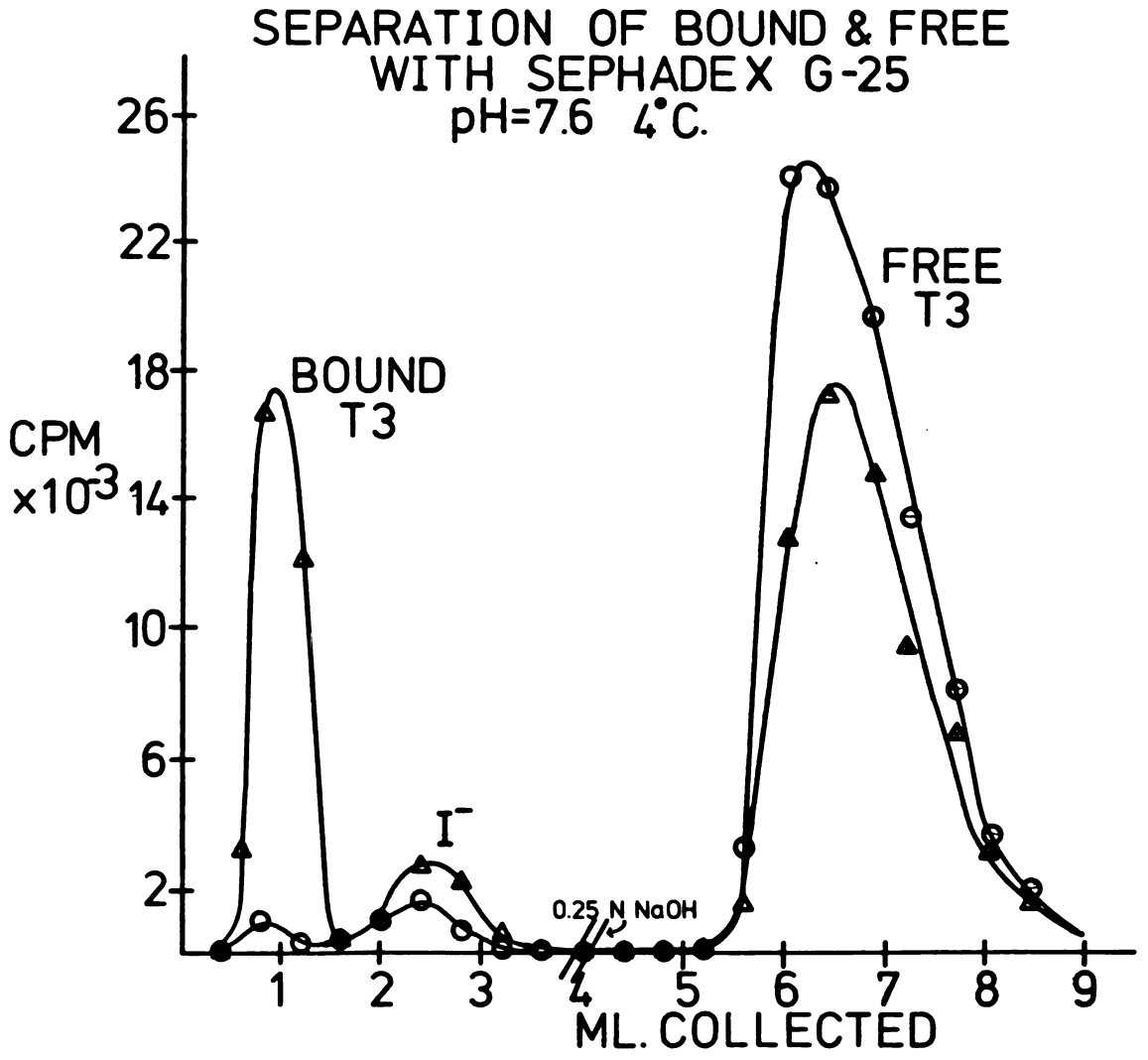

SEPARATION OF BOUND & FREE
WITH SEPHADEX G-25
pH=7.6 4°C.
CPM
x10⁻³
26
22
18
14
10
6
2
BOUND
T3
FREE
T3
I⁻
0.25 N NaOH
1
2
3
4
5
6
7
8
9
ML. COLLECTED

Figure 4-2.

in the excluded volume (1.6 ml) and represents macro-molecular-bound [^{125}I]T_3. The second peak at 2.5 ml represents [^{125}I]iodide. The elution buffer must be changed to 0.25 N NaOH in order to elute the free cpm between 5.5 ml and 8.5 ml.

The procedure described above can be modified as follows for: (a) determination of the time required to reach equilibrium (kinetic assay), (b) determination of the equilibrium dissociation constant and nuclear receptor site concentration (Scatchard assay), and (c) determination of the relative binding affinity of a series of thyroid hormone analogs.

For determination of kinetic parameters, two sets of tubes are prepared as above for determination of total and non-specific binding. However, in this assay, each tube is started at a different time and when the assay is finished, all of the tubes are cooled to 4^{o} for separation of bound and free [^{125}I]T_3. The resulting data is plotted as specifically bound counts versus time as in figure 4-3. This plot indicates that for a 25^{o} incubation the amount of specifically bound cpm reaches 95% of the equilibrium plateau level in two hours. Also, the kinetic rate constants k_1 and k_{-1} may be determined as described in Chapter Six. After determination of the time required to reach equilibrium at a particular temperature, a Scatchard analysis may be used to determine the equilibrium association constant (K_a) and the concentration of hormone binding sites (P_t) in each incubation tube.

The Scatchard plot[60] is a plot of bound/free [^{125}I]T_3 versus the concentration of bound [^{125}I]T_3 as in the following equation:

$$B/F = K_a (P_t - B)$$

Normally, both the ordinate and abcissa would be divided by the total concentration of binding protein so that the abscissa intercept would yield the number of binding sites per protein molecule. Since the

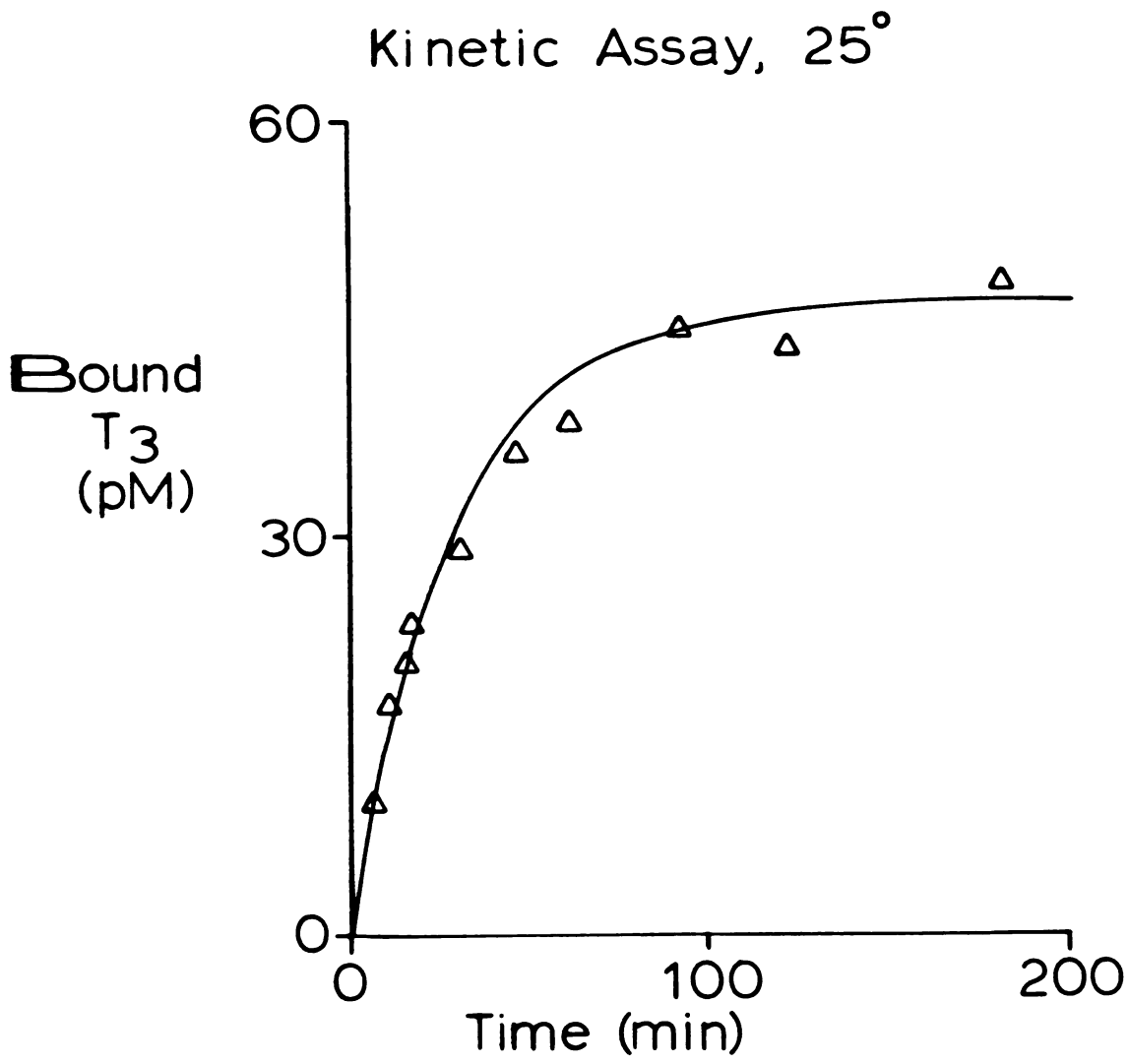


Figure 4-3.

nuclear extract is a mixture of many different proteins in addition to the nuclear receptor for thyroid hormone, it is impossible to determine the molarity of the nuclear receptor protein. Therefore, the abscissa intercept yields the total concentration of hormone binding sites but indicates nothing about the number of sites per receptor molecule. In order to obtain the data for Scatchard analysis, the same procedure as for the kinetic and direct assays is employed except that the amount of added [^{125}I]T_3 is varied from 10 times less than the K_d to 10 times more than the K_d. Again, a parallel set of tubes is incubated for 2 hr at 25^{o} with 1000-fold excess of unlabeled-T_3 to determine non-specific binding. Determination of bound and free cpm allow calculation of the B/F ratio for each point. The calculation of bound molarity from the counts per minute data will be described.

When a shipment of [^{125}I]T_3 is received, it is accompanied by a specific activity (914 μCi/μg) and an assay date on which that specific activity is valid. It is important to consider what occurs over the two months after that date that experiments can be performed.

Radioactive decay of the ^{125}Iodine in the 3'-position to the stable element $^{125}_{52}$Tellurium occurs by gamma emission (0.35 Mev) and electron capture. The recoil energy of 0.35 x 10^6 ev (8.07 x 10^5 kcal) is enough to insure that the molecule which was [^{125}I]triiodothyronine is no longer hormonally active due to the loss of iodine or complete disintegration. In order to determine if this radioactive decay contributes significantly to lowering the T_3 concentration, the percentage of molecules in the original solution which contains ^{125}Iodine must be calculated.

The specific activity of 914 μCi/μg does not represent 100% incorporation of ^{125}I at the 3'-position. Since the specific activity due to 100% incorporation of ^{125}I can be calculated, it is trivial to determine

the original percentage of the incorporation of ^{125}I.

Calculation of the decay constant α:

$$\ln \frac{N_o}{N} = \alpha t \quad \text{or} \quad \alpha = \frac{\ln 2}{t_{1/2}}$$

Since $t_{1/2}$ = 60 days, $\alpha = 0.0115 \text{ days}^{-1}$.

Calculation of the disintegrations that would occur per minute if all the molecules in a mole of T_3 were labeled:

$$-\frac{dN}{dt} = \alpha N = 0.0115 \times 6.061 \times 10^{23} \text{ molecules/mol}$$

$$-\frac{dN}{dt} = 6.97 \times 10^{21} \text{ disint./day}$$

$$\text{or } -\frac{dN}{dt} = 4.84 \times 10^{18} \text{ dpm}$$

$$\text{or } -\frac{dN}{dt} = 2.18 \times 10^{12} \ \mu\text{Ci}$$

Since there are 645 grams per mol of $[^{125}I]T_3$

$$-\frac{dN}{dt} = \frac{2.18 \times 10^{12}}{645} \ \mu\text{Ci/g}$$

Finally, the specific activity of a preparation of $[^{125}I]T_3$ if all of the molecules were labeled would be:

3,380 μCi/μg

The specific activity received from N.E.N. is 914 μCi/μg.

Since $\frac{914}{3,380} = 0.27$, there must be 27% of the molecules labeled.

Therefore, 27% of the molecules in the solution obtained from New England Nuclear are labeled. If the solution is used for one half-life (60 days) then 13.5% of the molecules in the original solution would be gone. This would be a large error in the estimation of the concentration of triiodothyronine based on counts per minute.

It is also important to correct for the cpm due to $[^{125}I]$iodide. This correction changes with the age of the hormone preparation and can

be estimated to be 3% at time zero and 2 to 3% per month after that. In this work the following equations were developed to calculate the bound molarity from raw counts per minute for use in a PROPHET computer procedure. (see Appendix I).

I. To calculate the factor for each day to account for basic radioactive decay:

$$F = \frac{1}{E\ (2.22 \times 10^9\ \text{dpm/mCi})(\text{S.A.})\ V}$$

Where:

F = the factor $(\frac{\text{moles/liter}}{\text{cpm}})$

E = radioactive counting efficiency (generally 74%)

S.A. = specific activity of preparation (mCi/mol)

V = volume of the appropriate sample (liters)

II. To correct for X% of [^{125}I]iodide:

$$F_c = F - (F \times X\%/100)$$

Where:

F_c = Factor corrected for $^{125}I^-$ (mol/liter/cpm)

X% = percentage $^{125}I^-$ in the sample

III. Finally, to calculate the molarity of a given sample with a measured activity in counts per minute (cpm):

$$M = (F_c \times e^{\alpha t})(\text{cpm}) - \left[(F_c \times e^{\alpha t})(\text{cpm})(.27(1 - e^{-\alpha t}))\right]$$

Where:

$\alpha = 0.0115\ \text{days}^{-1}$

t = no. of days past assay date

$e^{\alpha t}$ = ratio of molecules at time 0/molecules at time t

M = molarity of sample (mol/liter)

0.27 = 27% of molecules labeled ÷ 100

$e^{-\alpha t}$ = ratio of [^{125}I]molecules which have decayed in time t

$(.27(1-e^{-\alpha t}))$= fraction of total molecule population which has disappeared at time t.

The last equation can easily be applied to a computer procedure for calculating the molarity of bound counts to be used in the abscissa of a Scatchard plot. A typical Scatchard plot is seen in figure 4-4. The triangles represent one out of five experiments conducted to determine the mean association constant and standard deviation. The correlation coefficient of 0.985 indicates that a straight line gives a good fit to the points and there is only one class of independent binding sites. The standard free energy is calculated from the thermodynamic equation:

$$\Delta G^{o} = -RT \ln K_a$$

R represents the gas constant 1.9872 cal/mol^{o}K and T is the temperature in ^{o}K. P_t is the average number of moles of binding sites in 100 µl of nuclear extract (0.30 mg total protein) determined from the x-axis intercept. The graph which is inset (figure 4-4) is a plot of specifically bound molarity versus free molarity, and the triangles indicate that the binding reaction is saturable. The circles on the inset graph are a plot of the counts bound from the non-specific control tubes which contained a 1000-fold excess of unlabeled T_3. The graph shows that this non-specific binding is non-saturable since there is no plateau as in the case of the specifically bound hormone.

After the equilibrium association constant for [^{125}I]triiodothyronine binding to nuclear receptor has been determined, the binding affinity of

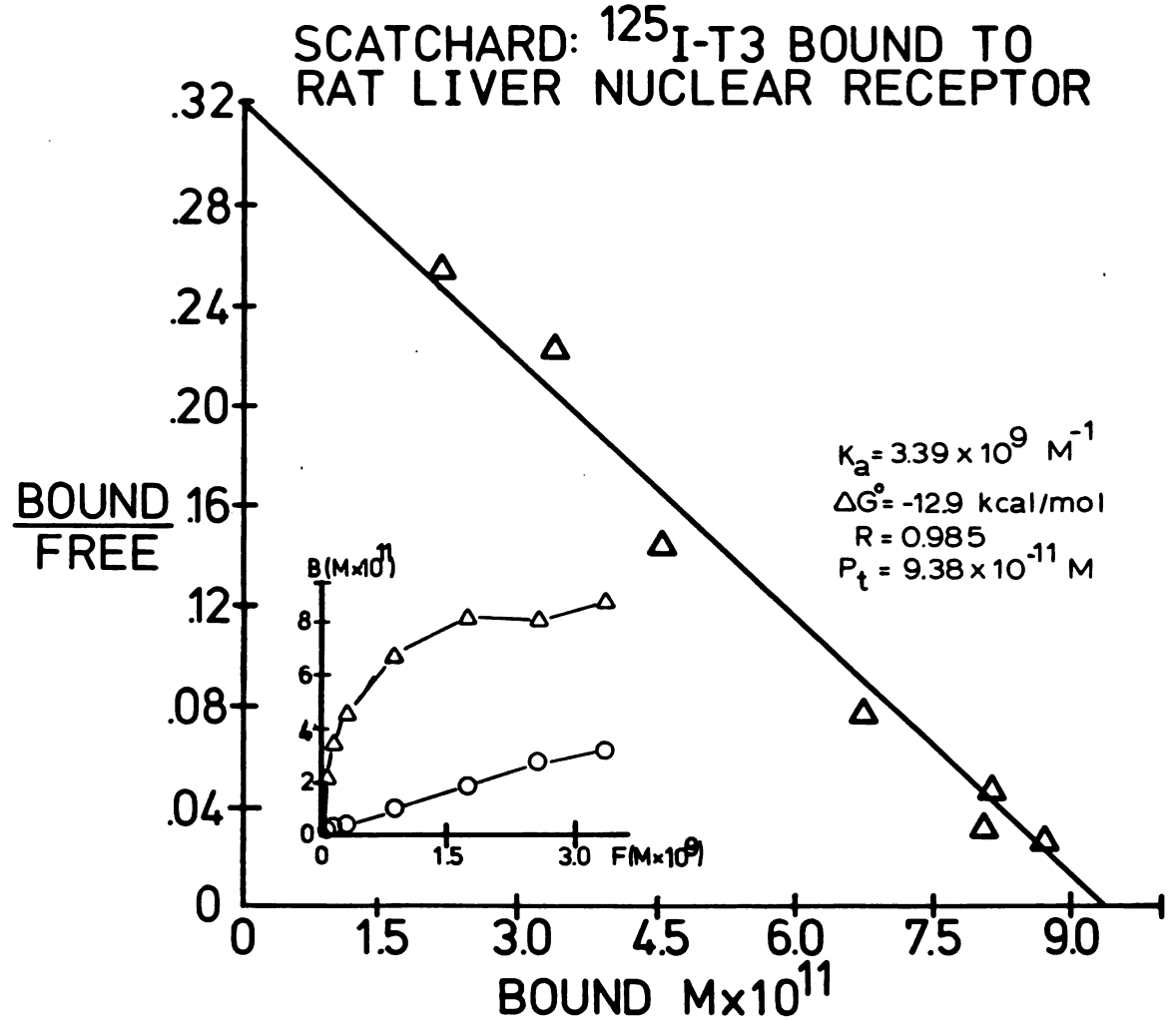

SCATCHARD: 125I-T3 BOUND TO
RAT LIVER NUCLEAR RECEPTOR
BOUND / FREE
.32
.28
.24
.20
.16
.12
.08
.04
0
0
1.5
3.0
4.5
6.0
7.5
9.0
BOUND Mx10^11
Ka = 3.39 x 10^9 M^-1
ΔG° = -12.9 kcal/mol
R = 0.985
Pt = 9.38 x 10^-11 M
B(Mx10^11)
8
6
4
2
0
0
1.5
3.0
F(Mx10^9)

Figure 4-4.

unlabeled analogs can be measured with a simple equilibrium competition binding assay. The assay uses a tracer dose of [^{125}I]T_3 (½ of the K_d at 25^o) with a constant amount of nuclear extract and an increasing amount of unlabeled thyroid hormone analog. Parallel incubation tubes are started at 25^o, allowed to incubate for 2 hr, and cooled to 4^o for separation of bound and free [^{125}I]T_3. A PROPHET computer procedure was written to analyse the data according to the method of Koerner, Surks, and Oppenheimer using the following equation:[61]

$$\frac{\text{Free}}{\text{Bound}} = \frac{1}{K_{a_3} \cdot P_t} + \frac{\frac{K_{a_A}}{K_{a_3}} (\text{concn analog added})}{P_t}$$

Where:

K_{a_3} = equilibrium association constant for T_3

K_{a_A} = equilibrium association constant for cold analog

P_t = total moles per liter of hormone binding sites

A derivation of this equation is given in Appendix II.

A typical plot of the competition binding assay for two cold analogs is seen in figure 4-5. The triangles represent a line of steeper slope and higher affinity for unlabeled triiodothyronine and the circles represent a line of smaller slope and lower affinity for thyroxine. The total moles per liter of binding sites (P_t) can be calculated from the Y-axis intercept and the known K_a for T_3. The ratio of equilibrium association constants for the unlabeled analog and T_3 can be obtained from the slope and P_t. The unlabeled analog association constant can be calculated from this ratio and the known K_a for T_3. Using this method the binding affinity of fifty unlabeled analogs has been determined and is presented later in this chapter.

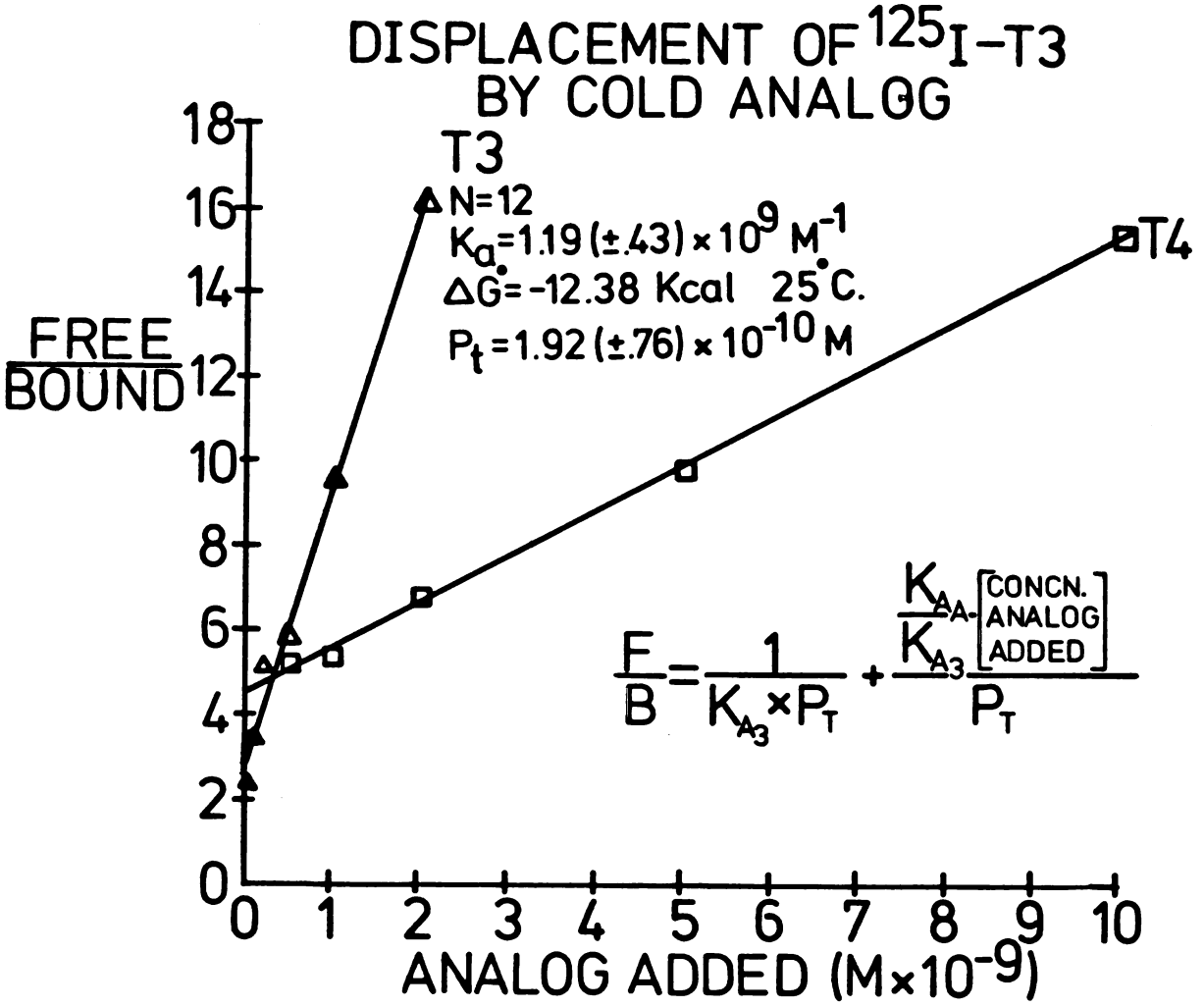

DISPLACEMENT OF 125I-T3
BY COLD ANALOG
T3
N=12
Ka=1.19 (±.43) x 10^9 M^-1
ΔG°=-12.38 Kcal 25°C.
Pt=1.92 (±.76) x 10^-10 M
T4
FREE
BOUND
18
16
14
12
10
8
6
4
2
0
0 1 2 3 4 5 6 7 8 9 10
ANALOG ADDED (M x 10^-9)
F/B = 1/(KA3 x PT) + (KAA [CONCN. ANALOG ADDED])/PT

Figure 4-5.

IN VIVO RAT ANTIGOITER BIOASSAY

Many methods have been developed to test the *in vivo* potency of thyroid hormone analogs, such as rat oxygen consumption,[62] tadpole metamorphosis,[1,63-66] jellyfish *Aurelia* metamorphosis,[2] and serum cholesterol levels.[67-69] However, the most reliable and extensively used method which involves feedback control by the thyroid hormones of pituitary thyrotropin secretion, is called the "antigoiter bioassay". An extensive description and history of the antigoiter assay has been prepared by Dietrich.[34] This discussion will be limited to the basic principles of the rat antigoiter assay.[71]

The presence of an "antithyroid" drug such as thiouracil or propylthiouracil in the diet of rats, induces a thyroid hormone deficiency by blocking the biosynthesis and release of T_3 and T_4 from the thyroid. In response to the lowered circulating thyroid hormone levels, thyrotropin is released from the pituitary producing increased thyroid tissue development and circulation and eventually leads to an enlarged thyroid gland called a goiter. This goiter is well-formed within the ten day length of the bioassay. Graded doses of either the reference compound (T_3 or T_4) or the analog to be tested are administered daily by subcutaneous injections. The relative activity of an analog is estimated (based on a standard log dose *vs.* 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 drug-induced goiter.

The three new thyroid hormone analogs that have been tested in the rat antigoiter assay for this study all contain hydrogen instead of hydroxy at the 4'-position. The *in vivo* assay provides a measure of

the metabolic conversion of the 4'-deoxy analog to the corresponding 4'-hydroxy analog by comparison with the *in vitro* binding affinity of the 4'-deoxy analog. The bioassay results for 3,5-diiodo-3'-(fluoro, chloro, and bromo)-4'-deoxy-L-thyronine and a discussion of the correlation between *in vivo* activity and *in vitro* binding affinity will be presented in the next section.

CORRELATION BETWEEN IN VITRO BINDING AND IN VIVO ANTIGOITER ACTIVITY

Whenever a new putative "receptor" is found, there have been five important criteria, defined by Cuatrecasas,[72] which must be met in order to establish its physiological relevance. The thyroid hormone nuclear receptor was found to meet all of the following criteria: (1) The binding sites should be finite in number and therefore, saturable. This was shown first by Oppenheimer and coworkers in 1972.[12] (2) The hormone binding should have tissue specificity consistent with biological specificity. Limited-capacity binding sites have been found in all tissues studied in the rat, however, the capacity in tissues generally considered to be non-responsive to thyroid hormone (testis and brain) was very low compared to thyroid responsive tissues (anterior pituitary, liver, and heart).[40] (3) Hormone binding should be of high-affinity and consistent with its physiologic concentrations. The K_d reported here for T_3 is 8.4×10^{-10} M. Since the typical endogenous serum concentration of T_3 in rats is 1.3×10^{-9} M, the hormone dissociation constant appears to be very close to the physiologic concentration.[73] (4) The binding of the hormone to the receptor should be reversible. All of the competitive analog binding studies show that binding is reversible.[56] (5) The *in vitro* hormone binding interaction with the receptor should correlate

with known steric and structural specificity determined from *in vivo* thyromimetic activity and *in vitro* binding to intact nuclei. In two recent studies, Dietrich *et al.*[36,56] reported a highly significant quantitative correlation between *in vivo* antigoiter bioassay activity and *in vitro* binding to the solubilized rat liver nuclear receptor for 26 thyroid hormone analogs. In the current study, the solubilized receptor binding data has been expanded and corrected to account for a change in the mean equilibrium association constant for T_3 from 1.29 x 10^9 M^{-1} to 1.19 x 10^9 M^{-1}. The current value of K_a = 1.19 (±0.43) x 10^9 M^{-1} is based on twelve determinations over a two year period involving ten different solubilized hepatic nuclear protein preparations.

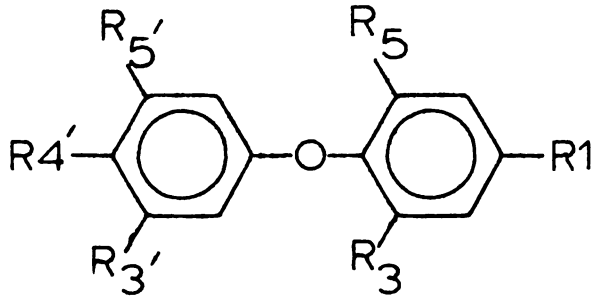
The PROPHET computer was used to derive a quantitative correlation for 32 analogs between the *in vivo* rat antigoiter bioassay activity (BA) and the *in vitro* solubilized receptor binding affinity (BS). Utilizing the analogs of Table 4-1 and the data of Table 4-2, eq 4-1 was derived.

$$\begin{aligned} \log(BA) = &+ 0.251 + 0.906\ (\pm 0.067)\ \log(BS) \\ &+ 1.428\ (\pm 0.193)\ I4'H \\ &+ 0.599\ (\pm 0.207)\ I4'OCH_3 \end{aligned} \qquad \text{eq 4-1}$$

$N = 32 \quad R = 0.935 \quad S = 0.381$

The indicator variable I4'H and $I4'OCH_3$ were entered to account for the *in vivo* metabolic hydroxylation of 4'-deoxy compounds and for O-demethylation of 4'-OCH_3 compounds. Analogs which had either 4'-H or 4'-OCH_3 were assigned a value of 1 for the respective indicator variable and all other analogs were assigned 0. One exception, data point #19 = 4'H-3', 5'Me_2T_2 of Table 4-2 was assigned I4'H = 0 because of the apparent steric inhibition by the 3'5'-methyl groups of the *in vivo* hydroxylation of this analog. A stepwise development of eq 4-1 is presented in Table 4-3. All

Table 4-1. Structures and Activities of Thyroid Hormone Analogs Used in Deriving Equation 4-1



4—1

Data Point No.	Abbreviation	DL	R_1	R_3	R_5	$R_{3'}$	$R_{5'}$	$R_{4'}$	Anti-goiter[a] %L-T_3	Soluble Binding[b] %L-T_3
1	4'H-T2	L	ALA	I	I	H	H	H	1.24[c]	0.012
2	T2	L	ALA	I	I	H	H	OH	0.81	0.089
3	4'NH2-T2	L	ALA	I	I	H	H	NH2	0.036	0.01
4	4'H-T3	DL	ALA	I	I	I	H	H	27.12	0.26
5	T3	L	ALA	I	I	I	H	OH	100.0	100.0
6	4'OMe-T3	L	ALA	I	I	I	H	OMe	11.25	1.62
7	4'H-3'Me-T2	DL	ALA	I	I	Me	H	H	2.71	0.24
8	3'Me-T2	L	ALA	I	I	Me	H	OH	14.47	3.55
9[e]	4'OMe-3'Me-T2	L	ALA	I	I	Me	H	OMe	---	0.185
10[e]	4'H-3'iPr-T2	L	ALA	I	I	iPr	H	H	---	0.534
11	3'iPr-T2	L	ALA	I	I	iPr	H	OH	142.1	92.53
12	4'OMe-3'iPr-T2	L	ALA	I	I	iPr	H	OMe	19.0	7.39
13	3'sBu-T2	L	ALA	I	I	sBu	H	OH	79.9	84.82
14	4'OMe-3'sBu-T2	L	ALA	I	I	sBu	H	OMe	21.0	1.41
15[e]	4'H-3'tBu-T2	DL	ALA	I	I	tBu	H	H	---	0.34

Table 4-1. (Continued)

Data Point No.	Abbreviation	DL	R_1	R_3	R_5	$R_{3'}$	$R_{5'}$	$R_{4'}$	Anti-[a] goiter %L-T_3	Soluble[b] Binding %L-T_3
16	3'tBu-T2	L	ALA	I	I	tBu	H	OH	21.7	9.23
17	4'OMe-3'tBu-T2	L	ALA	I	I	tBu	H	OMe	2.35	0.29
18	3'nPr-T2	L	ALA	I	I	nPr	H	OH	39.5	25.99
19	4'H3'5'Me2T2	DL	ALA	I	I	Me	Me	H	0.054	0.155
20	3'5'-Me2-T2	L	ALA	I	I	Me	Me	OH	9.04	0.917
21[e]	4'OMe3'5'Me2-T2	L	ALA	I	I	Me	Me	OMe	---	0.369
22[e]	4'H-3'NO2-T2	DL	ALA	I	I	NO_2	H	H	---	0.041
23	3'NO2-T2	L	ALA	I	I	NO_2	H	OH	0.32	0.244
24	4'NH2-3'Me-T2	L	ALA	I	I	Me	H	NH_2	0.036	0.033
25	4'NH23'5'Me2-T2	L	ALA	I	I	Me	Me	NH_2	0.13	0.045
26[e]	3'5'iPr2-T2	L	ALA	I	I	iPr	iPr	OH	---	1.195
27	RT3	L	ALA	I	H	I	I	OH	0.125	0.189
28	33'-T2	L	ALA	I	H	I	H	OH	0.25	0.747
29	3'F-T2	DL	ALA	I	I	F	H	OH	0.65[d]	0.202
30	3'Cl-T2	L	ALA	I	I	Cl	H	OH	4.88	4.05
31	3'Br-T2	L	ALA	I	I	Br	H	OH	23.78	17.25
32	T4	L	ALA	I	I	I	I	OH	18.1	14.4
33	4'H-3'F-T2	L	ALA	I	I	F	H	H	1.39	0.015
34	4'H-3'Cl-T2	L	ALA	I	I	Cl	H	H	7.78	0.129
35	4'H-3'Br-T2	L	ALA	I	I	Br	H	H	18.0	0.258
36[e]	3'iPr-5'Cl-T2	L	ALA	I	I	iPr	Cl	OH	---	56.96
37[e]	3'iPr-5'Br-T2	L	ALA	I	I	iPr	Br	OH	---	23.8
38[e]	3'iPr-355'-T3	L	ALA	I	I	iPr	I	OH	---	13.46

Table 4-1. (Continued)

Data Point No.	Abbreviation	DL	R_1	R_3	R_5	$R_{3'}$	$R_{5'}$	$R_{4'}$	Anti-[a] goiter %L-T_3	Soluble[b] Binding %L-T_3
39	3'5'Cl2-T2	L	ALA	I	I	Cl	Cl	OH	3.8	4.015
40[e]	3'5'Br2-T2	L	ALA	I	I	Br	Br	OH	1.58	5.495
41[e]	D-T3	D	ALA	I	I	I	H	OH	7.5	62.77
42[e]	35Et2-3'-Tl	DL	ALA	Et	Et	I	H	OH	---	0.649
43	35Me2-3'-Tl	L	ALA	Me	Me	I	H	OH	0.9	0.54
44	35Me2-3'iPr-TO	L	ALA	Me	Me	iPr	H	OH	3.6	0.469
45[e]	35iPr2-3'-Tl	DL	ALA	iPr	iPr	I	H	OH	0.0	0.138
46[e]	35Et2-3'5'-T2	DL	ALA	Et	Et	I	I	OH	---	0.099
47[e]	TRIAC	-	AC	I	I	I	H	OH	6.43	282.5
48[e]	TRIPROP	-	PROP	I	I	I	H	OH	3.58	234.5
49[e]	TRIFORM	-	FORM	I	I	I	H	OH	0.081	8.47
50[e]	TRIBUTYR	-	BUTYRI	I	I	H	OH		0.73	14.4

a. Relative to L-T3 = 100 in the rat antigoiter assay, literature values being recalculated on a molar basis.[34]

b. Relative to L-T3 = 100, $K_a = 1.19 \times 10^9\ M^{-1}$ at 25°. No correction was made for DL analogs. All values determined for this study.

c. DL analog compared to an L analog. Corrected to L-value by dividing by 0.58.

d. DL analog relative to DL-T3. Corrected to compare with DL analog relative to L-T3 by multiplying by 0.58.

e. Not used in correlation of antigoiter activity and binding affinity.

Table 4-2. Data Used in the Formulation of Equation 4-1 Correlating *In Vivo* Antigoiter Activities (BA) with *In Vitro* Binding to Solubilized Rat Hepatic Nuclear Protein (BS) for Thyroid Hormone Analogs

Data Point No.	Abbreviation	log(BS) Obsd.	log(BA) Obsd.	log(BA) Calcd.	log(BA) Dev.
1	4'H-T2	-1.923	0.093	-0.062	0.155
2	T2	-1.051	-0.092	-0.701	0.609
3	4'NH2-T2	-2.000	-1.440	-1.560	0.117
4	4'H-T3	-0.585	1.430	1.150	0.285
5	T3	2.000	2.000	2.060	-0.063
6	4'OMe-T3	0.210	1.050	1.040	0.012
7	4'H-3'Me-T2	-0.620	0.433	1.120	-0.684
8	3'Me-T2	0.550	1.160	0.750	0.411
11	3'*i*Pr-T2	1.966	2.150	2.030	0.120
12	4'OMe-3'*i*Pr-T2	0.869	1.280	1.640	-0.358
13	3'*s*Bu-T2	1.928	1.900	2.000	-0.096
14	4'OMe-3'*s*Bu-T2	-0.538	-1.320	0.985	0.337
16	3'*t*Bu-T2	0.965	1.340	1.130	0.211
17	4'OMe-3'*t*Bu-T2	-0.538	0.371	0.362	0.009
18	3'*n*Pr-T2	1.415	1.600	1.530	0.064
19	4'H-3'5'Me2-T2	-0.810	-1.270	-0.483	-0.785
20	3'5'Me2-T2	-0.038	0.956	0.217	0.739
23	3'NO2-T2	-0.613	-0.495	-0.304	-0.191
24	4'NH2-3'Me-T2	-1.481	-1.440	-1.090	-0.352
25	4'NH2-3'5'Me2-T2	-1.347	-0.886	-0.969	0.083

Table 4-2. (Continued)

Data Point No.	Abbreviation	log(BS) Obsd.	log(BA) Obsd.	log(BA) Calcd.	log(BA) Dev.
27	RT3	-0.724	-0.903	-0.405	-0.499
28	33'-T2	-0.127	-0.602	0.136	-0.738
29	3'F-T2	-0.695	-0.187	-0.378	0.191
30	3'Cl-T2	0.607	0.688	0.801	-0.113
31	3'Br-T2	1.237	1.380	1.370	0.005
32	T4	1.158	1.260	1.300	-0.043
33	4'H-3'F-T2	-1.824	0.143	0.026	0.117
34	4'H-3'Cl-T2	-0.889	0.891	0.873	0.018
35	4'H-3'Br-T2	-0.588	1.260	1.150	0.110
39	3'5'Cl2-T2	0.604	0.580	0.798	-0.218
43	35Me2-3'-Tl	-0.268	-0.046	0.009	-0.054
44	35Me2-3'iPr-TO	-0.329	0.556	-0.047	0.603

Table 4-3. Stepwise Development of Equation 4-1

Eq No.	Equation and Statistical Data
4-2	log (BA) = +0.576 + 0.709 log (BS) R = 0.784 S = 0.644 $F_{(1),1,30}$ = 47.89 (99% vs. mean)
4-3	log (BA) = +0.343 + 0.909 log (BS) + 1.339 I4'H R = 0.915 S = 0.426 $F_{(1),1,29}$ = 39.34 (99.9% vs. eq 4-2)
4-1	log (BA) = +0.251 + 0.906 log (BS) + 1.428 I4'H + 0.599 I4'OMe R = 0.935 S = 0.381 $F_{(1),1,28}$ = 8.35 (99.2% vs. eq 4-3)

of the variables except I4'OMe were entered at >99.9% confidence level as indicated by the F statistic.[74] I4'OMe was entered at the 99.2% confidence level. A plot of the logarithm of the antigoiter activity (%L-T_3) is presented in figure 4-6. The straight line with a log (BS) regression coefficient of 0.906 and an intercept 0.251 represents the best fit for 22 analogs (open triangles) that do not have a 4'-H or 4'-OMe substitution. Dietrich and co-workers reported a log (BS) regression coefficient of 0.757 and an intercept of 0.274 for a similar correlation with 17 analogs that did not have 4'-H or 4'-OMe substitution.[36] The larger log (BS) regression coefficient for eq 4-1 indicates that the correlation between *in vivo* and *in vitro* activity is closer to a 1:1 correlation. The deviation from the previous work,[36] is due to a recent recalculation of the binding affinity data to reflect a change in the average value of K_a for T_3 from 1.29×10^9 to 1.19×10^9 M^{-1} and the addition of 5 more analogs to the correlation. The deviations of the intercepts from 0.0 and of the log (BS) regression coefficient from 1.0 reflect not only the minimal accuracy of some of the *in vivo* data but also the differences in analog plasma protein binding, metabolism, elimination, and 4'-OH ionization effects *in vivo*. The middle straight line in figure 4-6 represents a slope of 0.906 and an intercept of 0.251 plus the regression coefficient for the indicator variable I4'OMe (0.511). This line indicates that for four analogs (crosses), the percent *in vivo* activity is greater than the corresponding percent *in vitro* activity. This can be accounted for by O-demethylation of the 4'-OMe to form the 4'-OH analog which has higher activity in both assay systems.

The upper straight line represents the six analogs (open circles) with a 4'-H substitution. For these analogs the percent *in vivo* activity

Correlation Between In VIVO and In VITRO Thyroid Hormone Activity

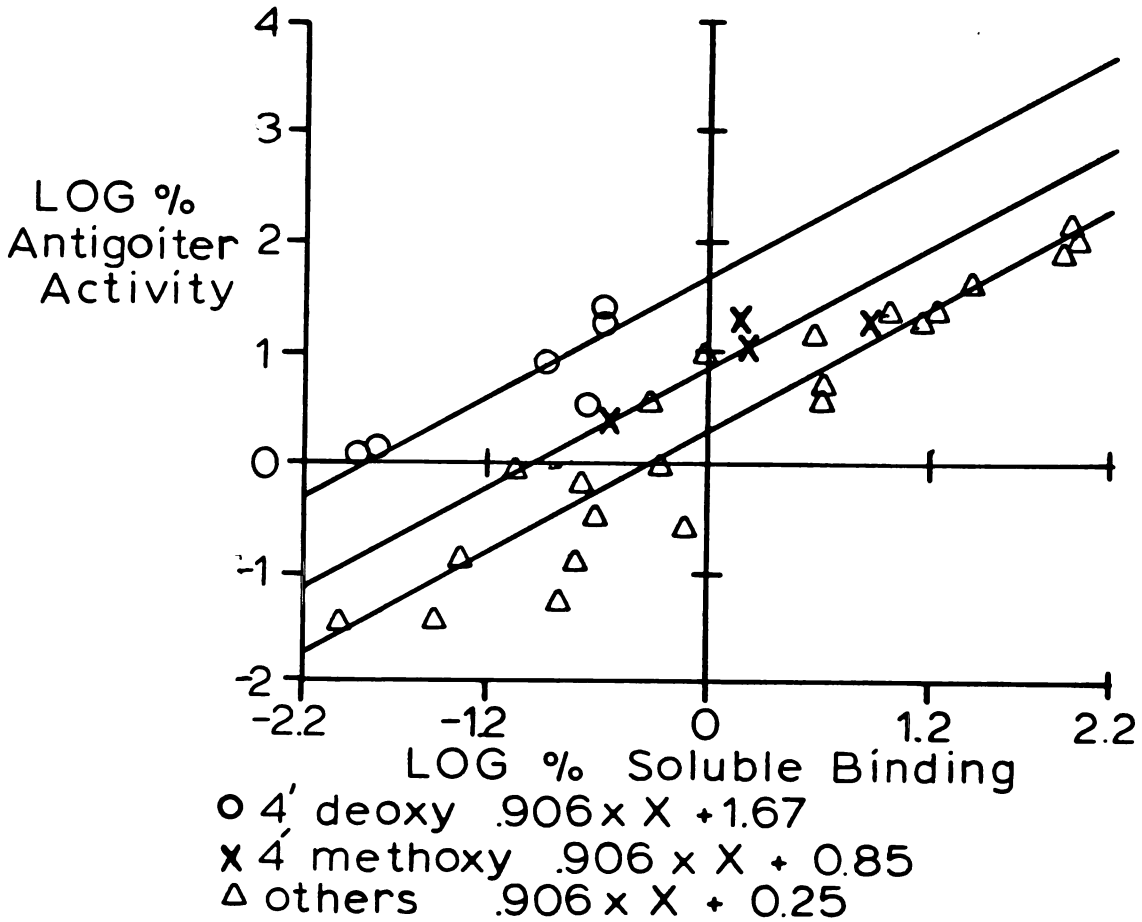


Figure 4-6.

is considerably higher than the percent in vitro activity presumably due to the ease of p-hydroxylation of the 4'-position of these analogs. One exception is the 3',5'-dimethyl-4'-deoxy-3,5-diiodo-L-thyronine (#19, Table 4-1) which has a lower antigoiter activity than binding affinity. This 4'-deoxy analog is probably not extensively hydroxylated in vivo because of the steric hinderance of the two methyl groups ortho to the position of hydroxylation, which would inhibit aromatic hydroxylation.

It thus appears that in vivo antigoiter activities (BA) correlate well with in vitro binding to the solubilized rat liver nuclear receptor (BS), with adjustments made for in vivo metabolism of 4'-deoxy (I4'-H) and 4'-OMe (I4'-OMe) analogs. Eq 4-1 further supports the physiological relevance of this T_3 receptor as an initial mediator of in vivo hormonal activity.

Chapter Five: Molecular Interactions Between Thyroid Hormone and the Solubilized Rat Liver Nuclear Receptor

Previous studies of *in vivo* and *in vitro* thyroid hormone activity have focused on the determination of the structural features of the thyroxine molecule required for activity.[38,42,71] These studies have determined that the following structures will enhance thyromimetic activity: (1) A hydroxyl group in the 4'-position is required. (2) Binding to the nuclear receptor and biological activity *in vivo* are greater for 3'-mono than 3',5'-disubstituted iodothyronines. (3) The 3'-position can contain various halogen atoms or alkyl groups for which the distal conformation is preferred, without appreciable loss of thyromimetic activity. (4) The ether linkage can be replaced by sulfur or a methylene group. (5) Thyromimetic activity is directly related to the ability of the 3 and 5 substituents to confine the diphenyl ether nucleus to two readily interconvertible conformers of approximately equal energy in which the two phenyl

rings are mutually perpendicular. (6) The alanine side chain can be replaced by acetic or propionic acid without significant loss of thyromimetic activity.

The present analog binding studies were chosen to obtain information about the strength of the molecular binding interactions between these required structures and the nuclear receptor site. These binding studies with the solubilized rat liver nuclear receptor have allowed us to: (a) determine the magnitude and directionality of hydrogen bond formation in the hormone-receptor complex; (b) determine the strength and size limit of hydrophobic and dispersion force interactions of the 3,5,3'-substituents with the receptor; and (c) define the probable type of electrostatic interaction between the alanine side chain and charged groups on the receptor.

PARTITIONING THE FREE ENERGIES OF BINDING INTO SUBSTITUENT CONTRIBUTIONS

In the previous chapter it was shown that there is a 1:1 correlation between *in vivo* and *in vitro* thyromimetic activity. The binding affinity of 55 thyroid hormone analogs has been measured as in figure 4-5 and presented as a percentage of L-T_3 in Table 4-1. Since the equilibrium association constant (K_a) of L-T_3 is known, the K_a of the 55 analogs can be calculated. From the following equation (5-1) the free energies of binding (ΔG^o) can also be calculated and are presented in Table 5-1.

$$\Delta G^o = -RT \ln K_a \qquad \text{eq 5-1}$$

This free energy, which is released upon formation of the T_3-receptor

Table 5-1. Binding Free Energies for Thyroid Hormone Analogs from Competition Binding Assay

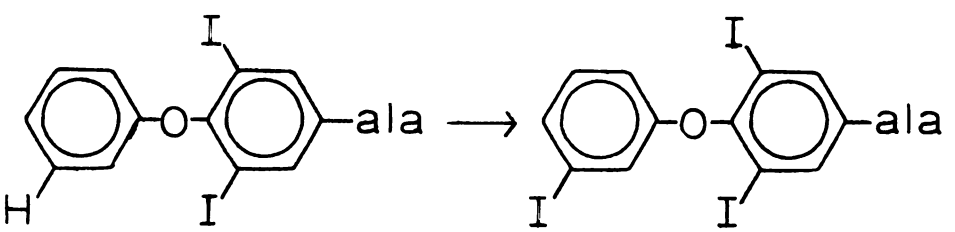
Data Point No.	Abbreviation	$-\Delta G^o$ (kcal/mol)	Data Point No.	Abbreviation	$-\Delta G^o$ (kcal/mol)
1	4'H-T2	7.00(±0.03)	23	3'NO2-T2	8.81(±0.02)
2	T2	8.24(±0.17)	24	4'NH2-3'Me-T2	7.62(±0.09)
3	4'NH2-T2	6.92(±0.06)	25	4'NH2-3'5'Me2-T2	7.80(±0.09)
4	4'H-T3	8.85(±0.38)	26	3'5'iPr2-T2	9.75(±0.02)
5	T3	12.38(±0.18)	27	RT3	8.66(±0.12)
6	4'OMe-T3	9.93(±0.13)	28	33'-T2	9.48(±0.01)
7	4'H-3'Me-T2	8.80(±0.42)	29	3'F-T2	8.70(±0.35)
8	3'Me-T2	10.40(±0.09)	30	3'Cl-T2	10.48(±0.17)
9	4'OMe-3'Me-T2	8.65(±0.19)	31	3'Br-T2	11.33(±0.24)
10	4'H-3'iPr-T2	9.28(±0.19)	32	T4	11.23(±0.08)
11	3'iPr-T2	12.33(±0.17)	33	4'H-3'F-T2	7.14(±0.02)
12	4'OMe-3'iPr-T2	10.83(±0.17)	34	4'H-3'Cl-T2	8.44(±0.04)
13	3'sBu-T2	12.28(±0.11)	35	4'H-3'Br-T2	8.85(±0.10)
14	4'OMe-3'sBu-T2	9.85(±0.05)	36	3'iPr-5'Cl-T2	12.04(±0.07)
15	4'H-3'tBu-T2	9.01(±0.11)	37	3'iPr-5'Br-T2	11.52(±0.10)
16	3'tBu-T2	10.96(±0.05)	38	3'iPr-355'-T3	11.19(±0.09)
17	4'OMe-3'tBu-T2	8.91(±0.15)	39	3'5'Cl2-T2	10.47(±0.06)
18	3'nPr-T2	11.58(±0.08)	40	3'5'Br2-T2	10.66(±0.10)
19	4'H3'5'Me2-T2	8.54(±0.02)	41	D-T3	12.09(±0.29)
20	3'5'-Me2-T2	9.60(±0.07)	42	35Et2-3'-T1	9.39(±0.01)
21	4'OMe3'5'Me2-T2	9.06(±0.05)	43	35Me2-3'-T1	9.28(±0.07)
22	4'H-3'NO2-T2	7.76(±0.18)	44	35Me2-3'iPr-T0	9.20(±0.03)

Table 5-1. (Continued)

Data Point No.	Abbreviation	$-\Delta G^{o}$ (kcal/mol)
45	35iPr2-3'-T1	8.48(±0.05)
46	35Et2-3'5'-T2	8.28(±0.36)
47	TRIAC	12.99(±0.03)
48	TRIPROP	12.88(±0.17)
49	TRIFORM	10.91(±0.07)
50	TRIBUTYR	11.23(±0.09)
51	Thyroxamine	< 6.41
52	3'-T1	5.00(±0.15)
53	3'5'-T2	4.10(±0.13)
54	3-T1	6.39(±0.34)
55	T0	< 5.00

complex, is a sum of many small free energy contributions from the various functional groups in T_3 interacting with the amino acid side chains located in the receptor site. In order to obtain a better perspective on the mechanism of hormone action, it is important to know the quantitative contribution of the various functional groups in T_3 to the binding free energy.

For example, the association constant for 3,5,3'-triiodo-4-phenoxy-DL-phenylalanine (5-1) was measured from competition binding studies (figure 4-5) as $K_a = 3.09 \times 10^6\ M^{-1}$ at 25^o and the corresponding free energy is calculated to be $\Delta G^o = -8.85\ (\pm 0.38)$ kcal/mol. Similarly, 3,5-diiodo-4-phenoxy-L-phenylalanine (5-2) shows a binding free energy of -7.00 (±0.03) kcal/mol. Therefore, the enhancement in binding ($\Delta\Delta G$) from addition of a 3'-iodine to the unsubstituted outer ring is $\Delta G^o_{3'\text{-I}} - \Delta G^o_{ref} = -1.85\ (\pm 0.41)$ kcal/mol.



5-2 5-1

$\Delta G^o = -7.00\ (\pm 0.03)$ kcal/mol $\Delta G^o = -8.85\ (\pm 0.38)$ kcal/mol

$$\Delta\Delta G_{3'\text{-I}} = -8.85 - (-7.00) = -1.85 \text{ kcal/mol}$$

This analysis gives a measure of the contribution of a 3'-iodine to the binding free energy. This contribution is probably the result of a sum of "hydrophobic bonding" (described in Chapter Six) and close range van der Waals dipole-induced dipole attraction.

3'-Substitution:

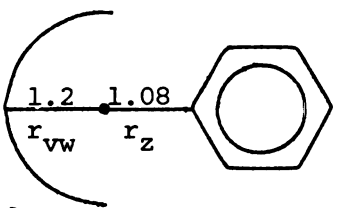
For a series of eight 3'-substituents, Table 5-2 presents the $\Delta\Delta G_{3'\text{-X}}$

and the corresponding 3'-SIZE > H. The parameter 3'-SIZE > H (based on bond distances, van der Waals radii, and conformational considerations) is an estimate of the average distance a 3'-substituent extends out from the 3'-position further than hydrogen.[36] Utilizing the bond distances from benzene substitution,[77] an estimate of 2.0 Å[75] for the van der Waals radii of a CH_2 or CH_3 group, and van der Waals radii of Bondi,[76] 3'-SIZE > H values were calculated as follows: First, the distance (r_z) was calculated to the furthest out non-hydrogen atom from the 3' carbon atom. For 3' substituent = H, r_z = 1.08 Å.[77] The appropriate heteroatom, H, or CH_2 van der Waals radius was then added to r_z to give $r_{3'}$ = approximate average van der Waals size of a 3' substituent extending out from the 3' carbon. The following two examples illustrate the calculation of 3' SIZE > H:

I. 3'-H substitution

Since: C-H (aromatic) bond distance = r_z = 1.08 Å

and the van der Waal radius of H = r_{vw} = 1.2 Å



Therefore: $r_{3'}$ = 2.28 Å

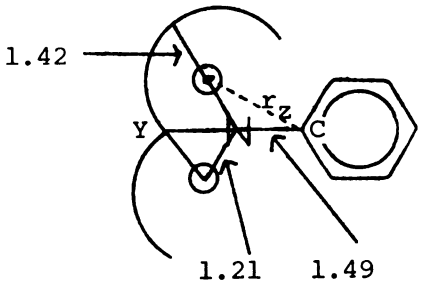
and 3'SIZE > H = 2.28 - 2.28 = 0

II. 3'-NO_2 substitution

C-N (Φ-NO_2) bond distance = 1.49 Å

N-O (NO_2) average bond distance = 1.21 Å

van der Waals radius of O = 1.42 Å



First, the distance r_z to the furthest out non-hydrogen atom is calculated:

$$r_z = \sqrt{\left[1.49 + 1.21\ (\cos\ 120)\right]^2 + \left[1.21\ (\sin\ 120)\right]^2}$$

$$r_z = 2.34\ \text{Å}$$

Next, the distance Y-C which measures the distance that the NO_2 substituent extends out along the C-N axis is calculated:

$$\text{Y-C} = \sqrt{(1.42)^2 - \left[1.21\ (\sin\ 120)\right]^2} + 1.49 + 1.21\ (\cos\ 120)$$

$$\text{Y-C} = 3.05\ \text{Å}$$

Since the NO_2 extends out $r_z + r_{vw}$ (O) in two directions and Y-C in only one direction, the average distance that the NO_2 substituent extends out would be:

$$r_{3'} = (3.76 + 3.76 + 3.05)/3 = 3.52\ \text{Å}$$

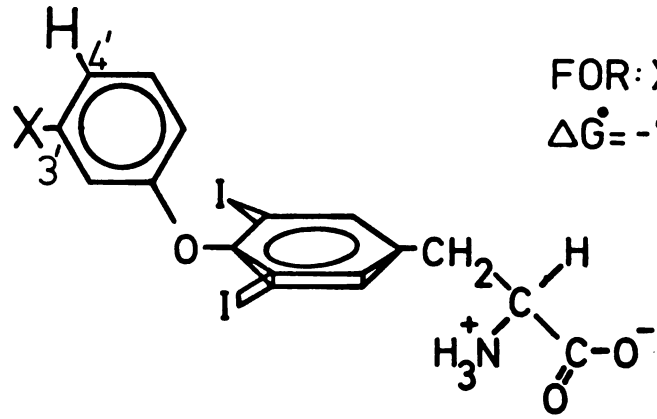
Therefore, to calculate 3'SIZE > H:

$$\text{3'SIZE} > \text{H} = r_{3'} - r_H$$

$$= 3.52 - 2.28 = 1.24\ \text{Å}$$

A direct measure of the strength of the interaction between a 3'-substituent and the receptor is seen in Table 5-2. These values of $\Delta\Delta G$

STRENGTH OF 3'-X INTERACTION WITH RECEPTOR



FOR: X=H

$\Delta\dot{G}$ = -7.00 (±.0) Kcal

$\Delta\Delta G = \Delta G_X - (-7.00)$

X	ΔΔG	3'-SIZE > H Å
F	-0.14 (±.05)	0.45
NO_2	-0.76 (±.21)	1.24
CL	-1.44 (±.07)	1.15
CH_3	-1.80 (±.45)	1.25
BR	-1.85 (±.13)	1.50
I	-1.85 (±.41)	1.83
CH_3>CH CH_3	-2.28 (±.22)	1.90
$(CH_3)_3$-C	-2.01 (±.14)	2.23

Table 5-2

have a negative sign which indicates that the 3'-substituent produces enhanced binding affinity. The strength of the 3'-X interaction ranges from -0.14 kcal/mol for fluorine to -2.28 kcal/mol for isopropyl. Except for NO_2 and tBu, the $\Delta\Delta G$ roughly increases with increasing size of the 3'-substituent. This can be ascribed to a size-limiting hydrophobic binding association for the 3' group, a relationship which was noted for binding to intact nuclei,[78] as well as for in vivo rat antigoiter activities,[79] in related series. The 3'-nitro substitution has a much smaller favorable interaction than would be expected from its size. However, since nitro is not a very hydrophobic group (π_{Benz} = -0.38) it would not be expected to produce a hydrophobic or dispersion force bond that was as strong as an alkyl or halogen substitution. The 3'-isopropyl substitution appears to be the size limit of favorable interaction for a 3' substituent. Since 3'-t-butyl is the only 3'-alkyl substituent with a third non-hydrogen α-carbon branch, apparently this extra bulk adds a negative steric influence to this group which results in a lowering of $\Delta\Delta G$.

4'-Hydroxyl:

The strength of the 4'-OH interaction with the receptor has been determined by subtracting the binding free energy of a 3'-substituted-4'-hydroxyl-3,5-diiodo-thyronine from the binding free energy of a 3'-substituted-4'-deoxy-3,5-diiodo-thyronine and is presented in Table 5-3.

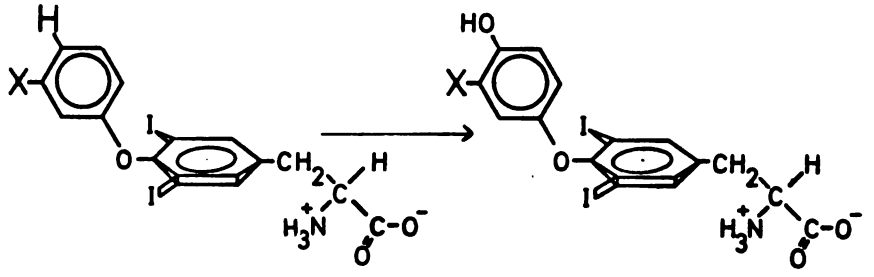
The addition of a 4'-OH to the unsubstituted outer ring increases the binding affinity by -1.24 kcal/mol. Within the series of 3'-alkyl thyronines the enhancement in binding affinity due to addition of the 4'-OH increases, reaching a maximum at isopropyl (-1.24 to -3.05) then decreasing with the more bulky tertiary butyl group (-1.95). The high binding affinities of these 3'-alkyl thyronine (phenolic pK_a ca. 10), compounds

in which the 4'-OH group is essentially un-ionized at pH = 7.6, implies that the 4'-OH group acts as a proton donor in hydrogen bond formation with the receptor. A similar trend is seen in Table 5-3 for the 3'-halogen substituted compounds. The enhancement in binding due to the addition of a 4'-OH increases with the size of the 3' group from fluorine (-1.56) to iodine (-3.53). Thus, for all 3'-substituted compounds except NO_2, the strength of the 4'-OH interaction with the receptor is greater than for the 3'-unsubstituted compound (-1.24). The positive correlation between size of the 3' substitution and its enhancement in the strength of the 4'-OH interaction implies that the effect of the bulky 3'-substituent is to orient the 4'-OH away from the 3' side to a position cis to the 5' side which produces a more favorable donor hydrogen bond. The very low $\Delta\Delta G$ for a 3'-NO_2 substitution supports this conclusion since a nitro group forms a very strong intramolecular hydrogen bond with the 4'-OH which would orient the 4'-OH away from the 5' side of the outer ring. Finally, Table 5-3 indicates that for 3'-substitution of similar size, the 3'-halogen has a greater $\Delta\Delta G$ than the 3'-alkyl. This is probably due to the inductive electron withdrawing effect of the halogens which would tend to weaken the O-H bond of the 4'-OH making it a slightly better hydrogen bond donor.

5'-Substitution:

The fact that 3',5',3,5-tetrasubstituted thyronines have lower in vivo and in vitro activies than the corresponding 3',3,5-trisubstituted analogs has been noted and wondered about for many years. Previous studies have shown that for a given substituent, the 3'-monosubstituted compound has a higher in vivo[20,21] and in vitro[42] activity than the corresponding 3',5'-disubstituted compound.

STRENGTH OF 4'-OH INTERACTION WITH RECEPTOR



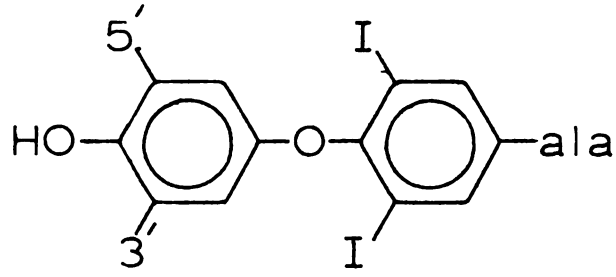
$\Delta\Delta G_X = \Delta G^{\circ}_{OH_X} - \Delta G^{\circ}_{H_X}$ FOR: X=H $\Delta\Delta G_H = (-8.24) - (-7.00) = -1.24$ Kcal

X	ΔΔG	X	ΔΔG	X	ΔΔG
H	-1.24(±.20)	NO_2	-1.05(±.20)	F	-1.56(±.37)
CH_3	-1.60(±.51)			CL	-2.04(±.21)
$(CH_3)_2$-CH	-3.05(±.36)			BR	-2.48(±.34)
$(CH_3)_3$C	-1.95(±.16)			I	-3.53(±.56)

Table 5-3

A similar relationship has been found for binding to the solubilized nuclear receptor as seen in Table 5-4.

Table 5-4. The Negative Influence of 5'-Substitution on the Binding Free Energy



5'	$\Delta\Delta G = \Delta G^{o}_{3'5'} - \Delta G^{o}_{3'}$ $\Delta\Delta G$(kcal/mol)	5'SIZE>H[a] (Å)
Cl	+0.01 (±0.23)	1.15
Me	+0.80 (±0.16)	1.25
Br	+0.67 (±0.34)	1.50
I	+1.15 (±0.26)	1.83
iPr	+2.58 (±0.22)	1.90

a. Calculated in a fashion analogous to 3'SIZE > H (see text pp 61-62.)

The change in the free energy ($\Delta\Delta G$), when a 5'-substituent is added to a 3'-substituted-3,5-diiodo-thyronine, is a direct measure of the energy lost due to steric repulsion. Chlorine substitution in the 5'-position results in a negligible loss of binding free energy (+0.01). However, as the size of the 5' substituent is increased, the loss due to 5' substitution becomes greater except for 5'-bromine substitution. This is probably due to the greater polarizability of bromine which would cause less steric repulsion than the hard sphere of a 5'-methyl.

3,5-Substitution:

Some of the early theories of thyroid hormone activity ascribed a unique functional role to the 3,5-halogen atoms.[80,81] One early attempt to measure biological activity with 3',5',3,5-tetramethyl-DL-thyronine and 3,5-dimethyl-3'-isopropyl-DL-thyronine failed due to an erroneous synthesis.[82] It has been shown, using sterically constrained analogs, that the 3'-distal conformation is preferred to the 3'-proximal conformation for thyromimetic activity.[83] However, it was not until Jorgensen and associates[84] unambiguously synthesized the 3,5-dimethyl-3'-isopropyl-L-thyronine, and found that it was active *in vivo*, that the role of the 3,5-substituents in the production of thyromimetic activity became clear. It has subsequently been concluded that thyromimetic activity is directly related to the ability of the 3 and 5 substituents to confine the diphenyl ether nucleus to two approximately equal energy, readily interconvertible conformers, in which the two phenyl rings are mutually perpendicular.[85]

Table 5-5 presents the binding free energy of 3'-iodo-3,5-hydrogen-thyronine subtracted from the binding free energy of four 3'-iodo-3,5-disubstituted-thyronines. This is a direct measure of the respective 3,5-substituents interaction with the receptor. This study presents the first measurement of the thyromimetic activity of the 3,5-diethyl-3'-iodo-DL-thyronine.[86]

Table 5-5. Strength of 3,5 Substituent Interaction with the Nuclear Receptor

	3'T_1			
3,5-Substitution	Me	Et	I	iPr
$\Delta G^{o}_{3,5}-\Delta G^{o}_{3'-T_1}=\Delta\Delta G$	-4.28(±0.22)	-4.39(±0.16)	-7.38(±0.33)	-3.48(±0.20)

From Table 5-5 it can be concluded that there is a very strict conformation and size limit for optimal 3,5-substitution. The strength of the 3,5-dimethyl and 3,5-diethyl interaction with the receptor is drastically less than the 3,5-diiodo. This is because the 3,5 substitutions not only participate in hydrophobic bonding but also are responsible for maintaining a perpendicular conformation of the outer ring. The methyl and ethyl substitutions are not bulky enough to constrain the diphenyl ether nucleus in a mutually perpendicular conformation.[85] Therefore, their interactive effect on the 3'-iodine is not as great as 3,5-diiodo substitution (-7.38). Also, there appears to be a strict size limit for 3,5-disubstitution since the 3,5-diisopropyl interaction with the receptor is so low (-3.48).

Amino Acid Side Chain:

Based on the pK_a values for tyrosine (pK_{a1} = 2.20, pK_{a2} = 9.11)[87] the alanine side chain of 3,5,3'-L-thyronine would be a zwitterion at pH = 7.6. Therefore, the potential for ionic bond formation exists for both the carboxylate and protonated amino portions of the amino acid. An analog without the alanine side chain was not available for this study, so a direct measure of its contribution to the binding free energy cannot be calculated. However, the acetic, and propionic acid analogs, triiodothyropropionic acid (triprop) and triiodothyroacetic acid (triac), show ΔG values -0.50 and -0.61 kcal/mol more negative than that of T_3 (-12.38), respectively. A rare sample of 3,5,3',5'-tetraiodothyropropylamine (thyroxamine) was obtained from Dr. Rosalind Pitt-Rivers for measurement of binding affinity to the solubilized nuclear receptor. The affinity of this analog, which contains no carboxylate anion, was so low (<-6.4 kcal/mol) that it could not be measured within the solubility limits of

the compound. These results indicate that the most favorable ionic interaction of the alanine side chain is formation of electrostatic bonds between the carboxylate anion and some positively charged group on the receptor. The positively charged alpha-NH_3^+ group has an unfavorable effect on binding. The relatively low affinities of the shorter side chain analog, 3,5,3'-triiodothyroformic acid (-10.91 kcal/mol), and of the longer side chain analog, 3,5,3'-triiodothyrobutyric acid (-11.23 kcal/mol), indicate that the ionic binding site on the receptor has strict steric and size limitations, as has been previously seen for the rest of the molecule.

Summary:

The present study has determined the strength of the molecular interactions between thyroid hormone analogs and the solubilized rat liver nuclear receptor.

It has been shown that L-triiodothyronine forms a donor hydrogen bond from the 4'-hydroxyl in the direction of the 5'-side of the outer ring which adds -1.24(±0.20) kcal/mol of binding free energy. This contribution from the phenolic hydroxyl can be enhanced by interaction with the 3'-iodine. The amount of this enhancement will be calculated in the next section. The 3'-substituent also participates in a hydrophobic association with a size limit at 3'-isopropyl and a contribution of 3'-iodine to the binding free energy of T_3 of -1.85(±0.41) kcal/mol.

The binding site on the 5'-side of the outer ring is sterically limited to 5'-substitution by substituents smaller than chlorine, because of the drastic loss in binding free energy when bromine, iodine, or isopropyl groups are substituted.

The binding site around the 3,5-substituents will accomodate groups

no larger than iodine and the requirement for orientation of the outer ring in a conformation perpendicular to the inner ring prevents substitution of groups smaller than iodine for maximal activity. The 3,5-diiodo substituents add -7.38(±0.33) kcal/mol of binding free energy from hydrophobic bonds and their positive interactive effect on the conformation of the outer ring.

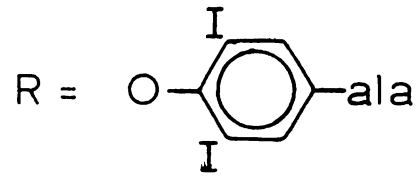
Finally, it has been shown that the alanine side chain adds a substantial free energy of binding through an eletrostatic interaction with the receptor probably involving the carboxylate anion of T_3 and a positively charged amino acid side chain of the receptor.

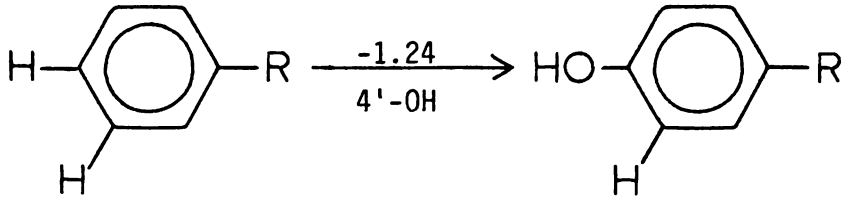
THE INTERACTIVE TERM: SECOND ORDER PARTITIONING TO DETERMINE THE INTERACTION BETWEEN SUBSTITUENTS

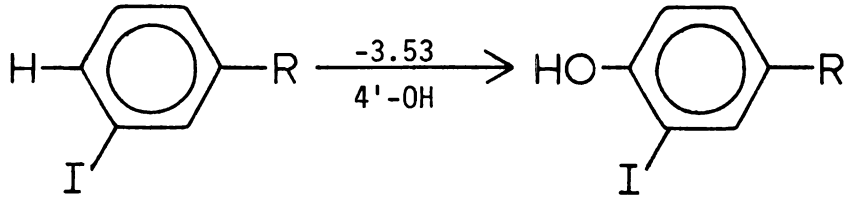
Calculation of the magnitude of the interaction of one substituent upon another was an original idea developed for this study. The following example will illustrate this type of calculation: From Table 5-3 it can be seen that addition of a 4'-hydroxyl to 4'-H-T_2 (5-3) produces -1.24 (±0.20) kcal/mol from donor hydrogen bond formation. Also it can be seen that addition of the 4'-hydroxyl to 4'-H-T_3 produces -3.53(±0.56) kcal/mol from donor hydrogen bond formation. The large difference between the contribution of the 4'-OH to the binding free energy when hydrogen is the 3'-substituent and when iodine is the 3'-substituent (-2.29(±0.76) kcal/mol) is a measure of the interaction of the 3'-iodine on the strength of the hydrogen bond between the 4'-hydroxyl and the receptor (see figure 5-1).

From Table 5-6 it can be seen that for the 3'-alkyl substitutions the direct enhancement of binding free energy by the 3'-substituent increases with size, presumably due to orientation of the 4'-OH toward

Figure 5-1. Calculation of the Interactive Effect of the 3'-Iodo on the Strength of the 4'-Hydroxyl Interaction with the Receptor







Interactive effect (I→OH) = -3.53 - (-1.24) = -2.29 kcal/mol

Table 5-6. Strength of 3'-Substituent Interaction on the Contribution of 4'-OH to the Binding Free Energy of 3'-X-3,5-diI-Thyronines (kcal/mol)[a]

X	Interactive Term	X	Interactive Term	X	Interactive Term
Me	-0.36(±0.71)	NO_2	+0.19(±0.40)	F	-0.32(±0.57)
iPr	-1.81(±0.56)			Cl	-0.80(±0.41)
tBu	-0.71(±0.36)			Br	-1.24(±0.54)
				I	-2.29(±0.76)

a. Calculated from the data of Table 5-1.

the 5'-side of the outer ring. The tertiary butyl substitution is so bulky that it could be interacting with the receptor to prevent the proper alignment of the outer ring for optimal hydrogen bond distances. The positive sign of the interactive term for the 3'-nitro indicates that it weakens the 4'-OH interaction with the receptor. This is probably due to the strong intramolecular hydrogen bond formation between the nitro and the 4'-OH which would orient the 4'-OH away from the 5'-side of the outer ring. For 3'-halogen substitution of a size similar to 3'-alkyl substitution (ie. compare Me and Cl, or iPr and I) the interactive effect is much larger. The inductive electron withdrawing properties of the halogens would result in a more favorable hydrogen bond formation with the receptor by decreasing the electron density of the O-H bond.

In the previous section the strength of the 3,5-substituent interaction was presented. By measuring the binding affinity of several partially iodinated thyronines the contribution of the 3-iodine, the first iodine added to the inner ring, can be calculated separately from the contribution of the 5-iodine, the second iodine added to the inner ring, and the strength of their respective interactions on the contribution to binding affinity by the 3'-iodine can be determined. The schematic diagram in figure 5-2 illustrates the breakdown of binding free energies for partially iodinated thyronines. The binding free energy of L-thyronine (T_0) was not determined exactly because the limit of solubility (2×10^{-4} M) at pH = 7.6 precluded its measurement at a concentration (4×10^{-3} M) which should show displacement of [^{125}I]T_3. Its binding free energy (-3.15 kcal/mol) was estimated by subtracting the contribution of a 3'-iodine to the binding free energy of 3,5,3'-triiodo-4'-deoxy-DL-thyronine (Table 5-2, -1.85 kcal/mol) from the binding free energy of 3'-iodo-DL-thyronine

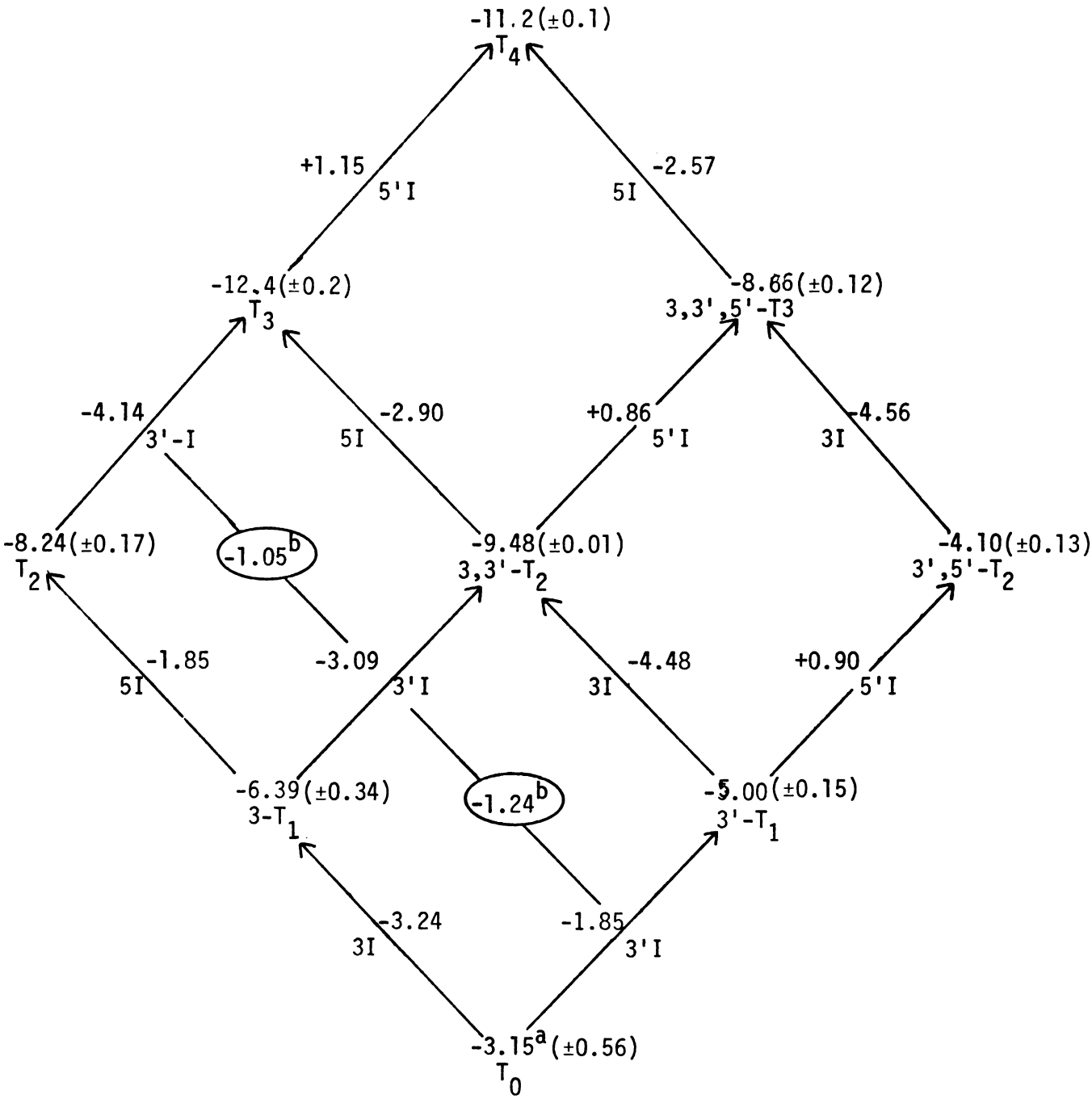
(Table 5-1, -5.00 kcal/mol).

The diagram in figure 5-2, developed by Dr. Jorgensen, is a rather concise method of presenting the individual contributions of the 3,5,3' and 5' iodines to the binding free energy of T_3 and T_4. The numbers above each compound abbreviation represent the binding free energy of that compound in kcal/mol. The numbers above headings labeled 3I, 5I, 3'I, or 5'I are the contributions of the respective substituent to the binding free energy of the compound above and in the direction of the arrow from the respective heading. The circled numbers in the center of the bottom and left-hand quadrants are the interactive energies for the effect of 3 and 5-iodines on the energy of addition of a 3'-iodine to the thyronine outer ring.

It can be seen that for mono, di, and non-substituted thyronines the addition of a 3-iodine (ave -4.09 kcal/mol) is more energetically favorable than addition of a 5-iodine (ave -2.44 kcal/mol). As discussed previously, the addition of a 5'-iodine is unfavorable in all cases (ave +0.97). The energy contributed by addition of a 3'-iodine ranges from -1.85 to -4.14 kcal/mol. This is because the alignment of the 3'-iodine in the "distal" binding pocket depends on the orientation of the outer ring in a conformation which is mutually perpendicular to the inner ring. Since the 3 and 5 iodines are responsible for this alignment, they would be expected to have an interactive effect on the additional binding energy of a 3'-iodine. This effect can be calculated and is represented by the circled numbers in the center of the lower and left-hand quadrants of figure 5-2. It can be seen that the presence of a 3-iodine increases the binding contribution of a 3'-iodine by -1.24 kcal/mol, and the presence of 3 and 5 iodines increase the binding contribution of a 3'-iodine by

Figure 5-2. Changes in Binding Free Energy

for Various Partially Iodinated Thyroid Hormone Analogs



a. Estimated Value as described in text p 71.

b. Interactive term for the effect of 3 and 5 iodines on addition of 3'-iodine.

-2.29 kcal/mol [-1.05 + (-1.24)].

Summary

The use of first and second order partitioning of the free energy of binding of thyroid hormone analogs to the rat liver nuclear receptor has allowed the direct calculation of the strength of the interaction of analog substituents with the binding site of the nuclear receptor. The total free energy released when L-triiodothyronine binds to the nuclear receptor is -12.4(±0.2) kcal/mol. If the binding free energy of specific group contributions is added as seen in figure 5-3, then the sum should be -12.4 kcal/mol. From figure 5-3, it can be seen that the sum of the individual group contributions to the binding free energy of T_3 (-12.4(±1.8) kcal/mol) is not significantly different from the measured binding free energy for T_3 (-12.4(±0.2) kcal/mol).

The following features of the molecular interaction between thyroid hormone analogs and the solubilized rat liver nuclear receptor have been determined: (1) The 4'-hydroxyl participates in a donor hydrogen bond oriented toward the 5'-side of the outer ring and adds -1.2(±0.2) kcal/mol of binding free energy. (2) The 3'-substituent participates in hydrophobic and van der Waals bonding, with a size limit at isopropyl, and adds to the strength of the 4'-hydroxyl interaction. The contribution of a 3'-iodine is (-1.85(±0.41) + (-2.29(±0.76))) = -4.1(±1.2) kcal/mol. (3) The optimal 3,5-substituents are iodine atoms which can contribute an average of -2.5(±0.7) kcal/mol per iodine. This value contains the interactive effect on orientation of the outer ring as well as the direct contribution to binding by the 3,5-iodine atoms and the aromatic rings. (4) The alanine side chain probably participates in an electrostatic attraction

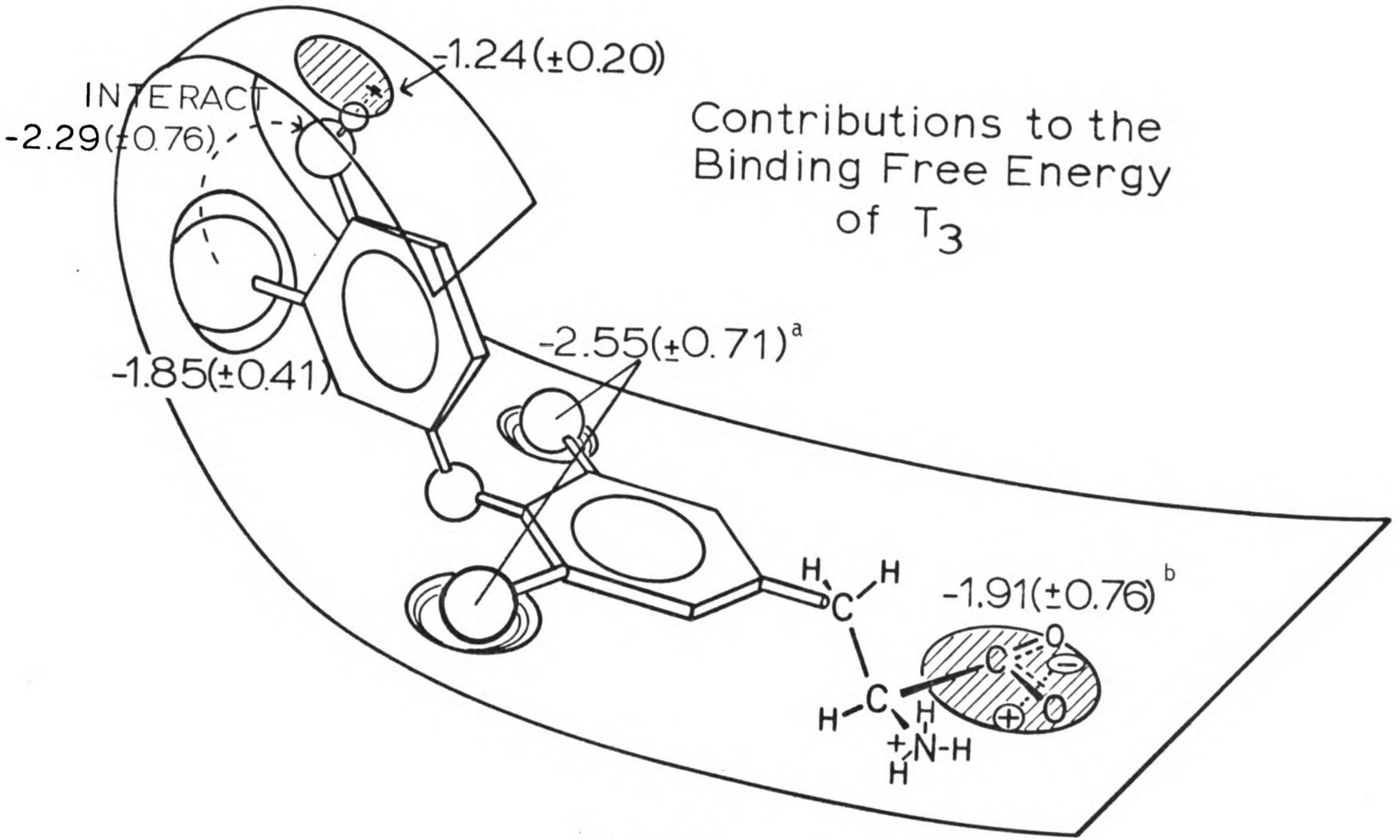


Figure 5-3.

[a]The contribution of 3 and 5-iodine atoms was calculated as the average of the individual contributions of 3-I = -3.24 kcal/mol and 5-I = -1.85 kcal/mol.

[b]Contribution of alanine side chain and diphenyl ether nucleus was calculated by subtracting the contribution of a 4'-OH (-1.24 kcal/mol) from the binding free energy of T_o.

between the carboxylate anion and a positively charged amino acid side chain in the receptor and adds -1.9(±0.8) kcal/mol to the binding free energy.

Chapter Six: Thermodynamics and Kinetics

It has been shown that the free energy released by the T_3-receptor complex can be partitioned into individual substituent contributions to the overall binding reaction. The strength of the molecular binding interactions of various functional groups in the thyroid hormone has been determined. Several intermolecular forces which contribute to the binding free energy have been discussed such as: hydrogen-bond, electrostatic, and close range van der Waals forces. In the gas phase these are the forces which predominantly govern the interaction between molecules. According to Jencks, the strong charge-solvating and hydrogen-bonding ability of water tends to reduce the possibility of obtaining large binding energies from these forces.[94] Chothia has also shown that there are large losses in entropy produced by restriction of translational and rotational degrees of freedom in typical ligand-protein complexes.[97] Since it has been shown in Chapter Five that the free energy released when T_3 binds to

the receptor is relatively large, it is necessary to describe another type of molecular interaction called "hydrophobic bonding".

In the previous chapter the strength of the binding interaction of a 4'-OH group was attributed to energetically favorable hydrogen-bond formation with the receptor. This $\Delta\Delta G$, like any free energy, is comprised of a contribution from enthalpy and entropy. The enthalpic contribution ($\Delta\Delta H$) is a measure of the actual change in energy upon breaking hormone-water and receptor-water hydrogen bonds and reforming them between hormone-receptor and water-water. The entropic contribution ($\Delta\Delta S$) is a measure of the change in the state of order of the system, and contains, in part, the contribution of the 4'-OH substituent to hydrophobic bonding.

In this chapter the enthalpic and entropic contributions to the binding free energy are analyzed by examining the temperature dependence of the equilibrium association constant and the kinetic rate constants. The translational and rotational entropy which is lost upon binding of T_3 to the receptor, is calculated in order to determine the magnitude of the favorable contribution to entropy by the solvent-mediated hydrophobic forces. Kinetic rate constants are determined from computer fitted curves and second order linear plots using the kinetic data of T_3-receptor complex formation. The activation parameters, $\Delta G^{\ddagger}$, $\Delta H^{\ddagger}$, and $\Delta S^{\ddagger}$ are calculated and used to obtain insights into the nature of the transition state for T_3-receptor complex formation. Finally, the magnitude of the rate constants are analyzed to derive information on the nature of the binding site.

TEMPERATURE DEPENDENCE STUDY OF THE RECEPTOR BINDING ASSOCIATION CONSTANT

Methods:

The equilibrium association constant for 3,5,3'-triiodo-L-thyronine binding to the solubilized nuclear receptor was determined from the slope of the best fit straight line to Scatchard assay data at six temperatures in the range 4-34^{o}. In order to determine the length of time required to reach equilibrium, a kinetic study was performed first. The Scatchard assays were then incubated for a time sufficient to reach 95% of the equilibrium bound [^{125}I]T_3 level at a temperature which was maintained at $\pm 1^{o}$. The amount of [^{125}I]T_3 added for each assay spanned a concentration range of (0.1-10) K_d at 25^{o}. Nuclear extract which had been partially purified with the QAE-Sephadex A-50 column (Chapter Three), was used in this study.

At 34^{o} and 25^{o} with incubations containing very low concentrations of [^{125}I]T_3, it was found that protein degradation caused a lowering of the B/F ratio as seen in figure 6-1. A control experiment was performed to determine if pre-incubation of the QAE-purified nuclear extract at 34^{o} caused a significant lowering of the specifically bound counts. The bar graph in figure 6-2 shows that if the QAE-purified nuclear extract is pre-incubated in the absence of [^{125}I]T_3 at 34^{o} for various time periods followed by incubation with [^{125}I]T_3, the resulting specifically bound counts are significantly lower than if the QAE-purified nuclear extract is not preincubated. The effect shown in figure 6-1, which is seen only at 34^{o} and 25^{o}, is common in protein-ligand assays because many binding proteins are much more stable with bound ligand than without. Since this solubilized nuclear receptor preparation contains other proteins (eg. proteolytic enzymes) in addition to the binding protein, it is reasonable that at high temperatures and very low ligand concentration there would

Figure 6-1. Scatchard Assay at 34° for 30 min

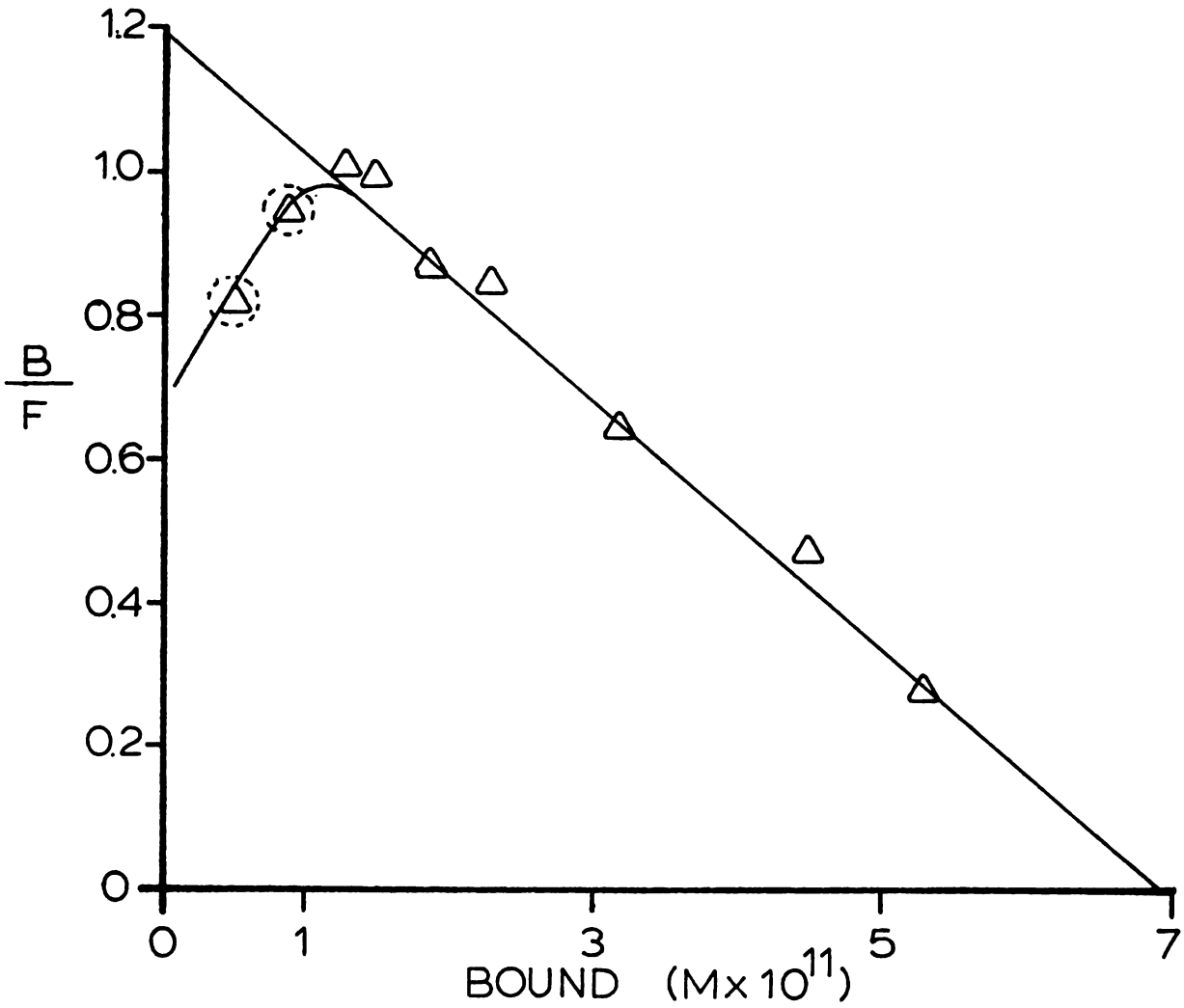
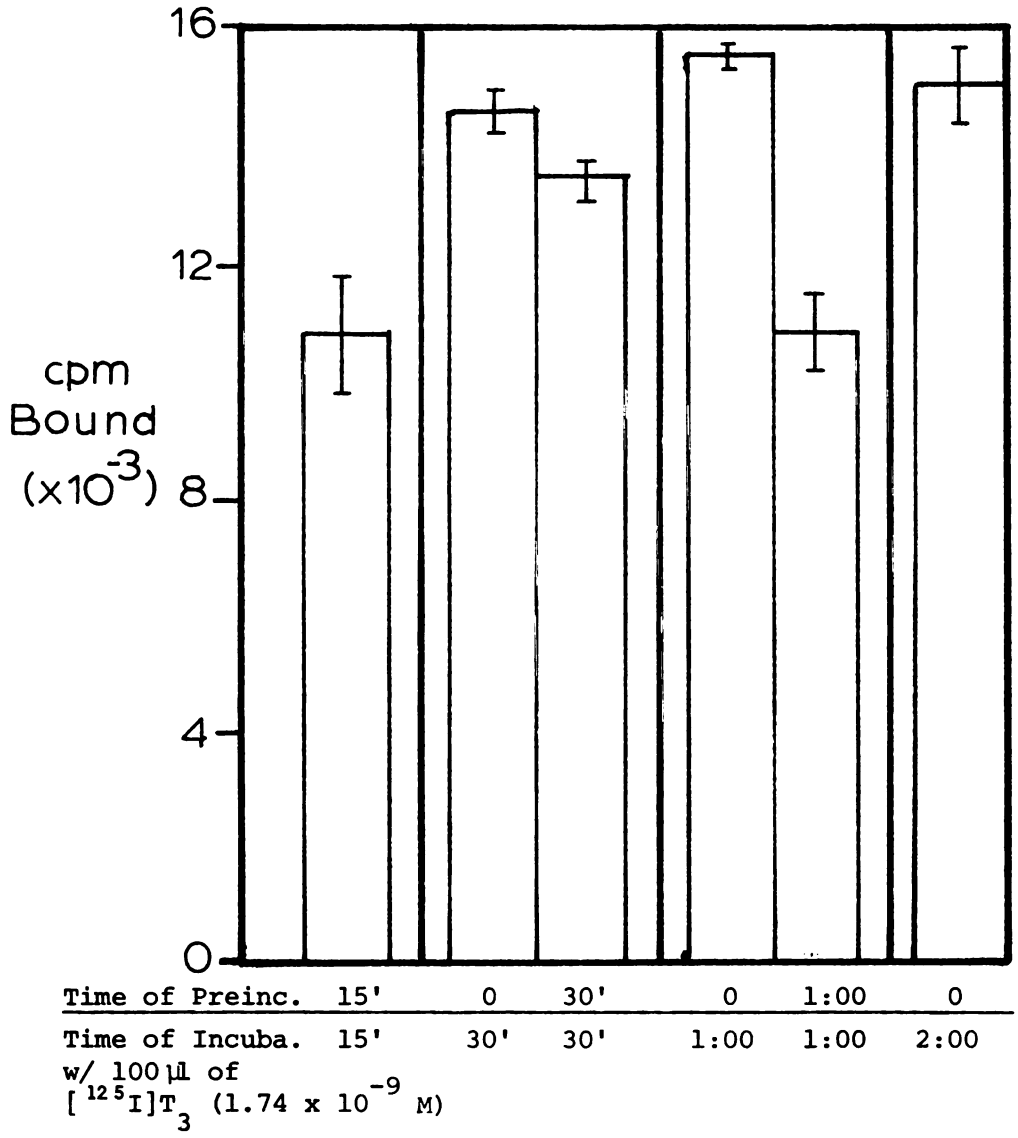


Figure 6-1.

Figure 6-2. Effect of Preincubation at 34° on Specifically Bound Counts



be some degradation. Chamness states that probably many reports of positive cooperativity in steroid-receptor interactions have arisen in this way.[88] For this study, the low concentration points which fall obviously lower than the best-fit line to the rest of the points have not been used in the final determination of the slope and intercept.

Results:

Three separate sets of duplicate Scatchard assays were performed for 34^{o} and 25^{o}, and an average of the three slopes and intercepts was determined. In addition, duplicate Scatchard assays were performed at four other temperatures and the averages at all temperatures are plotted in figure 6-3. The fit of the data points to the least squares line at 4^{o} has a correlation coefficient of 0.865 which indicates that a straight line at 4^{o} may not be an accurate representation of the data. The data points at 4^{o} show a decreasing slope which could be due to: (a) experimental error, (b) the presence of two independent sites with different association constants (which is unlikely at 4^{o} since this behavior was not seen at higher temperatures), or (c) the presence of two sites which interact with negative cooperativity at 4^{o} but not at 10^{o} to 34^{o}. For this study these questions have not been resolved and the association constant at 4^{o} will be taken as the slope of the best fit straight line to the experimental data points. A summary of the equilibrium association data is compiled in Table 6-1 and is used to determine the enthalpic and entropic contributions to the binding free energy.

If the fundamental thermodynamic equations 6-1 and 6-2 are combined and rearranged as follows, an expression for the temperature dependence of the equilibrium association constant is obtained in equation 6-3.

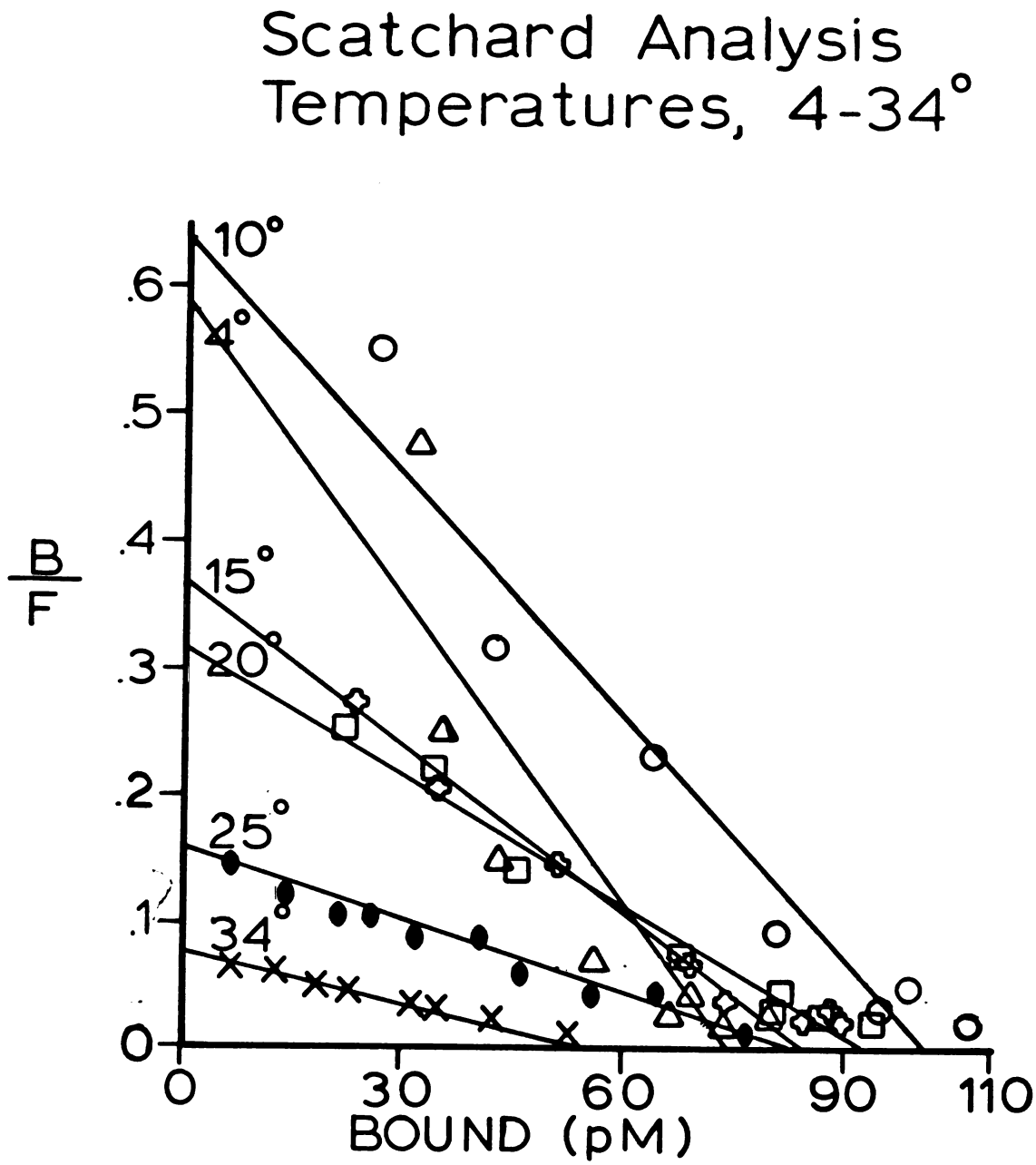

Scatchard Analysis
Temperatures, 4-34°
B/F
10°
4°
15°
20°
25°
34°
.6
.5
.4
.3
.2
.1
0
0
30
60
90
110
BOUND (pM)

Figure 6-3.

Table 6-1. Temperature Dependence Data Obtained from the Best Fit Line by Linear Regression for the Scatchard Analysis of T_3 Binding to the Nuclear Receptor

Temperature					
^oC[a]	^oK	$K_a(M^{-1})$[b]	Pt (nM/mg protein)[c]	R[d]	Time of Incubation (hr)
34	307	(1.17 ± 0.52)[e]$\times10^9$	1.20	0.993	0.5
25	298	(1.98 ± 1.52)[e]$\times10^9$	1.57	0.990	2.0
20	293	$(3.39\pm0.24)\times10^9$	1.79	0.985	8.0
15	288	$(4.44\pm0.15)\times10^9$	1.59	0.998	19.5
10	283	$(6.10\pm0.79)\times10^9$	2.00	0.954	24.0
4	277	$(7.72\pm1.83)\times10^9$	1.42	0.865	48.0

a. Temperatures maintained at $\pm1^o$.

b. Association constant determined for ^{125}I-L-triiodothyronine binding to QAE-Purified Nuclear Extract (Chapter 3).

c. Pt = total moles per liter of binding sites per mg total protein. From Lowry protein determination: 1.05 mg protein/ml of QAE-purified nuclear extract. Each incubation contained 50 μl of QAE-nuclear extract.

d. Correlation coefficient from linear regression on data points for average B/F vs Bound by PROPHET computer.

e. Mean and standard deviation based on three separate duplicate assays at each temperature.

$$\Delta G^{o} = -RT \ln K_{eq} \qquad \text{eq 6-1}$$

$$\Delta G^{o} = \Delta H^{o} - T\Delta S^{o} \qquad \text{eq 6-2}$$

Combining these equations yields:

$$-RT \ln K_{eq} = \Delta H^{o} - T\Delta S^{o}$$

Dividing both sides by -RT yields:

$$\ln K_{eq} = -\frac{\Delta H^{o}}{RT} + \frac{\Delta S^{o}}{R} \qquad \text{eq 6-3}$$

Equation 6-3 states that <u>if</u> ΔH^{o} and ΔS^{o} are constants <u>independent of temperature</u>, within the temperature range studied, the logarithm of the equilibrium constant is a simple linear function of the reciprocal temperature. It has been shown for steroid binding to the glucocorticoid receptor[90] and for non-polar solutes in water,[91] that there is a negative change in heat capacity upon association, and consequently both ΔH^{o} and ΔS^{o} decrease as the temperature increases. However, as can be seen in figure 6-4, ΔH^{o} and ΔS^{o} are independent of temperature (in the temperature range studied) for L-T_3 binding to the nuclear receptor. A similar result was obtained for L-T_3 and L-T_4 binding to the thyroxine-binding globulin (TBG) as reported by Korcek and Tabachnick.[92]

The data from Table 6-1 is plotted in figure 6-4 as the logarithm of K_a versus the reciprocal of the temperature ($1/^{o}K$). The slope of the best fit straight line ($d(\ln K_{eq})/d(1/T)$) is equal to $-\Delta H^{o}/R$ and the Y-axis intercept is equal to $\Delta S^{o}/R$. The graph in figure 6-4 is consistent with Le Chatelier's principle, which states that increasing the temperature of an equilibrium mixture causes the reaction to proceed in the direction which absorbs heat. This means that the K_a measured at 34^{o} is expected to be smaller than the K_a measured at 25^{o} because the reaction $P + T_3 \rightleftharpoons PT_3$ is exothermic (ΔG^{o} = -12.68 kcal/mol) and an increase

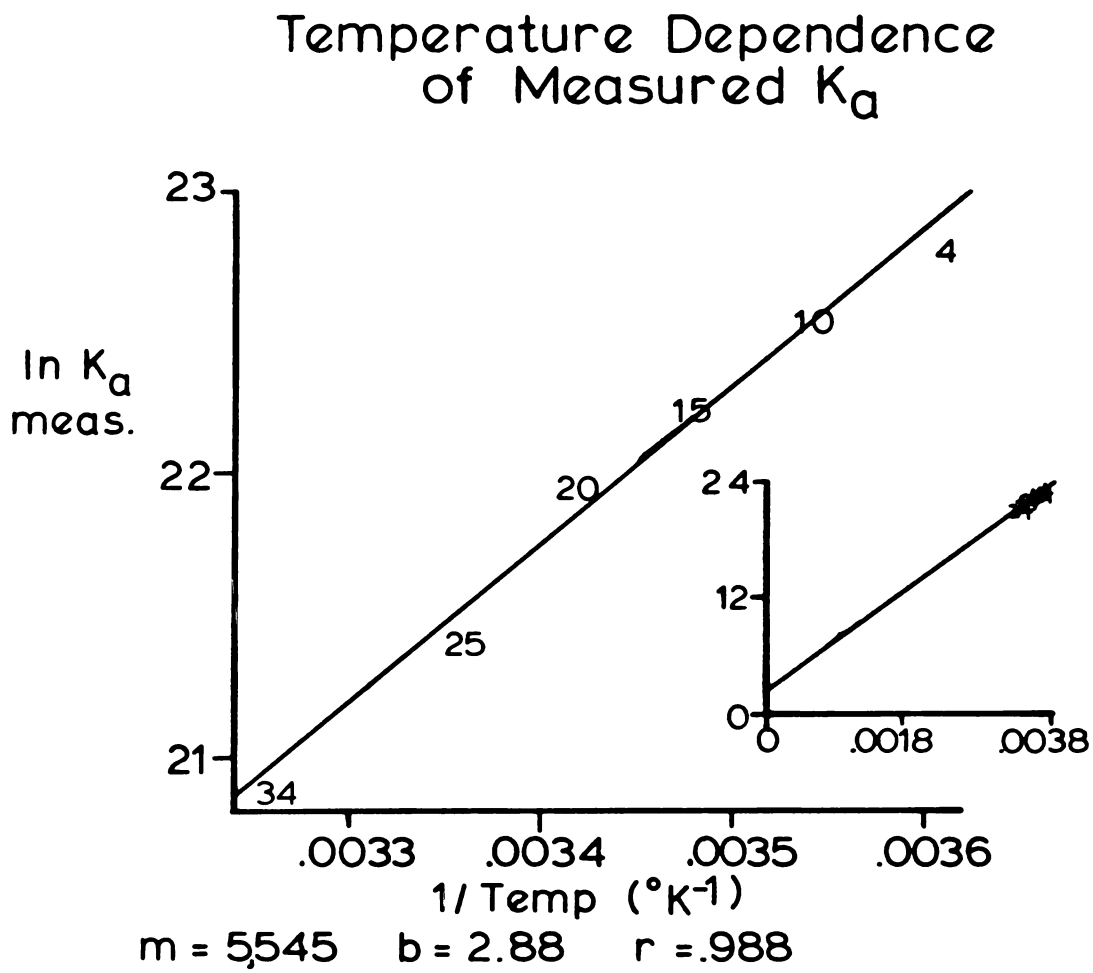

Temperature Dependence
of Measured K_a
In K_a
meas.
23
22
21
4
10
15
20
25
34
24
12
0
0
.0018
.0038
.0033
.0034
.0035
.0036
1/ Temp (°K^{-1})
m = 5.545 b = 2.88 r = .988

Figure 6-4.

in temperature causes the equilibrium to shift toward the reactants.

The following Table (6-2) contains the values of the thermodynamic state functions G, H, and S for L-[^{125}I]T_3 binding to the solubilized nuclear receptor.

Table 6-2. State Functions Calculated from the Slope and Intercept of the Thermodynamic Plot in Figure 6-6

ΔG^{o}[a] (Kcal/mol)	ΔH^{o}[b] (Kcal/mol)	ΔS^{o}[b] (cal/mol ^{o}K)	$T\Delta S^{o}$ (Kcal/mol)	R[c]
-12.73 (±1.69)	-11.02 (±0.84)	+5.72 (±2.88)	+1.71 (±0.86)	0.989

a. Calculated from $\Delta G^{o} = \Delta H^{o} - T\Delta S^{o}$ at 25^{o}. Standard deviation calculated from error in $\Delta H^{o} + \Delta S^{o}$.

b. Calculated from the slope and intercept and standard deviation of the slope and intercept of the best fit line as illustrated in figure 6-6. ΔH^{o} = -1.9872 x slope ΔS^{o} = 1.9872 x intercept.

c. Correlation coefficient of the best fit line as illustrated in figure 6-6.

Discussion:

The calculated $\Delta G^{o}_{T_3}$ in Table 6-2 is consistent with the measured $\Delta G^{o}_{T_3}$ (-12.68 kcal/mol) from the Scatchard analysis at 25^{o}. The large negative ΔH^{o} (-11.02 kcal/mol) and low positive ΔS^{o} (+5.72 eu.) both contribute favorably to the binding free energy. According to thermodynamics, the energy of the bonds broken minus the energy of the bonds formed is equal to ΔE and, for this discussion, to an acceptable approximation ΔH.[93] It can be seen that the excess energy of bonds formed in the T_3-receptor complex (-11.02 kcal/mol) represents a significant portion

of the binding free energy (-12.73 kcal/mol). For T_3 these bonds include: electrostatic (alanine side chain), hydrogen bond (4'-hydroxyl), and close range van der Waals forces (3',3,5-iodines) as shown in Chapter Five. The sum of $\Delta\Delta G$ for these interactions as calculated in Chapter Five (-12.39 kcal/mol) is larger than ΔH^0 because this sum contains a contribution from the change in entropy as well as enthalpy for these groups.

It is useful to qualitatively describe the molecular basis of hydrophobic bonding before discussion of the entropic contribution to the binding of T_3 to the receptor. While there is still much controversy whether hydrophobic forces are the result of positive attraction between solute molecules or a negative interaction with the solvent, it is more relevant to the discussion of entropy to consider the latter.

Hydrophobic interaction is described by Jencks as "a negative sort of force which results from the strong attraction of water molecules for each other and which is often associated with changes in the 'structure' of water in the neighborhood of the solute molecules."[94] Small strongly dipolar water molecules interact with strong cohesive forces through hydrogen bonds and other dipolar interactions and give rise to the large surface tension displayed by water. In order to introduce a non-polar solute into water, a cavity must be formed which absorbs energy in the breaking of water-water hydrogen bonds. To regain as much of this energy as possible, water has a tendency to surround the solute molecule and form the maximum possible number of hydrogen bonds. This process regains some of the lost energy, but since the water molecules now lie very close to the non-polar solute, normal hydrogen bonding in the direction of the solute is not possible. This leads to the formation of a region around the solute where the number of energetically favorable orientations of the water molecules is restricted, resulting in a decrease in the entropy

and an increase in the "structure" of the system.[94] This difficulty in placing a non-polar solute in water provides the driving force for the formation of the "hydrophobic bond". It generally requires less work to make one large cavity than two small cavities, so there will be a tendency for two large hydrophobic solute molecules in water to come together in a single cavity, just as oil droplets tend to coalesce. Since the structured water surrounding the non-polar solutes will be transferred to the bulk solvent when the solutes come together, there is a net increase in entropy as the solvent becomes more disordered.

Since there is a large portion of the T_3 molecule which has been described qualitatively,[71] and quantitatively[36] as hydrophobic, it would be expected that the entropic contribution to the binding free energy would be large and positive to account for the hydrophobic bonding of the 3',3,5-iodine substituents. As can be seen in Table 6-2, the value of ΔS^o (+5.7 eu) which contributes only -1.7 kcal/mol to the binding free energy does not meet this expectation. The following discussion of entropy addresses this problem.

Entropy is a measure of the degree of order of a system. The change in entropy of a system during a chemical reaction arises from a change in the order of the reactants, products, and solvent such as formation of a water molecules solvent cage around small ions or restriction of translational and rotational degrees of freedom when a small molecule ligand binds to a macromolecular protein. The second law of thermodynamics states that the entropy of the universe tends toward a maximum. For T_3 this means that the loss of its translational and rotational degrees of freedom when bound to the receptor requires the release of an equal or greater amount of energy from another source. Part or all of this energy

must come from "hydrophobic bonds".

In order to determine the magnitude of the free energy lost from restriction of translational and rotational degrees of freedom, the entropies of translation and rotation can be calculated from their respective molecular partition functions. Page and Jencks have shown that this theory gives reasonable estimates of the entropies of association for small organic molecules in non-polar solvents.[96] Janin and Chothia have used these calculations to determine the loss of translational and rotational energy in the trypsin-trypsin inhibitor complex.[97] The translational and rotational energy loss of the receptor in a bimolecular reaction would be expected to be minimal as the association of the receptor with the relatively small T_3 molecule would have very little effect on the translational and rotational entropy of the receptor as a whole. Using equations from Davidson[95] the following translational and rotational entropies were calculated:

Calculation of the Translational Entropy for T_3

Given: $S^o_t = 5/2\ R - \frac{F - F_o}{T}$ $\qquad \frac{F - F_o}{T} = -R \ln \frac{q}{n}$

Where: S^o_t = translational entropy

R = gas constant (1.9872 cal/mol ^oK)

$(F-F_o)/T$ = change in free energy/temperature

q/n = translational partition function per molecule

Calculation of q/n:

$$q/n = \left(\frac{2\pi 3kT}{h^2}\right)^{3/2} \frac{V}{n}$$

Where: m = mass of one molecule in grams

k = Boltzmann's constant (1.3804×10^{-16} erg/deg)

T = temperature in ^{o}K

h = Planck's constant (6.6252×10^{-27} erg sec)

V/n = volume (in cm^3)/molecule

and: $$m = \frac{M}{N} = \frac{650.1 \text{ g/mol}}{6.02322 \times 10^{23} \text{ molecules/mol}} = \frac{\text{M wt of } T_3}{\text{Avogadro's number}}$$

Since:

$$V \text{ (in } cm^3) = 1000 \times V' \text{ (in liters)}$$

$$n \text{ (in molecules)} = N \times n' \text{ (in moles)}$$

Then: $$\frac{V}{n} = \frac{1000}{N} \times \frac{V'}{n'} \quad \text{(for 1 M, } V'/n' = 1)$$

Therefore: $$\frac{q}{n} = \left[\frac{2\pi k}{h^2}\right]^{3/2} \left[\frac{1000}{N}\right] \left[\frac{M}{N}\right]^{3/2} \left[T\right]^{3/2}$$

$$\frac{q}{n} = 2.66 \times 10^{7}$$

and: $$S^{o}_{t} = 5/2\ R + R \ln (2.66 \times 10^{7})$$

$$S^{o}_{t} = 39 \text{ eu}$$

Calculation of Rotational Entropy for T_3

Given: $$S^{o}_{r} = 3/2\ R - \frac{F - F_o}{T} \qquad \frac{F - F_o}{T} = -R \ln q_{rot}$$

Where: S^{o}_{r} = rotational entropy for non-linear polyatomic molecule

q_{rot} = rotational partition function

Calculation of q_{rot}:

$$q_{rot} = \pi^{1/2} \left[\frac{8\pi^2 k}{h^2}\right]^{3/2} (I_x I_y I_z)^{1/2} T^{3/2}$$

Where:

I_x, I_y, I_z = principle moments of inertia for T_3 (g cm^2)

$$I_x' = 2835 \text{ awu/Å}^2$$

$$I_y' = 10326 \text{ awu/Å}^2$$

$$I_z' = 10924 \text{ awu/Å}^2$$

Since:

$$I(\text{in g cm}^2) = I' \text{ (in awu/Å}^2)/6.02322 \times 10^{39} \text{ molecules Å}^2/\text{mol cm}^2$$

Conversion Factor

$$= (1/6.02322 \times 10^{39})^{3/2} = 2.1392 \times 10^{-60}$$

Then:
$$q_{rot} = \pi^{\frac{1}{2}}\left[\frac{8\pi^2 k}{h^2}\right]^{3/2} (2.1392 \times 10^{-60}) (I_x'I_y'I_z')^{\frac{1}{2}} T^{3/2}$$

$$q_{rot} = 0.01483 (I_x'I_y'I_z')^{\frac{1}{2}} T^{3/2}$$

$$q_{rot} = 4.316 \times 10^7$$

and:

$$S^o_r = 3/2R + R \ln (4.316 \times 10^7)$$

$$S^o_r = 38 \text{ eu}$$

The translational and rotational entropies are added and multiplied by T to determine the magnitude of the energy lost from restriction of the six translational and rotational degrees of freedom when T_3 binds to the receptor.

$$T\Delta S_{t+r} = (39 \text{ eu} + 38 \text{ eu})(298\ ^oK) = 23 \text{ kcal/mol}$$

This value of $T\Delta S$ would be an unfavorable contribution to the binding free energy and would be the upper limit that would be expected to be lost due to restriction of translation and rotation. The value could be smaller since Page and Jencks have noted that in equilibrium complexes, some of the translational and rotational entropy lost in forming the complex is converted into low frequency internal motions which would tend to lower the amount of energy lost from translational and rotational

restriction.[96] These internal motions may contribute from 21 eu to 30 eu of residue entropy to the equilibrium complex.[90,96] If the energy loss from restriction of translation and rotation is recalculated, the resulting value is:

$$T\Delta S_{t+r} = (39 \text{ eu} + 38 \text{ eu} - 30 \text{ eu})\ (298\ ^{o}K) = 14 \text{ kcal/mol}$$

Therefore, it would be expected that -15.7 kcal/mol ($-T\Delta S^{o}$) be released in the binding event from some other entropic source such as "hydrophobic bonds". This is not an unreasonable energy to expect from solvent mediated hydrophobic bonding since Janin and Chothia have reported that hydrophobicity contributes -35 kcal/mol to the stability of the trypsin-inhibitor complexes.[97]

Summary:

The following information has been determined from the temperature dependence study of the equilibrium association constant for T_3 binding to the solubilized rat liver nuclear receptor: (1) There is good agreement between the measured ΔG^{o} for T_3 at 298° (-12.7 ($\pm$ 0.3) kcal/mol) and the ΔG^{o} calculated from the ΔH^{o} and ΔS^{o} (-12.7 ($\pm$1.7) kcal/mol) obtained from the temperature dependence studies. (2) Enthalpy contributes a large proportion of the equilibrium binding free energy which indicates that there is an overall gain of -11.0 ($\pm$0.8) kcal/mol of bond energy from bonds formed in the receptor-T_3 complex. (3) The small favorable entropic contribution to the binding free energy (-1.7 ($\pm$0.9) kcal/mol could be a measure of the difference between the favorable entropic contribution from solvent mediated hydrophobic bonds and the loss of the translational and rotational energy of the ligand (T_3) when bound to the receptor.

KINETIC RATE CONSTANTS: A WINDOW TO THE TRANSITION STATE FOR T_3-RECEPTOR COMPLEX FORMATION

The method for collection of the kinetic data has been described in Chapter Four and an example of a kinetic plot was seen in figure 4-3. The temperature dependence of the association and dissociation rate constants was determined at six temperatures in the range 4-34^{o}. All assays were cooled to 4^{o} for separation of bound and free cpm. QAE-purified nuclear extract ($P_t \simeq 1 \times 10^{-10}$ M) was used to measure bound versus time for all temperatures except 25^{o} for which sufficient data had been collected with nuclear extract (unpurified). To each incubation tube was added [^{125}I]T_3 ($\simeq 3.5 \times 10^{-10}$M), and at equilibrium there was from 30 to 50 pM of bound label. The assays at each temperature were run in duplicate or triplicate.

Results:

The average molarity of specifically bound [^{125}I]T_3 is plotted as a function of time as in figure 6-5. This graph shows that a similar equilibrium molarity is reached for each temperature except 34^{o} which reaches an equilibrium level that is half as much as the level reached at the other temperatures because of its lower K_a, and receptor degradation. As expected, the lower the temperature of the assay the slower the reaction reaches its equilibrium level.

Two different methods were used to determine the association and dissociation rate constants for the following reaction from the measured values of bound [^{125}I]T_3 versus time.

$$P + T_3 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} PT_3$$

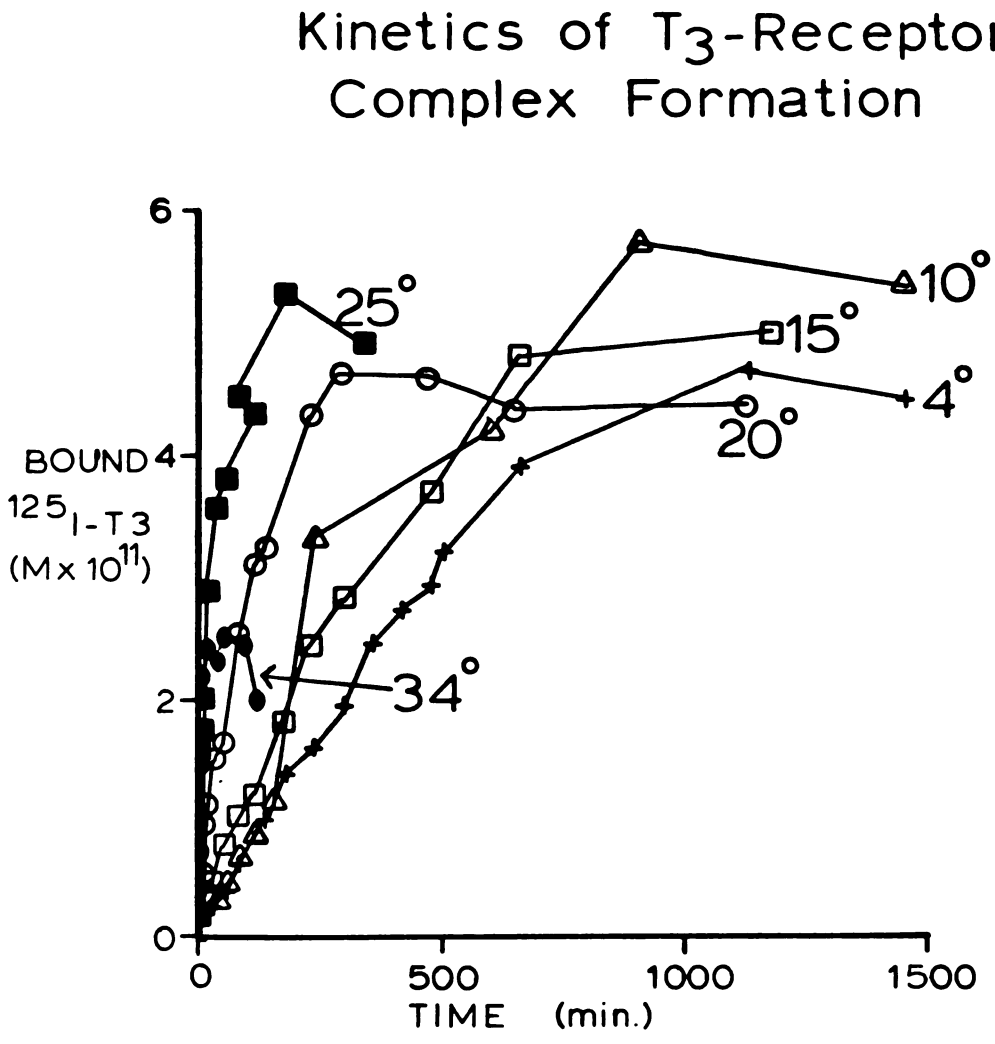

Kinetics of T3-Receptor
Complex Formation
BOUND
125I-T3
(M x 10^11)
6
4
2
0
0
500
1000
1500
TIME (min.)
25°
10°
15°
4°
20°
34°

Figure 6-5.

The first method utilizes a computer curve-fitting routine developed by Gary Knott and Douglas Reece at the N.I.H. This routine is obtained in a package called "MLab"[98] (modeling laboratory) and is on line as part of the PROPHET[99] system. The experimental data is fitted to the differential equation 6-4 to obtain k_1 and k_{-1}. Then this differential equation is numerically integrated and plotted as the best fit curve.

$$\frac{dPT_3}{dt} = k_1 (P_t - PT_3)(T_{3t} - PT_3) - k_{-1}(PT_3) \qquad \text{eq 6-4}$$

The second method involves a linear plot of the initial rates in order to determine k_1 by the second order rate equation:

$$\frac{1}{P_t - T_{3t}} \ln \frac{T_{3t}(P_t - PT_3)}{P_t(T_{3t} - PT_3)} = k_1 t \qquad \text{eq 6-5}$$

Where:

P_t = total concentration of receptor sites, determined from Scatchard analysis at the corresponding temperature

T_{3t} = total concentration of $[^{125}I]T_3$ added

PT_3 = concentration of bound $[^{125}I]T_3$ at time t

k_1 = second order association rate constant

t = time (min)

The results obtained by computer curve-fitting and by second order linear plot of initial rates are presented in figures 6-6 and 6-7 respectively. The curves in figure 6-6 show a good fit to the experimental data, which indicates that eq 6-4 is a reasonable model for T_3-receptor kinetic data. The data at 34^o produced a very "stiff fit", ie. the rate constant k_1 was so fast at 34^o that the computer could not converge on a solution to the rate constants unless constraints were applied.

Time(min) vs pM Bound ^{125}I-T_3

Fitted to $\frac{dPT_3}{dt} = k_1(P_t - PT_3)(T_{3t} - PT_3) - k_{-1}(PT_3)$

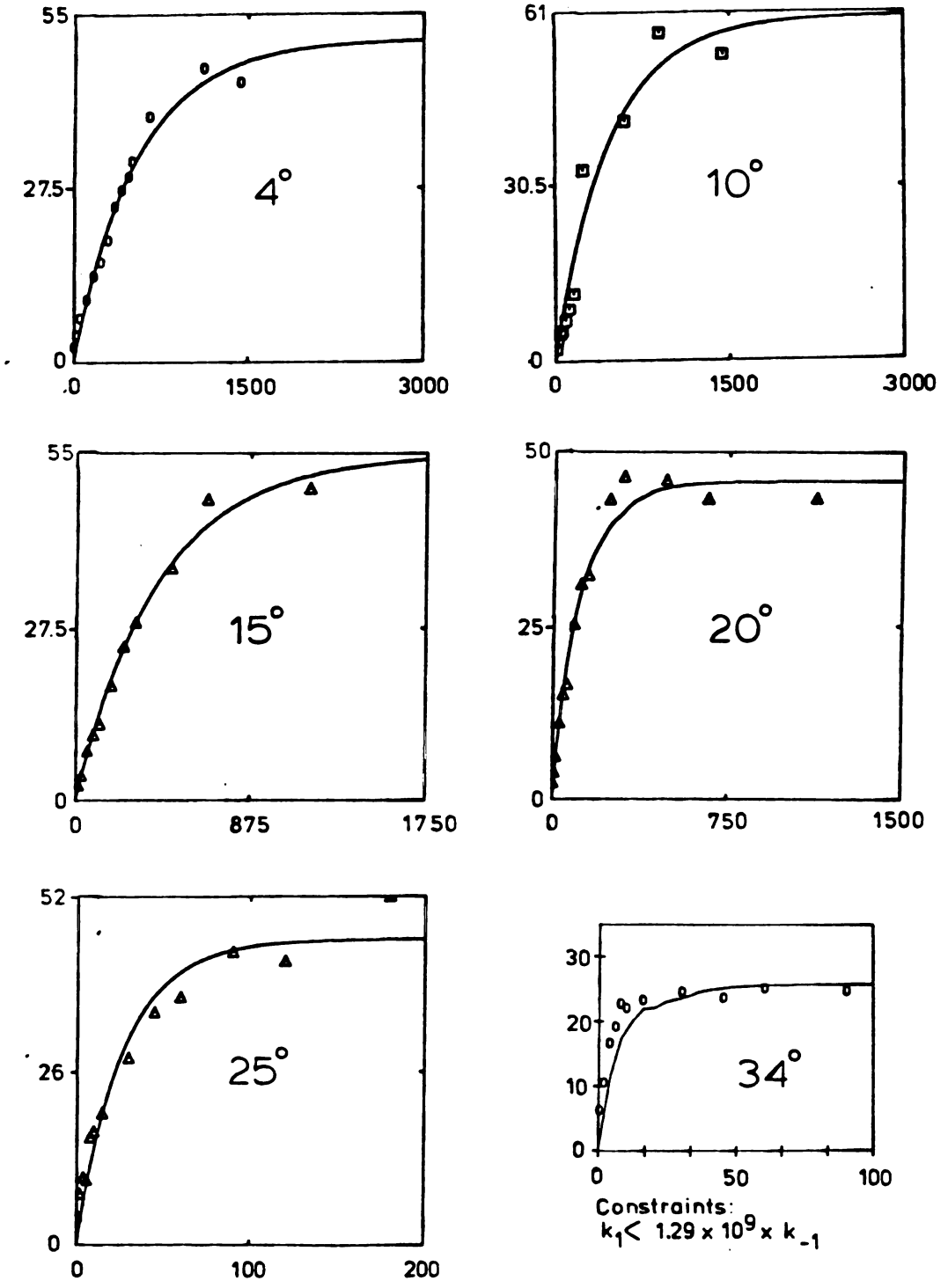


Figure 6-6.

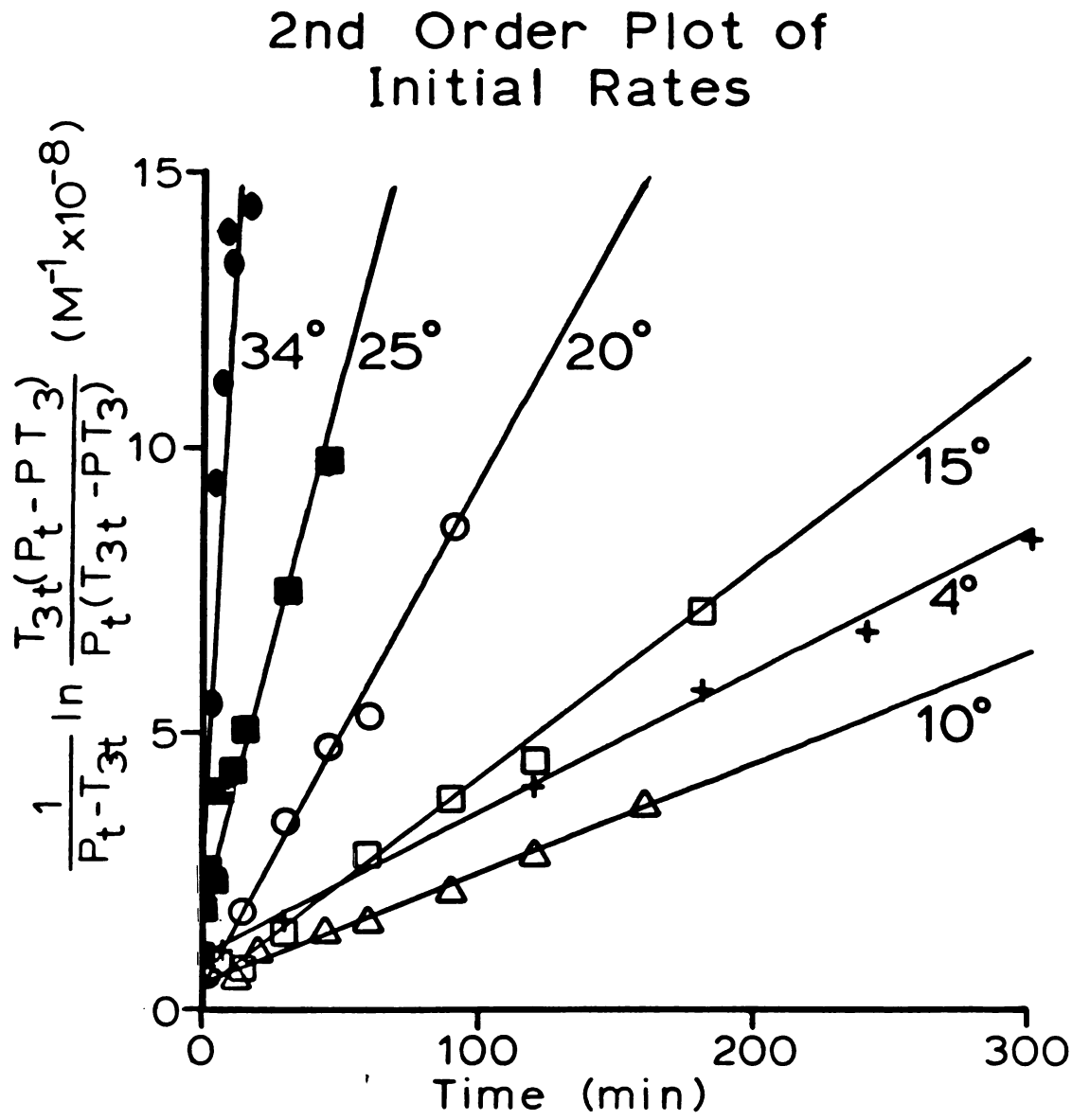

2nd Order Plot of
Initial Rates
$\frac{1}{P_t - T_{3t}} \ln \frac{T_{3t}(P_t - PT_3)}{P_t(T_{3t} - PT_3)}$ ($M^{-1} \times 10^{-8}$)
15
10
5
0
34°
25°
20°
15°
4°
10°
0
100
200
300
Time (min)

Figure 6-7.

The constraints were derived logically from the Scatchard data. The first constraint ($k_1>0$, $k_2>0$) is logical since rate constants cannot be less than zero. The second constraint ($k_1<1.29 \times 10^9 \times k_{-1}$) means that the computer can vary k_1 and k_{-1} until the fit converges as long as k_1/k_{-1} is less than 1.29×10^9 which is less than the equilibrium association constant at 25^o. Using these constraints a reasonably good fit without excess oscillation was obtained at 34^o.

The first seven data points at each temperature were used to plot the initial rates according to the integrated linear form of the second order rate equation as seen in figure 6-7. The slope of each line corresponds to the association rate constant (k_1). All of the rate constants obtained from the curve fitting routine and the linear plot are summarized in Table 6-3. Comparison of K_a (calcd) and K_a (meas.) shows that the equilibrium association constants calculated from the ratio k_1/k_{-1} (fitted) are similar to the values measured by Scatchard analysis as in figure 6-3.

It is interesting to examine the magnitude of the association rate constants. At 25^o, if the reaction was diffusion controlled as in the case of acetylcholinesterase antagonists binding to a surface site, it would be expected that the association rate constants would be approximately 7×10^{10} M^{-1} min^{-1}.[100] This is not observed for T_3 binding to the nuclear receptor. The rate constant at 25^o (4.78×10^7 M^{-1} min^{-1}) is 10^3 times slower than the surface site reaction. Spindler *et al.* also reported a similar value (4.7×10^7 M^{-1} min^{-1}) for T_3 binding to rat liver nuclei.[48] This can be interpreted in either one of two ways: (1) the binding site could be a deep channel which has specific conformational and orientational requirements for formation of the T_3-receptor complex, or (2) a conformational change might be required in

the receptor or T_3 before an energetically favorable complex can be formed. It is possible that both mechanisms could be responsible for the slow association rate. However, the possibility that T_3 binds to a simple surface site can be ruled out.

Temperature Dependence of Kinetic Rate Constants:

In order to obtain thermodynamic information about the nature of the transition state for T_3-receptor complex formation, the rate constants from Table 6-3 were plotted according to the Arrhenius equation:[101]

$$\ln k = \ln A - \frac{E_a}{RT} \qquad \text{eq 6-6}$$

The resulting plots of $\ln k_1$ versus $1/T$, and $\ln k_{-1}$ versus $1/T$ are seen in figure 6-8. The slopes of the best fit lines correspond to minus the activation energy (E_a) divided by the gas constant (R) and the intercepts correspond to ln A, where A is the frequency factor (min^{-1}). In order to calculate the activation parameters, $\Delta G^{\ddagger}$, $\Delta H^{\ddagger}$, and $\Delta S^{\ddagger}$, the following equations were used:[102]

$$\Delta H^{\ddagger} = E_a - RT \qquad \text{eq 6-7}$$

$$\Delta S^{\ddagger} = (\ln A - \ln(\frac{kT}{h} \times 60 \text{ sec/min})) \times R \qquad \text{eq 6-8}$$

$$\Delta G^{\ddagger} = \Delta H^{\ddagger} - T\Delta S^{\ddagger} \qquad \text{eq 6-9}$$

Where: ln A = intercept from Arrhenius plots in figure 6-10

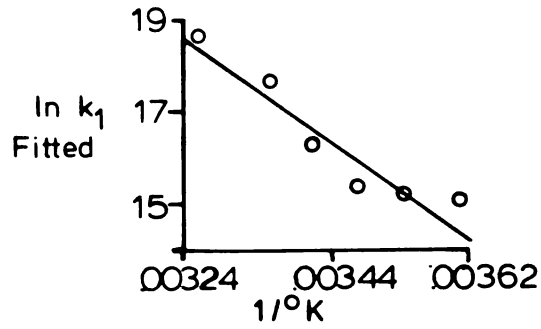
k = Boltzmann's Constant (1.3804×10^{-16} erg $^{o}K^{-1}$)

h = Planck's Constant (6.6252×10^{-27}

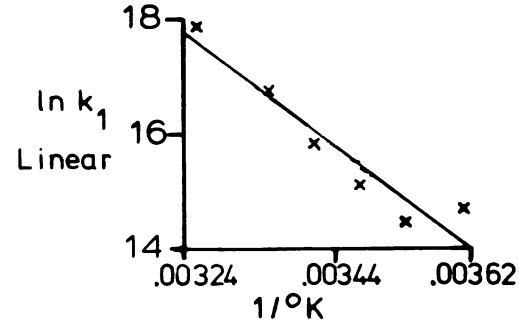
R = Gas Constant (1.9872 cal/mol ^{o}K)

A summary of the equilibrium and activation parameters is given in Table 6-4.

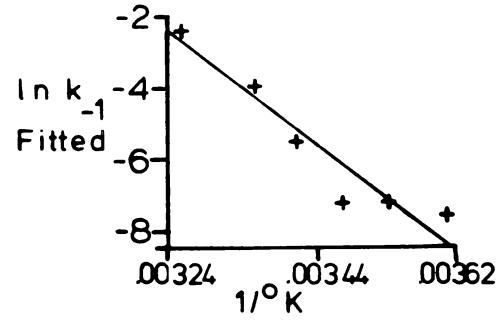
Arrhenius Plot of k_1 & k_{-1} from Linear & Fitted Data



r = .946
m = -11,157
b = 54.8
E_{a_1} = 21.17 Kcal/mole



r = .955
m = -9,823
b = 49.6
E_{a_1} = 19.52 Kcal/mole



r = .951
m = -16,027
b = 49.5
$E_{a_{-1}}$ = 31.85 Kcal/mole

Figure 6-8.

Table 6-3. Temperatures, Association and Dissociation Rate Constants and Calculated K_a for T_3 Binding to the Solubilized Nuclear Receptor

Temp (^oC)	k_1 fitted (M^{-1} min^{-1})[a]	k_{-1} fitted (min^{-1})	K_a calcd (M^{-1})[b]	K_a meas (M^{-1})	k_1 linear (M^{-1} min^{-1})[c]	R[d]
34	$(1.28\pm0.20)\times10^{8}$	$(9.90\pm1.49)\times10^{-2}$	1.29×10^{9}	1.17×10^{9}	$(5.71\pm1.80)\times10^{7}$	0.846
25	$(4.78\pm0.57)\times10^{7}$	$(2.01\pm0.36)\times10^{-2}$	2.39×10^{9}	1.98×10^{9}	$(1.90\pm0.14)\times10^{7}$	0.981
20	$(1.22\pm0.07)\times10^{7}$	$(4.25\pm0.38)\times10^{-3}$	2.87×10^{9}	3.39×10^{9}	$(8.97\pm0.40)\times10^{6}$	0.995
15	$(4.62\pm0.24)\times10^{6}$	$(7.20\pm1.27)\times10^{-4}$	6.42×10^{9}	4.44×10^{9}	$(3.73\pm0.17)\times10^{6}$	0.996
10	$(3.87\pm0.58)\times10^{6}$	$(7.73\pm s.90)\times10^{-4}$	5.01×10^{9}	6.10×10^{9}	$(1.97\pm0.10)\times10^{6}$	0.993
4	$(3.55\pm0.18)\times10^{6}$	$(5.16\pm1.05)\times10^{-4}$	6.88×10^{9}	7.72×10^{9}	$(2.51\pm0.09)\times10^{6}$	0.997

a. Final parameter values and standard errors from Mlab fitting of kinetic data to $dPT_3/dt = k_1(P_t - PT_3) - k_{-1}(PT_3)$.

b. Association constant calculated from $K_a = k_1/k_{-1}$.

c. Slopes and standard deviation of slopes from linear second order rate equation versus time as plotted in Figure 6-9.

d. Correlation coefficient for best fit straight lines as plotted in Figure 6-9.

Table 6-4. Summary of Equilibrium and Activation Energy Parameters

Determined from the Temperature Dependence of Equilibrium Constants and Kinetic Rate Constants[a]

Source of Temp Dependence Data	kcal/mol					
	ΔG^o	ΔH^o	$T\Delta S^o$	$\Delta G^{\ddagger}$	$\Delta H^{\ddagger}$	$T\Delta S^{\ddagger}$
Measured K_a	-12.7(±1.7)	-11.0(±0.8)	+1.7(±0.9)			
Calculated K_a[b]	-12.8(±3.6)	-9.7(±1.8)	+3.1(±1.8)			
Fitted k_1				+9.0(±7.7)	+21.6(±3.8)	+12.6(±3.9)
Linear k_1				+9.4(±6.1)	+18.9(±3.0)	+9.5(±3.1)
Fitted k_{-1}[c]				-21.8(±10.5)	-31.3(±5.2)	-9.5(±5.3)

a. Average energies were calculated for 298 ^oK from the data in Figure 6-10.

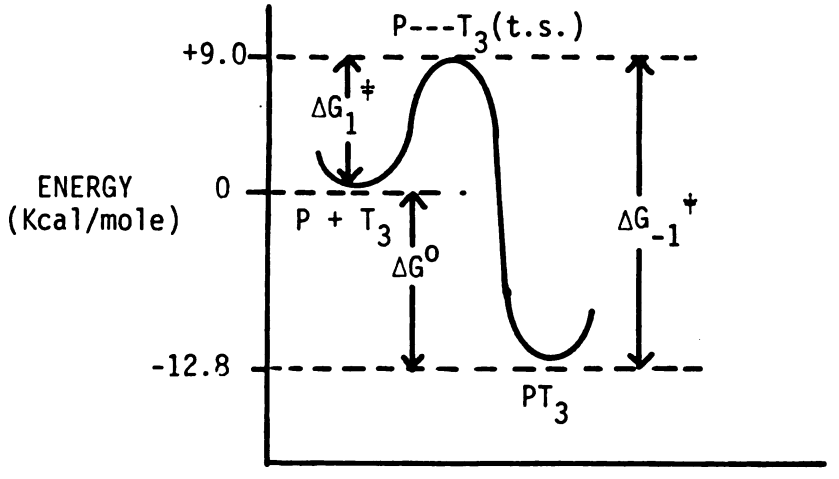
b. K_a is calculated from the ratio of fitted k_1/k_{-1}.

c. Values of $\Delta G_{-1}^{\ddagger}$, $\Delta H_{-1}^{\ddagger}$, and $T\Delta S_{-1}^{\ddagger}$ are calculated for the forward direction from transition state to products.

Discussion:

There is close agreement between the free energy, enthalpy, and entropy determined from the measured K_a values and from the K_a values calculated from the ratio of computer-fitted k_1 and k_{-1}. These parameters have been discussed previously.

The activation parameters may be used to make inferences about the nature of the transition state for T_3-receptor complex formation. The following figure shows a reaction coordinate diagram of the energies involved.



Reaction Coordinate Diagram at 25°

Figure 6-9.

The transition state (t.s.) is defined as an intermediate activated complex of higher energy than either the reactants or products. The diagram in figure 6-9 shows that the observed equilibrium free energy of binding (ΔG^o) is the sum of the two activation free energies $\Delta G^{\ddagger}_1$, and $\Delta G^{\ddagger}_{-1}$. The magnitude of these activation free energies is what determines the rate of the reaction in the forward and reverse direction. Since the magnitude of $\Delta G^{\ddagger}_{-1}$ is much larger than $\Delta G^{\ddagger}_1$, the off-rate (k_{-1}) is much slower than the on-rate (k_1). Since the product (T_3-receptor complex) is

-12.8 kcal/mol more stable than the reactants, at equilibrium there will be a large ratio of products to reactants.

The free energy barriers for reactants and products to reach the transition state contain a contribution from enthalpy and entropy. These have been measured and reported in Table 6-4 as $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$. Interpretation of the magnitude of these contributions to the activation free energy can provide information about the nature of the transition state. The results of these studies can be summarized as follows: (1) The large positive enthalpy of activation ($\Delta H^{\ddagger}_1$ = +21.6(±3.8) kcal/mol) indicates that the reactants absorb a large amount of energy to reach the transition state. This could be due to a conformation change in the receptor as T_3 enters the binding region and could result in the observed slow on-rate (k_1). (2) Entropy contributes -12.6(±3.9) kcal/mol to the activation free energy ($\Delta G^{\ddagger}_1$) which means that the transition state is more disordered than the reactants. Since this is opposite to what would be expected for two molecules reacting to produce one complex, this positive contribution to $\Delta S^{\ddagger}_1$ probably arises from the hydrophobic effect which disorders the solvent cage around two reactive non-polar solute molecules when the activated complex is formed. It is interesting that this hydrophobic effect contributes an energy close to that predicted for hydrophobic bonding (-14.7 kcal/mol) earlier in this chapter. (3) The large negative energy (-31.3 (±5.2) kcal/mol) contributed by enthalpy as the reaction proceeds from the transition state to the product is clearly the driving force for complex formation and represents the sum of the energy released in the formation of electrostatic, hydrogen, and dispersion force bonds between T_3 and the receptor. (4) The loss of -9.5(±5.3) kcal/mol in free energy due to the entropy change as the final complex is formed represents the

part of the free energy lost due to restriction of translational and rotational degrees of freedom of T_3 as well as locking the outer ring of T_3 into the distal conformation.

This chapter has presented the results of the first study of the temperature dependence of the kinetic rate constants and the equilibrium association constant for T_3 binding to the nuclear receptor from rat liver. It has shown that the predominant driving force for complex formation is contributed by the enthalpy change which represents a decrease in the binding free energy from formation of specific electrostatic bonds, hydrogen bonds, and dispersion force interactions. It is suggested that the trade off between favorable hydrophobic interactions and restriction of translational and rotational degrees of freedom results in a very small contribution of entropy to the binding free energy for T_3.

Chapter Seven: PROPHET Computergraphic Study of the Theoretical Binding Interactions between Thyroid Hormone and Human Plasma Prealbumin

Human plasma prealbumin is a highly stable tetrameric protein composed of four identical subunits of 127 known amino acid residues with an over-all molecular weight of 54,980.[103,104] Prealbumin acts as a storage and carrier system for the thyroid hormones and for retinol (vitamin A alcohol). The thyroid hormones are bound directly to prealbumin, whereas the vitamin is bound to a small carrier protein (retinol-binding protein) which in turn is bound to sites on prealbumin which are independent of the hormone binding sites.[105] Figure 7-1 presents a schematic drawing of the prealbumin tetramer.

The prealbumin tetramer has two hormone binding sites whose association constants for L-thyroxine (T_4) are 1.0×10^8 M^{-1} and 9.5×10^5 M^{-1}; L-3,5,3'-triiodothyronine also binds at these sites with an affinity

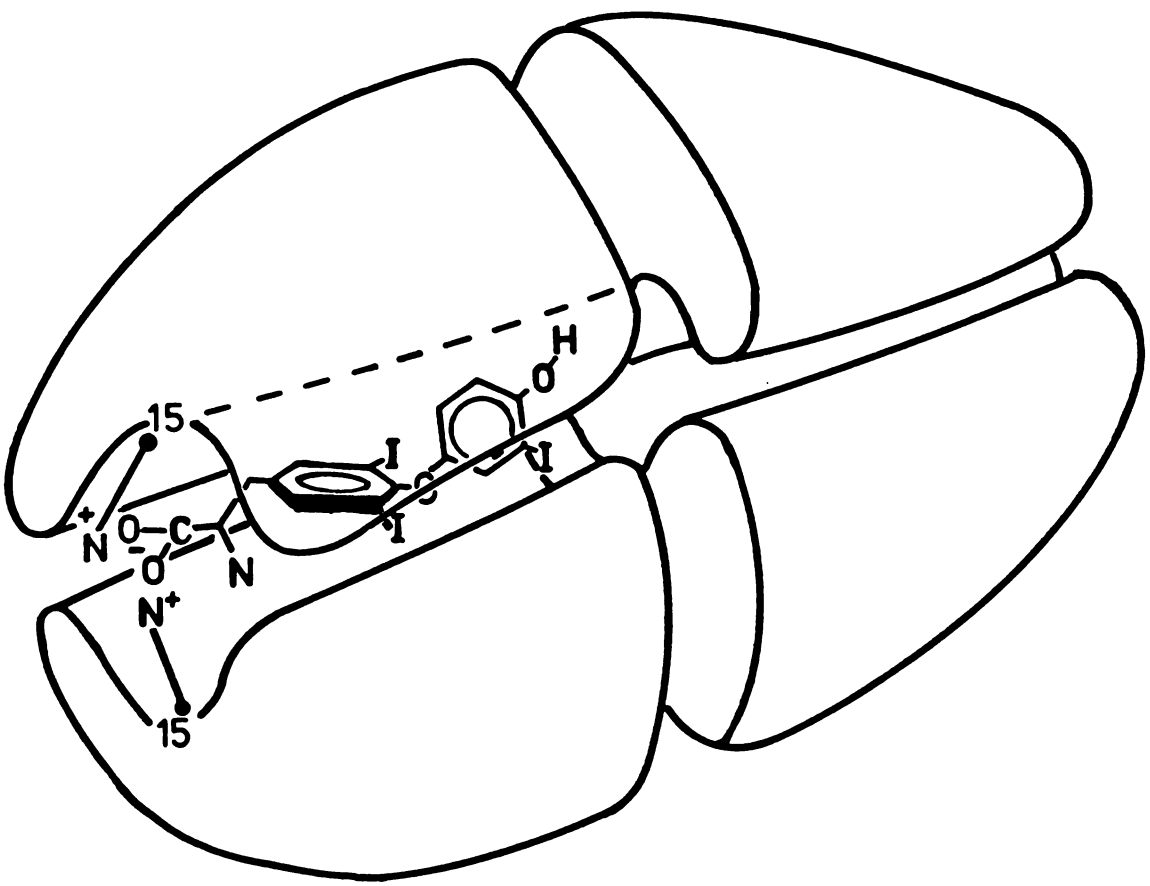


Figure 7-1.

about ten times less than T_4[106]. The differences between the two observed association constants for T_4 could be due to T_4 binding to two different classes of independent binding sites or to T_4 binding to two identical classes of binding sites that interact with negative cooperativity. Blake has shown that these two sites are structurally identical and are related by a two-fold axis of symmetry.[107] This evidence eliminates the first possibility since the sites are identical and it infers that the observed difference between the association constants is due to negative cooperativity as was earlier suggested by Ferguson et al.[106]

The mechanism of this negative cooperativity could be due to (1) direct ligand-ligand interaction (meaning that some part of the first T_4 molecule bound could be sterically inhibiting the binding of the second T_4 molecule) or (2) site-site interaction. The extensive β-structure of prealbumin, with adjacent chains and subunits interacting through hydrogen bonds, presents a potentially efficient mechanism for transmittal of conformational effects from one site to the other. Utilizing x-ray data at the 1.8 Å level, Blake has observed a constriction near the center of the binding channel (figure 7-1) which would prevent access from one site to the other. This indicates that the observed negative cooperativity could not be of the ligand-ligand type. Therefore, it can be concluded that binding of the first molecule of T_4 induces a conformational change that is transmitted to the second site and lowers the affinity for the second molecule of T_4.

Blake has used this evidence, and the observation of a third region on the prealbumin molecule which is complementary to the double-helix of DNA, to suggest that prealbumin could be used as a model for the thyroid hormone nuclear receptor.[107] He suggests that the binding of T_4 to prealbumin may alter the geometry and properties of the "DNA-binding" site

by translation of conformational change through a tyrosine residue which has its peptide involved in the β-structure of the hormone binding site and its hydroxyl involved in the hydrogen bonding network of the β-sheet that form the "DNA binding" site.

The evidence that prealbumin is a DNA binding protein that could be studied as a model for the thyroid hormone nuclear receptor is both circumstantial and meager. However, it is still interesting to examine the specific molecular interactions between substituents on the hormone and the amino acid side chains of a biologically important protein with which it associates in a structurally specific manner. This chapter presents the results of a computergraphic study which describes a theoretical binding interaction between L-T_3 and human plasma prealbumin.

METHODS

The x-ray coordinates of prealbumin at 2.5 Å resolution were generously supplied by Colin Blake at Oxford. The x-ray coordinates of L-3,5,3'-triiodothyronine were taken from the work of Cody.[108] The PROPHET system[99] (utilizing a DEC PDP-10 computer) was used via a remote Tektronics terminal. The PROPHET graphics prodecures which allowed independent movement and rotation of one or more graphic molecules were used to generate stereo-views of the T_3 prealbumin binding complex.

In order to convert the x-ray coordinates into a PROPHET molecule, the public procedures CRYST and CONNECT must be restored and compiled (RAC). A table is made containg the atom identifier (eg. Y105CA = Tyrosine 105 c-alpha), the atom type (eg. C = carbon), and the X, Y, and Z coordinates for the atoms of interest as shown in figure 7-2. Then the procedure CRYST is started by typing CALL CRYST (COORDINATE, 'PREALBUMIN'),

MK TBL COORDINATE

	1 ID	2 TYPS	3 X COORD	4 Y COORD	5 Z COORD
1	Y105CA	C	-8.08	9.75	15.26
2	T106CA	C	-5.25	7.45	14.31
3	I107CA	C	-3.32	7.9	11.11
4	A108CA	C	-.81	5.39	9.88

```
RAC PUBLIC CRYST
RAC PUBLIC CONNECT
CALL CRYST(COORDINATE,'PREALBUMIN')
```

Figure 7-2.

where COORDINATE is the name of the table that contains the x-ray coordinates and PREALBUMIN is the name used for the molecule which is generated. The resulting molecule is displayed and can be altered if needed within the procedure. The stereo-views of each molecule are made by placing a pair of molecule images side by side. One of the images is turned by 10^{o} around the Y-axis relative to the other. When these paired images are viewed so that one eye sees only one image and the other eye sees an image rotated by 10^{o}, a feeling of perspective is generated which allows one to study the spacial relationships between the substituents in the molecule. An illustration of this is seen in figure 7-3. When the morphine and trans-morphine molecules are viewed without a stereo viewer it is impossible to distinguish the stereochemistry of trans-morphine. In morphine, which is drawn as a ball and stick molecule, it can be seen that the piperidine ring projects out from the plane of the paper but the rest of the molecule lacks depth. However, when a stereo-viewer is used, the two molecules take on a three dimensional quality and the stereochemistry of both molecules is quite clear.

The prealbumin dimer (figure 7-4) shows the α-carbon backbone of two monomers which are highly organized structures containing two extensive β-sheets, each composed of four strands. The view in figure 7-4 is down the molecular Z-axis which shows the large central channel that has been shown by low resolution electron-density difference maps to be the binding site for the thyroid hormones.[103]

The prealbumin dimer can be modified by removing most of the bottom monomer and leaving only the portion of the β-sheet which is involved in the binding site. Then the side chain residues which line the binding channel, can be introduced by inserting them into the original table of x-ray coordinates and calling CRYST again. By adding T_3 to the image with

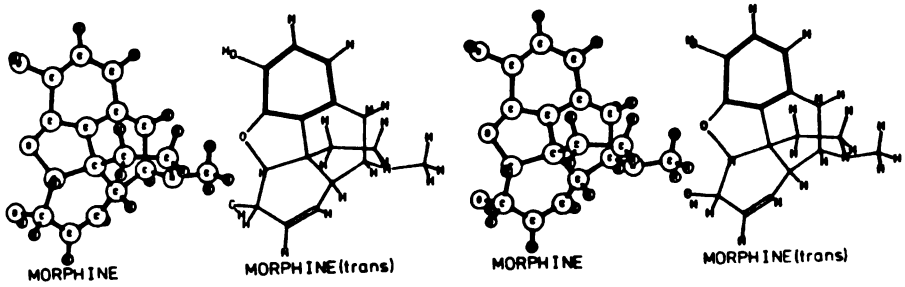


Figure 7-3.

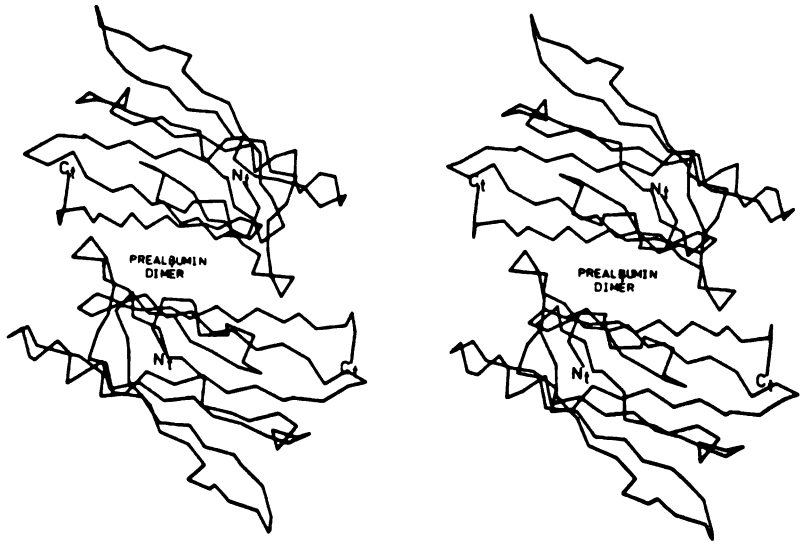


Figure 7-4.

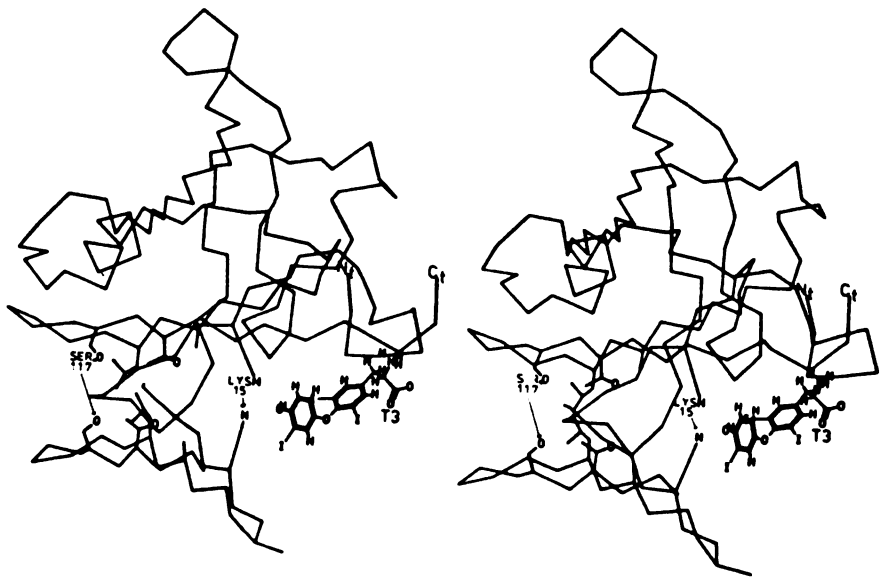


Figure 7-5.

the "MULTIMOLECULE" commands, the size relationship between prealbumin and T_3 can be seen as in figure 7-5, which is rotated 90^0 around the Y-axis from the view seen in figure 7-4.

ORIENTATION OF T_3 IN THE BINDING SITE

At high concentration, 8-anilino-1-naphthalene sulfonic acid (ANS) can compete with T_4 for the prealbumin binding site.[106] The structurally related and reactive 5-dimethyl-amino-1-naphthalene sulfonyl chloride (dansyl chloride)[109] and the T_4 derivative, N-bromoacetyl-T_4 ($BrAcT_4$),[110] applied as affinity labels, were shown to associate with the lysine-15 residues of prealbumin. To a lesser extent, reaction also occured between $BrAcT_4$ and the nearby methionine-13 and aspartate-18 residues. From this evidence, one can assume that ion-pair formation occurs between the sulfonate (ANS) or carboxylate (T_4) anionic residues of the ligands and the cationic lysine-15 residue of prealbumin and that these are complemented by additional non-ionic interactions at the binding site.

Using the methods described above, it has been possible to generate stereo-views of T_3 situated in the binding site of prealbumin. By manipulating a computergraphic display of the x-ray coordinates for L-T_3, the carboxylate side chain of T_3 was placed between the display of the ε-amino groups of the lysine-15 side chains that project into the entrance of the binding cavity. The triiododiphenyl residue was placed within the channel as observed in the low-resolution x-ray data.[107] The T_3 molecule was orientated to display potential binding interactions, without changing the x-ray coordinate positions obtained from ligand-free prealbumin.

RESULTS

Figure 7-6 shows a stereo-view, looking down the x-axis of the protein, of the resulting location of T_3. This is the same view as in figure 7-5 but most of the top monomer has been stripped away so that only the β-sheet and side chains involved in the binding site can be seen. The specific molecular interactions between T_3 and prealbumin, seen in figure 7-6, can be summarized as follows: (1) there is an ion-pair association of the carboxylate anion of T_3 and the cationic ε-amino group of the lysine-15 residues, (2) there is an ion-pair association of the amino cation of T_3 and the anionic δ-carboxylate group of one of the glutamate-54 residues, (3) the 3,5-iodine atoms and the inner phenyl ring are positioned in a size-limiting lipophilic region of the binding channel composed of the aliphatic amino acids valine-121, leucine-17, and alanine-108, and (4) the 4'-hydroxyl is positioned midway between the oxygens of serine-117 and threonine-119 which suggests that some form of hydrogen bond is formed in the T_3-prealbumin complex. It is interesting to note that due to the electron withdrawing effect of its 3',5'-iodine atoms, T_4 has much lower pK_a for the phenolic hydroxyl than T_3 ($pK_a(T_4) = 6.7$, $pK_a(T_3) = 8.5$).[111] Since T_4 binds an order of magnitude more tightly than T_3, it is suggested that the most favorable interaction of the 4'-hydroxyl is formation of an acceptor hydrogen bond between a proton from serine-117 or threonine-119 and the phenolate anion of T_3. It can also be seen that there is no steric hindrance at the 5'-position which is in contrast to the nuclear receptor. Figures 7-7 and 7-8 are views of the binding site looking down the molecular Y-axis and Z-axis respectively. These views further elaborate the precise orientation required by the T_3 molecule in order to maximize the potential binding interactions with

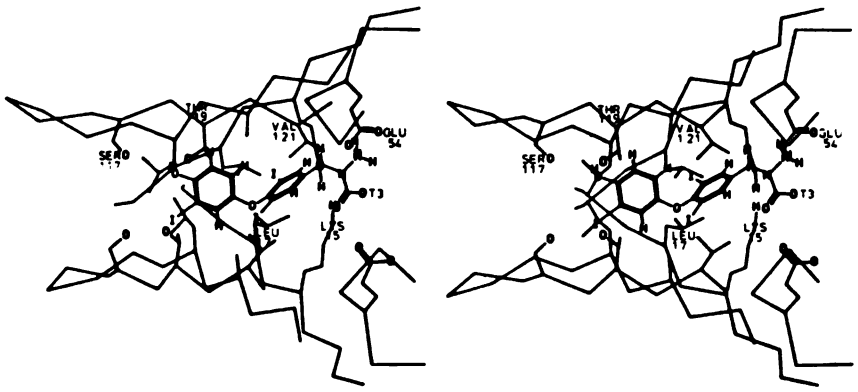


Figure 7-6.

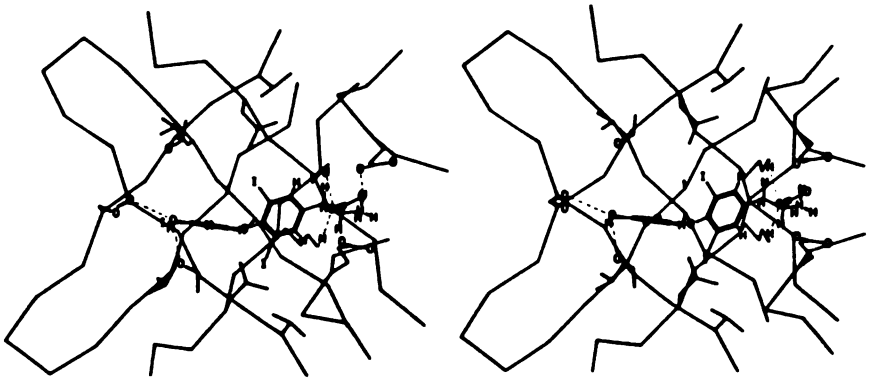


Figure 7-7.

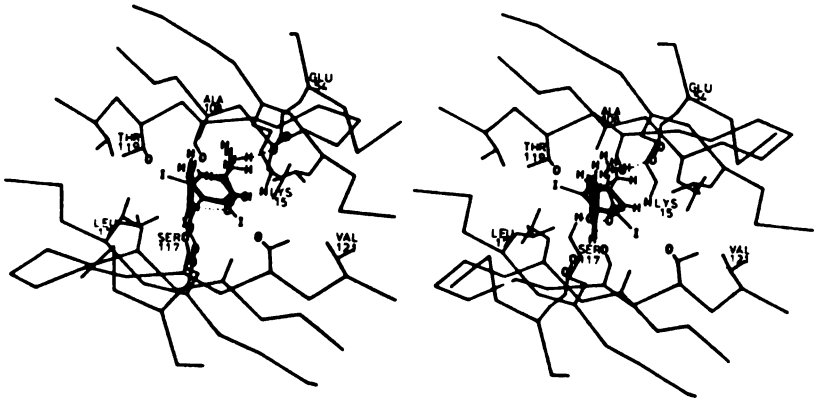


Figure 7-8.

side chain residues in prealbumin.

Table 7-1 lists interatomic distances which result from the combined crystallographic coordinates of T_3 and prealbumin in this model. It can be seen that there is a very good correlation between the distances measured in the model and those of experimentally determined or theoretically calculated values for similar interactions.

SUMMARY

This postulated model of binding between the thyroid hormones and prealbumin is the first specific representation of the molecular interactions between substituents on the hormone and the amino acid side chains of a biologically important protein with which it associates in a structurally specific manner. The model described in Chapters Five and Six for the molecular interactions between T_3 and the nuclear receptor are remarkably consistent with the model of T_3-prealbumin binding. The alanine binding region of prealbumin involves ion-pair formation between the carboxylate anion and the cationic ε-amino group of lysine-15. This corresponds to the evidence from nuclear binding studies that the carboxylate anion of T_3 forms an electrostatic bond to some positively charged amino acid residue in the receptor. It has been shown that there is a size-limited hydrophobic association of the 3,5-iodine atoms and the inner ring for both prealbumin and receptor binding. However, there are major differences between the receptor and prealbumin such as the fact that there is no evidence of two sites in the receptor. Also the inner polar region of prealbumin which contains serine-117 and threonine-119 shows binding characteristics in contrast to the nuclear receptor. The most favorable binding of the phenolic hydroxyl of T_3 to the nuclear

Table 7-1. Interatomic Distances for Proposed Interactions Between T_3 and Prealbumin Compared with Literature Interatomic Distances for Similar Interactions

Atoms Thyronine---------Protein	Model Interaction Distance, (Å)	Literature Interaction Distance, (Å)
Amino		
$-NH_2-H\cdots\cdots O-\overset{\overset{-}{O}}{\overset{\|}{C}}-$(Glu-54)	3.2	2.87[a]
Carboxylate		
$-\overset{\overset{O}{\|}}{C}-O^-\cdots\cdots H-\overset{+}{N}H_2-$(Lys-15)	2.8	2.87[a]
$3I\cdots\cdots\cdots C-\gamma-$(Leu-17')	3.6	---
$5I\cdots\cdots\cdots C-\gamma-$(Leu-17)	4.3	---
Phenolate		
$-O^-\cdots\cdots\cdots HO-$(Ser-117)	3.5	2.35[b]
$-O^-\cdots\cdots\cdots HO-$(Ser-119)	2.4	2.35[b]
$3'I\cdots\cdots\cdots\overset{H}{O}-$(Ser-117')	3.2	3.0[c]

[a]Downie, T.C., Speakman, J.C., J. Chem. Soc., 787, (1954) for ammonium hydrogen disalicylate hydrate.

[b]Dietrich, S.W., Ph.D. Dissertation for the minimal energy distance between oxygen atoms in the phenoxide-water hydrogen bonding interaction.

[c]Mulliken, R.S., Person, W.B., "Molecular Complexes", 319 (Wiley-Interscience, New York, 1969). For the $I_2\cdots$ethanol oxygen distance.

receptor involves donor hydrogen bond formation, whereas the plasma protein prealbumin favors ionization of the phenolic hydroxyl and formation of an acceptor hydrogen bond between the phenolate anion and serine-117 or threonine-119.

These differences are important biologically since prealbumin would then have a higher affinity for T_4 than T_3 and allow the release and translocation to nuclear receptors of the more biologically potent T_3 molecule. If the nuclear receptor resembles prealbumin with respect to binding at the core and transmission of conformational changes to a highly charged and polar surface, it would be an excellent means for inducing structural changes in the closely associated DNA as an initiating event in hormonal action. The possibility still exists that the similarity in binding characteristics between prealbumin and the nuclear receptor are coincidental and that the majority of the protein structures are different. Future studies which could help clarify this issure are summarized as follows: (1) A study of the temperature dependence of T_4 and T_3 binding kinetics to prealbumin should be performed so that the enthalpic ($\Delta H^{\ddagger}$) and entropic ($\Delta S^{\ddagger}$) contributions to the activation free energy could be compared with those of the nuclear receptor. It would be expected that the enthalpy of activation would be large and positive for prealbumin binding since there is a deep channel to penetrate, and conformational changes, which absorb energy, are postulated to occur. (2) If there is structural homology between prealbumin and the nuclear receptor, some evidence of subunit structure should be found for the nuclear receptor. (3) It should be proven that prealbumin is a DNA binding protein as suggested by Blake.

It has been shown that computergraphic techniques can produce a model of thyroid hormone binding which is consistent with available

crystallographic, affinity-labeling, and interatomic distance data. More research is required to determine if prealbumin binding can be used as a model for the thyroid hormone nuclear receptor.

Chapter Eight: Concluding Remarks and Future Studies

The last five years has seen a large increase in our knowledge of the molecular basis of thyroid hormone action. The discovery of the thyroid hormone nuclear receptor and the subsequent molecular biological studies have decreased the number of possible mechanisms of hormone action but have increased the number of known processes regulated by thyroid hormone to include fetal lung development, growth hormone synthesis in cell culture and biosynthesis of the Na^+ K^+ ATPase ion pump.[9] The present study has been at the periphery of this molecular biological research and most of the quantitative conclusions presented here have been qualitatively suggested previously. Nevertheless, a compact and consistent description of the quantitative molecular interactions between the thyroid hormones and the solubilized rat liver nuclear receptor has been presented.

In order to expand this thermodynamic study of T_3-receptor binding interactions, it would by interesting to determine the magnitude of the enthalpic and entropic contributions to the binding free energy contributed by hydrogen bond formation, hydrophobic association of 3 and 5-iodine atoms, and electrostatic interaction of the alanine side chain. The measured $\Delta\Delta G$ values presented in Chapter Five for substituent interaction with the receptor can be partitioned into the enthalpy $\Delta\Delta H$ and entropy $\Delta\Delta S$ contributions. For example, the $\Delta\Delta G$ for a hydrogen bond would be expected to contain an enthalpic contribution due to a decrease in enthalpy upon forming hormone-receptor bonds from hormone-water and receptor-water bonds and an entropic term which would be a measure of how the hydrogen bond effects the structure of water in the receptor site. This type of analysis has never been done before and would be exciting in the theoretical sense.

It would be very useful to have a specific covalent affinity label for the receptor. A potentially effective method of alkylation would involve synthesis of a T_3 analog in which the alanine side chain is substituted with an aldyhyde. If the receptor contains a positively charged amino group at the carboxylate binding region, as is the case for prealbumin, the aldehyde would be expected to form a Schiff base with the receptor which could be reduced to complete the covalent attachment. Such an analog could be easily labeled with radioactive ^{125}Iodine and could be used effectively in the purification of the receptor. By denaturing the labeled receptor with SDS-gel electrophoresis it could be determined if the receptor contains subunits of lower molecular weight than 60,000.

Finally, the hypothesis that the T_3 receptor is an enzyme with kinase activity should be investigated with a nuclear receptor preparation purified by affinity chromatography. Using these purified preparations

and a sensitive assay for kinase activity it could be shown very clearly if the nuclear receptor is responsible for the phosphorylation of non-histone protein.

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Appendix I

```
RBNDATA:PROCEDURE;
     /* THIS PROCEDURE PRINTS A TABLE OF BINDING ASSAY
DATA AFTER RECEIVING INPUT OF:  FINAL COLD ANALOG CONCN.
TOT. COUNTS ADDED, % I- CORRECTION,
NON-SPECIFIC COUNTS, AND SPECIFICALLY BOUND COUNTS.
DERIVED COLUMNS ARE:  CORRECTED TOT. COUNTS, PT3%-NS,
4/5 TOT. COUNTS, T3% FREE, AND FREE/BOUND.
     NEXT A LINE IS FITTED TO THE F/B VS ANALOG ADDED
AND STATISTICAL PARAMETERS ARE PRINTED.
     FINALLY A GRAPH IS GENERATED WHICH HAS
TWO CURVES;  POINTS AND BEST FIT LINE.  PRINTING IS
OPTIONAL. */


DCL (M,INT,PT) FLOAT;
DCL (RATIO,KAA,KDA,DG) FLOAT;
DCL T TEXT;
DCL BB(*) FLOAT;

TYPE '
WHAT IS THE % IODIDE CONTAMINATION';
INPUT IOD;
SET DERIVATION OF COL 4 OF DATAIN2 TO 'CELL(2)-((IOD/100)*CELL(2))';
TYPE '
ENTER NON-SPECIFIC COUNTS';
INPUT NS;
SET DERIVATION OF COL 5 OF DATAIN2 TO 'CELL(3)-NS';
MAKE TABLE TOM USING DATAIN2;
TYPE '
WHEN YOU TYPE "YES" YOU CAN ENTER YOUR DATA AS FOLLOWS:
      COL 1 = FINAL COLD ANALOG CONCN.
      COL 2 = TOT COUNTS ADDED
      COL 3 = BOUND COUNTS';
IF %YESANSWER('
TYPE YES WHEN READY TO ENTER DATA.') THEN EA;
FILL TOM;
DIS TOM COLS 1,5 TO 9 ROWS 1 TO 7;
IF %YESANSWER('TYPE YES TO CONTINUE') THEN EA;
T = %FITLINE(TOM,1,8,2,TRUE,TRUE,FALSE,BB);
PT = 1/(1.19E9*BB(2));
PTE = (1/(1.19E9*BB(2)))-(1/(1.19E9*(BB(2)+BB(6)))); TYPE '


    Several Spaces

PT=',PT,'+(',PTE,')M';
RATIO = PT*BB(1);
RATIOE = (PT*BB(1))-(PT*(BB(1)-BB(5))); TYPE '
% L-T3=',RATIO*100,'+(',RATIOE*100,')%';
KAA = 1.19E9*RATIO;
KAAE = (1.19E9*RATIO)-(1.19E9*(RATIO-RATIOE)); TYPE '
KAA=',KAA,'+(',KAAE,')M-1';
KDA = 1/KAA; TYPE '
KDA=',KDA;
DG = -.59218*LOG KAA;
DGE = (-.59218*LOG KAA)-(-.59218*LOG (KAA-KAAE)); TYPE '
DEL G=',DG,'+(',DGE,')KCAL';
IF %YESANSWER('
TYPE YES IF YOU WANT TO SEE A GRAPH OF
F/B VS ANALOG ADDED.') THEN
MAKE GRAPH GTOM FROM TOM AS COL 1 VS COL 8;
ADD CURVE TO GTOM FROM TOM AS COL 1 VS T;
EAD GTOM;
END;
```

Table Used in Procedure: RBNDATA

DATAIN2 0R X 13C

1	2	3	4 (D)
CONCN. ANALOG ADDED (M) <NUMBER>	TOT. COUNTS ADDED <NUMBER>	CPM BOUND T3 PT3* <NUMBER>	TOT COUNTS CORRECTED FOR IODIDE <NUMBER> CELL('TOT. COUNTS ADDED')-(5. /100)*CELL('TOT. COUNTS ADDED')

5 (D)	6 (D)	7 (D)
PT3*- NON-SPEC. <NUMBER> CELL('CPM BOUND T3 PT3*')-2553	4/5 TOT COUNTS <NUMBER> (4 /5)*CELL('TOT COUNTS CORRECTED FOR IODIDE')	T3* FREE CPM <NUMBER> CELL('4/5 TOT COUNTS')-CELL('CPM BOUND T3 PT3*')

8 (D)	9 (D)
T3*/PT3* <NUMBER> CELL('T3* FREE CPM')/CELL('PT3*- NON-SPEC.')	PT3*/T3* <NUMBER> 1 /CELL('T3*/PT3*')

10 (D)
CONCN. OF T3 FREE (M) <NUMBER> (2.82E-15 *CELL('T3* FREE CPM')-(.12 *(2.82E-15 *CELL('T3* FREE CPM')))

11 (D)
COLD T3 ADDED /1+PT3*/T3* <NUMBER> CELL('CONCN. ANALOG ADDED (M)')/(1 +CELL('PT3*/T3*'))

12 (D)
(T3*/PT3*)*PT- T3 <NUMBER> (CELL('T3*/PT3*')*1.92E-15)-CELL('T3*/PT3*')

13 (D)
FREE ANALOG CONCN.(M/L) <NUMBER> CELL('CONCN. ANALOG ADDED (M)')-(5.4E-10 -(((CELL('PT3*/T3*')/1.19E9)+- (CELL('PT3*/T3*')*CELL('CONCN. OF T3 FREE (M)'))))

Procedure Used to Calculate Moments of Inertia

```
MOINERTIA:PROCEDURE(CA,NATOM);
     /* THIS PROCEDURE ACCEPTS A MATRIX OF X-RAY COORDINATES
PLUS A FOURTH COLUMN CONTAINING THE AMU FOR THE ATOMS.
THE MATRIX SHOULD BE A TWO DIMENSIONAL FLOAT ARRAY.
THE PROCEDURE CALCULATES THE COORDINATES OF THE CENTER OF MASS AND
RECOMPUTES THE ORIGINAL COORDINATES SO THAT THE ORIGIN IS
AT THE CENTER OF MASS.
     NEXT A THREE BY THREE MATRIX IS CALCULATED THAT CONTAINS
 THE FOLLOWING:
                IXX  IXY  IXZ
                IYX  IYY  IYZ
                IZX  IZY  IZZ
THIS IS THE PRODUCT MATRIX AND IS PRINTED.  FINALLY THE PUBLIC
PROCEDURE EIGEN IS CALLED TO CALCULATE THE EIGENVALUES WHICH
CORRESPOND TO THE PRINCIPLE MOMENTS OF INERTIA. */

DCL EIGVAL(*) FLOAT;
DCL (PD(*,*),CA(*,*),EIGVEC(*,*)) FLOAT;
DCL (SM,SX,SY,SZ) FLOAT;
DCL NATOM FIXED;

/* CONVERT CA TO CA W/ ORIGIN AT CENTER OF MASS */

SM = 0.;
DO I = 1 TO NATOM;
   SM = SM+CA(I,4);
   END;
SX = 0;SY = 0;SZ = 0;
DO I = 1 TO NATOM;
   SX = SX+(CA(I,4)*CA(I,1));
   SY = SY+(CA(I,4)*CA(I,2));
   SZ = SZ+(CA(I,4)*CA(I,3));
   END;
DO I = 1 TO NATOM;
   CA(I,1) = CA(I,1)-(SX/SM);
   CA(I,2) = CA(I,2)-(SY/SM);
   CA(I,3) = CA(I,3)-(SZ/SM);
   END;
IF %YESANSWER('
DO YOU WANT TO SEE THE NEW MATRIX OF COORDINATES BASED ON THE
ORIGIN AT THE CENTER OF MASS?') THEN PRINT CA; ELSE

DO I = 1 TO 3;
   DO J = 1 TO 3;
      PD(I,J) = 0;
   END;
END;
DO I = 1 TO NATOM;
   PD(1,1) = PD(1,1)+(CA(I,4)*((CA(I,2)**2)+(CA(I,3)**2)));
   PD(2,2) = PD(2,2)+(CA(I,4)*((CA(I,1)**2)+(CA(I,3)**2)));
   PD(3,3) = PD(3,3)+(CA(I,4)*((CA(I,1)**2)+(CA(I,2)**2)));
   PD(1,2) = PD(1,2)+(CA(I,4)*CA(I,1)*CA(I,2));
   PD(1,3) = PD(1,3)+(CA(I,4)*CA(I,1)*CA(I,3));
   PD(2,3) = PD(2,3)+(CA(I,4)*CA(I,2)*CA(I,3));
   PD(2,1) = PD(1,2);
   PD(3,1) = PD(1,3);
   PD(3,2) = PD(2,3);
END;
EAD PD;
IF %YESANSWER('
TYPE YES WHEN READY TO OBTAIN THE PRINCIPLE MOMENTS
OF INERTIA.') THEN
CALL EIGEN(PD,EIGVAL,EIGVEC);
TYPE '
      Several Spaces

MOMENT OF INERTIA X =',EIGVAL(3);
TYPE '
MOMENT OF INERTIA Y =',EIGVAL(2);
TYPE '
MOMENT OF INERTIA Z =',EIGVAL(1);
END;
```

Output From MOINERTIA

36R X 4C

	1	2	3	4
1	-.7870294	-2.394389	2.636874	126.9
2	-.6970293	1.405611	-2.153126	126.9
3	4.622971	2.815611	.9668741	126.9
4	-3.097029	-1.974389	-.9931259	12.
5	-2.577029	-2.334389	.2568742	12.
6	-1.537029	-1.654389	.8168741	12.
7	-.9870294	-.5343891	.1568742	12.
8	-1.457029	-.1743891	-1.083126	12.
9	-2.517029	-.8643891	-1.633126	12.
10	-4.197029	-2.814389	-1.643126	12.
11	-5.587029	-2.594389	-1.043126	12.
12	-6.477029	-3.714389	-1.483126	14.
13	-6.197029	-1.264389	-1.393126	12.
14	-5.657029	-.2543891	-.9231259	16.
15	-7.277029	-1.254389	-1.993126	16.
16	-.01702937	.1756109	.8068741	16.
17	1.312971	.2356109	.2768742	12.
18	2.072971	1.265611	.6868741	12.
19	3.342971	1.415611	.1968741	12.
20	3.832971	.4856109	-.7731258	12.
21	3.032971	-.5343891	-1.143126	12.
22	1.742971	-.7243891	-.6331259	12.
23	5.102971	.6856109	-1.253126	16.
24	-3.017029	-3.174389	.7868742	1.
25	-2.907029	-.5443891	-2.583126	1.
26	-3.937029	-3.854389	-1.533126	1.
27	-4.247029	-2.554389	-2.693126	1.
28	-5.497029	-2.644389	.02687417	1.
29	-6.067029	-4.654389	-1.143126	1.
30	-6.547029	-3.724389	-2.563126	1.
31	-7.457029	-3.564389	-1.063126	1.
32	1.682971	1.975611	1.406874	1.
33	3.422971	-1.254389	-1.063126	1.
34	1.132971	-1.554389	-.9331259	1.
35	5.142971	.8756109	-2.083126	1.

```
TYPE YES WHEN READY TO OBTAIN
OF INERTIA.?Y€
```

3R X 3C

	1	2	3
1	4656.084	3162.112	1156.159
2	3162.112	9326.66	-544.9985
3	1156.159	-544.9985	10183.7

```
MOMENT OF INERTIA X = 10924.26
MOMENT OF INERTIA Y = 10326.41
MOMENT OF INERTIA Z = 2835.776 →
```

Appendix II

Modified Lowry Protein Determination[57]

1) Bovine serum albumin (BSA) standards are prepared from lyophilized BSA in the range of 0.01 mg/ml to 0.30 mg/ml.

2) A 0.1 ml aliquot of each standard and the samples are precipitated with an equal volume of 7% trichloroacetic acid at room temperature overnight.

3) The precipitate is separated from the TCA solution by centrifugation (5000rpm, Sorval RC-2, 4^{o}, 10 min). The TCA solution is decanted while trying to avoid contact with the skin.

4) Sodium hydroxide (0.1 ml of 1 M solution) is added to each tube to digest the protein.

5) ABC solution (0.9 ml) (0.2 ml of 1% $CuSO_4$, 0.2 ml of 2% Sodium Potassium Tartrate, 19.6 ml of 2% Na_2CO_3) is added and the tubes are vortexed and allowed to stand 10 min.

6) Next, 0.1 ml of 1 N Phenol reagent (Folin Ciocalteu solution solution diluted 1:1) is added to each tube and vortexed immediately. The color is allowed to develop 30 min.

7) Each sample is read against a blank at 750 mμ.

Derivation of Equation Used in Competition Binding Assay[61]

Given:

$$P + T_3^* \rightleftharpoons PT_3^*$$

$$P + A \rightleftharpoons PA$$

$$P_t = P + PA + PT_3^*$$

$$K_{a_A} = \frac{(PA)}{(P)(A)} \qquad K_{a_3} = \frac{(PT_3)}{(P)(T_3^*)}$$

Where:

P = unbound nuclear receptor protein

T_3^* = $[^{125}I]T_3$

A = unlabeled analog

PT_3^* = receptor bound to T_3^*

PA = receptor bound to A

P_t = total moles per liter of binding sites

Substituting for (P):

$$K_{a_A} = \frac{(PA)}{(A)(P_t - PA - PT_3^*)}$$

Rearrange and cancel:

$$\frac{1}{K_{a_A}} = \frac{(A)(P_t - PT_3^*)}{(PA)} - \frac{(A)\cancel{(PA)}}{\cancel{(PA)}}$$

Rearrange:

$$\frac{1}{K_{a_A}(P_t - PT_3^*)} + \frac{(A)}{(P_t - PT_3^*)} = \frac{(A)}{(PA)}$$

But:

$$\frac{(A)}{(PA)} = \frac{K_{a_3}}{K_{a_A}} \frac{(T_3^*)}{(PT_3^*)}$$

Substitute, rearrange, and cancel:

$$\frac{\cancel{K_{a_A}}}{K_{a_3}\cancel{K_{a_A}}(P_t - PT_3^*)} + \frac{\frac{K_{a_A}}{K_{a_3}}(A)}{(P_t - PT_3^*)} = \frac{(T_3^*)}{(PT_3^*)}$$

Two assumptions are made:

1) Since the concentration of the analog added is large compared to the tracer $[^{125}I]T_3$, the free analog concn ≃ concn of analog added.

2) As the concentration of analog added increases (PT_3^*) becomes small compared to P_t.

Therefore:

$$\frac{T_3^*}{PT_3^*} = \frac{1}{K_{a_3}(P_t)} + \frac{\frac{K_{a_A}}{K_{a_3}} \left| \begin{array}{c}\text{concn of} \\ \text{analog} \\ \text{added}\end{array} \right|}{P_t}$$