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STUDIES OF THYROID HORMONE INTERACTIONS WITH THE NUCLEAR RECEPTOR

by

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DISSERTATION

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To my father and late mother,

also Melissa and Peter.

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ABSTRACT

Selected thyroid-hormone analogs were compared for their binding affinity to the nuclear receptor solubilized from the nuclei of rat liver, to the altered nuclear receptor generated by heat treatment, and to thyroxine(T_4)-binding prealbumin (TBPA) by equilibrium competitive binding assay. Their binding patterns of, together with kinetic and thermodynamic properties of binding reaction were compared to clarify interrelationships among these thyroid-hormone-binding proteins, and to evaluate the usefulness of TBPA as a working model for the nuclear receptor (Blake and Oatley, 1977).

The binding of 3,5-diiodo-naphthyronines were studied with the nuclear receptor and TBPA to understand the stereochemical and electrostatic requirements at their outer aromatic rings and to look for possible leads of a thyroid hormone antagonist. The results show: (1) The additional bulky phenyl group of naphthyronine is accommodated by both proteins, but TBPA is better. (2) The intact 4'-hydroxyl group functions as a hydrogen-bond donor in nuclear binding and contributes substantially to binding free energy (3.44($\pm$0.14)Kcal/mol). By contrast, it might act as a H-bond acceptor in TBPA binding, probably as the phenoxide anion. (3) Location of the 4'-hydroxyl group at positions other than para causes marked decline in binding strength. (4) The unionized 4'-hydroxyl group does not contribute significantly to TBPA binding, and substitutions with $-NH_2$, $-OCH_3$ or $-CH_3$ do not change TBPA binding activity. However, such substitutions result in drastic decreases in nuclear binding activity. (5) The nuclear binding activity in vitro correlates well with antigoiter activity in vivo, whereas

TBPA binding activity does not. Therefore, there are substantial differences in binding requirements between the nuclear receptor and TBPA. Moreover, an extra phenyl group of naphthyronine does not produce antagonism at the nuclear receptor.

Scatchard analysis of the binding of $[^{125}I]L-T_4$ by TBPA exhibits biphasic characteristics and the Hill coefficient is 0.76. These data are consistent with negative cooperative, site-site interactions in tetrameric TBPA. Further studies of the dissociation of $[^{125}I]T_4$-TBPA complexes as a function of the concentration of unlabeled T_4 and various analogs added were performed according to the method of De Meyts. Dissociation kinetics were measured after 100-fold dilution at 4°C, in the absence or presence of unlabeled L-T_4. An enhanced dissociation rate $> 2.41 \times 10^{-2} min^{-1}$ is linearly related to the fractional occupancy of the second site by T_4. Thyroid hormone analogs containing 3' and 5'-iodine substituents accelerate dissociation; however, analogs containing only the 3'-iodine substituent impede it. Therefore, the 5'-iodine atom may play an instrumental role in the modulation of cooperative binding interactions. These modulatory influences can be either positive or negative. In addition, ligand dissociation enhancement can discriminate multiple interacting binding sites from multiple independent binding sites. Similar studies with $[^{125}I]$tri-iodothyronine(T_3) binding with the nuclear receptor were carried out. The absence of enhancing effect on dissociation by unlabeled L-T_3 might be due to the fact that the receptor is a monomeric protein. Therefore, the nuclear receptor and TBPA may also differ in their subunit structure.

However, analysis of the binding data of thirty hormonal analogs to the altered receptor show a close resemblance with thyroxine-binding globulin (TBG), in terms of binding requirements in the side-chain, the

outer and the inner ring, and with TBPA except the side chain. Based on (a) similarities in analog-binding characteristics; (b) common function as thyroid-hormone binding proteins; (c) high structural stability as evidenced by strong resistance to denaturing reagents; (d) similar molecular weights; and (e) possible role as DNA-binding proteins, it is likely that the nuclear receptor, TBG, and TBPA belong to the same evolutionary family with the altered receptor as the "missing link".

Further studies using twenty T_3 and T_4 analogs were performed to determine the electronic features of pockets containing 3',5',3,5 substituents, size-limit of the 3'-substituent, stereospecificity and optimal carbon-length of the side chain. The results show that: (1) hydrophilic 3'-, 3- or 5- substituents destabilize the nuclear binding; (2) the nuclear binding is stereospecific, i.e.L-isomer > D-isomer; and (3) the optimal length of the acidic side chain is between two and three carbons.

CHAPTER ONE : GENERAL INTRODUCTION

The hormonal substances biosynthetically produced in the thyroid gland and released into circulation, are known as iodothyronines or thyroid hormones. Iodothyronines consist of one outer and one inner iodinated phenyl ring joined by an ether linkage, and an alanine side chain connected to the inner phenyl ring. They have diverse physiological influences on differentiation, development, growth, cardiovascular function, metabolism and thermogenesis.

There have been a number of hypotheses regarding the mechanism of action of the thyroid hormones triiodothyronine(T_3) and thyroxine(T_4). In addition to the cell nucleus (Oppenheimer, 1979; Eberhardt et al., 1980), which is the major subject of this dissertation, the mitochondrion (Sterling, 1979 ab; Maxfield et al., 1981; Shears and Bronk, 1979). the plasma membrane (Goldfine et al., 1975 ab; Segal and Ingbar, 1979, 1980 ab), have been considered as potential targets. Dratman (1978) postulated that incorporation of iodothyronine into protein during biosynthesis can have specialized functions to affect metabolism. Earlier, Niemann (1950) developed a model of oxidation-reduction in electron transfer processes to account for the thyromimetic effects. Further, Szent-Györgyi (1957) proposed that thyroid hormones could increase the probability of electronic excitation into somewhat reactive triplet state by means of their heavy iodine atoms.

In any event, binding of a hormonal or drug molecule with macromolecules, which may be called "receptors" as envisioned by Ehrlich (1908) or "receptive substances" by Langley (1906), must be the first

step to produce subsequent biological responses. An understanding of
this receptor concept is critical to a meaningful interpretation of
ligand-binding data. The receptor can be conceived as a highly specific
recognition factor, usually a protein, of high binding affinity and low
binding capacity, present in or on cells in very small numbers, and can
lead ultimately to a generalized cellular response upon binding with
ligand molecules. In some instances, the receptor may turn out to be a
membrane-bound macromolecule, e.g. isulin or acetylcholine receptors;
alternatively, the receptor may be present in the cytosol, e.g. steroid
receptors; or the receptor may reside at the nucleus, e.g. thyroid hormone
receptors, as will be discussed below.

It is relevant to point out that while the interaction of some ligands
with their receptors could be dealt with conceptually, the practicalities
of isolating these recognition factors may be insurmountable. When this,
more often than not, is the case, how can one make it certain that he is
studying the "receptor" to the ligand of interest? Therefore, one must
decide upon the criteria useful for the purpose of evalution. Generally,
receptors tend to have the following properties: (1) high ligand affinity,
which usually means that there is substantial binding in the range near
the physiological concentration of the ligand in question; (2) saturabi-
lity (i.e.limited capacity); (3) ligand specificity (e.g.chirality, etc.);
(4) tissue specificity consistent with those organs, tissues, cells on
which the ligand is known to act; (5) correlation between binding ability
and biological potency.

A large body of information now suggests that the basic unit of
thyroid hormone action is the triiodothyronine-nuclear receptor complex
(Oppenheimer, 1979). On the basis of the work by Tata and Widnell (1966),

the concept that thyroid hormone might exert its stimulatory metabolic effects through an action on the cell nucleus was hinted. They observed an enhancement of the activity of Mg^{2+}-dependent ribosomal RNA polymerase. Subsequent studies searching evidence for selective localization of thyroid hormone in nuclear or other subfractions of cells were unfruitful. In 1972, Griswold et al. (1972) reported the presence of saturable T_4-binding sites in nuclei of tadpole liver. Schadlow et al. (1972) and Oppenheimer et al. (1972) also reported in vivo evidence for specific thyroid-hormone receptors in nuclei of anterior pituitary, liver and kidney, which are all responsive to thyroid hormone. In their studies, liver and kidney were fractionated after in vivo injection to the rats with tracer quantity of $[^{125}I]L$-T_3 and increasing amounts of unlabeled L-T_3. The percentage of radiolabeled L-T_3 bound to the nuclear fraction was found to decrease with increasing concentrations of unlabeled L-T_3. By contrast, no similar dose-response relationship could be seen for other subcellular fractions. However, if cells were not fractionated, the nuclear binding activity of L-T_3 could only be detected in anterior pituitary. This was attributed to the smaller number of receptors and to a greater contamination of non-receptor binding so that the nuclear binding was masked.

Using a limited number of analogs administered in vivo, Oppenheimer and coworkers (1973) observed a generally good correlation between their hormonal activity and ability to displace $[^{125}I]L$-T_3 from hepatic and heart nuclei, and Ka (L-T_3) = $4.7 \times 10^{+11} M^{-1}$. Some major discrepancies were that the relatively high binding affinity of D-T_3 to rat liver nuclei in vivo, as compared with its low hormonal activity, was noted without explanation; and that 3,5,3'-triiodothyroacetic acid (triac) showed equal binding affinity but low hormonal activity as compared with L-T_3.

The latter was subsequently shown as a result of rapid metabolism and excretion of triac in vivo (Goslings et al., 1976).

Techniques for in vitro studies were developed by subsequent work, including intact cultured cells or isolated nuclei from rat pituitary turmor-cell line (GH_1 cells)(Samuels and Tsai, 1974), and isolated nuclei from rat liver (Koerner et al., 1974; DeGroot and Torresani, 1975; Charles et al., 1975). Similar apparent equilibrium association constants (Ka) were measured for the high-affinity, limited-capacity binding sites in these preparations. For example, $Ka(L-T_3)=3.5 \times 10^{10} M^{-1}$ for intact GH_1 cells (Samuels and Tsai, 1973), $4.8 \times 10^9 M^{-1}$ for isolated GH_1 nuclei (Samuels and Tsai, 1974), $6.1 \times 10^9 M^{-1}$ (Koerner et al.,1974), $2 \times 10^9 M^{-1}$ (DeGroot and Torresani, 1975), $5.3 \times 10^9 M^{-1}$ (Spindler et al., 1975) for isolated rat-liver nuclei. Interestingly, Samuels et al. (1974) first demonstrated that a preparation of receptors by high-salt (0.4M Kcal) solubilization can be effectively used in assessment of the binding affinity of thyroid hormone analogs. Ka for $L-T_3$ in binding to the solubilized receptor was determined as $5.6 \times 10^9 M^{-1}$ in GH_1 cells, and $6.4 \times 10^9 M^{-1}$ in rat liver. These values of Ka of $L-T_3$ are within its serum concentration(3 nM). Therefore, the nuclear receptor meets the first and second criteria for being a receptor substance, as described before.

As will be mentioned in Chapters Two and Three,the third criterion of ligand-specificity has been partially characterized in vivo and in vitro by several laboratories (Koerner et al., 1974, 1975; Samuels et al., 1974 ab; Thomopoulos et al., 1974; DeGroot and Torresani, 1975; Latham et al., 1976; Silva et al., 1977; Bolger and Jorgensen, 1980). For example, L-form of T_3 is the preferred optical isomer in all preparations.

Furthermore, nuclear receptors have been detected in several tissues. Included in this list are anterior pituitary, liver, kidney, heart, lung, brain, spleen, testis (Oppenheimer, 1979). Anterior pituitary was found to have the highest concentration of binding sites, while testis and spleen the lowest. The apparent lack of responsivity of spleen and testis can be attributed to their low concentrations of nuclear receptors. The intermediate concentration of nuclear receptor in brain which has been shown unresponsive to thyroid hormones in terms of oxygen-consumption change may be responsible for their physiological actions in the central nervous system (CNS) such as brain development and function. A recent study also confirmed the effect of thyroid hormone on stimulation of synthesis of nerve growth factor (NGF) in the mature CNS which in turn can mediate CNS development (Walker et al., 1979).

Most importantly, excellent quantitative correlation between nuclear binding affinity of analogs in vivo as well as in vitro and their thyromimetic potencies in vivo has been developed, while exceptions can be attributed to metabolic or distributional differences among the analogs (Dietrich et al., 1977). Taken together, the physiological relevance of the nuclear receptor has been supported by all the evidence presented thus far. However, it is important to note that this cannot be used as clear-cut proof that these binding sites are the receptors, while short of reconstitution experiments with purified preparation of these binding sites.

Some physicochemical data of the nuclear receptor prepared by partial purification with Sephadex G-100 or QAE-A50 chromatography have been provided by Latham and associates (1976). The receptor was shown to be a nonhistone acidic protein by studies with proteolytic enzymes, nucleases and by pH-extraction profiles. Its molecular weight was estimated as

50,500 by gel chromatography and gradient sedimentation studies. Thereby, the frictional coefficient was calculated as 1.4, and indicated a rather asymmetric shape for the receptor macromolecule. Further, the binding reaction of thyroid hormone with the nuclear receptor can be inhibited by sulfhydryl reagents (DeGroot et al., 1974; Eberhardt et al., 1979a).

As will be described in more detail in Chapter Four, Part II, a significant proportion of the receptors are found associated with chromatin, but at least 90% of them can be extracted from chromatin by high salt $(0.4M$ KCl or $0.2M(NH_4)_2SO_4)$, and the receptor-L-T_3 complex can reassociate in vitro with DNA (MacLeod and Baxter, 1975; 1976). At present, it remains unclear whether or not the nuclear receptor binds directly to DNA in vivo. However, it has been established that thyroid hormone does not increase the binding of nuclear receptors to chromatin (Spindler et al., 1975, Surks et al., 1975; DeGroot et al., 1976; Bernal et al., 1978). Consequently, the phenomenon of translocation to nucleus by hormonal binding in the steroid-hormone cytosolic receptor has no counterpart in the thyroid-hormone nuclear receptor.

Unlike the case with polypeptide and catecholamine hormones, thyroid hormones do not appear to regulate extensively the levels of nuclear receptors. However, Samuels et al. (1976) did report that addition of L-T_3 to GH_1 cells causes a dose-dependent depletion of nuclear receptor up to a maximun of 20-50%. They also reported that the dose-response curve for receptor resembles that for stimulation of synthesis of growth hormone and suggested that this depletion by L-T_3 may be created by the action of the hormone per se. By contrast, no evidence of negative or positive regulation of receptor concentrations by the hormone was found in liver (Spindler et al., 1975; Surks et al., 1975; Bernal et al., 1978). However, the

receptors have been shown to be regulated in a negative fashion during starvation, partial hepatectomy and glucagon administration (Dillman et al., 1978). More recently, the reduction of the number of nuclear receptors by n-butyrate or other aliphatic carboxylic acids has been found and ascribed to the inhibitory effect of these compounds on chromatin-associated deacetylases of GH_1 cells (Samuels et al., 1980). This inhibition is reflected in the increased acetylation of core histones. In contrast, the normal concentration of receptors is concomitant with the restoration of normal state of acetylated core histones, which have been hypothesized to maintain the nucleosome structure of chromatin (Kornberg, 1974; Klug et al., 1980).

Although numerous attempts to purify the nuclear receptor have been made over the years, purification to homogeneity has not yet been achieved. A number of laboratories have reported the loss of $L-T_3$ binding ability through the course of various standard isolation procedures, including molecular gel-sieving and affinity chromatography (Eberhardt et al., 1980). Nevertheless, Eberhardt and coworkers (1979b) have demonstrated that the further purified receptors can recover their lost binding activity with $L-T_3$ by adding back purified core histones (H_2A, H_2B, H_3, H_4) which lack $L-T_3$-binding activity per se. This type of reconstitution experiment has also been demonstrated with a partially purified preparation of nuclear receptors after dilution (Eberhardt et al., 1979b). These authors have proposed a model of the receptor in which a "core" receptor subunit containing $L-T_3$ binding site can interact with a regulatory subunit, possibly histone or histone-like molecules. Although many downstream effects of thyroid hormone have been established, the immediate events after the hormone binds with nuclear receptors still remain as a black box. So far,

the earliest detectable response is a stimulation of ribonucleic acid
(RNA) synthesis (Tata and Widnell, 1966). More specifically, hormone-
mediated increases in messenger RNA (mRNA) levels has been exhibited in
the case of growth hormone in rat pituitary cells (Martial et al., 1977ab;
Seo et al., 1977), α_{2u}- globulin in rat liver (Kurtz et al., 1976),
malate dehydrogenase in rat liver (Towle et al., 1980) and carbamyl phos-
phate synthetase I in tadpole liver (Mori et al., 1979). Other early
observations reported include increased RNA polymerase activity (Tata and
Widnell, 1966; DeGroot et al., 1977, 1980), stimulated nuclear kinase
activity (Kruh and Tichonicky, 1976), increased phosphorylation of proteins
(Coleoni and DeGroot, 1980), enhanced phosphorylation of histones (Morris
and Cole, 1980) augmented synthesis of poly(A)$^{+}$RNA (Dillman et al., 1978)
and altered chromatin template activity (Baxter, et al., 1979, DeGroot and
Rue, 1980).

A number of gene products are known to be regulated by thyroid hormone,
and only a long, long list would suffice to mention every gene product.
Many of these products have been reviewed (Eberhardt et al., 1980). Thus,
only those identified more recently are listed below: myocardial DNA poly-
merase α (Limas, 1979), ventricular myosin V_1 (Flink et al., 1979; Hoh et al.,
1979), large and small subunits of renal Na^{+}K^{+}-ATPase (Lo and Lo, 1980),
cytochrome p-450 (Leakey et al., 1979), hepatic histidase (Armstrong and
Feigelson 1980), lactate dehydrogenase (Suleiman and Vestling, 1979),
vitellogenin (Huber et al., 1979), glycolipid: adenosine 3'-phosphate-5'-
phosphosulfate sulfotransferase(s) and 2',3'-cyclic nucleotide 3'-phos-
phohydrolase (Bhat et al., 1981), casein (Houdebine et al., 1978) and carbonic
anhydrase B (Taniguchi et al., 1978).
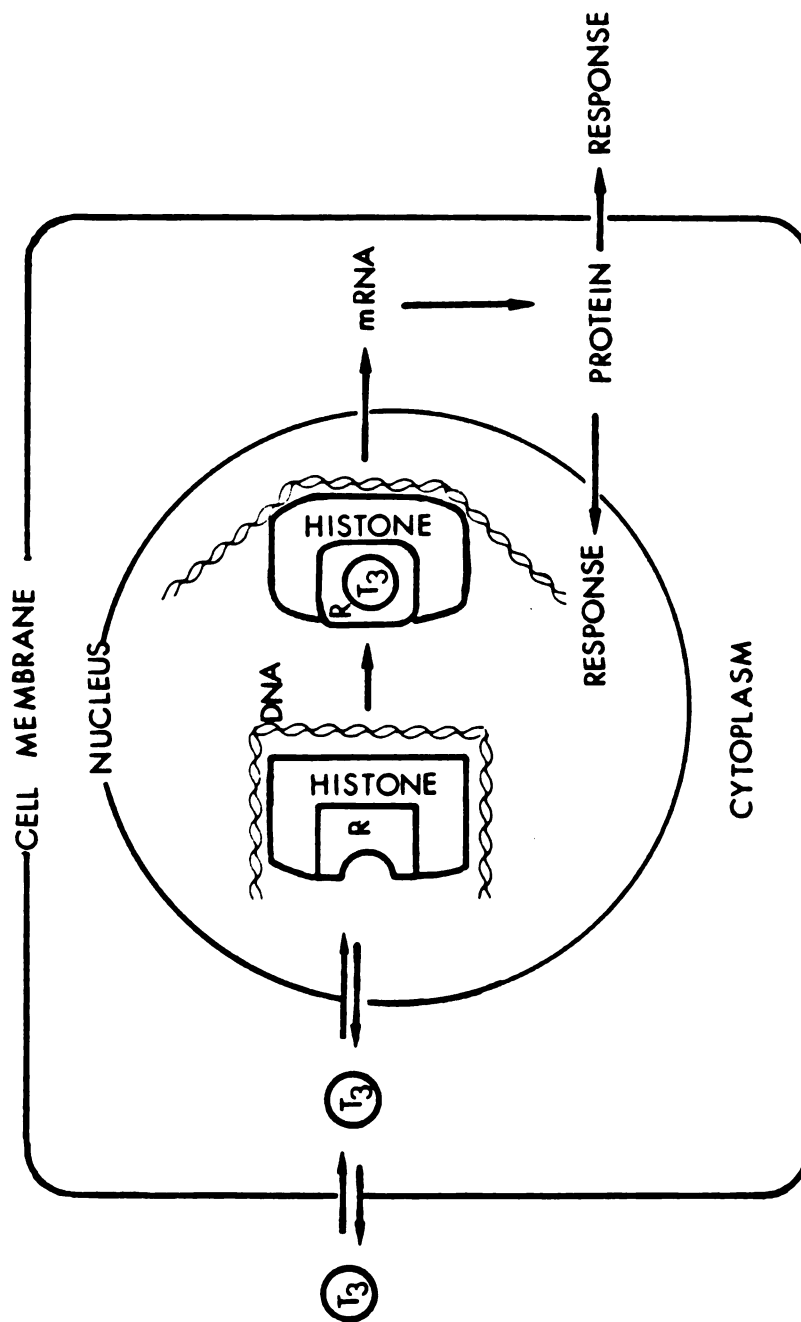
Fig. 1-1. Working model for the mechanism of thyroid hormone action in a responsive cell.

Based on all the information described thus far, a working model, as shown in Fig. 1-1, can be constructed to illustrate the hypothesized mechanism of action of thyroid hormone at the cellular level.

In addition to the nuclear receptor, several other proteins that bind thyroid hormone in various subcellular compartments, such as cytoplasm, mitochondria, and plasma membrane, have been studied as possible sites for the initiation of thyroid hormone action. In these systems, either higher than physiological concentrations of thyroid hormone are needed to observe response or the binding affinity of hormonal analogs can not correlate with their thyromimetic potency. Therefore, a major role for these proteins to act as receptors to elicit physiological effects has been not so well established. (Eberhardt et al., 1980).

Serum proteins, thyroxine-binding globulin (TBG) and thyroxine-binding prealbumin (TBPA), are of particular interest to us, because they share a common function as thyroid-hormone-binding proteins, and have already been purified and well characterized. For instance, even X-ray crystallography has been employed to obtain the three-dimensional structure for TBPA. More detailed description of these proteins will be presented in Chapters Two, Four and Five.

As shown by the X-ray data, TBPA has contained binding sites for thyroid hormone, and putative sites for DNA. Thus Blake and Oatley (1977) hypothesized that TBPA could be a working model for the nuclear receptor. Since TBPA has not been demonstrated to bind DNA, two immediate questions are noted. First, how useful is this working hypothesis to guide drug-design studies? Second, are there any evolutionary relationships possible between the nuclear receptor and TBPA?

This thesis will focus on comparative studies of the binding patterns of thyroid-hormone analogs with the solubilized nuclear receptor, TBPA, and TBG, and their thermodynamic and kinetic properties.

Chapter Two presents binding studies to both the nuclear receptor and thyroxine-binding prealbumin (TBPA) to assess the effectiveness of the Blake's hypothesis. A series of twelve 3,5-diiodo-naphthyronines with variations in attachment sites of the hydroxyl group, and in 4'-substituents are utilized to understand the stereochemical requirement at the outer aromatic rings. Preparation of solubilized receptor is described. A method to derive binding affinity from raw binding data is discussed. Comparative analysis of the derived data is made. Correlation between binding strength and antigoiter potency is examined for both proteins.

Chapter Three covers a concise discussion of various techniques frequently used in binding studies. Twenty thyroid-hormone analogs containing different substitutions in the side-chain, inner and outer rings are employed to probe the binding site of the nuclear receptor. Analogs with hydrophilic 3,5-substituents or three aromatic rings are assayed for the first time. The effect of dehydroxylation at the 4'-position on binding is also investigated. The size and electronic atmosphere of the pocket binding to the 3'-group of the hormone is assessed.

Chapter Four explains an experimental approach, capable of distinguishing two possible models to account for a concave upward Scatchard curve. We study changes in dissociation kinetics of $[^{125}I]T_4$ binding to TBPA of which the subunit structure has been elucidated and known to exhibit negative cooperativity. A mathematical model is constructed to illustrate the dissociation kinetics of a two-binding-site protein. As

far as we know, this is the first unambiguous study with a well-defined system to clear up a heated controversy over the years in the field of hormonal or drug receptors. This controversy centers around the question as how to identify the existence of negative cooperativity. Similar studies with $[^{125}I]T_3$ are also performed with the nuclear receptor. Implications of the data obtained as to the subunit structure of the nuclear receptor are suggested.

Chapter Five describes thermodynamic and kinetic analysis of an altered nuclear receptor generated by heat treatment. pH dependence of $L-T_3$ or $L-T_4$ binding to this altered receptor is examined. Temperature dependence of the equilibrium association constant and dissociation kinetics are measured. ΔH, ΔS parameters are calculated by van't Hoff plot. In addition, thirty analogs are used to delineate their binding characteristics with the altered nuclear receptor. The contribution to binding free energy by each additional group is estimated. Comparison of these data with those of thyroxine-binding globulin (TBG) and TBPA are made to clarify their interrelationships.

Chapter Six, the last of this dissertation, presents epilogue and prospects.

CHAPTER TWO : COMPARATIVE STUDY OF NAPHTHYRONINE BINDING TO THE NUCLEAR RECEPTOR AND THYROXINE BINDING PREALBUMIN

I. Introduction

Recently, intensive studies of the thyroid hormones have been pursued to elucidate the exact nature of their thyromimetic activity. One of the approaches is to search for analogs that can inhibit (antagonists) or stimulate (agonists) thyroid-gland functions. In particular, it would be of great interest to identify antagonists; to date none have not been found, in spite of a substantive effort. This approach has provided numerous synthetic thyroid drugs (Jorgensen, 1978a). The evaluation of biological activity of a variety of thyroid hormone analogs serves as a means to understand the structural features related to hormone responses. In vitro, such an evaluation is usually carried out with binding assays using their receptors from cell nuclei, either intact or solubilized, as described in Chapter One. This is because there is a good correlation between binding and the biological activity.

In this chapter, emphasis will be on the solubilized nuclear receptor and TBPA systems,since both are representative binding proteins in the nuclei of target cells and the circulation respectively. The aim of this chapter is to provide evidence to compare the relationship between these two types of binding. Detailed description of the nuclear receptor has been presented in Chapter One. Therefore, only relevant details about TBPA are reviewed below and in Chapter Four, Part I.

A number of properties of TBPA have been measured. This protein has a tetrameric subunit structure. Its molecular weight of 55,000 is

close to that of the rat liver nuclear receptor (50,500). The binding
studies of thyroid hormones with TBPA are especially attractive to pursue
for the following reasons. The primary structure (amino acid sequence)
has been determined by Kanda et al. (1974). The secondary, tertiary and
quarternary structures have also been clarified by Blake et al. (1974, 1977,
1978) using the method of X-ray crystallography. TBPA also binds retinol-
binding protein (RBP)(Van Jaarsveld et al., 1973; Raz et al., 1970). The
binding domains of thyroid hormones and RBP do not seem to overlap, because
the binding of the former does not interfere with that of the latter, and
vice versa (Horwitz and Heller, 1974). Unlike most other serum proteins,
TBPA is unusual in that it contains no carbohydrate. Moreover, TBPA has
exhibited thymic-hormone-like activity (Burton et al., 1978). It is worth
mentioning that TBPA is the first hormone-binding protein for which such
detailed description of structure at molecular level has been documented.

By the low-resolution X-ray analysis, Blake and Oatley (1977) have
indicated that T_3 or T_4 are exclusively bound to TBPA at two symmetry-
related sites lying within a central channel. The surface of this channel
is compactly organized by the polypeptide chains of the inner four stranded
β-sheet of each subunit. This channel takes a shape of a cynlinder about
25 Å long each as illustrated in Fig. 2-1. Tentative assignments of inter-
acting patches along the binding channel have been made for key substituents
in thyroid hormones, based on low-resolution data: (a) the two phenyl rings
of the hormone are nearly perpendicular, as proposed by Jorgensen (1964);
(b) the 4'-hydroxyl group of the hormone interacts, via a well-defined water
molecule, with hydroxyls of Ser-117 and Thr-119 in a hydrophilic patch; (c)
each of the 3',5'-iodines is situated between and in close proximity with
Leu-110 and Leu-117; (d) each of the 3,5-iodines is within a pocket surrounded
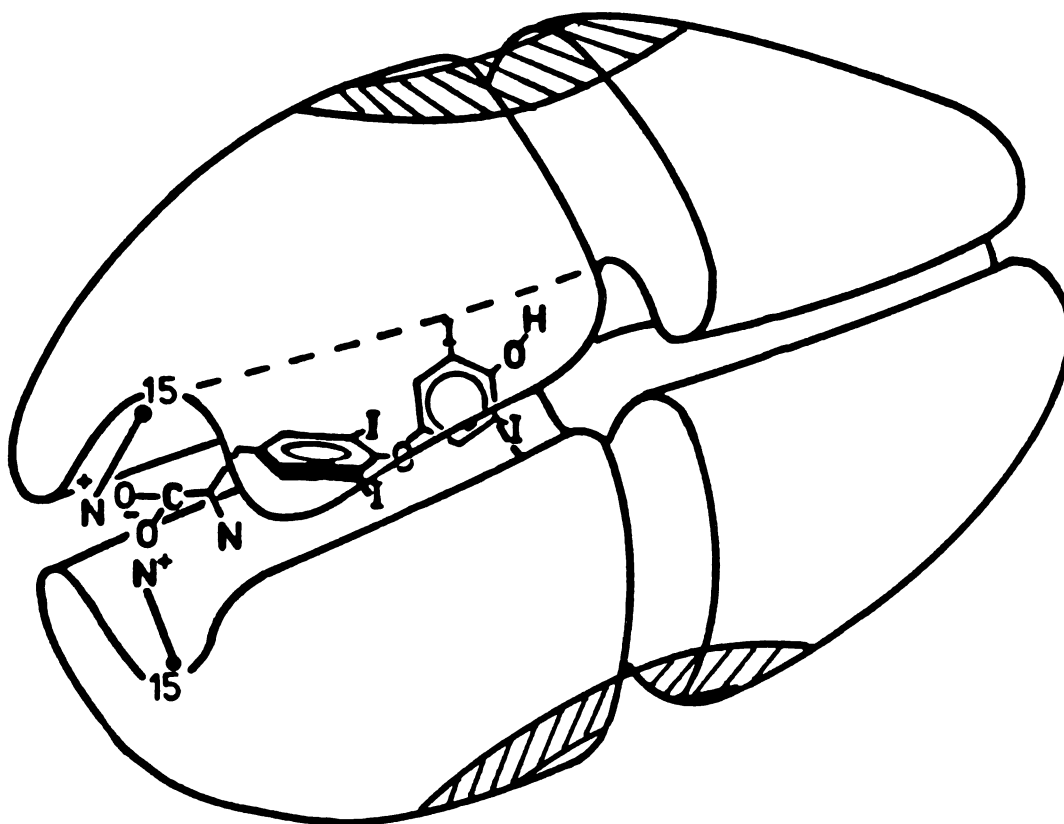
Fig. 2-1. Schematic representation derived from x-ray crystallographic studies of TBPA, which illustrates the interaction of four identical subunits by forming a channel through the interior of the molucule, where two identical thyroid hormone binding sites are located. The channel narrows at the center of the molecule. Although the binding sites are identical, T4 binds in a mode of negative cooperativity; occupation of T4 at the first site presumably alters the conformation of the second site, which results in a lower affinity for the second T4 binding. Depicted on the upper and lower side of the molecules is a symmetrical β-pleated-sheet structure that is relatively rich in amino acids with ionic side chains and that also contains tryptophan molecules. Based on computer-graphic modeling studies, this site has been proposed to be a DNA binding site (adopted from Blake and Oatley, 1977).

by the methyl groups of Thr-106, Ala-108 and Val-121 and β- and γ-methylenes of Lys-15; (e) the α-carboxylate and the α-ammonium groups interact with Lys-15 and Glu-54 respectively.

In previous (Jorgensen, 1978b) studies a comparative analysis of binding data of nuclear receptors and TBPA has been performed. Elements that contribute to binding activity have been identified qualitatively and quantitatively. The main features are:

(1) The T_4 binding affinity is closely similar in TBPA($5.5 \times 10^7 \sim 2.3 \times 10^8 M^{-1}$) and nuclear receptor($6.7 \times 10^7 \sim 1.7 \times 10^8 M^{-1}$)(Jorgensen, 1978b). In contrast, T_3 binds much more avidly to the nuclear receptor ($1.19 \times 10^9 \sim 5.3 \times 10^9 M^{-1}$) than to TBPA($2.0 \times 10^6 \sim 1.2 \times 10^7 M^{-1}$)(Jorgensen, 1978b).

(2) The same stereospecificity, i.e. L form > D form,is preferred by both molecules.

(3) Similarity in contribution to binding activity is observed for the 3 and 5 groups and for the side chains.

(4) Different influences are detected for 3',5' substituents. Binding to the nuclear receptor is enhanced by 3'-alkyl and -halogen groups, but is decreased by 5'-groups. By contrast, binding to TBPA is influenced favorably by the same 3',5' substituents.

(5) It is possible that the 4'-hydroxyl group of the hormone acts as a hydrogen-bond donor in binding to the receptor, while it may be an acceptor in binding to TBPA.

In addition to the hormonal binding sites in TBPA, another striking finding by Blake and Oatley is that the surface of TBPA tetramer contains symmetrical depressions that are complementary in conformation to double-helical DNA. However, there has been no experimental evidence that TBPA does bind to DNA. Low affinity DNA binding with TBPA might occur without

being detected under the conditions that receptor binding with DNA is demonstrated (MacLeod and Baxter, 1975). Based on the common features in hormone-binding behavior, possible DNA-binding sites, molecular weight, and acidic proteins, Blake has proposed that TBPA may serve as a model for studying the thyroid hormone binding with their nuclear receptor. Irrespective of whether or not the two molecules are similar in features other than the hormone-binding sites, the nuclear receptor and TBPA have the previously mentioned similarities in their hormone-binding properties, and are in any case worth a further comparative examination.

This chapter describes binding studies of both the nuclear receptor and TBPA, using a series of 3,5-diiodo-naphthyronines with variations in attachment loci of the hydroxyl group, and also several different 4'-substituents.

$$R = H, OH, OCH_3, CH_3, NH_2$$
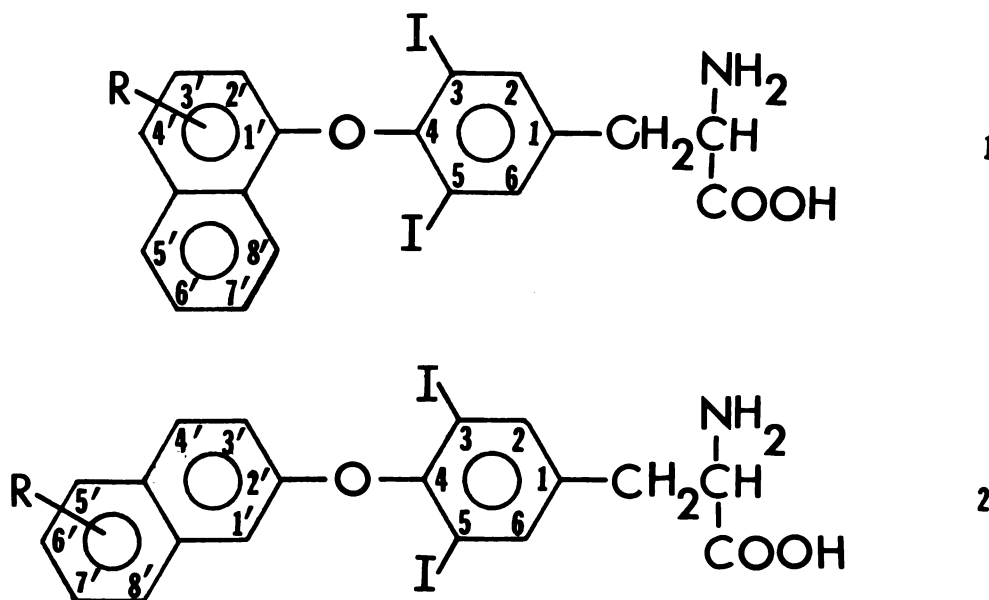
(1) 1'-3,5-Diiodonaphthyronines

(2) 2'-3,5-Diiodonaphthyronines

Our purpose is two-fold. Firstly, we hope to understand their stereo-chemical and electrostatic effects on binding to both proteins in terms of: (a) the orientation of the hydroxyl group on the outer rings; (b) various 4'-substituents; and (c) extra bulkiness of the outer aromatic ring. The importance of an intact hydroxyl group at 4'-position of thyroid hormones has been recognized (Bolger and Jorgensen, 1980), but there is little information concerning the relationship between the hydroxyl orientation and binding affinity. In addition, very little is known about the influence of 4'-substitution on TBPA binding. Moreover, the extra bulkiness of an additional aromatic ring in naphthyronines should provide us a probe to investigate the binding cavity in that region. Secondly, we also want to correlate antigoiter activity in vivo with nuclear receptor and TBPA binding activity. It is hoped that comparison of these data should shed some light to evaluate the effectiveness of TBPA to serve as a model of the nuclear receptor.

II. Thyroid Hormones

$[^{125}I]$Triiodo-L-thyronine and $[^{125}I]$-L-thyroxine(1000~1250 μCi/μg) were obtained from New England Nuclear (Boston, MA). This hormone pre-paration usually contained 3% of free $[^{125}I]$iodide. Due to the short half-life of ^{125}I ($t_{\frac{1}{2}}$=60 d.) which decays via emission (0.35Mev) and electron capture to generate ^{125}Te , each sample was used within 2 months from the supplier's assay date. When necessary, concentration corrections were made for radio labeled hormones to account for the two possible sources of errors mentioned above. L-T_4, and L-T_3 were from Sigma Chemical Co. (St. Louis, MO), The remaining compounds were prepared in this laboratory. The purity of the compounds was examined by TLC on silica gel plates

(supplied by Whatman-Quantum, Inc., Clifton, NJ); or by high pressure liquid chromatography (HPLC) with C-18 reverse phase column (supplied by Altex Co., Berkeley, CA) using a solvent system of acetonitrile: tetrahydrofuran:H_3PO_4(pH3.5, titrated with triethanolamine)(28:5:67).

III. <u>Solubilization of Nuclear Receptor</u>

The solubilized nuclear receptor was prepared as described by Latham <u>et al</u>. (1976) with modifications. Male Sprague-Dawley rats (250~350 g/rat, approximately 10 g tissue/liver, supplied by Simonsen Laboratories, Gilroy, CA) were anesthetized lightly with petroleum ether. Before laparatomy, the liver was thoroughly perfused with phosphate buffer saline (PBS) (supplied by Gibco) via the portal vein. The liver was then removed, and immediately stored in liquid nitrogen cryogenic container (MVE SX-34). Nuclei were prepared by a method modified from the procedure developed for extraction of RNA polymerase (Goldberg, <u>et al</u>., 1977). Frozen liver of 100 g was quickly pulverized with a cooled pestle, 200 ml of warm (35°C) solution A(0.34 M sucrose, 0.24 mM spermine·4HCl, 15 mM $MgCl_2$, pH7.6) was added. With stirring, the mixed solution was thawn to a final temperature of 4°C. The liver pieces were collected by a coarse nylon mesh, and transferred to 300 ml of icy cold Homogenization Solution B (2.1 M sucrose, 0.1 mM permine· 4HCl, 6.5 mM $MgCl_2$, pH7.6). The mixture was then homogenized by a Tekmar homogenizer (Cincinnati, OH) at maximum speed for three one-minute periods, separated by one minute of cooling. This preparation contained intact nuclei, cell debris, but no intact cells under a light microscope by staining with 1% crystal violet. The mixture was then removed of larger pieces of connective tissue by gently squeezing through a layer of miracloth (Calbiochem). Each Sorvall GSA-rotor bottle was filled with approximately 150 ml

of the liver preparation. Another 125 ml of 2.1 M sucrose solution was added to the bottom of the bottles with a separatory funnel so that the liver prep would float on top. The nuclei were pelleted by centrifugation (25,000xg, 90 min, 4°C) in a Sorvall RC-5B with a GSA-rotor (diameter 14.61 cm). The nuclear pellet was obtained by aspirating the cell debris and sucrose solution from the top. The pellets were washed by gently suspending in 50 ml of Washing Buffer C(20 mM Tricine, 2 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.6 containing 0.5% Triton X-100(Sigma)), and re-centrifugation (500xg, 10 min, 4°C). After decanting the supernatant, the final nuclear pellets were each resuspended in 20 ml of icy cold Resuspension Buffer D (20 mM Tris, pH 8.0, 0.25 M sucrose, 1 mM EDTA, 0.1 mM dithiothreitol, 5% glycerol). Saturated ammonium sulfate (4M, 1.05 ml) was added to make a final concentration of 0.2 M. The suspension was then sonicated with a microtip sonicator, at 95 watts for two 15 sec periods, separated by one minute of cooling. Finally, the sonicated mixture was centrifuged (45,000xg, 30 min, 4°C) and the supernatant was collected. This was designated as nuclear crude extract and stored as 2-5 ml fractions in a liquid nitrogen tank. The binding activity of the extract was not lost by freezing or prolonged storage for six months. Endogenous triodothyronine in the nuclear extract was determined by the method described by Latham et al. (1976). The level (less than 1 pM) was negligible as compared with the amount used (0.1 nM) in binding studies. Further purification can be carried out with G-100 gel chromatography, Sephadex QAE-50 anion exchange gel chromatography or affinity chromatography according to the methods of Apriletti et al. (1980) and Latham et al. (1976).

IV. SDS Polyacrylamide Gel Electrophoresis of TBPA

TBPA was obtained from Dr. Y.L. Hao, American National Red Cross Blood

Research Laboratory (Bethesda, MD). Its purity was examined by SDS poly-acrylamide gel electrophoresis with a 10% separating gel, and a 5% stacking gel. The gels were prepared according to the method of Davis (1964). TBPA samples were first denatured with 6 M guanidium·HCl overnight (Branch et al., 1971). Guanidium·HCl treated TBPA (20 µl(20 µg), containing 2% SDS, 5% β-mecaptoethanol, 9% glycerol, 0.002% bromophenyl blue, was applied to each well. Electrode buffer was 0.025 M Tris, 0.19 M Glycine, 0.1% SDS, pH 8.6, as described by Laemmli (1970). Electrophoresis was carried out at room temperature for 4 hours at a constant current of 20 mA. The slab gel was then stained with Coomassie blue solution, destained with 7.5% glacial acetic acid and 5% isopropanol. Molecular weight was estimated by the known mole-cular-weight standards according to Weber and Osborn (1969). The molecular weight markers used were lysozyme (13,900), bacteriophage T_4 gene product 32(24,000), gene product 45(35,000), hexokinase(51,000) and bovine serum albumin(68,000). The results are shown in Fig. 2-2 and 2-3, and will be discussed in more details in Chapter Four.

V. Equilibrium Binding Assays for Scatchard Analysis

To avoid errors arising from radioactive decay and contamination of [^{125}I]iodide in [^{125}I]T_3 or [^{125}I]T_4, stock solutions (5×10^{-8}M for L-T_3, and 5×10^{-7}M for L-T_4) were prepared by mixing trace amount of [^{125}I]T_3 or [^{125}I]T_4 in a solution of known amount of unlabeled thyroid hormone. The accuracy of measurement was ±0.0001 mg by a Cahn electrobalance. In these preparations the [^{125}I]-labeled hormone constituted less than 1% of the total hormone. In order to determine the total [^{125}I]-labeled hormone which included that bound specifically and that nonspecifically by the receptor , two parallel series of incubation tubes(12x75 cm) were used with or without the addition

Upper-lower-gel interface

Dye front
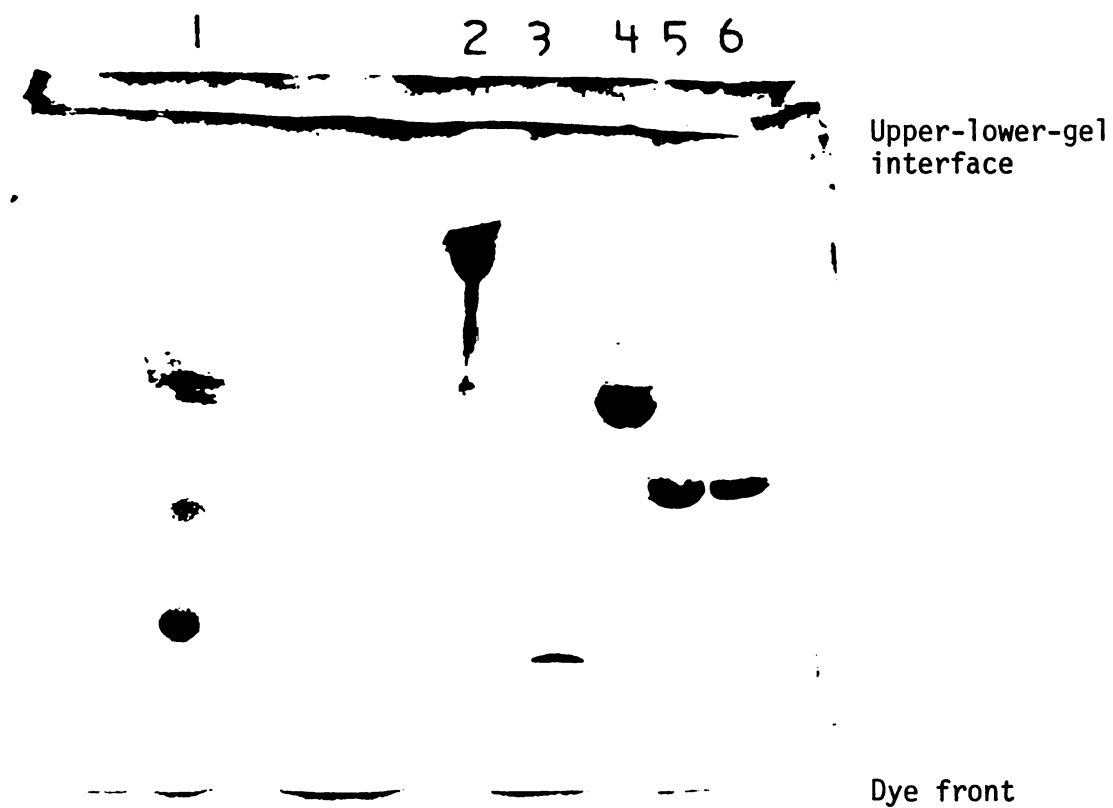
Fig. 2-2. SDS polyacrylamide-Gel-Electrophoresis Diagram.
1. TBPA 2. Bovine serum albumin(BSA) 3. Lysozyme
4. Hexokinase 5. Bacteriophage T_4 gene 32 product
6. Gene 45 product
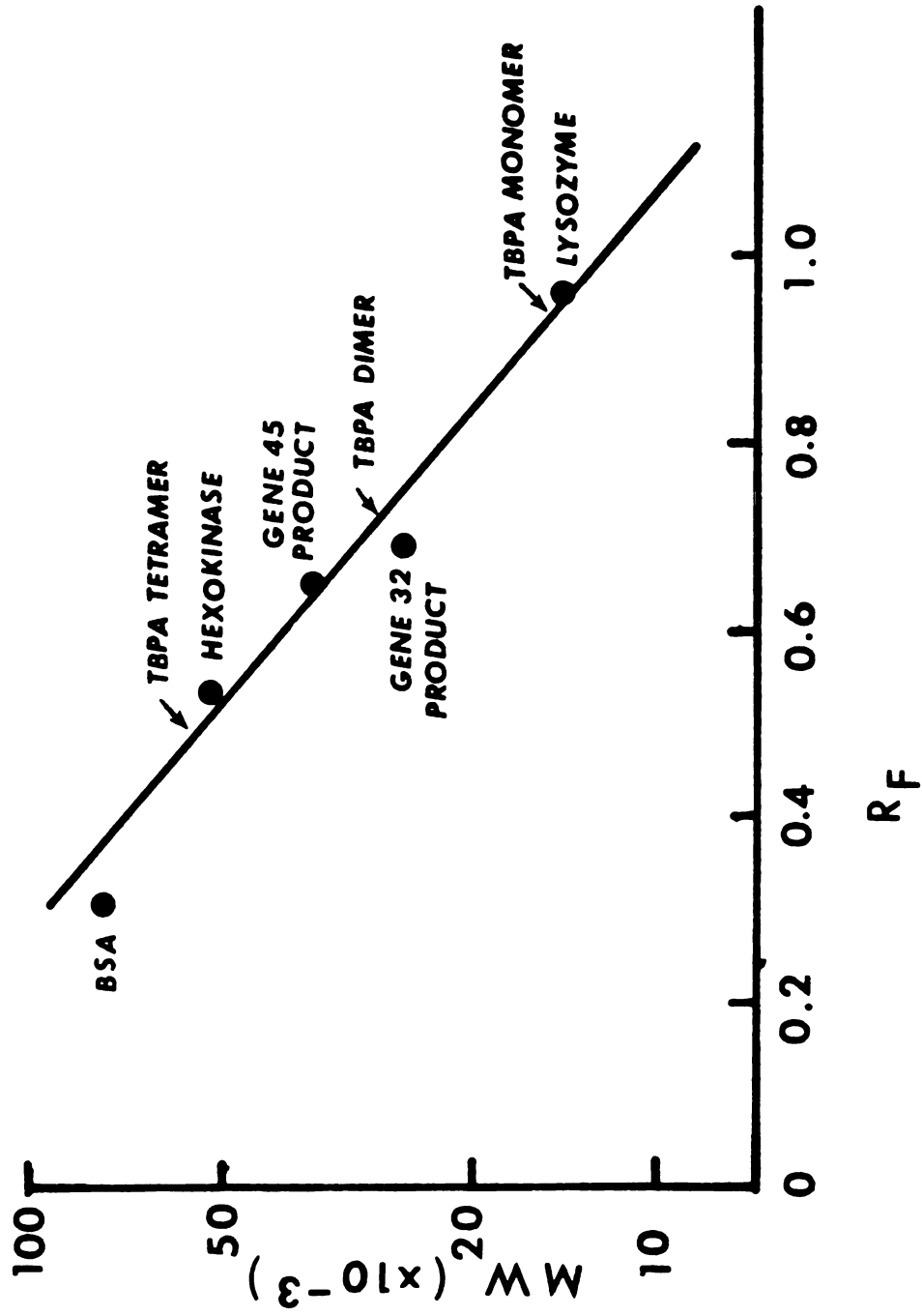
Fig. 2-3. Semilog plot for molecular weight determination. Arrows were used to indicate different states of TBPA association.

of a 1000~10000-fold excess of unlabeled L-T$_3$(2×10^{-5}~ 2×10^{-6}M). Each tube contained various doses of [^{125}I]-labeled hormone (60,000 ~ 1,800,000 cpm). Incubation buffer (0.05 M sodium phosphate, 0.2 M ammonium sulfate, 0.1 mM dithiothreitol, 1 mM EDTA, 5% glycerol, pH 7.6) was added to a final incubation volume of 0.5 ml together with 50 µl of nuclear crude extract. These tubes were incubated at 25±1°C for 2 hours. Separation of the bound from the free hormone was achieved by gel filtration through a Sephadex G-25 (medium) column (bed volume=2.0±0.1 ml) preequilibrated with the phosphate buffer at 2-4°C. To minimize any dissociation during separation, the elution time was reduced to less than 30 sec by applying mild pressure. The radioactivity of bound hormone was then determined with a Searle Auto-Gamma spectrometer with an efficiency of 74%. The specific binding was calculated by subtracting the radioactivity due to nonspecific binding from that due to the total bound hormone. The amount of free hormone was obtained by subtracting the radioactivity due to the total bound hormone from the radioactivity due to the total hormone added. The ratio of bound/free ligand was then calculated from the ratio of the radioactivity for the specifically bound hormone to that of the free hormone. The molarity of bound hormone was derived from the ratio of the radioactivity of the specifically bound hormone to that of the total hormone, times the molarity of the added total hormone. The data were fitted by the Scatchard equation:

$$\frac{\text{Bound}}{\text{Free}} = K_A (P_T - B)$$

where K_A = the affinity constant of the hormone to the nuclear receptor

P_T = the total concentration in molarity of the nuclear receptor binding sites in an incubation tube

B = the concentration in molarity of the specifically bound hormone

The fitting was performed by using Public Procedure LINEAR FIT of the PROPHET computer system, which is an integral part of the National Institutes of Health's Chemical/Biological Information Handling Program (Raub, 1974).

According to the Scatchard equation, receptor binding sites can be measured directly from the X-intercept of the plot. The results are presented in Fig.2-4. The K_A's for L-T_3 and L-T_4 were measured as $6.1 \times 10^8 M^{-1}$ and $2.0 \times 10^8 M^{-1}$ respectively from the slope of the Scatchard plot. These values are in agreement with previously published data, ranging from $5.6 \times 10^8 \sim 5.3 \times 10^9 M^{-1}$ for L-T_3, and $6.71 \times 10^7 \sim 5.2 \times 10^8 M^{-1}$ for L-T_4 (Koerner et al., 1974; DeGroot and Torresani, 1975; Latham et al., 1976; Bolger and Jorgensen, 1980). As demonstrated in Fig. 2-4, L-T_4 had more binding sites than L-T_3. This also confirmed earlier observations by Latham et al. (1976) that upon storage the receptor molecules can be converted to a form with unchanged binding affinity and number of binding sites for L-T_4. However, there is a reduced number of binding sites (whereas binding affinity does not decrease) for L-T_3. This converted receptor is presumably an aggregated product of the nuclear receptor, since its binding activity is often in the excluded fractions of G-100 or QAE Sephadex chromatography. Further discussions on this sort of converted (altered) receptors are in Chapter Five.

VI. Competitive Binding Assays

The competitive binding assay for the nuclear receptor was performed as follows. Each assay tube contained crude receptor preparation (~200 pM binding sites per 0.3 mg of protein) and a constant amount of $[^{125}I]T_3$(~0.1 nM, 100,000~150,000 cpm) in the incubation buffer as described previously. The mixture was incubated at 25°C for 2 hours to reach equilibrium (Bolger and Jorgensen, 1980). The competitive assay for TBPA was performed as follows.
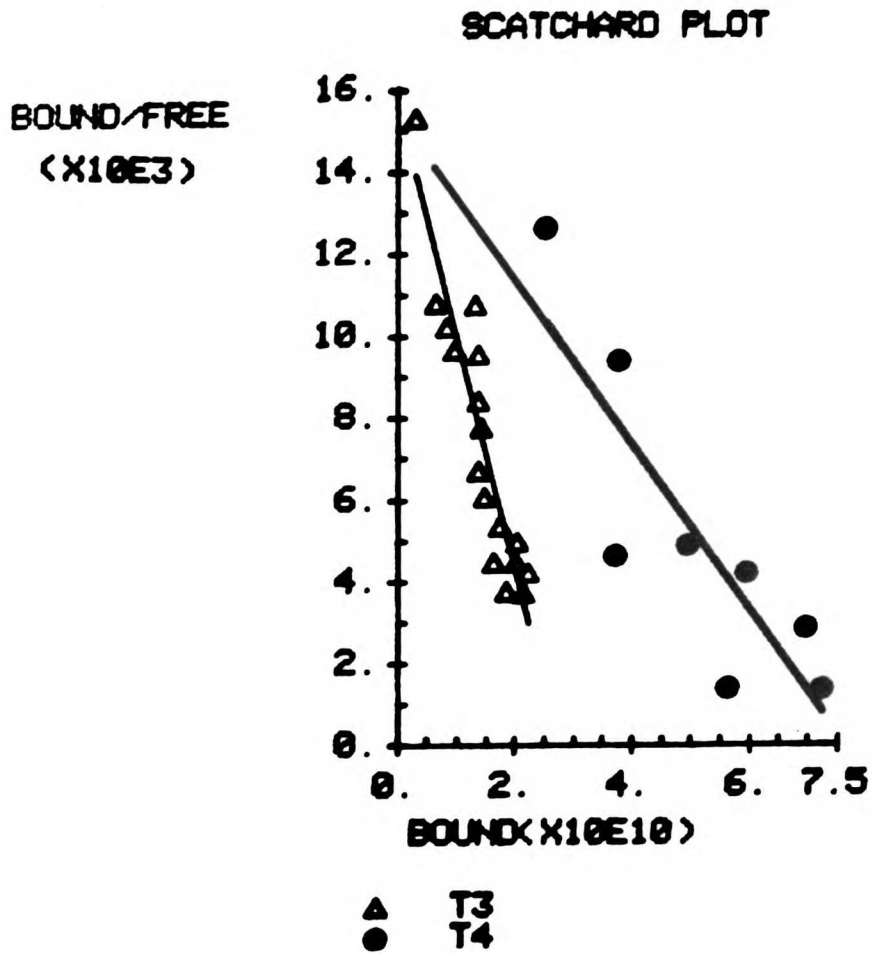
Fig. 2-4. Scatchard plot for nuclear binding of L-T$_3$ and L-T$_4$.
The binding assay was performed at 25°C in the phosphate
buffer at pH 7.6.

Each assay tube contained 70 ng of protein which had approximately 2.5 nM binding sites, and a constant amount of $[^{125}I]T_4$ in the buffer solution (0.1 M Tris, 0.1 M NaCl, 1 mM EDTA, pH 7.6). Each unlabeled hormone analog was weighed with a Cahn electro-balance to prepare a desired stock solution (0.5 or 0.05 mM) in methanol with trace of ammonium hydroxide(5%). Organic solvents are known to denature proetin molecules. However, it was deter-mined that the presence of methanol, up to 20% in the incubation mixture , had no effect on the outcome of our binding studies. Further serial dil-utions of the stock solution were often prepared with the buffer solution to reduce methanol content (<20%). The final analog concentrations (six of them for each analog) covered a range of 10-fold above and 10-fold below the K_d expected from previous quantitative-structure-activity-relationship (QSAR) results. When the measured K_d for an analog deviated much (greater than 10-fold difference) from the expected value, a similar experiment was repeated with analog concentrations within the ten fold range of the meas-ured K_d. This is because only concentrations in the neighborhood of an analog's K_d conform to the linear portion of the Michaelis-Menten binding curve, and the equation used to derive K_A for hormone analogs is valid in this region. Nonspecific binding was approximately 5 ~ 10% of the total bound $[^{125}I]$-labeled hormone as measured by the amount of radioactivity of $[^{125}I]$-labeled hormone remained in the presence of 1,000 ~ 10,000 fold excess of unlabeled $L-T_3$(in nuclear binding experiments) or unlabeled $L-T_4$ (in TBPA binding experiments). The remaining procedures were similar to those used in the preceeding section.

VII. Competition Binding Data Analysis

A PROPHET programming procedure(COMPBIND) written in PL/1 language was adopted from Bolger (1977). Some modifications were made for simul-

taneous input of a large amount of data. COMPBIND is shown in Appendix I. Based on the method of Koerner et al. (1974), the following equation was employed to analyze the data:

$$\frac{Free}{Bound} = \frac{K_T}{[P_0]} + \frac{\frac{K_T}{K_X}[Analog\ Concentration\ X]}{[P_0]}$$

where K_T = the equilibrium dissociation constant of $L\text{-}T_3$ or $L\text{-}T_4$

K_X = the equilibrium dissociation constant of analog X

$[P_0]$ = the total concentration of hormone binding sites

A derivation of this equation is shown in Appendix II.

The assumptions in the derivation of the equation are:

(1) The concentration of radiolabeled hormone free is much smaller than the K_d of that hormone. Thus, $[^{125}I]$-labeled hormone bound is negligible as compared to P_0.

(2) The added concentration of analog X is a good approximation of the concentration of free X.

These two conditions were in general met in our experiments. On one hand, the concentration of radiolabeled hormone used was about 0.1 nM, but K_d's(1.0 nM for $L\text{-}T_3$ in nuclear binding; 20 nM for $L\text{-}T_4$ in TBPA binding) were significantly larger. On the other hand, since the total amount of analog X was in excess as compared with P_0, and the radiolabeled hormone in use was the thyroid hormone with the highest binding affinity in each binding protein assay, it followed that the amount of X displacing $[^{125}I]$-labeled hormone was a rather small fraction of X added. A typical competition binding analysis is illustrated in Fig. 2-5. The ratio of the slope to Y-intercept generates the desired association constant (K_A) for each competing analog. In this example, K_A was 7.92×10^6 and $6.85 \times 10^5 M^{-1}$ for
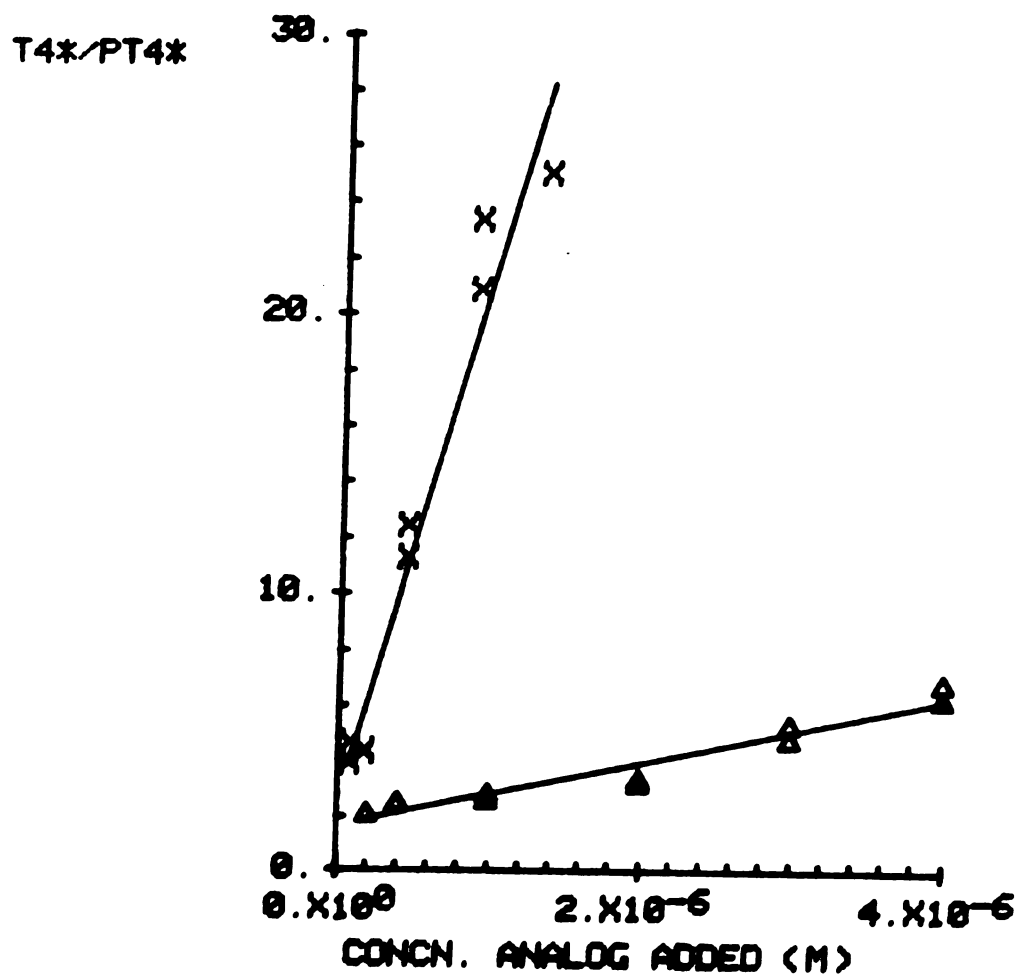
Fig. 2-5. Competition binding assay was performed with TBPA for 4'-OH-1'-Naphthyl(Δ) and 4-OH-3'-Br-1'-Naphthyl(χ) analogs at 37°C in Tris buffer,pH 7.6.

TABLE 2-1 Nuclear Receptor Binding, TBPA Binding and Antigoiter Assay Data for 1'- and 2'-3, 5-diiodonaphthyronines

R3'
R4'
R5'
R7'
I
O
CH_2
CH
NH_2
COOH
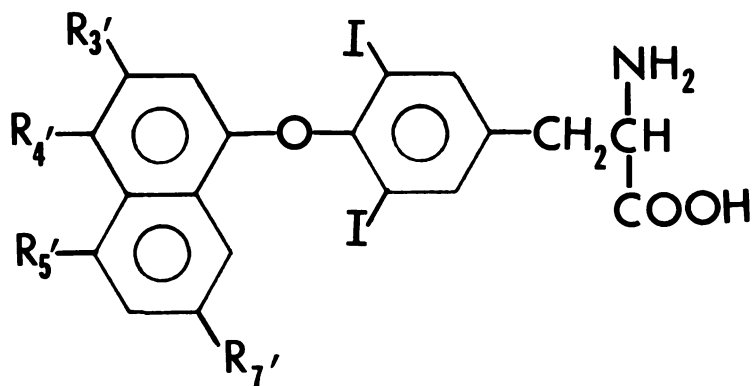
No.	DL	$R_{4'}$	$R_{3'}$	$R_{5'}$	$R_{7'}$	Relative Nuclear Receptor Activity $\%L\text{-}T_3{}^a$	Relative TBPA Activity $\%L\text{-}T_4{}^b$	Relative Antigoiter Activity $\%L\text{-}T_3{}^c$
1	L	OH	H	H	H	63.6(±3.4)	1.38(±0.12)	18.08
2	DL^f	OH	Br	H	H	46.4(±6.8)	19.2(±2.6)	5.24
3	DL^f	OCH_3	H	H	H	0.25(±0.015)	1.1(±0.32)	0.89
4	DL^f	H	H	OH	H	0.46(±0.07)	1.42(±0.18)	< 0.036
5	L	H	H	H	OH	0.021(±0.006)	0.32(±0.05)	< 0.036
6	L	CH_3	H	H	H	0.41(±0.018)	1.18(±0.13)	< 0.036
7	L	NH_2	H	H	H	0.051(±0.01)	1.73(±0.12)	0
8	DL^f	H	H	H	H	0.16(±0.01)	0.23(±0.03)	0.57

TABLE 2-1 (Continue)
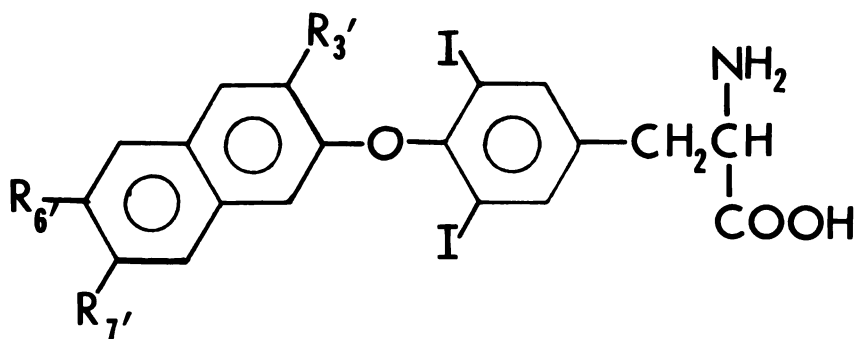
No.	DL	$R_{4'}$	$R_{3'}$	$R_{6'}$	$R_{7'}$			
9	L	H	H	OH	H	0.21(±0.02)	1.13(±0.08)	0.36
10	L	H	H	H	OH	0.19(±0.05)	0.58(±0.04)	0
11	L	H	OH	H	H	0.0048(±0.0008)	1.54(±0.09)	<0.036
12	DL[f]	H	H	H	H	0.018(±0.006)	0.19(±0.04)	0.036
13	L-T_2	OH	H	H	H	0.09[d]	0.14(±0.01)	0.3
14	L-T_2	NH_2	H	H	H	0.01[e]	0.14(±0.01)	

[a] $K_{L-T_3} = 1.19 \times 10^9 \ M^{-1}$

[b] $K_{L-T_4} = 4.97 \times 10^7 \ M^{-1}$

[c] based on data from Jorgensen et al. (1978b)

[d,e] based on data from Bolger and Jorgensen (1980)

[f] no correction was made for DL racemic analogs

TABLE 2-2 Free Energy ΔG of Nuclear Receptor Binding, TBPA Binding,

and Comparison of Substituent Contribution (ΔΔG)
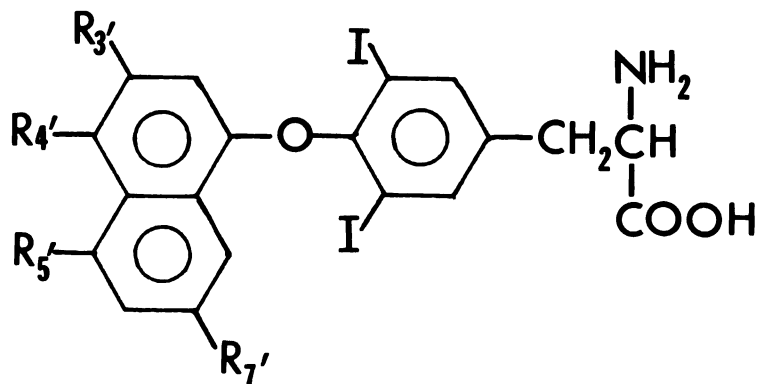
No.	DL	$R_{4'}$	$R_{3'}$	$R_{5'}$	$R_{7'}$	Nuclear Receptor Binding free energy		TBPA Binding free energy	
						ΔG^a	$\Delta\Delta G^b$	ΔG^a	$\Delta\Delta G^b$
1	L	OH	H	H	H	-12.11(±0.08)	-3.56(±0.13)	-7.96(±0.05)	-1.08(±0.11)
2	DL	OH	Br	H	H	-11.92(±0.09)	-3.37(±0.14)	-9.52(±0.17)	-2.64(±0.23)
3	DL	OCH_3	H	H	H	-8.82(±0.04)	-0.27(±0.09)	-7.82(±0.20)	-0.94(±0.45)
4	DL	H	H	OH	H	-9.01(±0.09)	-0.46(±0.14)	-7.98(±0.08)	-1.10(±0.14)
5	L	H	H	H	OH	-7.36(±0.22)	1.19(±0.27)	-7.09(±0.10)	-0.21(±0.16)
6	L	CH_3	H	H	H	-9.04(±0.05)	-0.49(±0.10)	-7.86(±0.07)	-0.98(±0.13)
7	L	NH_2	H	H	H	-7.89(±0.12)	0.66(±0.17)	-8.09(±0.04)	-1.21(±0.10)
8	DL	H	H	H	H	-8.55(±0.05)	0.00	-6.88(±0.06)	0.00

TABLE 2-2. (Continue)
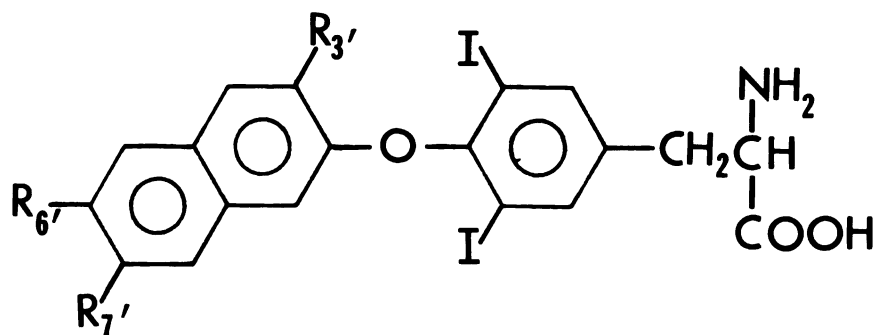
No.	DL	$R_{3'}$	$R_{6'}$	$R_{7'}$	ΔG^a	$\Delta\Delta G^c$	ΔG^a	$\Delta\Delta G^c$
9	L	H	OH	H	-8.72(±0.06)	-1.39(±0.44)	-7.84(±0.04)	-1.34(±0.29)
10	L	H	H	H	-8.66(±0.05)	-1.33(±0.43)	-7.44(±0.04)	-0.94(±0.29)
11	L	OH	H	H	-6.49(±0.11)	0.84(±0.49)	-8.02(±0.04)	-1.52(±0.29)
12	DL	H	H	H	-7.33(±0.38)	0.00	-6.50(±0.25)	0.00

a
$\Delta G = -RT$ In K, T=298°K

b
$\Delta\Delta G = \Delta G(X'-OH/or\ 4'-X') - \Delta G(Deoxy-1'-analog)$

c
$\Delta\Delta G = \Delta G(X'-OH) - \Delta G(Deoxy-2'-analog)$

4'-OH-3'-Br-1'-(3,5-diiodo)naphthyronine and 4'-OH-1'-(3,5-diiodo) naphthyronine, respectively, with TBPA.

3,5-Diiodonaphthyronines containing variations in: (1) the location of the hydroxyl group on outer aromatic rings; and (2) 4'-substituents were employed to determine their binding activities to the nuclear receptor and TBPA by competitive binding assay. These compounds can further be divided into two subgroups with the ether linkage at either 1'-(or α-) or 2'-(or β-) position of the naphthyl moiety. The results of their relative affinities (K_A's) are listed in Table 2-1, based on L-T_3=100% in nuclear binding, or L-T_4=100% in TBPA binding. The free energy changes(ΔG's) in the binding reaction and the free energy contributions of each individual hydroxyl group ($\Delta\Delta$G's) are listed in Table 2-2; where ΔG's were derived from a thermodynamic equation, $\Delta G=-RT\ln K_A$, at T=298°K; and $\Delta\Delta$G's were calculated by comparing with reference compounds, either deoxy-1'-analog in the 1'-series, or deoxy-2'-analog in the 2'-series.

VIII. Role of Naphthyl Ring in Binding

First of all, it is interesting to note that even the 4'-hydroxyl (No. 1, 63.6% of the binding activity of L-T_3) and the 4'-hydroxyl-3'-bromo (No. 2, 46.4% of L-T_3) naphthyl analogs could bind almost as well with the nuclear receptor as did L-T_3. To further determine the role of additional phenyl ring in the binding, the binding data for analogs with and without it were compared. The results are presented in Fig. 2-6. It can be seen that extra stabilization (-0.58 Kcal/mol) of hormone-receptor complexes by the second aromatic ring in naphthyl analogs was not so app-reciable when containing 3'-bromine atom. By contrast, the bulky extra ring contributed more (-3.88 Kcal/mol) to nuclear receptor binding in the

	ΔΔG(Nuclear Receptor)	ΔΔG(TBPA)

I

HO—⬡—R
Br

-0.58 Kcal/mol   -1.85 Kcal/mol

II

HO—⬡⬡—R
Br

III

HO—⬡—R

-3.88 Kcal/mol   -1.35 Kcal/mol

IV

HO—⬡⬡—R

$$R = -O-\overset{I}{\underset{I}{\bigcirc}}-CH_2\overset{NH_2}{\underset{COOH}{CH}}$$
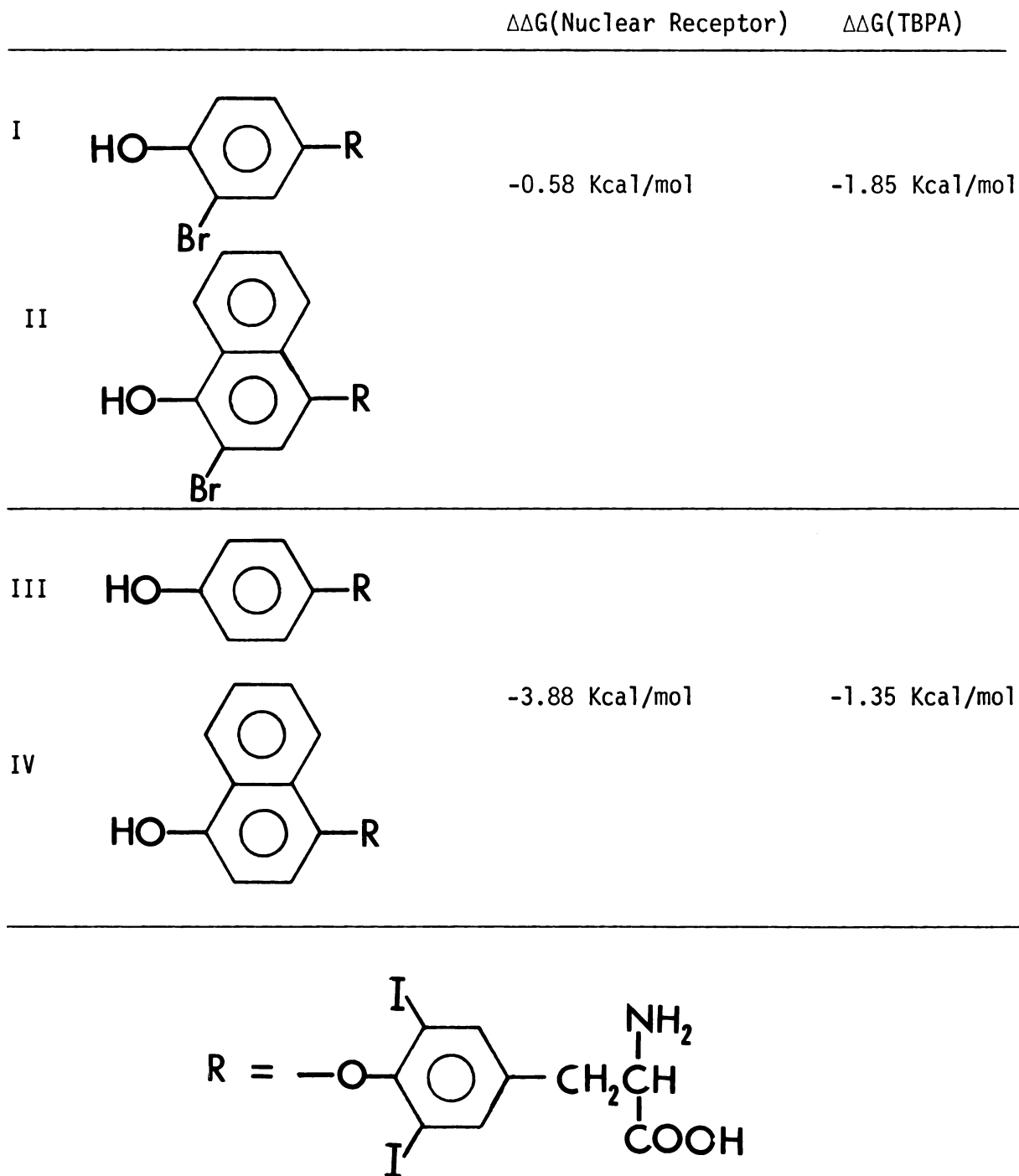
Fig. 2-6. Binding strength of the 2', 3'-fused phenyl ring

ΔΔG = ΔG(II) - ΔG(I)   or

ΔΔG = ΔG(IV) - ΔG(III)

absence of the 3'-bromine. The results indicated that the binding domain of the nuclear receptor might be less flexible. This inflexibility might have caused a trend of decreasing binding affinity with increasing size of 5'-halogen substituent as shown in a series of 3'-iPr-5'-halogen-T_2 analog, i.e. 3'-iPr-5'-H-T_2 (92.5% based on L-T_3=100%)>3'-iPr-5'-Cl-T_2(57.0%)>3'-iPr-5'-Br-T_2(23.8%)>3'-iPr-5'-I-T_2(13.5%)(Bolger and Jorgensen, 1980). However, TBPA was more accommodative so that the extra bulk could bind equally well, regardless of the size of 5'-substituents.

IX. Positional Preference of the Outer-Ring Hydroxyl in Binding

In the 1'-series, the feasible positions of the hydroxyl group are 4',5', or 7'; whereas in the 2'-series they are 3',6', or 7'. As indicated in Table 2-2, it is noteworthy to point out that addition of a 4'-hydroxyl group to the unsubstituted outer-rings of the naphthyronine nucleus decreased the nuclear binding free energy by 3.44 Kcal/mol. In the 1'-naphthyl series, the hydroxyl at 7'-position (No. 5)(ΔG=+1.30 Kcal/mol, relative to deoxy-1'-3,5-diiodonaphthyronine) was quite unfavored, whereas compounds with the hydroxyl at 5'-position (No. 4) showed less marked decline in binding activity. In the 2'-naphthyl (or β-naphthyl) series, 6'-hydroxyl-2'-naphthyl analog (No. 9) which is analogous to 4'-hydroxyl-1'-naphthyl compound in the 1'-naphthyl series, had a higher affinity than 7'-hydroxyl-2'-naphthyl compound (No. 10) which is analogous to 5'-hydroxyl-1'-naphthyl compound in the 1'-naphthyl series. Therefore, a para position for the hydroxyl group and the ether bridge is essential to possess significant nuclear receptor binding avidity. It also suggests that the binding reaction probably undergoes via hydrogen bonding, dipole-ion,or dipole-dipole electrostatic interactions. In contrast, all the hydroxyl groups except the 4'-hydroxyl in 4'-hydroxyl-3'-bromo-1'-naphthyronine exerted little

effect on TBPA binding as shown in Table 2-1.This indicated that an interplay of several factors was responsible for the high binding affinity of the bromine-containing compound. Among them are phenoxide formation resulted from inductive effect of halogens, charge-transfer complexation, and Van der Waals interaction. The former two could not be mimicked by aliphatic or aryl substituents. When the data of nuclear and TBPA binding assays were compared in the case of 3'-hydroxyl-2'-naphthyl compound (No. 11) had the second largest difference (+0.98 Kcal/mol in nuclear binding, while -1.08 Kcal/mol in TBPA binding). This was consistent with our earlier view that the hydroxyl group is less important in TBPA binding in the absence of halogen atoms, whereas the orientation of this intact hydroxyl group is essential in nuclear binding.

X. Effect of Various Substituents at 4'-Position on Binding

As shown in Table 2-1 and 2-2, substitutions of methoxy, amino, and methyl (Nos. 3,6,7) for the 4'-hydroxyl did not influence significantly the binding to TBPA, indicating that the requirement at 4'-site in TBPA binding is nonspecific. A similar tendency could also be demonstrated by the result that 4'-amino and 4'-hydroxyl-T_2 analogs (Nos. 13, 14) had similar binding affinities (0.14% of L-T_4 for both). This nonspecificity could be further characterized by a lack of specific electrostatic, hydrophobic or Van der Waals interactions, based on the following aspects: (1) An amino group has a potential to carry a positive charge at physiological pH; (2) A hydroxyl group can lose a proton and become negatively charged; (3) A methoxy group can have both hydrophobic and dipole-dipole interactions; and (4) A methyl group can have only hydrophobic and Van der Waals bonding. By contrast, there were drastic changes in nuclear binding by these substituents, as illustrated in Table 2-1. This suggested that there

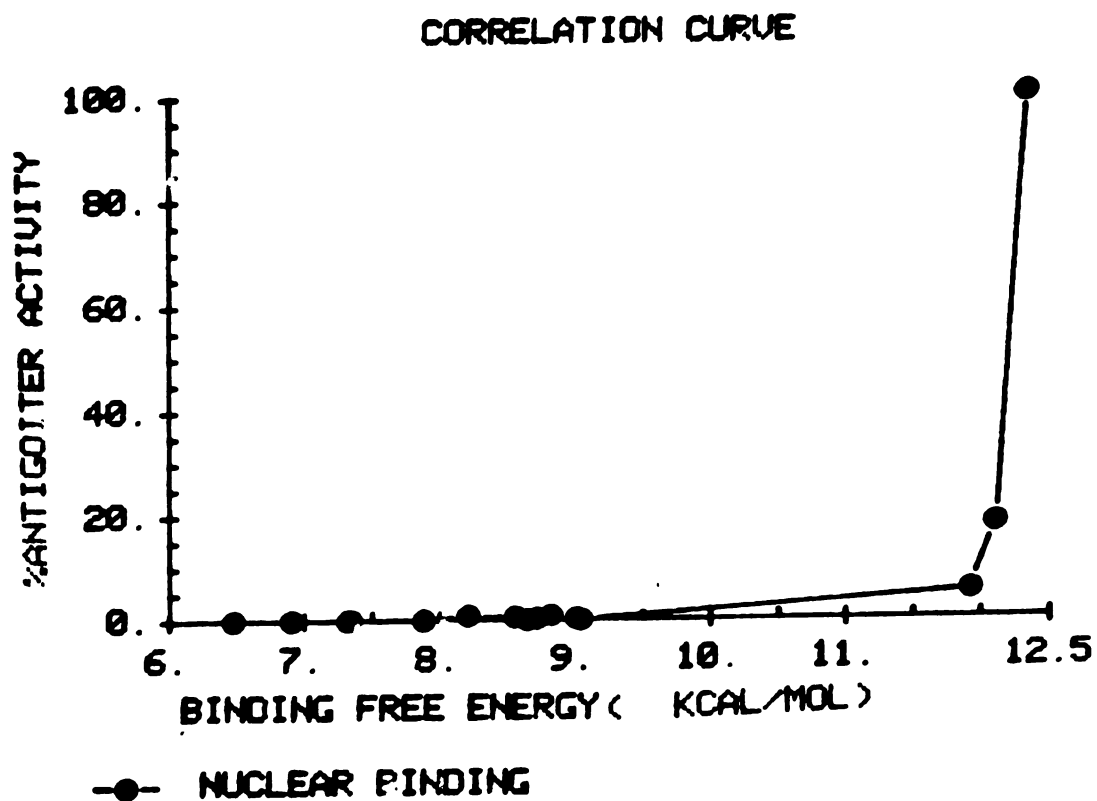Fig. 2-7. Correlation plot of structure-activity data from nuclear receptor binding assay. Rat antigoiter activity of naphthyronines was plotted as a function of their nuclear binding free energy.
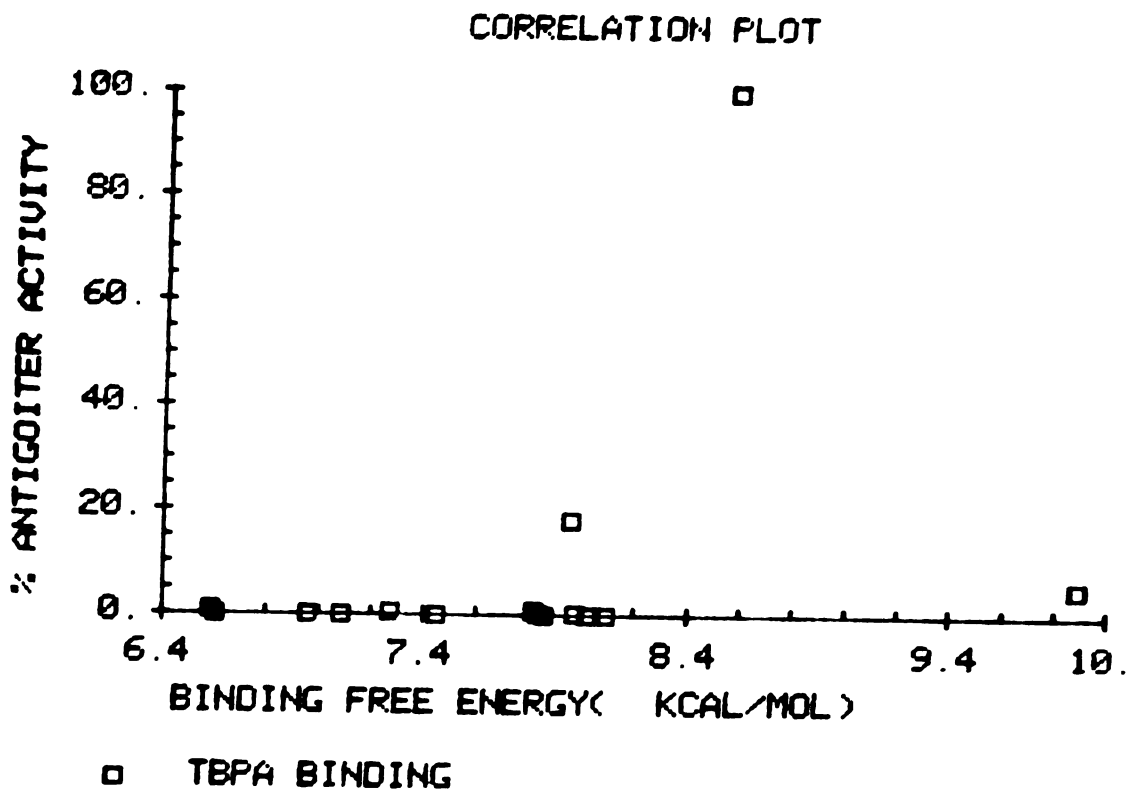
CORRELATION PLOT

Fig. 2-8. Correlation plot of structure-activity data from TBPA binding assay. Rat antigoiter activity of naphthy-ronines was plotted as a function of their TBPA binding free energy.

was a substantial difference in the microenvironment in TBPA and the nuclear receptor for the binding of 4'-substituents of thyroid hormone analogs.

XI. Correlation Between Binding Activity and Thyromimetic Potency

Correlation between receptor binding affinity in vitro and biological activity in vivo is one of the most important requirements to support the existence of such a receptor. Measurement by antigoiter activity in the rat has been widely accepted among several in vivo bioassay methods. In this bioassay, the disease state of goiter in the rat is induced by anti-thyroid drugs, such as thiouracil or propyl-thiouracil. The thyromimetic potency of an analog is defined as the molar ratio of the amount of the analog to that of a standard reference hormone $(L-T_3)$ needed to produce comparable decrease in thyroid gland weight. In Fig.'s 2-7 and 2-8, the data of thyromimetic potency (reported by Jorgensen et al., 1962, 1971) were plotted as a function of binding free energy of analogs. It is noted that a good correlation existed in nuclear receptor binding. By contrast, no apparent relationship could be found in TBPA binding. Therefore, these results supported the idea that nuclear receptor is one of the target sites to elicit physiological responses of thyroid hormones.

As can be seen in Tables 2-1 and 2-2, analogs in the 1'-series exerted stronger interactions than did parallel analogs in the 2'-series in terms of nuclear receptor binding, TBPA binding and thyromimetic activity. This suggested that overall stereochemistry of the 1'-analogs produces more efficient binding to the active sites of protein molecules.

XII. Discussion

In this chapter, we examined the binding activities of thyroid-hormone analogs containing bulky naphthyl moieties, and hydroxyl groups at various orientations. The results show that both the nuclear receptor and TBPA

have pockets to accommodate the bulky naphthyl group, and that the hydroxyl group must have the adequate orientation and ionization state to achieve maximal binding.

Since the conformation of a drug (or hormone) molecule plays an important role in molecular interactions with receptors, can the high affinity of some naphthyl analogs to the nuclear receptor be related with the conformational effects of the naphthyl substituent?

The iodo-diphenyl ether structure of thyroid hormones has been proposed by Zenker and Jorgensen (1959) as a preferred conformation in which the planes of aromatic rings are perpendicular and bisect each other, while locked in a 120-degree angle. Thus, two nonequivalent positions as depicted in Fig. 2-9, can be distinguished: the 3'-position defined as "distal" to the diiodo-phenylalanine ring, and the 5'-position as "proximal". This proposal was later confirmed by the synthesis of 3,5-diiodothyronines that contain bulky groups in the 2'-position. Rotational barriers by steric interactions between the 2'-alkyl group and voluminous 3,5-iodine atoms help fix the 3'-group at the "distal" conformation which has been shown to have higher binding affinity. Likewise, the second ring of 3,5-diiodo-naphthyronines is constrained in the distal form. It has been suggested by Bolger and Jorgensen (1980) that one of the effects of a bulky 3'-substituent is to orient the hydrogen atom of the 4'-hydroxyl group toward the 5'-side, producing a better hydrogen donor to the receptor. The bulkiness of this extra ring in naphthyronines might function in a similar manner. Additionally, this aromatic substituent could also enhance Van der Waals forces as well as produce minor steric repulsions. The bromine atom at 3'-position in 4'-OH-3'-Br-naphthyronine (No. 2) lowered nuclear binding affinity. To achieve this, this bromo substituent had to be confined to the

DISTAL   PROXIMAL

a

b

c

d

$$R = CH_2CH(NH_2)COOH$$
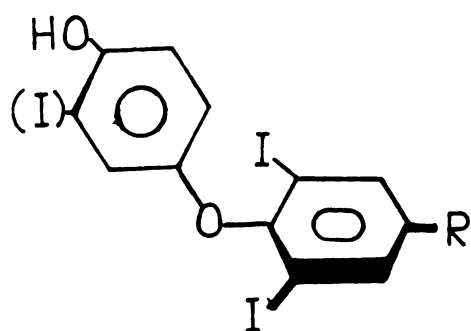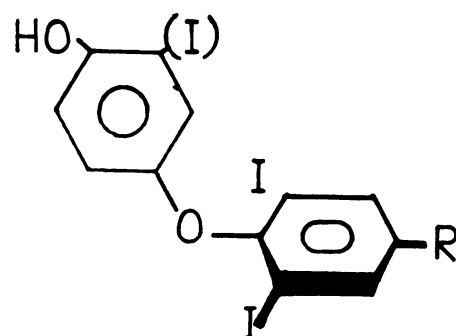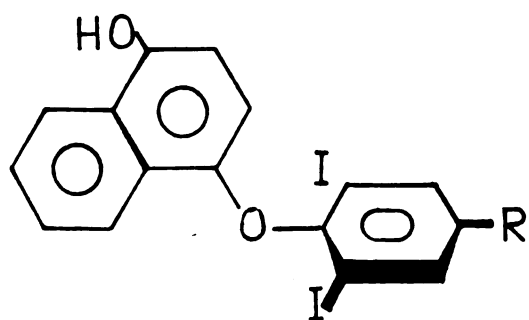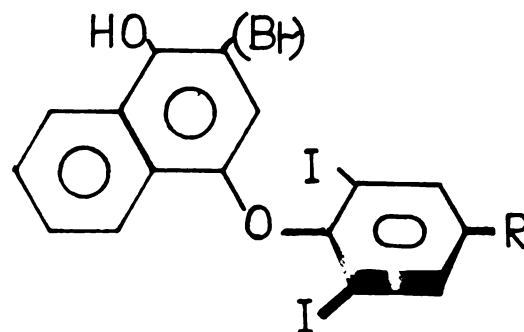
Fig. 2-9. Two minimal-energy conformations are possible for T_3 or analogs that are mono-substituted in the 3'-position.
(a) The distal conformer with the single outer-ring 3'-substituent oriented away from the inner-ring.
(b) The proximal conformer with the same substituent directed toward the inner-ring.
(c) 3,5-Diiodonaphthyronine with 2',3'-fused aromatic ring fixed in the distal conformation.
(d) 3'-Bromo-3,5-diiodonaphthyronine, in which the bromine atom is forced to be in the proximal conformation.

proximal site in the presence of a naphthyl outer-ring. The diminishing binding ability was probably due to the size-dependent negative effect of the proximal group which interfered with the 4'-hydroxyl's capacity for hydrogen bonding, and possibly direct steric hindrance to nuclear binding.

On the contrary, 4'-OH-3'-Br-naphthyronine had the highest binding affinity to TBPA among naphthyronines as shown in Table 2-1. It has been thought that 4'-OH of thyroid hormones is primarily responsible for binding to TBPA through formation of phenoxide ion, since T_4 is almost completely ionized at physiological pH(pK_a=6.73)(Jorgensen, 1978a). The difference in pK_a between T_4 and T_3 (pK_a=8.45) has been implicated as one reason to produce the ten-fold decrease in binding activity for T_3. Inductive effect of 3'-bromine made the formation of phenoxide ion more readily. As a phenoxide ion, the bromo-analog might act like a proton acceptor in TBPA binding. Further, studies have been performed to indicate that the C-OH bond lengths in several thyromimetic compounds are correlated with their acidic strengths (pK_a), and the bond length decreases with increasing acidity (Cody, 1978). Comparison of these compounds shows that the average C-O length for the 4'-OH bond in T_3 is 1.38 $\overset{o}{A}$, and in T_4 is 1.32 $\overset{o}{A}$ or 1.37 $\overset{o}{A}$, depending on whether the 4'-OH is ionized or not. In addition, the $C_{3'}$-$C_{4'}$-$C_{5'}$ angle is contracted, and the $C_{3'}$-$C_{4'}$, $C_{4'}$-$C_{5'}$ bond lengths and angles at both the $C_{3'}$ and $C_{5'}$ sites are enlarged in the tetroiodo analogs, as illustrated in Fig. 2-10. In the absence of electronegative elements like bromine, our data indicate that 4'-OH did not contribute much to TBPA binding. Further, this group could be substituted by $-NH_2$(or $-NH_3^+$), $-OCH_3$, or $-CH_3$ without causing much change in binding affinity. By contrast, the binding requirement for the nuclear receptor is different. Unionized 4'-OH was a better competitor, perhaps being a hydrogen-bonding donor. Generally speaking,
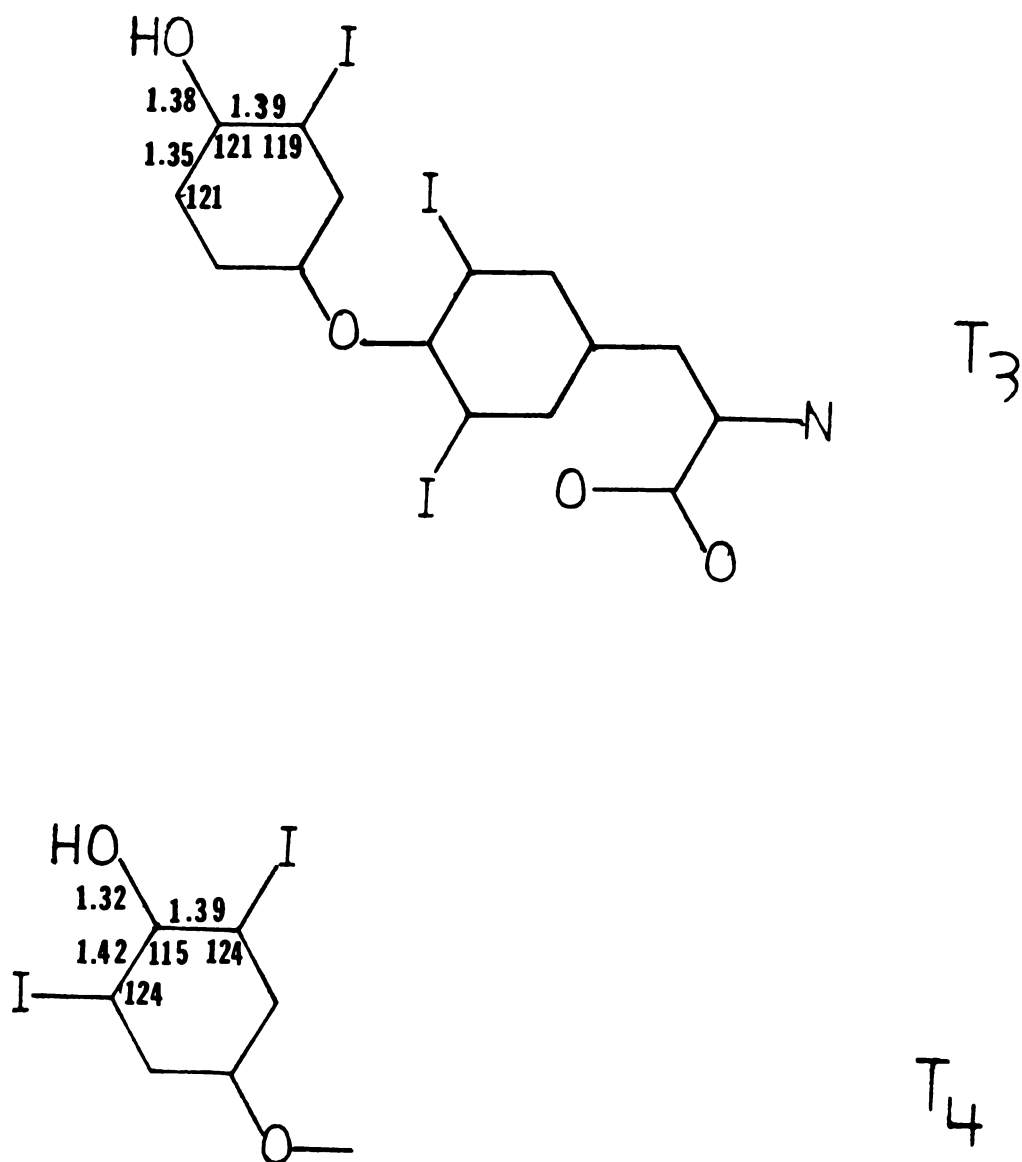
Fig. 2-10. Stereochemical differences in outer phenyl ring of
T₃ and T₄ structures (After Cody,1978). The bond length is in Å,
whereas the bond angle is in degrees.

hydrogen-bonding contributes 0.5~1.0 Kcal per mole in biological system (Jencks, 1965). Thus, hydrogen bonding could only account for part of the binding-free-energy contribution (-3.44 Kcal/mol) by the 4'hydroxyl group of 4'-OH-1'-naphthyl analog (No. 1)(relative to deoxy-1'-analog (No. 8)). It is likely that some unknown factors also are involved. Although nuclear binding with naphthyronines displays a good correlation with their thyro-mimetic potencies, some discrepancy between the in vivo and in vitro data is present. For instance, the nuclear binding activity of 4'-OH-3.5-diiodo-naphthyronine (No. 1, 63.55% of L-T$_3$) was not paralleled by the antigoiter activity (18.08% of L-T$_3$). This might be attributed to the differences in absorption, distribution, metabolism and elimination in in vivo studies. Nonetheless, the nuclear binding assay still offers a quick, inexpensive, efficient method to provide significant information for drug-design studies.

For a long time, this laboratory has been interested in clues that could lead to the designing of a thyroid-hormone antagonist that might have clinical usefulness. Thus far, analogs that bind to the receptor and inter-fere with thyromimetic effects have not been identified. In the case of β-adrenergic receptors, the primary structural difference between the agonist epinephrine and the antagonist propranolol is the substitution of a hydro-phobic double-ring for a single-ring. It is conceivable that an interplay of the hydroxyl position and this additional aromatic ring may lead to an antagonist. However, there has not been a definitive proof of it. Since some of the naphthyronines have fair nuclear binding affinity, but very little or no biological activity, e.g. 4'-CH$_3$-1'-naphthyl, 5'-OH-1'-naphthyl and 7'-OH-2'-naphthyl, we collaborated with Dr. Goldfine at Mt. Sinai Hospital, San Francisco, to test for possible antagonist effects. A method measuring [^{14}C]glucose utilization or [^{3}H]nucleotide incorporation into DNA with

tissue-cultured cells was used. So far promising leads have not been identified. However, we have identified 7'-OH-2'-3, 5-diiodo-naphthyronine as a partial agonist.

HO—
HO—〇—CHOHCH$_2$NHCH$_3$ Epinephrine
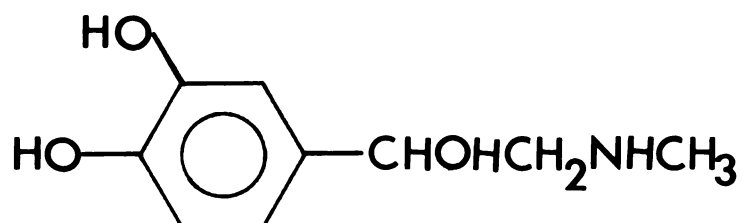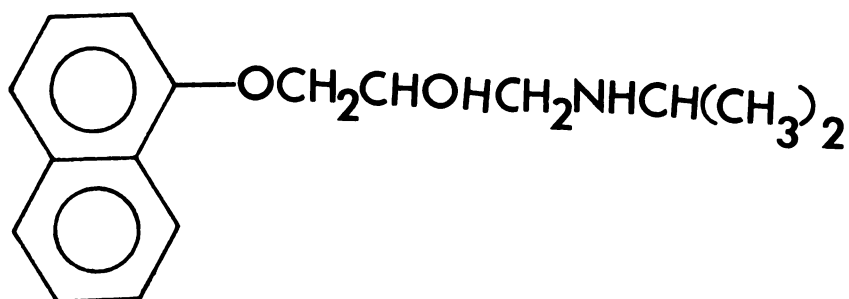
〇〇—OCH$_2$CHOHCH$_2$NHCH(CH$_3$)$_2$ Propranolol

In summary, this chapter presents comparative binding studies of 3,5-diiodo-naphthyronines with rat-liver nuclear receptors and TBPA. The following features are noted:

(1) The bulk of an additional outer aromatic ring of the naphthyl moiety is accommodated by both proteins, but TBPA is better.

(2) Location of the hydroxyl group at positions other than para causes marked decline in nuclear binding strength.

(3) The unionized 4'-hydroxyl group does not contribute significantly to TBPA binding, and substitutions with $-NH_2$, $-OCH_3$, or $-CH_3$ do not change the binding activity to TBPA. By contrast, such substitutions result in drastic decreases in nuclear binding activity.

(4) The intact 4'-hydroxyl group functions as a hydrogen-bond donor in nuclear binding and contributes substantially to binding free energy. By contrast, it might act as a H-bond acceptor in TBPA binding, probably as a phenoxide anion.

(5) The nuclear binding activity <u>in vitro</u> correlates well with antigoiter activity <u>in vivo</u> , whereas TBPA binding activity does not.

Therefore, there are distinctive differences between the nuclear and TBPA binding.

CHAPTER THREE :   STUDIES OF BINDING SITES IN HEPATIC NUCLEAR
RECEPTORS USING SYNTHETIC THYROID HORMONE
ANALOGS AS MOLECULAR PROBES

I. Introduction

In Chapter Two, binding studies using both the nuclear receptor and TBPA were carried out with naphthyl analogs which contain structural variations at the outer aromatic rings. In order to further clarify the binding specificities in the inner ring and the side-chain, nuclear binding studies were performed with thyro-active analogs which contain various structural features in this chapter.

As described in the preceeding chapter, interest in the mechanism of action of thyroid hormone has been rekindled by the recent discovery of specific nuclear binding sites for thyroid hormones triiodothyronine(T_3) and thyroxine(T_4) (Oppenheimer et al., 1972). The nuclear binding assay has provided a potentially convenient method, not only for biochemists to characterize the receptor protein(s), but also for medicinal chemists to innovate new analogs as either agonists or antagonists.

The specificity of this binding assay operates on the basis of favorable thermodynamic or kinetic state of the resultant hormone-receptor complexes. Because of this strict stereochemical requirement, the assay is presumably not interfered by the impurities in the partially purified receptor preparation.

The effectiveness of the binding assay lies in the differentiation of the free from the bound ligands. To achieve this differentiation, the most commonly used methods are: (1) equilibrium dialysis; (2) gel (or filter disc) filtration; and (3) fluorometric method. The gel filtration method has been

employed throughout in this dissertation.

The method of equilibrium dialysis operates by the use of a semi-permeable membrane which only allows free ligands with small molecular weight to penetrate from one compartment to the other. The penetration of receptor molecules is prevented by their high molecular weight. A true equilibrium state can be reached in this method, because there is no disturbance in the course of ligand separation. However, it is a time-consuming approach, and creates experimental errors due to loss of ligands by membrane adsorption.

Based on changes in ligand fluorescence upon binding, the fluorometric method doen not require separation of free from bound ligands and thus has also a true equilibrium. This method is frequently applied. Nonetheless, its use is limited, when the ligand in question is not fluorescent or when no fluorescent synthetic analogs are readily available. Unfortunately, highly fluorescent analogs of thyroid hormones with sufficient affinity have not been prepared.

The gel filtration (or filter discs of various materials, e.g. nitro-cellulose) method is the most efficient, as long as highly sensitive marker ligands(radioactively or otherwise labeled) are provided. The only drawback is that the separation process disturbs the equilibrium. Nevertheless, this method can still produce a state of quasi-equilibrium depending on the speed of separation. Errors can thus be minimized by the mild pressure applied to accelerate the passage of incubation mixture through gel columns. The half-life of $[^{125}I]T_3$-receptor complexes is within a range of 100-1530 min, depending on experimental conditions, as will be discussed in Chapter Four; while the separation process takes only < 30 sec. Therefore, it has been found that the data are comparable by both gel-filtration and

equilibrium dialysis for the same ligands.

As discussed previously, several laboratories have studied the nuclear receptor by in vivo and in vitro analog-binding assays (Oppenheimer et al., 1973; Koerner et al., 1975; Bolger and Jorgensen, 1980). Their results have delineated the major structural features concerning ligand-receptor binding, as described in Chapter Two. However, it is clear that this picture is far from being complete yet. In this chapter, twenty analogs were selected to define more clearly the requirement of molecular inter- action between thyroid hormones and the nuclear receptor. It was expected that this binding study could allow us to:

(a) determine stereospecificity and optimum carbon-length in T_4 analogs.

(b) define the size-limit of the 3'-substituent in 4'-deoxy analogs.

(c) define the electronic aspect of pockets containing 3',5',3,5 sub-
   stituents in the nuclear receptor.

II. Results and Discussion

The relative abilities of thyroid-hormone analogs to compete with [^{125}I]L-T_3 for binding to partially-purified soluble nuclear receptor are listed in Table 3-1. The data marked with an * , were obtained from ear- lier work of this laboratory. They were included for comparison with the present data. In this study, our goal was focused on examining nuclear binding by selected analogs: (1) DL optical isomers (Nos. 1-4); (2) amino acid side-chain carboxylate analogs and metabolites of T_4 (Nos. 5-12); (3) 3'-substituents of 4'-deoxy analogs (Nos. 13-19); (4) 3'-substituents of 3,5-dialkyl analogs (Nos. 24-28); (5) 3,5-substituents of hydrophilic or electrostatic character (Nos. 29,30); and (6) three-ring compound (4"-OH-3"-iPr-4"-phenoxy-T_3, No. 33)

TABLE 3-1. Structure and Binding Activities of Thyroid Hormone Analogs to the Solubilized Nuclear Receptor from Rat Liver

No.	DL	Name	R_1	R_3	R_5	$R_{2'}, R_{3'}$	$R_{4'}$	$R_{5'}, R_{6'}$	Nuclear Receptor Binding Activity %L-T$_3$[a]	Binding Free Energy $-\Delta G$[b]
1	D	D-T$_4$	Ala[c]	I	I	I	OH	I H	1.91(±0.12)	10.03(±0.04)
2*	L	L-T$_4$	Ala	I	I	I	OH	I H	14.4	11.23(±0.08)
3*	D	D-T$_3$	Ala	I	I	I	OH	H H	62.77	12.09(±0.29)
4	L	L-T$_3$	Ala	I	I	I	OH	H H	100	12.38(±0.18)
5		Tetraform	Form	I	I	I	OH	I H	9.83(±0.98)	11.00(±0.06)
6*		Triform	Form	I	I	I	OH	H H	8.47	10.91(±0.07)
7		Tetrac	Ac	I	I	I	OH	I H	83.49(±3.93)	12.27(±0.03)
8*		Triac	Ac	I	I	I	OH	H H	282.5	12.99(±0.03)
9		Tetraprop	Prop	I	I	I	OH	I H	57.70(±3.58)	12.05(±0.04)
10*		Triprop	Prop	I	I	I	OH	H H	234.5	12.88(±0.17)
11		Tetrabutyr	Bu	I	I	I	OH	I H	98.26(±10.37)	12.38(±0.07)

TABLE 3-1 (Continue)

No.	DL	Name	R_1	R_3	R_5	$R_{2'}$	$R_{3'}$	$R_{4'}$	$R_{5'}$	$R_{6'}$	Nuclear Receptor Binding Activity %L-T_3[a]	Binding Free Energy -ΔG[b]
12*		Tributyr	Bu	I	I	H	I	OH	H	H	14.4	11.23(±0.09)
13	L	4'-HT_3	Ala	I	I	H	I	H	I	H	4.57(±1.05)	10.54(±0.14)
14	L	4'H-3'iPr-T_2	Ala	I	I	H	iPr	H	H	H	2.62(±0.40)	10.16(±0.10)
15	L	4'H-3'Et-T_2	Ala	I	I	H	Et	H	H	H	0.43(±0.20)	9.14(±0.32)
16	DL	4'H-3'CF_3-T_2	Ala	I	I	H	CF_3	H	H	H	0.40(±0.03)	9.10(±0.06)
17	L	4'H-3'COOH-T_2	Ala	I	I	H	COOH	H	H	H	0.0025(±0.0005)	6.10(±0.16)
18	L	4'H-3'OH-T_2	Ala	I	I	H	OH	H	H	H	0.013(±0.004)	7.09(±0.16)
19*	L	4'H-T_2	Ala	I	I	H	H	H	H	H	0.012	7.00(±0.03)
20	L	m-T_4	Ala	I	I	H	OH	I	H	I	0.023(±0.0005)	7.41(±0.01)
21	L	4'CH_3-m-T_2	Ala	I	I	H	OH	CH_3	H	H	0.046(±0.00)	7.83(±0.00)
22*	L	3'Me-T_2	Ala	I	I	H	Me	OH	H	H	3.55	10.40(±0.09)
23*	L	L-T_2	Ala	I	I	H	H	OH	H	H	0.089	8.24(±0.17)
24	L	3'iPr-3,5Me_2T_2	Ala	Me	Me	H	iPr	OH	H	H	0.36(±0.03)	9.04(±0.05)
25	L	3'nPr-3,5Me_2T_2	Ala	Me	Me	H	nPr	OH	H	H	0.22(±0.05)	8.74(±0.15)
26	L	3'sBu-3,5Me_2T_2	Ala	Me	Me	H	sBu	OH	H	H	0.61(±0.06)	9.35(±0.07)
27*	L	3,5Me_2-T_1	Ala	Me	Me	H	I	OH	H	H	0.54	9.28(±0.07)
28	DL	3'iPr-3,5Et_2T_0	Ala	Et	Et	H	iPr	OH	H	H	0.68(±0.04)	9.39(±0.04)
29	L	3'iPr-3,5(COOH)$_2T_0$	Ala	COOH	COOH	H	iPr	OH	H	H	0.0025(±0.0009)	6.10(±0.26)
30	L	4'OMe-3'iPr-3,5(CN)$_2$-T_0	Ala	CN	CN	H	iPr	OMe	H	H	0.0083(±0.0016)	6.81(±0.13)

TABLE 3-1 (Continue)

No. DL	Name	R_1	R_3	R_5	$R_{2'}$	$R_{3'}$	$R_{4'}$	$R_{5'}$	$R_{6'}$	Nuclear Receptor Binding Activity %L-T_3[a]	Binding Free Energy -ΔG[b]
31* L	3'-iPr-T_2	Ala	I	I	H	iPr	OH	H	H	92.53	12.33(±0.17)
32* L	4'OMe-3'-iPr-T_2	Ala	I	I	H	iPr	OMe	H	H	7.39	10.83(±0.17)
33	4"OH-3"iPr-4"Phenoxy-T_3	Ala	I	I	H	I		H	H	1.73(±0.42)	9.97(±0.16)

a
K_A(L-T_3)=1.19x10^9 M^{-1}, at 25°C. No corrections were made for DL racemic mixture.

b
-ΔG=RT lnK_A. R=1.987 cal/mol°K , T=298°K.

c
The abbreviations used were : Ala-alanine; Form=formic, Ac=acetic; Prop=propionic; Bu=butyric.

*
The symbol * indicated the binding results of Bolger and Jorgensen (1980).

A. Chirality, Ionic Character and Length of Side Chain

Analogs (Nos. 1-4) were used to study the effect of chirality on binding. As shown in Table 3-1, the D-isomer of T_4(D-T_4)(No. 1) had 13.3% the binding activity of its L-isomer (L-T_4)(No. 2). The D-isomer of L_3(D-T_3) (No. 3) had 62.77% of the binding activity of its L-isomer (L-T_3) (No. 4). Therefore, L-form was the preferred optical isomer in nuclear binding. This suggested that the L-isomer could achieve an optimum spatial orientation for the ammonium (NH_3^+) and carboxylate (COO^-) groups at the chiral carbon of alanine side-chain.

The contributions to binding free energy by difference in optical activity (L or D) or addition of 5'-iodine atom to T_3 isomers are shown in Fig. 3-1. It can be seen that an additional 5'-iodine atom to D-T_3 (No. 3) caused a decrease in affinity by 2.06(±0.33) Kcal/mol. In contrast, the same substituent added to L-T_3 (No. 4) caused a smaller decrease by 1.15 (±0.26) Kcal/mol. Therefore, D-form was sterically more sensitive to addition of 5'-I than was L-form in nuclear receptor binding. Conversion from L-isomer to D-isomer lost binding energy by only 0.29(±0.47) Kcal/mol for T_3, while 1.20(±0.12) Kcal/mol for T_4.

In TBPA binding a similar stereospecificity (L>D) has been reported (Andrea et al., 1980). Based on the X-ray data of Blake et al. (1977,1978), Andrea et al.,(1978) postulated from simple theoretical calculations that this steric preference results from an interplay of electrostatic and sterically repulsive interactions. The electrostatic force involves inter-action of Lys-15 of TBPA with the carboxylate of L-T_4. The steric influence comes from bulky side chains of Leu-117 and Val-121 of TBPA which force the smallest substituent H on the α-carbon of L-T_4 to point toward

$$D\text{-}T_3 \xrightarrow[\text{+ 5'-I}]{2.06(\pm 0.33)} D\text{-}T_4$$

0.29($\pm$0.47) L $\rightarrow$ D

1.20($\pm$0.12) L $\rightarrow$ D

$$L\text{-}T_3 \xrightarrow[\text{1.15}(\pm 0.26)]{\text{+ 5'-I}} L\text{-}T_4$$
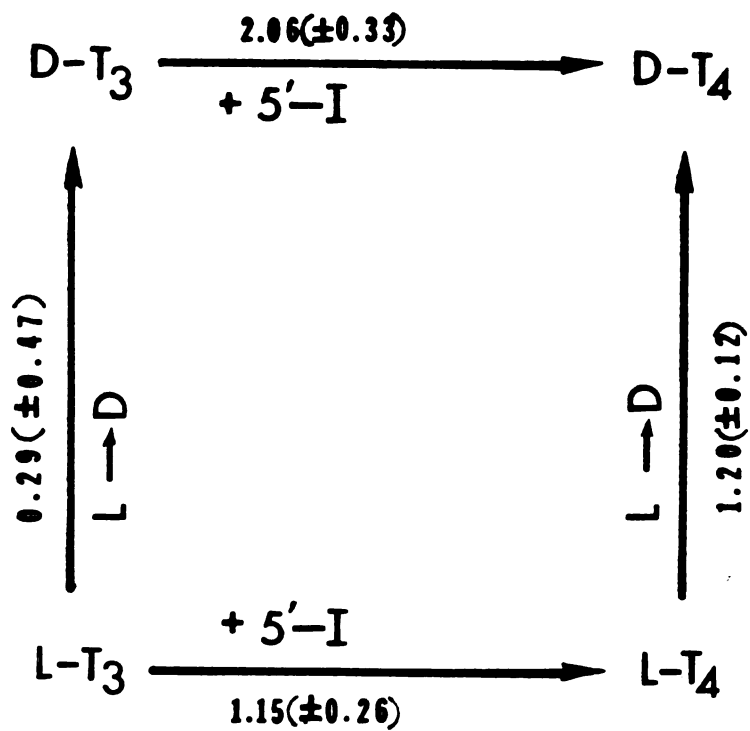
Fig. 3-1. Schematic diagram illustrating free energy contributions (Kcal/mol) to changes by addition of 5'-iodine atom or chirality at α-carbon of the amino acid side-chain.

them. This concept might be applied to the nuclear receptor binding. Since the difference in nuclear binding affinity between the L- and D-isomers was less, the effect of the interacting forces to cause optical preference was probably more moderate.

As shown in Table 3-1, the desamino (or acidic) T_4-analog substitutions (Nos. 5,7,9,11)contributed favorably to binding. Except for the tetraform (No. 5), all desamino analogs bound more firmly to the nuclear receptor than did L-T_4. Based on X-ray structure data of thyroid hormones, Cody (1978) has pointed out that part of the higher contribution to binding free energy by these desamino analogs is probably due to the lack of the conformation-restricting effect of the absence of the amino group. When the binding free energy (-$\Delta G°$) of desamino analogs was plotted as a function of side-chain length (Fig.3-2), a chain length of two or three carbons was found to be optimal in both the desamino-T_3 and the desamino-T_4 series. It was also clear that there was less of a destabilizing effect on binding with four-carbon desamino-T_4 analog than with comparable desamino-T_3 analog. The 5'-iodine atom of desamino-T_4 analog could produce a conformational change upon binding so that the carboxylate group of the analog could interact more effectively with some positively charged residue(s) of the receptor, e.g. Lys, His, or Arg.

B. Hydrophobic and Electronic Interactions of 3'-Substituents

Hydrophobicity is usually expressed by a parameter π . This is measured as the ability of a substituent to influence the partition coefficient of a reference compound between two immicible solvent layers, e.g. octanol and water (Hansch et al., 1973). π can be used to measure the size of a substituent, because the two variables are often correlated. Fig. 3-3 depicts
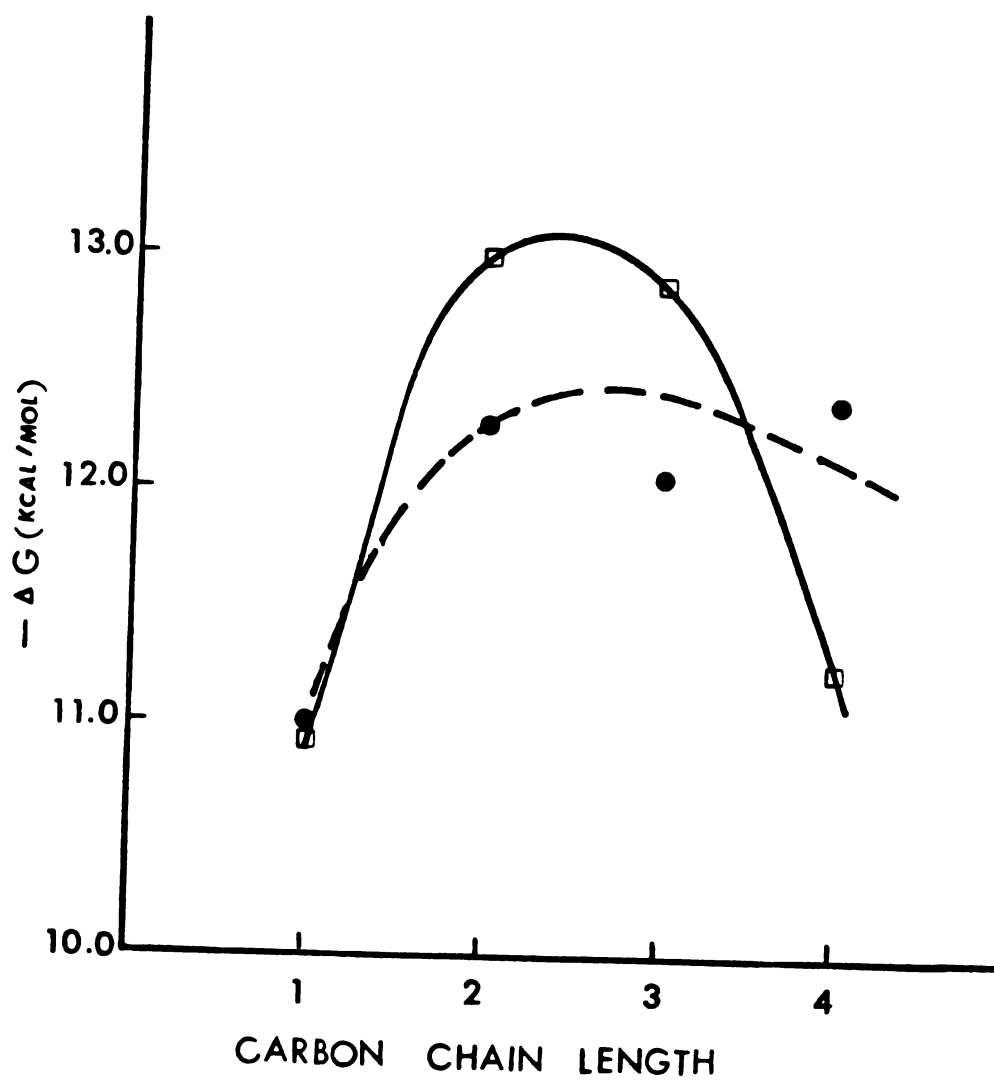
Fig. 3-2. The free energy change of binding (-ΔG) was plotted as a function of carbon chain length of amino acid portion. (—□—) representing T_3 series; (--●--) representing T_4 series.

the contribution to binding free energy by 3'-substituents as a function of their π value. The contributions to binding free energy were calculated as relative to reference analogs. The reference analog was 4'-H-T$_2$ (No. 19) for the 4'-deoxy series, and L-T$_2$ (No. 23) for 4'-OH series. As can be seen in Fig.3-3, there was a parabolic relationship. In the deoxy series, the free energy contribution increased in the order of CF$_3$ (No. 16), Et (No. 15), and 4'-H-T$_3$ (No. 13) with which a maximum was reached as shown in Table 3-1. As the size of the 3'-substituent was further increased, a decrease in binding energy contribution was seen with 3'-sBu. Our results supported the concept that a hydrophobic pocket is formed to contain the 3'-substituent up to a certain size like the iodine or isopropyl group.

Several 3,5-dimethylated analogs containing 3'-substituents (Nos. 24-26) were also studied. Because of the less bulky methyl group (Van der Waals volume = 13.7 cm^3/mol, Bondi (1964)) on the inner phenyl ring, there is more degree of freedom in the dihedral angles between ring planes as compared with 3,5-iodine analogs (Van der Waals volume of iodine = 19.6 cm^3/mol). Thus, 3'-s-butyl(-9.35 Kcal/mol) could contribute more than could 3'-n-propyl or isopropyl (-8.74, -9.04 Kcal/mol respectively) by reducing the degrees of freedom of conformation. However, the binding reaction of 3'-s-butyl was not so favorable in the 4'OH or 4'-deoxy-3,5-T$_2$ series, as described above.

In Fig. 3-4, the same data used for Fig.3-3 were plotted against electronic parameter σ , which correlates with the strength of electron-donating (-σ) or electron-withdrawing(+σ) properties (Hansch et al., 1973). There was no identifiable relationship between these two variables. Therefore, in contrast with prealbumin binding, dipolar or electrostatic contribution by the 3'-group was not critical in nuclear binding. Moreover, hydrophilic 3'-replacements, such as -COOH (No. 17) highly destabilized the binding,

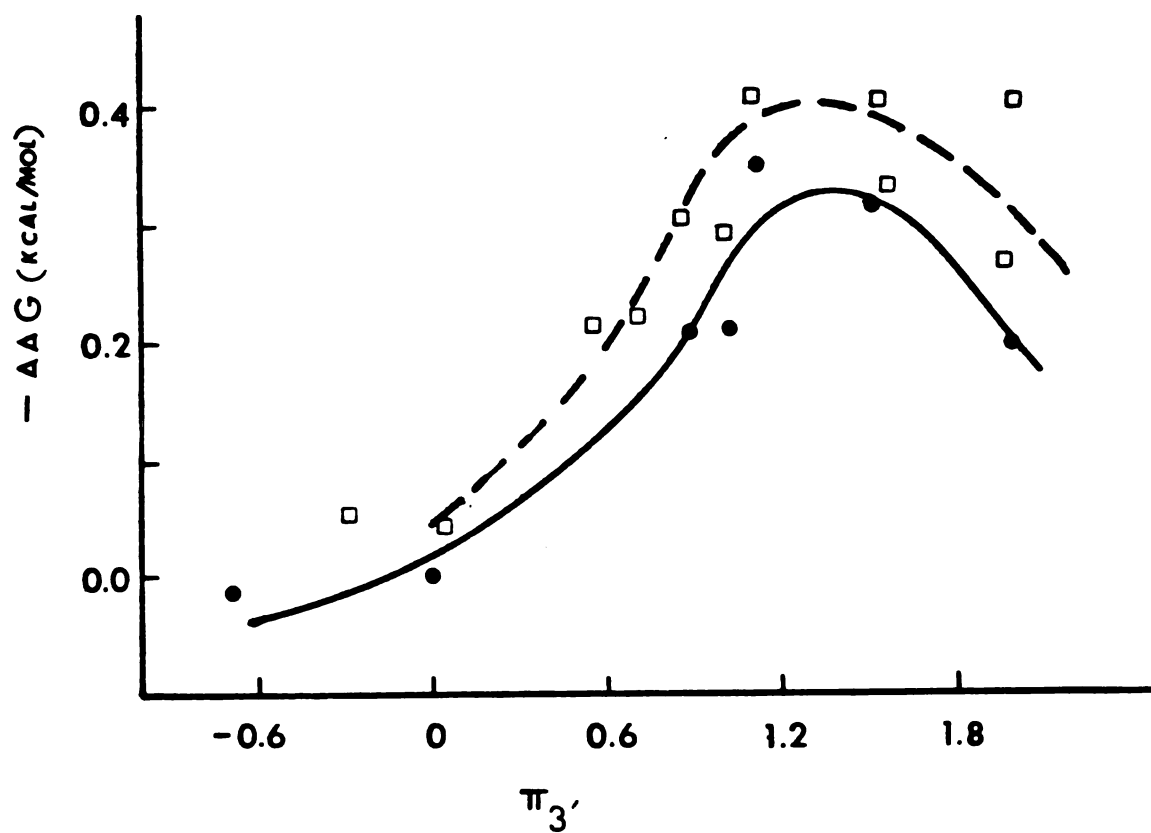Fig. 3-3. Correlation plot of free energy contribution (- ΔΔG)
(in Kcal/mol) vs. hydrophobicity parameter π of the 3'-substituent,
(—●—) denoting T_3 series; (--□--) denoting T_4 series.
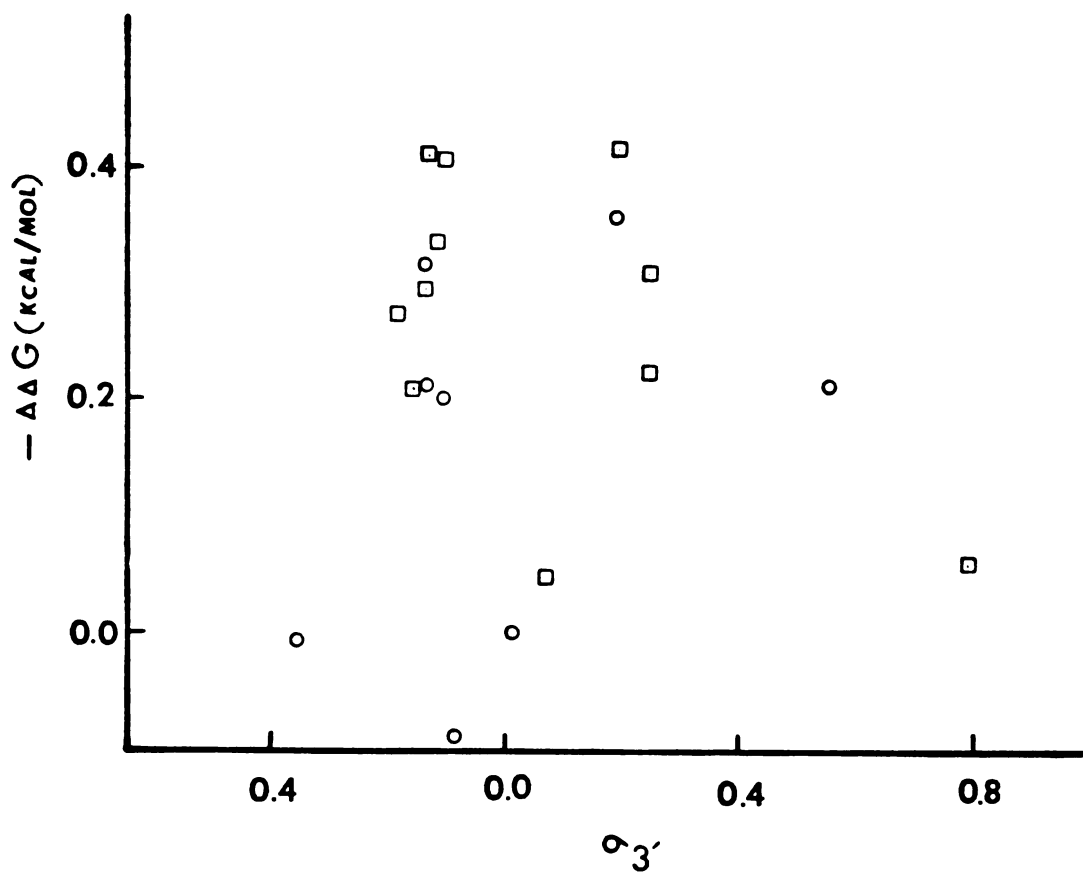
Fig. 3-4. Correlation plot of free energy contribution (- $\Delta\Delta G$) (in Kcal/mol) vs. electronic parameter σ of the 3'-substituent, (□) representing T_3 series; (O) representing T_4 series.

whereas -OH (No. 18) contributed little to binding.

C. Importance of the Location of Outer-Ring Hydroxyl Group

A very strict stereochemical requirement for the location of the phenolic hydroxyl group in nuclear binding has been discussed in the preceeding chapter. Here further evidence is presented to stress this point. By comparing the ΔG's of 4'H-3'OH-T_2 (No. 18) and 4'-H-T_2 (No. 19), the 3'-OH group made a contribution of -0.09($\pm$0.19) Kcal/mol to nuclear binding. By contrast, the 4'-OH contributed -1.24($\pm$0.20) Kcal/mol, which is twelve times as much as the 3'-OH contribution. Other 3'-OH analogs like m-T_4 (No. 20) and 4'-CH_3-m-T_2 (No. 21) also showed low binding affinity to the nuclear receptor (0.023% and 0.046% respectively). Consequently, the hydroxyl group on the outer ring of thyroid hormones is required to be located at 4'-(or para) position.

Comparison of the binding free energy of m-T_4(-7.41 Kcal/mol) (No. 20) with that of 4'H-3'OH-T_2 (-7.09 Kcal/mol) (No. 18) was performed. The two iodines at 4' and 6' positions added only -0.32($\pm$0.17) Kcal/mol, in striking disparity with the contribution of 3'-iodine of L-T_3(-4.14 Kcal/mol). In addition, the contribution of 4'-CH_3 was estimated as -0.74($\pm$0.16) Kcal/mol by comparing the binding free energy of 4'-CH_3-m-T_2 (7.83$\pm$0.00 Kcal/mol) (No. 21) with that of 4'H-3'-OH-T_2 (7.09$\pm$0.16 Kcal/mol) (No. 18).

Further, a very similar curve was also obtained for the 4'-OH series. This 4'-OH series was characterized by a constant difference in the range of 0.6~0.97 Kcal/mol over the 4'-deoxy series, Bolger and Jorgensen (1980) have termed this difference as interactive contribution by the 3'-substituent to orient the hydroxyl group for stronger hydrogen-bonding.

D. 3,5 Substitutions on the Inner Ring

The preparation of halogen-free compounds with alkyl substitutions has been of considerable interest. It has been reported that 3'-isopropyl-3, 5-dimethyl-L-thyronine is thyromimetically active and retains moderate nuclear binding avidity. More importantly, this analog can permeate through the placenta to stimulate fetal surfactant synthesis (Jorgensen, 1978b). In this study, 3'-isopropyl analogs containing 3,5-dialkyl substitutions of methyl (No. 24) and ethyl (No. 28) groups were examined by nuclear binding assay. The results indicated that the ethyl analog was a better binder than was the methyl analog, probably due to increased hydrophobic bonding and to its conformation-constraining effect on the two phenyl rings. In 3'-iodo-3, 5-dialkyl analogs, the 3'-iodo-3,5-diisopropyl analog has been demonstrated to have lower binding affinity to the receptor as compared with the 3-iodo-3, 5-diethyl analog (Bolger and Jorgensen, 1980). Because of this strict limit of size, 3'-isopropyl-3,5-diisopropyl analog could also be predicted to have lower affinity.

Few studies have focused on hydrophilic limitations for substituents in the 3 and 5 positions in binding to hepatic nuclear receptors. As presented in Table 3-1, we measured the binding free energy for 3-iPr-3, 5-$(COOH)_2$- and 4'-OMe-3'-iPr-3, 5-$(CN)_2$-analogs (No. 29,30) as -6.10($\pm$0.26) and -6.81($\pm$0.13) Kcal/mol respectively. When comparison was made with 3'-iPr-3, 5-I_2-analog (No. 31), 3,5-dicarboxyl substitution caused a loss of 6.24 Kcal/mol. Similarly, 3,5-dicyano substitution made a loss of 4.02 Kcal/mol as compared with 4'-OMe-3'-iPr-T_2 analog (No. 32). Because both carboxyl and cyano substituents are polar in nature, these results strongly suggested that binding regions for 3,5-substituents were composed of primarily hydrophobic residues of the nuclear receptor. This requirement

appears to be in common with that in TBPA binding (Blake and Oatley, 1977).

E. Binding Activity of a Three-Ring Analog

The binding free energy of an interesting three-ring compound, 4"OH-3"-isopropyl-phenoxy-T_3 (No. 33), was measured as -9.97(±0.16) Kcal/mol. Surprisingly, this analog had a moderate binding activity (1.73±0.42%). It is interesting to see how this compound could be located in the binding site.

Using comparative analysis, Bolger and Jorgensen (1980) have partitioned -ΔG's into contributions by substituents: each of the 3,5-iodine atoms adds -3.4(±0.7) Kcal/mol;4'-OH -1.2(±0.2) Kcal/mol;3'-iPr -2.8(±0.2) Kcal/ mol; 3',4' interactive energy -0.6(±0.5) Kcal/mol; and carboxylate -0.2 (±0.8) Kcal/mol. However, this kind of breakdown is not internally consistent, because the contribution by the alanine side chain is somewhat underestimated. If thyroxamine(-ΔG=6.41 Kcal/mol), a decarboxylated analog, should lose only 0.2(±0.8) Kcal/mol by deletion of the side-chain carboxyl group, L-T_4 would have a binding free energy of -6.61 Kcal/mol, too low a value as compared with its ΔG of -11.23 Kcal/mol from competetive binding study. Nevertheless, this partition method can provide a semi-quantitative scenario for the purpose of drug design studies. If the partitioning model proposed by Bolger and Jorgensen is qualitatively correct, then the first two rings could make a contribution of -8.0 Kcal/ mol; the remaining -2.0 Kcal/mol could be accounted for by the contribution of the third ring containing alanine.

To summarize, this study has provided deeper insight into the following structural aspects of hepatic nuclear binding:

(1) The nuclear binding is stereospecific, i.e. L-form binds more firmly than does D-form. Furthermore, D-form is sterically more sensitive

to addition of 5'-I than is L-form.

(2) Lengthwise, two or three carbons are optimal in both desamino -T_4 and -T_3 series.

(3) There is a good parabolic correlation between the hydrophobicity parameter π for the 3'-substituents and the contribution to binding free energy,but not the electronic parameter σ. This indicates that a complementary hydrophobic pocket is present in the nuclear receptor to contain 3'-substituents up to the size of iodine or the isopropyl group. Moreover, hydrophilic 3'-, 3- or 5- substituents like -OH, -CN and -COOH have a destabilizing effect on binding.

(4) Additional evidence is provided to support a very strict stereochemical requirement of the location of phenolic hydroxyl group. This is demonstrated by the low binding activity of m-T_4 and 4'CH_3-m-T_2.

(5) 4"OH-3"isopropyl-phenoxy-T_3 analog, a three-ring compound, shows a moderate binding activity of 1.73% (L-T_3=100%).

CHAPTER FOUR : PART I -- NEGATIVE COOPERATIVE BINDING OF L-THYROXINE AND
ANALOGS BY HUMAN THYROXINE-BINDING PREALBUMIN: A MODEL FOR
INHIBITORY ALLOSTERIC RECEPTOR-LIGAND INTERACTIONS

I. Introduction

Numerous studies of the binding of a variety of hormones and drugs
to putative receptors have been reported. The binding isotherm is generally
analyzed by expressing the bound/free ratio of the labeled ligand as a
function of the concentration of the ligand that is bound to the receptors,
i.e. a Scatchard plot (Scatchard , 1949). In some cases, this plot is
linear. The affinity constant, K_a, can be derived from the slope of the
plot, and the homogeneous receptor site concentration from the X-intercept.
However, the plot is nonlinear in other cases. Then the question about
how K_a and the receptor site concentration should be derived is truly crucial.

Different models have been proposed to rationalize concave upward
Scatchard plots of the insulin binding data to various receptor systems
(Garvin et al., 1973; Kahn et al., 1974; Reichler et al., 1976; Freychet
et al., 1977). The best known among them are :

(i) multiple classes of binding sites that are distinguishable, inde-
pendent, and have different binding affinities.

(ii) single class of binding sites that are identical and interactive
with negative cooperativity (anti-cooperativity); namely, the next
site has less affinity for the same ligand than the preceeding site
(Conway et al., 1968; Levitzki et al., 1969). By equilibrium or
steady-state data alone, these two models cannot be distinguished.

Other complications can also generate a nonlinear Scatchard plot.
For example, incomplete elimination of nonspecific binding (low-affinity,

high-capacity) can mask nonspecific binding sites as the second sites. Different binding affinities due to (1) heterogeneous subpopulations of the ligand (different conformations, different degree of polymerization, etc.) for the receptor (Nichol et al., 1969; Laiken et al., 1971), (2) heterogeneous subpopulations of the receptor (different degree of polymerization or depolymerization, presence of absence of effector associated with the receptor, etc.), can cause non-linear binding isotherms (Jacobs et al., 1976; Levitzki et al., 1974, 1975). Other problems, for example, ligand-induced stabilization of complexation, or poor measurement of free hormone, can also create false binding curves symbolic of positive cooperativity.

Using a method analogous in principle to the study of the positive-cooperativity kinetics of hemoglobin (Olson et al., 1972; Gibson et al., 1975), De Meyts and coworkers demonstrated the presence of site-site interactions of a type consistent with negative cooperativity, upon binding of insulin to its receptors (De Meyts et al., 1976) and also the absence of cooperativity upon binding of growth hormone to its receptors of the same cells (De Meyts et al., 1973). These reports have shown that the dissociation of [^{125}I]insulin bound to cultured human lymphocytes and mouse liver plasma membrane is enhanced by addition of unlabeled insulin after induction by 100-fold dilution. This dissociation enhancement has been interpreted to mean that the increased binding site occupancy in the insulin receptors produces negatively cooperative interactions which facilitate dissociation of the bound [^{125}I]insulin (i.e. larger dissociation rate constant). However, the validity of this approach has been challenged by a number of investigators (Frazier et al., 1974; Verrier et al., 1974; Cuatrecasas et al., 1975; Limbird et al., 1975, 1976; Caron et al., 1976; Kohn et al., 1976; Pollet et al., 1977; Reichler et al., 1977; Powell-Jones et al., 1979;

Saltiel et al., 1980).

Cooperativity phenomena, positive or negative, play an important role in biological systems, such as binding of oxygen to hemoglobin (positive cooperativity), and binding of glyceraldehyde-3-phosphate to its dehydrogenase (negative cooperativity). In recent years, cooperativity behavior has been claimed for the binding of hormones and drugs to their receptors (De Meyts et al., 1973, 1976; Frazier et al., 1974; Limbird et al., 1975; Caron et al., 1976; Limbird et al., 1976). Unlike hemoglobin, independent physical, chemical data (e.g. protein structure determination by X-ray crystallography) regarding molecular events are virtually nonexistent for all hormone (or drug)-receptor systems.

In constrast, X-ray structure data have been obtained for human thyroxine binding prealbumin (TBPA) which carries about 15-30% of circulating L-T_4 (Blake et al., 1974, 1977, 1978). TBPA is composed of four identical subunits containing 127 amino acid residues, arranged in tetrahedral symmetry. The nature of interactions between thyroid hormones and TBPA has been well studied (Ferguson et al., 1975; Cheng et al., 1977; Andrea et al., 1980). On the basis of X-ray studies, there are two identical hormone binding sites for L-T_4. These two sites form a narrow end-to-end channel of 4 Å in diameter near the constricting center and 10 Å at the peripheral entrance. "Negative cooperativity" has been hypothesized for TBPA from the observation of a concave, upward Scatchard plot of L-T_4 binding to TBPA (Ferguson et al., 1975; Blake et al., 1977).

Unlike insulin receptors which are on the membrane surface, TBPA is a purified plasma protein, well characterized and free from other interferences (e.g. effectors, non-specific receptors, etc.). TBPA is indeed an ideal model for testing the "negative cooperativity" hypothesis by the

kinetic approach of De Meyts.

In this study, we present data addressing the following questions:

(1) Is the degree of dissociation enhancement concentration-dependent?

(2) Does the degree of dissociation enhancement parallel the binding affinity of thyroid hormone analogs?

(3) Is the degree of dissociation enhancement binding-site occupancy dependent?

Furthermore, we present critical discussions on the literature regarding nonlinear Scatchard plots.

II. Materials and Methods

A. Prealbumin

This protein was obtained from Dr. Y.L. Hao, American National Red Cross Blood Research Laboratory (Bethesda, MD). Its purity was shown by SDS gel electrophoresis. The molecular weight of prealbumin was taken to be 55,000 (Branch et al., 1971). Prealbumin concentrations were determined spectroscopically at 280 nm ($E_{1\ cm}(1\%)=14.1$)(Raz et al., 1969).

B. Thyroid Hormones

T_4 was purchased from Sigma Chemical Co. (St. Louis, MO). T_3 was from U.S. Biochemical Co. (Cleveland, OH). Tetraac was from Travenol Laboratory (Los Angeles, CA). Triac was a gift from Smith Kline and French Laboratories (Philadelphia, PA). The remaining compounds were prepared in our laboratory. All the compounds were determined to be pure by elution through a reverse-phase C-18 column (Altex, Berkeley, CA) using acetonitrile:tetrahydrofuran: H_3PO_4 (titrated to pH=3.5 with triethanolamine) (28:5:67).

$[^{125}I]T_4$(1250 μCi/μg) was obtained from New England Nuclear (Boston, MA). Corrections were made in order to calculate the true concentration of

a given sample of $[^{125}I]T_4$ from the measured radioactivity as described. (Bolger et al., 1980).

C. Prealbumin Equilibrium Binding Assay

The binding studies followed those described (Bolger and Jorgensen, 1980), with modification. To determine: (i) the total amount of radioactive hormone that was bound to prealbumin and (ii) the amount of radioactive hormone that was bound nonspecifically, two parallel series of incubation tubes (12x75 mm) were prepared. The nonspecifically-bound hormone was measured as remaining radioactivity in the presence of 10,000-fold excess of unlabeled $T_4(2.0x10^{-5}M)$. In each tube there was a tracer dose of $[^{125}I]T_4(0.4$ nM, 100,000 cpm). Incubation buffer (0.1M Tris, 0.1M NaCl, 1mM EDTA, pH=7.6) was used to make the final volume 0.5 ml after addition of 50 µl of prealbumin (final concentration= 23 nM, 63 µg of protein). The tubes were incubated at 35±1°C for 4 hours. Separation of the bound from the free hormone by gel filtration through a small Sephadex G-25 (medium) column (bed volume=2.0±0.1 ml, equilibrated in the Tris buffer) at 2-4°C, to minimize experimental error due to dissociation. Elution time was reduced to less than 30 sec by applying mild pressure. The amount of bound $[^{125}I]T_4$ was then determined with a Searle Auto-Gamma spectrometer (efficiency=74%). The amount of specific binding was calculated by subtracting the radioactivity for nonspecific binding from the radioactivity for the total bound hormone. The amount of free hormone was calculated by subtracting the radioactivity for the total bound hormone from the radioactivity for total radioactive hormone added.

D. Scatchard Plot of T_4-Prealbumin Binding

The data were fitted by using a model for two distinguishable and

independent sites (Eq. 1) which is mathematically equivalent to a model

for two identical sites with interactive cooperativity (Eq. 2).

$$\frac{\bar{\nu}}{F} = \frac{K_1}{1 + K_1 F} + \frac{K_2}{1 + K_2 F} \qquad (\text{Eq. 1})$$

$$\frac{\bar{\nu}}{F} = \frac{2 K (1 + \alpha KF)}{1 + 2 KF + \alpha K^2 F^2} \qquad (\text{Eq. 2})$$

$$\alpha = \frac{4 K_1 K_2}{(K_1 + K_2)} \qquad (\text{Eq. 3})$$

Where $\bar{\nu}$ is the average number of L-T_4 molecules bound per prealbumin

molecule; F is the concentration of free L-T_4 in moles per liter; K_1 and

K_2 are the association constant of L-T_4 at the high- and low- affinity sites,

respectively; K is the association constant for the binding of L-T_4 to one

of the two equivalent sites; α is an interactive factor.

Setting equivalence for Eqs. (1) and (2)

$$\frac{K_1}{1 + K_1 F} + \frac{K_2}{1 + K_2 F} \equiv \frac{2 K (1 + \alpha KF)}{1 + 2KF + \alpha K^2 F^2}$$

$$\frac{(K_1 + K_2) + 2K_1 K_2 F}{1 + (K_1 + K_2)F + K_1 K_2 F^2} \equiv \frac{2K + 2\alpha K^2 F}{1 + 2KF + \alpha K^2 F^2}$$

Therefore, $K_1 + K_2 = 2 K$ ------------------------------------(Eq. 4)

$K_1 K_2 = \alpha K^2$ ------------------------------------(Eq. 5)

Solve α from Eqs. (4) and (5)

$$\alpha = \frac{4\ K_1 K_2}{(K_1 + K_2)^2} \text{---(\ Eq.\ 3\ )}$$

The fitting was carried out by using Public Procedure FITFUN on the PROPHET computer system, Chemical/Biological Information Handling Program, National Institute of Health, Bethesda, MD (Raub, 1974).

E. Dissociation Kinetics

1. <u>Optimization of Dilution</u> The technique of De Meyts <u>et al</u>.(1973 was followed with modification. Prealbumin was preequilibrated with $[^{125}I]T_4$ at 37±1°C for 4-6 hours in the absence and presence of $2x10^{-5}$M unlabeled T_4. In order to optimize the degree of dilution, different dilutions (x1, x5, x10, x20, x50, x100) with the above preequilibrated samples were obtained in duplicates. Thirty minutes after respective dilution, the remaining bound radioactivity was determined by gel filtration as described. Nonspecific binding was determined from the radioactivity of the sample with $2x10^{-5}$M unlabeled T_4 and subtracted from the radioactivity of the sample without $2x10^{-5}$M unlabeled T_4. The results (Fig. 4-3) show that 100-fold dilution was adequate for our system.

2. <u>Effects of Unlabeled T_4</u> Dissociation kinetics at 2-4°C in the presence of various concentrations of unlabeled T_4 were obtained by 100-fold dilution of preequilibrated prealbumin-$[^{125}I]T_4$ mixture (60 µl) in the Tris buffer (5940 µl) alone or containing various amount of unlabeled T_4 ($2x10^{-9}$M, $2x10^{-8}$M, $2x10^{-7}$M, $2x10^{-6}$M). After the indicated periods of dissociation at 2-4°C, duplicate aliquots (0.4 ml) were filtered as described above. Nonspecific binding was corrected as described. Dissociation rate constants were calculated according to the first-order kinetics.

3. <u>Effects of Binding Site Occupancy</u> To examine the dissociation rate

at different binding site occupancies (or molar ratios of protein/ligand),

as described by De Meyts <u>et</u> <u>al</u>. (1973) and Olson <u>et</u> <u>al</u>. (1972), 90 μl

of various concentrations of prealbumin $(3.3 \times 10^{-7} M \sim 6.6 \times 10^{-9} M)$ was pre-

equilibrated (37±1°C, 4-6 hr) with 10 μl of $[^{125}I]T_4 (1.9 \times 10^{-8} M)$. Disso-

ciation kinetics at 2-4°C was obtained by 100-fold dilution of the res-

pective preequilibrated mixture (30 μl) in the Tris buffer (2970 μl) alone

or containing unlabeled $T_4 (2 \times 10^{-7} M)$. After indicated periods of disso-

ciation, duplicate aliquots (0.4 ml) were filtered to determine the total

bound radioactivity as described above. Nonspecific binding was corrected

correspondingly. The dissociation rate constant was calculated according

to the first-order kinetics.

4. <u>Effects of Thyroid Hormone Analogs</u> Dissociation kinetics at 2-4°C

in the presence of various analogs $(2 \times 10^{-7} M)$ was also obtained for tetraac,

$r-T_3$, $L-T_3$ and triac, in addition to T_4. Procedures were the same as

described for unlabeled T_4 .

III. <u>Results</u>

A. <u>Equilibrium Binding of $[^{125}I]T_4$ to Prealbumin</u>

The data are shown in a Scatchard plot which displays bound/free

$[^{125}I]T_4$ as a function of the $[^{125}I]T_4$ bound in Fig. 4-1. The nonlinearity

is in agreement with previously published results (Ferguson <u>et</u> <u>al</u>., 1975;

Cheng <u>et</u> <u>al</u>., 1977; Andrea <u>et</u> <u>al</u>., 1980). The fitted parameters were

$2.82 \times 10^{+7} M^{-1}$ for the association constant K and 0.13 for the interactive

factor α . The corresponding values of parameters K and α, measured by

other investigators are $K=1.89 \times 10^{+7} M^{-1}$, α=0.25 (Andrea <u>et</u> <u>al</u>., 1980);

$K=5.05 \times 10^{+7} M^{-1}$, α= 0.04 (Ferguson <u>et</u> <u>al</u>., 1975); and $K=8.13 \times 10^{+7} M^{-1}$, α=0.06

(Cheng <u>et</u> <u>al</u>., 1977). Differences in buffer, pH, ionic strength and
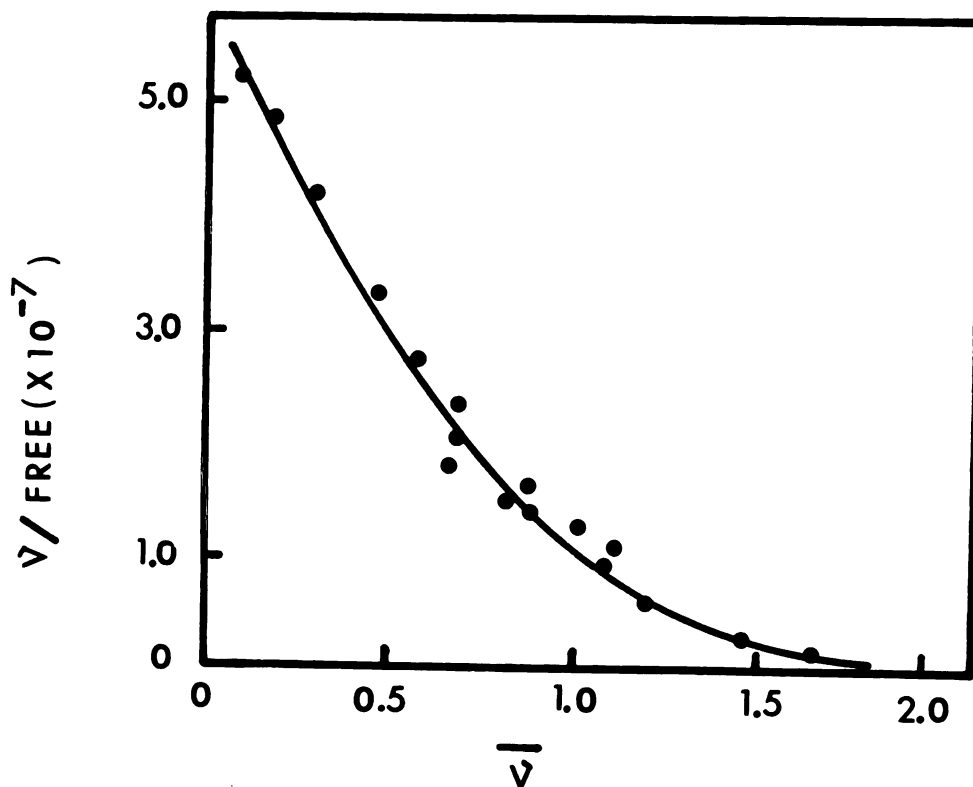
Fig. 4-1. Scatchard plot of $[^{125}I]T_4$ binding to prealbumin at pH 7.6, 35±1°C, in 0.1 M Tris Buffer containing 0.1M NaCl and 1mM EDTA. (●) experimental data; (—) best fit curve, K_1 = 5.46(±3.26)x10^{7}M^{-1} and K_2 = 1.86(±0.70)x10^{6}M^{-1}.
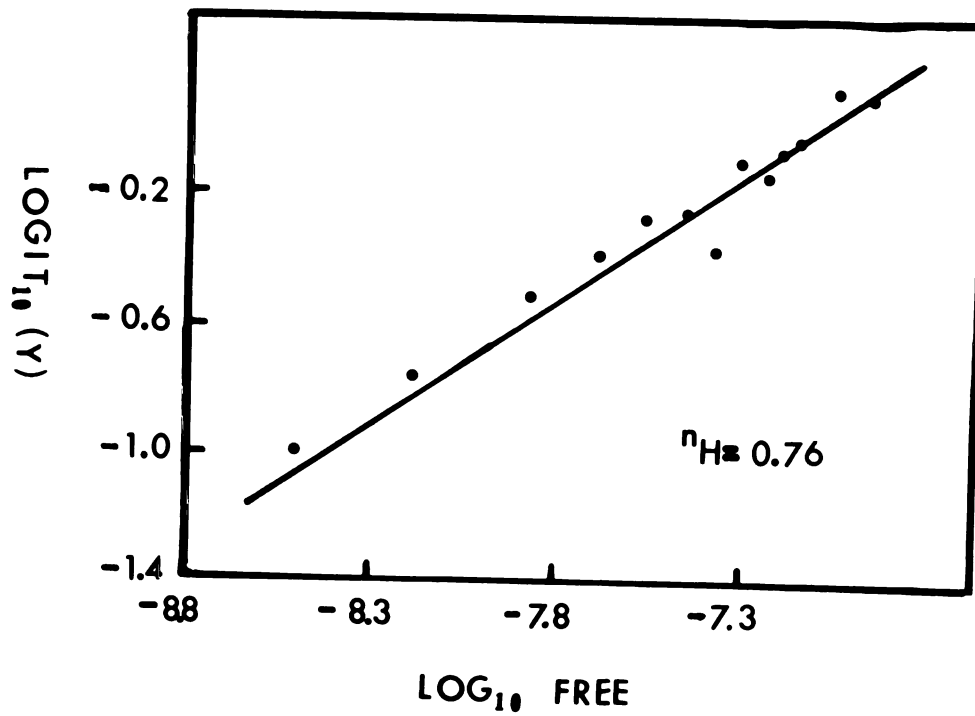
Fig. 4-2. Hill plot of $[^{125}I]T_4$ binding to prealbumin. The data were the same as described in the legend to Fig.4-1. (●) experimental data, (—) best fit curve. n_H=0.76. $Logit_{10}(Y)=log_{10}[Y/1-Y]$, where Y is the fractional receptor occupancy.

temperature may contribute to the differences in the parameters between our experiment and those of other investigators. The interaction energy defined as -RT lnα is thus 1.21 Kcal/mole (at T=298°K).

Another useful quantitative measurement of the extent of cooperative interaction among the sites is a Hill plot. In such a plot the binding data are delineated as log Y/1-Y (i.e. fraction of sites bound/fraction of sites free) versus log free ligand, where Y is the degree of saturation. The slope of the plot gives n_H, the Hill coefficient. If n_H=1.0, there is a single class of binding sites; if n_H<1.0, there is negative cooperativity; if n_H>1.0, there is positive cooperativity. In the case of prealbumin, n_H was 0.76 as shown in Fig.4-2. This is in support of the "negative cooperativity" hypothesis.

B. Optimization of Dilution Factor

The curve in Fig. 4-3 shows the percentage of $[^{125}I]T_4$ bound 20 min after dilution. The percentage bound at 1:50 dilution is not appreciably different from that at 1:100 dilution, in agreement with computer studies (De Lean and Rodbard, 1979) which indicate that a 100-fold dilution results in virtually complete prevention of rebinding.

C. Effects of Chasing Doses of Unlabeled T_4 and Fractional Occupancy at the Second Binding Site

Fig. 4-4 shows that no appreciable dissociation enhancement occurred until the concentration of unlabeled L-T_4 reached 10^{-7}M. In contrast to insulin where a further increase in concentration results in a decrease in the amount of radioactivity remaining bound, increasing concentrations of unlabeled L-T_4 produced increasing dissociation.
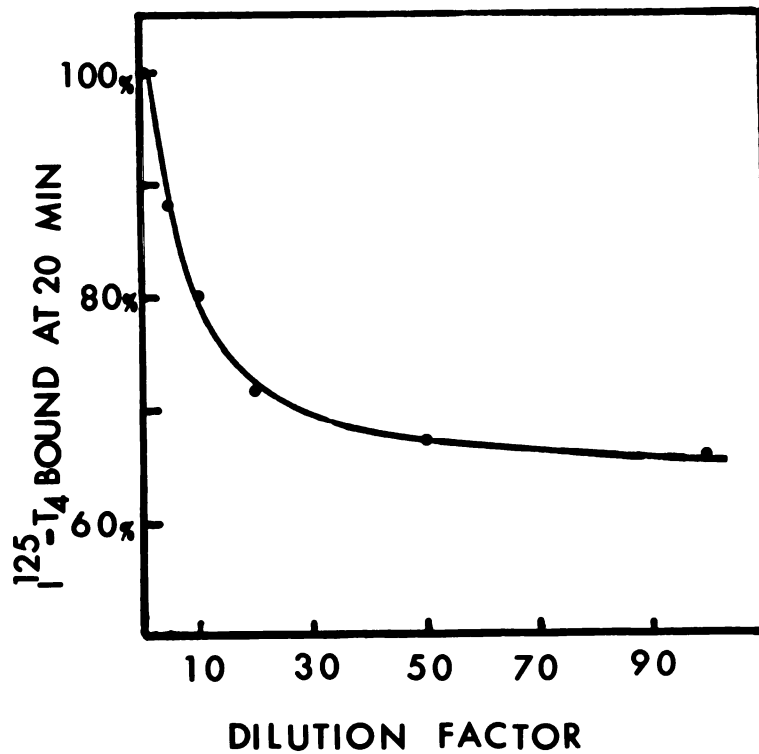
Fig. 4-3. Effect of dilution on the dissociation of $[^{125}I]T_4$. Prealbumin was incubated with $[^{125}I]T_4(3\times10^{-9}M)$ at $35\pm1°C$ for 1 hour, after which various aliquots ($10\mu l$ to $200\mu l$) were transferred to a series of tubes that contained the Tris buffer (final volume=1.0 ml) in the absence or presence of unlabeled $L-T_4(2\times10^{-5}M$ to measure nonspecific binding). After 20 min at $2-4°C$, duplicate aliquots (0.4 ml) were filtered through preequilibrated Sephadex G-25(medium) mini-columns to separate the bound from the free $[^{125}I]T_4$; the radioactivity was counted. The remaining radioactivity in the eluted fraction after correction for nonspecific binding was plotted as a function of the dilution factor during the dissociation. Each point was a mean of the duplicates; duplicates differed by less than 5%.
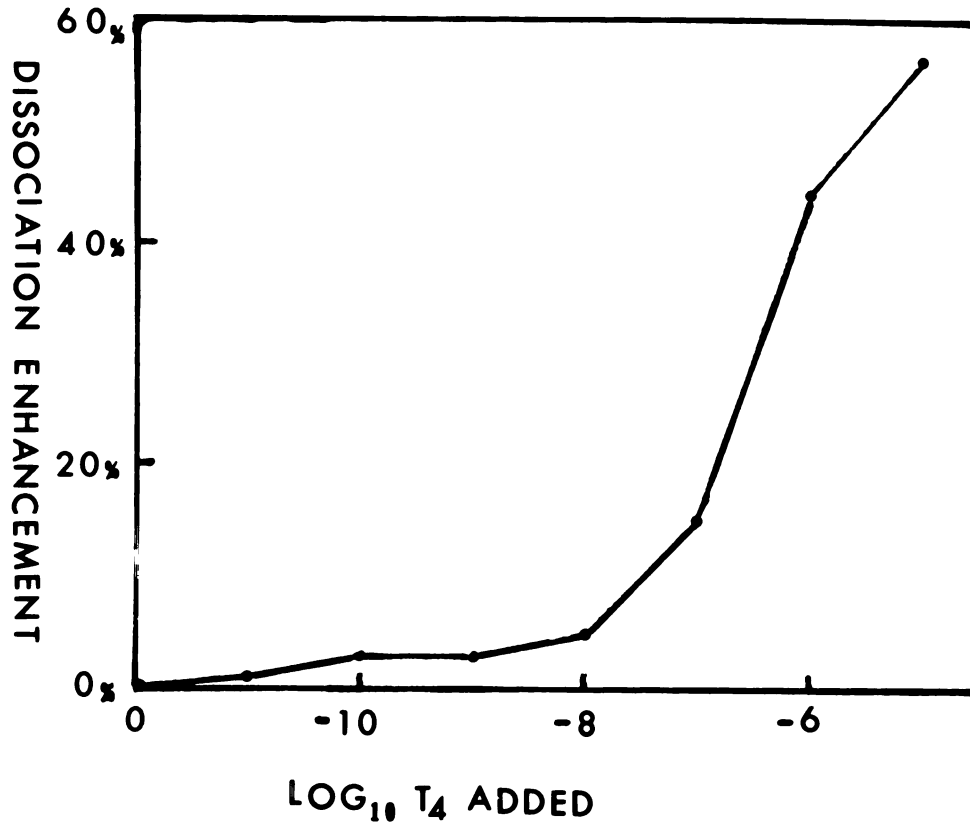
Fig. 4-4. Effect of unlabeled L-T_4 concentration on the dissociation of [^{125}I]T_4 bound to prealbumin. The binding of [^{125}I]T_4 to prealbumin was the same as described in the legend to Fig.4-3. 60 µl of the equilibrated [^{125}I]T_4-prealbumin complex were transferred to 5940 µl of Tris buffer at pH 7.6 in the absence or presence of unlabeled L-T_4(10^{-11}∼ 10^{-6}M). Nonspecific binding was measured in the presence of 2×10^{-5}M unlabeled L-T_4. After 30 min at 2-4°C, the bound [^{125}I]T_4 was separated from the free [^{125}I]T_4 as described in the legend to Fig.4-3. The radioactivity was counted. After correction for nonspecific radioactivity, the dissociation enhancement due to unlabeled L-T_4

$$= \left(1 - \frac{[^{125}I]T_4 \text{ bound with 100x dilution + unlabeled L-}T_4}{[^{125}I]T_4 \text{ bound with 100x dilution only}} \right) \times 100$$

was plotted as a function of the concentration of unlabeled L-T_4.

To linearize the data, the logarithm of [bound radioactivity at any time t]/[bound radioactivity at time 0] was plotted against time. Fig.4-5 illustrates the dissociation curves for the dissociation of the preequilibrated prealbumin-[^{125}I]T$_4$ complex after 100-fold dilution, combined with simultaneous addition of various amounts of unlabeled T$_4$. In the absence of L-T$_4$, the dissociation curve had a slope of 2.41×10^{-2}/min which was the dissociation rate constant (k_{-1}) according to 1st order kinetics. With increasing amounts of unlabeled L-T$_4$, the slope was constant until it reached 2×10^{-7}M, where k_{-1} was 4.27×10^{-2}/min, 80% more than the k_{-1} in the absence of L-T$_4$. Since concentrations less than 1×10^{-8}M did not generate appreciable dissociation enhancement, ligand exchange or unstirred Noyes-Whitney layers were not likely to facilitate dissociation enhancement (De Meyts et al., 1976; Pollet et al., 1977). The trend of increasing k_{-1} with increasing amount of unlabeled L-T$_4$ leveled off as shown in both Figs. 4-4 and 4-5. From the fitted values α and K, the association constant for the second site was $\alpha K = 3.67 \times 10^6 M^{-1}$, corresponding to a Kd of 2.7×10^{-7}M. Thus when L-T$_4$ concentration was around Kd (or Km, the Michaelis constant), half of the available sites would be occupied. Consequently, a sharp increase in k_{-1} at a L-T$_4$ concentration of 2×10^{-7}M was observed. Our results also indicate that more of the second binding sites occupied would accelerate the dissociation rate.

To further analyze any relationship between the dissociation rate constant and the fractional occupancy of L-T$_4$ at the second site while the first binding site was occupied by [^{125}I]T$_4$, the dissociation rate constant in the absence of L-T$_4$ was normalized to 100%. The ratios of the dissociation rate constants at various concentrations of L-T$_4$ were plotted as a function of corresponding fractional occupancy of the second site.
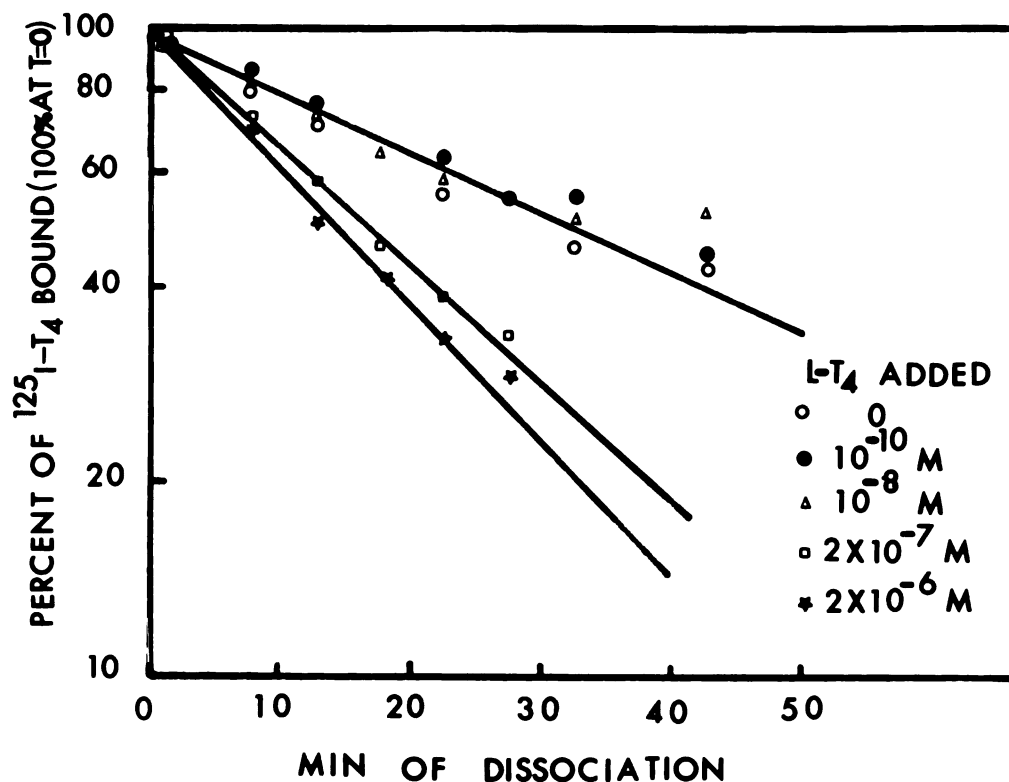
Fig. 4-5. Effect of unlabeled L-T$_4$ on the dissociation rate constants. The binding was studied as described in the legend to Fig.4-3. For each concentration of unlabeled L-T$_4$, an aliquot (60 μl) of equilibrated mixture was transferred to a series of tubes containing 5940 μl of the Tris buffer in the absence or presence of unlabeled L-T$_4$(10^{-10}~ 2x10^{-6}M). At intervals, duplicates from tubes were filtered as described in Fig.4-3; and the radioactivity was measured. After correction for nonspecific radioactivity, the radioactivity in the filtered fraction, expressed as a percentage of the radioactivity at time=0 min(onset time of the 100x dilution), was plotted as a function of the time elapsed after the dilution of the system. The dissociation rate constant was derived from the slope of this plot.
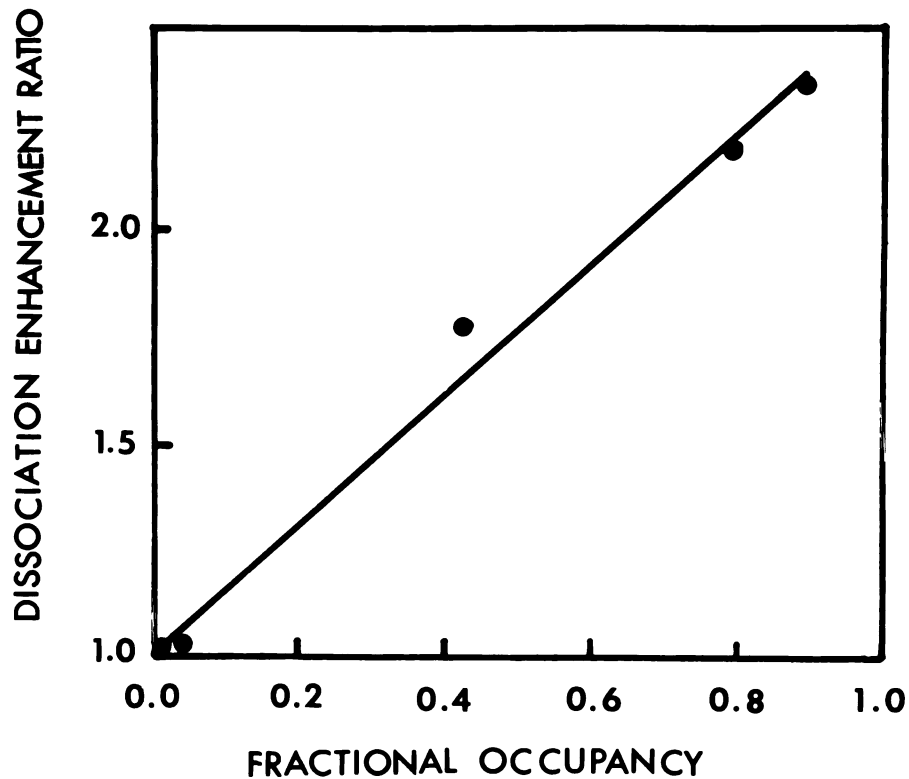
Fig. 4-6. Effect of fractional occupancy at the second binding site on the dissociation at the first binding site. The data in Fig.4-5 were employed to derive this plot. The fractional occupancy (the fraction of the second binding sites bound by unlabeled L-T_4 in the subpopulation of TBPA with the first site bound by [^{125}I]T_4) was calculated by using total sites=3.29×10^{-9}M and affinity constant=α K=3.67×10^6M^{-1}, as determined by the fitted Scatchard plot in Fig.4-1. Dissociation enhancement ratio was defined by the ratio

= dissociation rate constant in the presence of unlabeled T_4
   dissociation rate constant in the absence of unlabeled T_4

The data were fitted by the PROPHET program as described in the section of Materials an Methods. The correlation coefficient(r) was 0.995.

The linear curve in Fig. 4-6 suggested that the magnitude of dissociation enhancement of $[^{125}I]T_4$ bound at the first site was directly related to the fractional occupancy at the second site, and thus implied negative cooperative interaction between the two sites.

D. Effect of the First Site Occupancy on Dissociation

The ratios of dissociation rate constants in the presence and absence of $2 \times 10^{-7} M$ unlabeled $L-T_4$ versus molar ratios of protein/ligand (or the first site occupancy) are shown in Fig. 4-7. The prealbumin/$[^{125}I]T_4$ molar ratio spanned over a 100-fold range of 0.175-17.5, while the ratios of dissociation rate constants (dissociation enhancement ratios) varied from 1.24~4.51. In the presence of $2 \times 10^{-7} M$ unlabeled $L-T_4$, the dissociation rate constant increased with increasing prealbumin/$[^{125}I]T_4$ molar ratio. In the absence of unlabeled $L-T_4$, however, the dissociation rate constant at a prealbumin/$[^{125}I]T_4$ ratio of 17.5(1.09×10^{-2}/min) was much smaller than the values for other occupancies ($3.08~4.71 \times 10^{-2}$/min). The slower dissociation rate constant was probably in part due to the retention effect by the excess of prealbumin. It has been reported that when a solution of binding protein and its ligand is dialyzed against a large volume of ligand-free medium, the rate of exit of the ligand from the protein-containing compartment can be extremely slow, much slower than the rate observed in the absence of protein (Silhavy et al., 1977).

E. Effect of Thyroid Hormone Analogs on Dissociation

We next examined the relative effectiveness to render dissociation enhancement with tetrac, r-T_3, L-T_3 and triac. Previous studies indicated that tetrac (676%)binds several-fold better, and r-T_3 (32.9%) less better than $L-T_4$(100%). In Fig. 4-8, tetrac, as expected, yielded the greatest
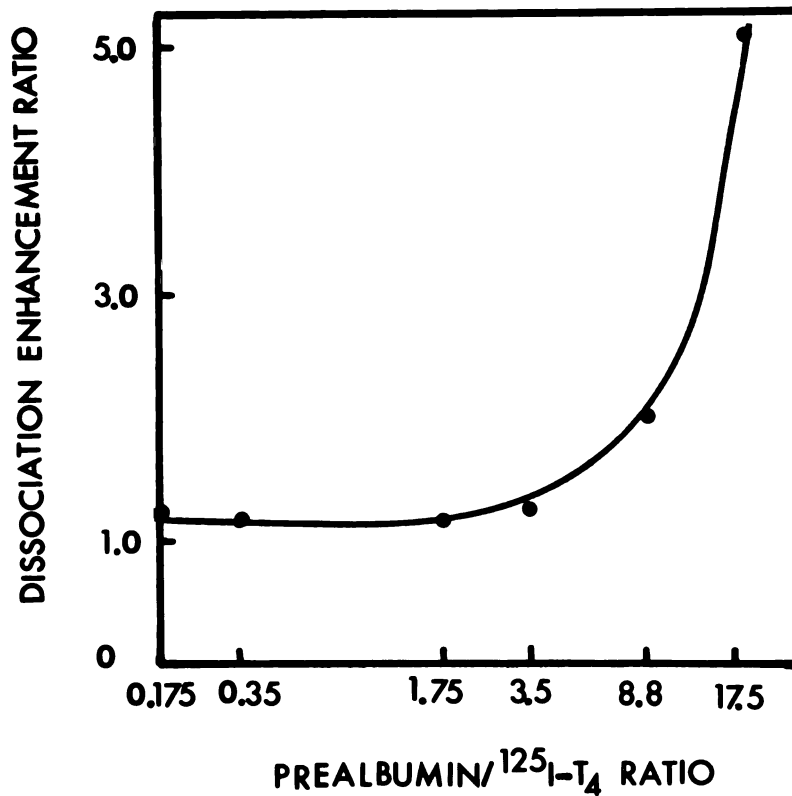
Fig. 4-7. Effect of fractional saturation of prealbumin binding sites by unlabeled L-T$_4$ on the dissociation of [^{125}I]T$_4$. [^{125}I]T$_4$(1.9x10^{-8}M) was incubated with various concentrations of prealbumin(3.0x10^{-7}M ~ 3.0x10^{-9}M) for 2 hours, and diluted 100-fold in the presence of unlabeled L-T$_4$(2x10^{-7}M), or absence (i.e.dilution only). The dissociation rate constants were derived from the slope of each dissociation curve as described in Fig. 4-5. The dissociation enhancement ratio was derived from the ratio of the dissociation rate constants in the presence and absence of unlabeled L-T$_4$, and was plotted as a function of prealbumin/ [^{125}I]T$_4$ ratio.
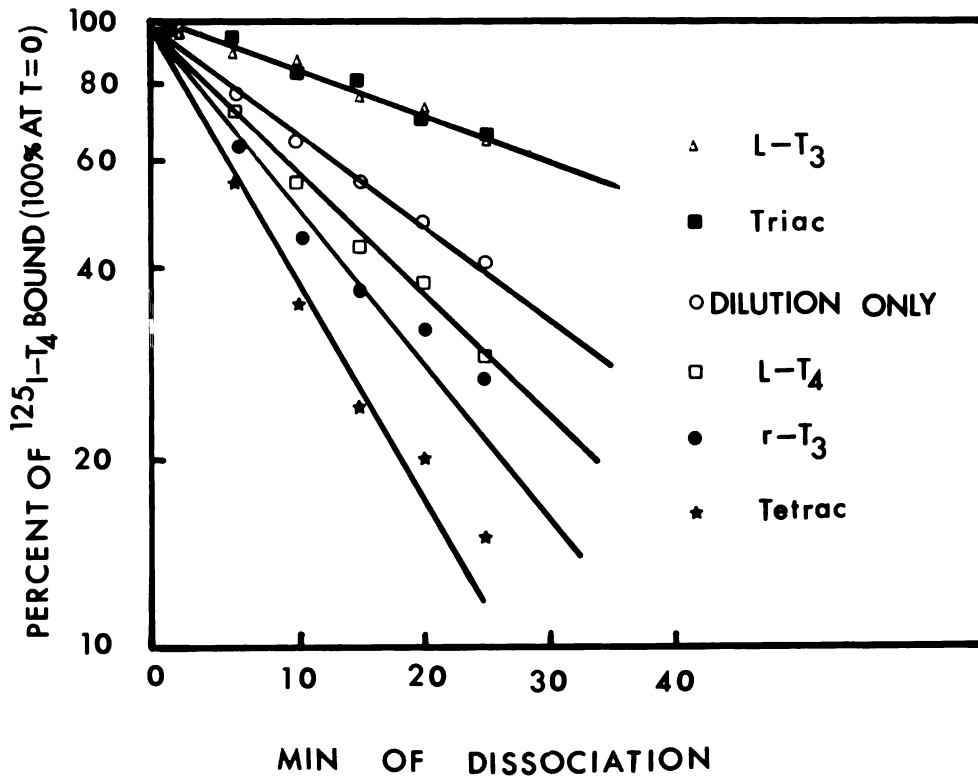
Fig. 4-8. Effect of thyroid hormone analogs on the dissociation of
[^{125}I]T$_4$. The binding and dissociation procedures were the same
as described in the legend to Fig. 4-5, except that unlabeled
analogs were used, and the concentration was fixed at 2×10^{-7}M.

dissociation enhancement among the analogs tested. However, r-T_3 was more effective than L-T_4 in producing dissociation enhancement at the same concentration. The second site might still prefer deaminated analogs such as tetraac; and yet for 3',5'-iodine analogs, it might prefer 3-iodine to 3,5-iodine analogs, due to the steric hinderance arising from the 5'-iodine on the inner ring for any conformational change as a consequence of negative cooperativity. This experiment again demonstrated that the occupant at the second site of prealbumin triggered the accelerated release of [^{125}I]T_4 bound at the first site.

Contrary to the enhancing effect of tetrac and r-T_3, L-T_3 and triac of which binding affinities to prealbumin are 9.2% and 19.7% of L-T_4, respectively (Andrea et al., 1980), impeded the dissociation process. Since the only common structural difference between the group including L-T_4, tetrac, and r-T_3, and the group including L-T_3 and triac was the 5'-iodine on the outer ring of the thyroxine backbone, this reversal of cooperativity from negative to positive illustrated the importance of the 5'-iodine, in addition to promoting binding affinity (Andrea et al., 1980).

IV. Discussion

Both thermodynamic and kinetic studies have been carried out to characterize the nature of binding of thyroid hormones to human plasma prealbumin. Most interestingly, the results show that analogs with only the 3'-I can impede the dissociation of [^{125}I]T_4 bound to TBPA. This is the first demonstration of reversal of negative to positive cooperativity in TBPA. By contrast, analogs containing both 3'- and 5'- iodine atoms can enhance the dissociation rate. The X-ray studies indicate that the 5'-I is in contact with Leu-17 and Leu-110 (Blake and Oatley, 1977).

Moreover, Oatley and coworkers have found that the 5'-I is surrounded by more hydrophilic environment than is the 3'-I (unpublished results of Oatley et al.). It is possible that the 5'-iodine atom causes easier formation of a phenoxide ion which in turn may be the trigger of the negative allosteric effect. Nevertheless, other contributions of the 5'-I, e.g. Van der Waals interactions, charge-transfer effect, and steric effect, can not be excluded. The detailed mechanistic features remain to be elucidated.

The results also show that the dissociation rate of $[^{125}I]T_4$ bound is dependent on the occupancy of the second binding site. Taken together, these findings are interpreted to indicate the existence of negative cooperativity between the two identical sites of prealbumin as revealed by X-ray studies (Blake et al., 1974, 1978; Blake and Oatley, 1977). Consequently, enhancement of dissociation of bound radioactive ligand by unlabeled ligand is a useful demonstration of negative cooperativity for receptor-ligand (or enzyme-substrate) systems.

In a two-site protein such as prealbumin, Fig. 4-9 illustrates the dissociation scheme after induction of 100-fold dilution. Assuming first-order kinetics, then as shown in Appendix III,

$$B(t) = P_{II}e^{-k_{-1}^{II}\,t} + [P_{II}(\,1 - e^{-k_{-1}^{II}t}\,) + P_I]\,e^{-k_{-1}^{I}t} \text{---------}(\text{ Eq. 6 })$$

where B(t) is the concentration of labeled ligand bound at time t after the induction by 100-fold dilution.

P_{II} is the population percentage at binding equilibrium with two sites bound .

P_I is the population percentage at binding equilibrium with one site bound .

POPULATION RATIO

$$P_{II} \qquad \frac{\alpha K^2 F^2}{1 + 2KF + \alpha K^2 F^2}$$

$$P_I \qquad \frac{2KF}{1 + 2KF + \alpha K^2 F^2}$$

$$P_o \qquad \frac{1}{1 + 2KF + \alpha K^2 F^2}$$

□ = subunit with filled binding sites

○ = subunit with vacant binding sites

▼ = $[^{125}I]T_4$

k^I_{-1} = dissociation rate constant for $[^{125}I]T_4$ when the two binding sites of TBPA are bound

k^{II}_{-1} = dissociation rate constant for $[^{125}I]T_4$ when only one binding site is bound
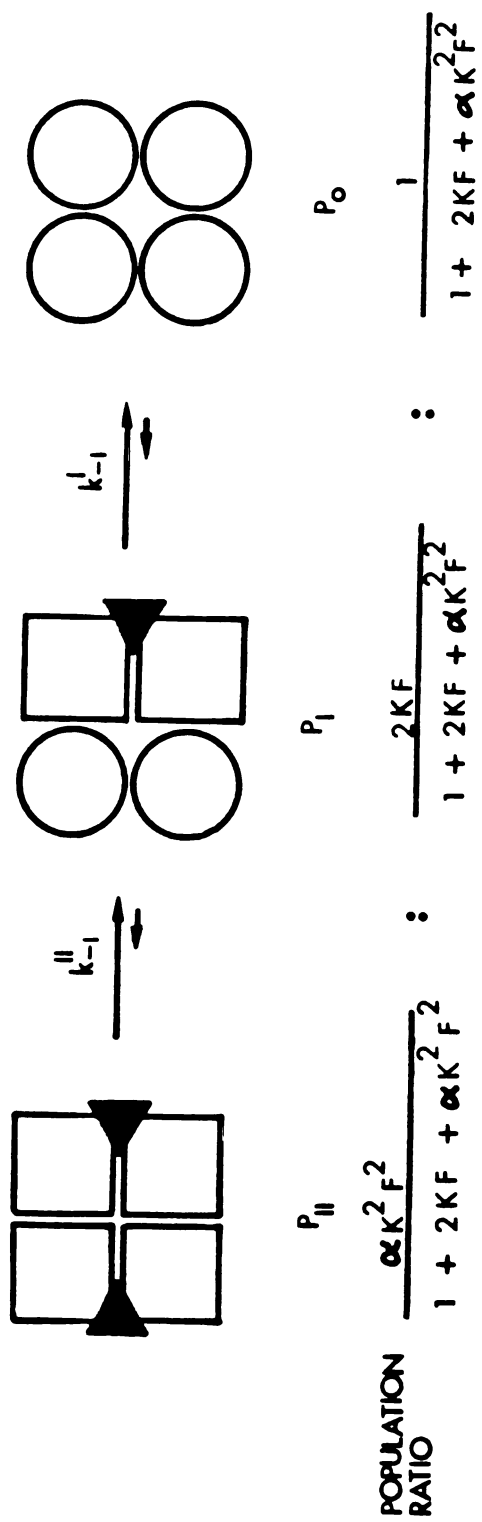
Fig. 4-9. A plausible scheme for the dissociation kinetics of $[^{125}I]T_4$ bound to TBPA. Intermediate step is illustrated as a mixed state with subunits in two conformations. The conformational change has to be ligand-induced. Because of the 100-fold dilution and an excess of unlabeled ligand, rebinding is almost, if not completely, prevented.

k_{-1}^{II} is the dissociation rate constant of P_{II} species.

k_{-1}^{I} is the dissociation rate constant of P_{I} species.

In our experimental conditions the prealbumin/$[^{125}I]T_4$ ratio was about 7:1, so that

$$P_{II} \ll P_I < P_0$$

Therefore Eq. 6 can be simplified to be

$$B(t) = P_I \; e^{-k_{-1}^{I} \; t}$$

for the case without the unlabeled L-T_4.

However, in the presence of unlabeled T_4, a substantial portion of P_I was converted to P_{II}' where the two binding sites of TBPA are again saturated; one by radiolabeled T_4, and the other by unlabeled T_4. In this case, k_{-1}^{II} was the dissociation rate constant to be measured, instead of k_{-1}^{I}. Since the dissociation rate constant was larger with addition of unlabeled T_4, it was concluded that $k_{-1}^{II} > k_{-1}^{I}$. The magnitude of dissociation rate constant was dependent on the concentration of P_{II}' which, in turn, was determined by the concentration of unlabeled T_4. Our studies confirm that the dissociation generally followed first-order kinetics (Figs. 4-5 and 4-6). However, in reports of other laboratories (De Meyts et al., 1973; Powell-Jones et al., 1979) the dissociation curves are not linear. Much larger ratios of receptor/ligand could result in a substantial increase of P_{II} and thereby a combination of two dissociation rate constants, k_{-1}^{II} and k_{-1}^{I}. In recent years, the generality of this kinetic approach has been criticized in four categories (Frazier et al., 1974; Verrir et al., 1974; Cuatrecasas et al., 1975; Limbird et al., 1975, 1976; Abou-Issa and Reichert, 1976; Caron et al., 1976; Pollet et al., 1977; Reichler et al., 1977;

Powell-Jones et al., 1979; Saltiel et al., 1980):

(1) Dissociation enhancement by unlabeled ligand is not always demon-
strated with receptor systems which have been claimed to have con-
cave upward Scatchard plots and negative cooperativity (Limbird
et al., 1976; Abou-Issa and Reichert, 1976; Pollet et al., 1977;
Reichler et al., 1977). The nonlinearity of these Scatchard plots
could be attributed to a mixture of both specific (high-affinity,
low-capacity) receptor sites and nonspecific (low-affinity, high-
capacity) sites due to incomplete correction for the nonspecific
binding. The ratio of [capacity of the first site]/[capacity of
the second site] often deviates from 1:1 (Abou-Issa and Reichert,
1976; Powell-Jones et al., 1979; Saltiel et al; 1980). Even if
there are high- and low-affinity sites, no dissociation enhancement
is likely to be detected with concentrations of unlabeled ligand
much less than the K_m for the second site (Pollet et al., 1977).

(2) Binding constants determined by kinetic studies are more consistent
with data determined by equilibrium studies, based on a model of
multiple independent classes of binding sites (Frazier et al., 1974;
Caron and Lefkowitz, 1976; Pollet et al., 1977; Powell-Jones et al.,
1979). However, first-order kinetics based on a one-site model is
used to derive binding constants for multi-site receptors. With
such a simplified model the contributions by the labeled ligand at
the second site to both dissociation and association are not accounted
for.

(3) Binding-site occupancy does not affect the magnitude of dissoc-
iation enhancement (Pollet et al., 1977; Powell-Jones et al., 1979).
Again this could have been due to insufficient concentration of

TABLE 4-1   Other Factors that can Generate Nonlinear
Scatchard Plots

Ligand	Receptor
1. polymerization (Nichol et al., 1969)	1. aggregation or
2. conformational isomers (Laiken and Nemethy, 1971)	2. deaggregation (Levitzki, 1974; Levitzki et al., 1975)
3. Ligand-ligand interactions (Ferguson et al., 1975)	3. mobile receptors (Jacobs and Cuatrecasas, 1976)

unlabeled ligand to produce a substantial fractional occupancy at the second site.

(4) Dissociation enhancement occurs in some receptor systems with linear Scatchard plots (Verrier et al., 1974; Limbird et al., 1975; Saltier et al., 1980). The reason is not clear. Nonetheless, the linearity of the Scatchard plot needs to be reexamined for reproducibility with more variation of fractional occupancy.

In addition to the two best known reasons to render a nonlinear Scatchard plot, other factors are summarized in Table 4-1. These possibilities can be ruled out by the following facts in the case of $L-T_4$ binding to prealbumin.

(1) Since $L-T_3$, an analog of $L-T_4$, does not produce in nuclear binding a nonlinear Scatchard plot (Bolger and Jorgensen, 1980), polymerization of hormones, conformational isomers, or isotope effects are unlikely to contribute to the curvilinear nature in the Scatchard plot of $L-T_4$ binding to prealbumin (Nichol et al., 1969; Laiken and Nemethy, 1971)

(2) The ligand-ligand interactions, e.g. steric hindrance or electrostatic repulsion (Ferguson et al., 1975),are not favored in the case of prealbumin, because of the structural isolation by the narrowness (4 Å) at the center of prealbumin; and the fact that the electrostatic charges of the zwitter ion are at the entrances of binding channels, which are far apart and related by the C_2 symmetry as shown by X-ray structure studies (Blake et al., 1974, 1977, 1978).

(3) TBPA is known to be exceptionally resistent to acids, bases and denaturing reagents (Branch et al., 1971, 1972; Nilsson et al., 1975).

No aggregation or deaggregation would occur under our experimental
conditions. Molecular weight determination by equilibrium sedi-
mentation also shows that the tetrameric structure is predominent
under physiological conditions (Branch et al., 1971). In addition,
Sephadex G-200 chromatography demonstrates only one single peak
with a molecular weight corresponding to the tetramer (Rask et al.,
1971).

(4) TBPA used in our study was in the purified, homogeneous form as
verified by SDS polyacrylamide gel electrophoresis in Chapter Two,
thus no such effector (or effectors) as described in "mobile receptor"
theory would be present (Jacobs and Cuatrecasas, 1976).

It is noteworthy that the concept of dissociation enhancement is very
important and diagnostically useful in receptor studies. Firstly, the
linearity of a Scatchard plot may not be truly linear, due to experimental
errors or lack of enough data points extending to the side of full occupany
of binding sites. If it is indeed linear, there should be no dissociation
enhancement in the presence of high enough concentration of unlabeled ligand;
otherwise any detection of dissociation enhancement would indicate the pos-
sibility of a nonlinear curve. Secondly, the presence of a nonlinear
Scatchard plot may be due to heterogeneous receptors or simply contamination
by nonspecific binding, other than the possibility of negative cooperativity
for homogeneous receptors. For heterogeneous receptor or nonspecific binding
there would be no dissociation enhancement.

In the binding of oxygen to hemoglobin, Monod, Wyman, and Changeux (res-
ponsible for MWC model) (Monod et al., 1965) demonstrated that cooperativity
can be explained in a very elegant but simple manner by assuming that a

small fraction of deoxyhemoglobin exists in the quarternary oxy state

(R-state) which binds oxygen more avidly than the deoxy state (T-state).

Upon binding one mole of oxygen the concentration of the oxy state is

increased,since oxygen binds preferentially to it and shifts the equili-

brium toward it. When a sufficient number of oxygen molecules are bound,

the oxy state is sufficiently enriched to be the major state in solution,

so that subsequent binding is more favored. This model is further gener-

alized to include other allosteric proteins by making the following assum-

ptions:

(i) The proteins are multimeric.

(ii) At equilibrium such proteins exist in either of the two conform-
ational states, T(tense), the predominent form when unbound; and
R(relaxed), when bound. The two states differ in the energies and
number of non-covalent bonds between subunits so that the T state is
constrained as compared to the R state. No intermediate state is
allowed (i.e. all or none).

(iii)The R state has a higher binding affinity for ligands than the T state.

(iv) All binding sites in each state are symmetrically related and equiv-
alent, and have the same binding constants.

In the MWC "all or none" model the assumption that is most open to criticism

is about the symmetry of binding sites. By reducing the number of inter-

mediate states, the model is at best an approximation to reality. But this

weakness is also the strength of the model, because it simplifies the

problem. Proteins in the category, to name a few, are aspartate transcar-

bamylase, deoxy thymidine kinase, pyruvate kinase, in addition to hemoglobin.

The other well known model (KNF "sequential"model) was hypothesized by

Koshland, Nemethy and Filmer (1966). The KNF model does not require symmetry

for binding sites, but uses another simplifying feature. It requires that
the transition from the T to R state be a sequential process. The confor-
mation of each sub-unit changes in turn as it binds ligand and there is no
abrupt switch from one state to another.

The two assumptions in the KNF model are:

(i) In the absence of ligands the multimeric protein exists in one
     conformation.

(ii) Upon binding there is a ligand-induced conformational change in the
     sub-unit to which the ligand is bound. This change may be trans-
     mitted to neighboring vacant subunits via quarternary contacts.

By considering intermediates the KNF model is more general but is often
somewhat more complex mathematically. Negative cooperativity cannot be
explained by the MWC "all or none" model, because the binding of the ligand
can only enrich the subpopulation of the high-affinity R state, but not
that of the low-affinity T state. The KNF model explains negative cooper-
ativity by induced affinity change originated from conformational change
of the protein upon binding the ligand. Negative cooperativity is thus a
diagnostic test for the KNF model. In a recent study, Irace and Edelhoch
(1978) found a blue shift in protein UV absorption with prealbumin, and a change in
the total absorption   confined to the binding of the first thyroxine
molecule. Further, although a blue shift was observed in the absorption
spectrum of thyroxine, absorbance change was the same for the first and
second bound molecule. These data have been interpreted in terms of two
identical and interacting sites on prealbumin and explain the origin of the
differences in binding affinities as a result of a conformational change.
Furthermore, fluorescence queching and increased tryptic digestion in the
presence of thyroxine were also in agreement with the above interpretation.

The data discussed thus far concerning dissociation enhancement by unlabeled

thyroxine and conformational change upon binding thyroxine reflect that

the KNF "sequential" model is more acceptable than the MWC "all or none" model.

Negative cooperativity in hormone-receptor binding has also been reported

for nerve growth factor (Frazier et al., 1974), thyrotropin-releasing hormone

(Kohn et al., 1976) and β-adrenergic catecholamines (Limbird et al., 1975,

1976). Nonetheless, what is the physiological relevance of having negative

cooperativity? It has been reasoned that negative cooperativity provides

a mechanism by which receptor binding is much more enhanced at low concen-

tration of hormone in contrast to the cases of positive cooperativity or to

normal Michaelian cases; but becomes damped at high concentration as illus-

trated in Fig. 4-10.
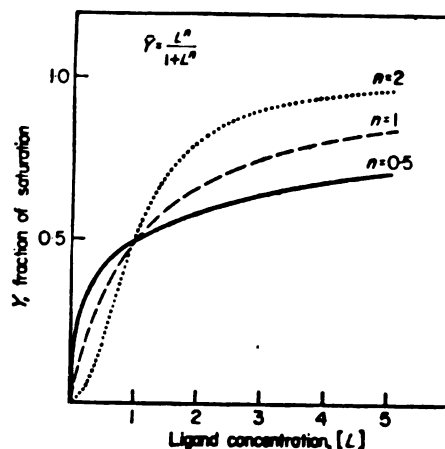
Fig. 4-10. The behavior of ligand saturation curves in Michaelian,
positively co-operative and negatively co-operative systems. The
plots were constructed from the equation $Y=L^n/(1+L^n)$ where n
assumes the values n=1 (Michaelian), n=2 (positively co-operative)
and n=0.5 (negatively co-operative).(After Levitzki, 1975).

Further, only low ligand occupancy is needed to elicit maximal response, and thus most receptors remain as "spare receptors". Intermolecular negative cooperativity as originated from conformational change can be further generalized to cover cases in which conformational change is transmitted via "clustering" of receptors on surface of "fluid" membrane (Levitzki et al., 1974, 1975). As with all these classes of receptors the conformational change which impedes further binding of the hormone could well be the molecular event triggering subsequent steps in the chain of physiological responses initiated by hormone binding.

In conclusion, our study confirms the general validity of the kinetic approach by De Meyts, to detect negative cooperativity for insulin receptors (De Meyts et al., 1973, 1976). This kinetic approach rests on the finding of enhancement of dissociation of bound radiolabeled ligand from its receptors in the presence of unlabeled ligand during dilution-induced dissociation. In our study, it is demonstrated with human plasma prealbumin (TBPA) which has a concave, upward Scatchard plot and a Hill coefficient less than unity that

(1) there is dissociation enhancement of bound $[^{125}I]T_4$ with a chasing dose greater than $10^{-7}M$.

(2) the magnitude of dissociation enhancement is dependent on the fractional occupancy of binding sites.

(3) the magnitude of dissociation enhancement is also related to relative binding affinities of thyroid hormone analogs to prealbumin, and 5'-iodine on the outer ring of the thyroid hormone backbone may be instrumental in modulation of binding cooperativity.

Prealbumin indeed is an ideal model for studies of inhibitory allosteric effects on ligand-binding to receptors by the kinetic approach of dissociation enhancement.

CHAPTER FOUR : PART II -- IMPLICATIONS ON THE QUARTERNARY STRUCTURE
OF THE NUCLEAR RECEPTOR FROM STUDIES OF LIGAND
DISSOCIATION ENHANCEMENT MEASUREMENT

I. Introduction

As discussed earlier, TBPA has been proposed as a model for the nuclear thyroid hormone receptor, based on similarities in ligand binding behavior, possible role as DNA binding proteins, and molecular weight. Since we have discussed ligand behavior in detail, we here put our emphasis on the latter two aspects.

The thyroid hormone receptor is generally believed to be a DNA-associated binding protein. Since Surks et al. (1973) found that specifically bound thyroid hormones are associated with the chromatin in the nucleus, several subsequent studies have supported this viewpoint. For instance, Charles et al. (1975) attempted to treat chromatin containing T_3 binding capacity with formaldehyde, which fixes histones and a small percentage of the acidic proteins to the DNA, but does not fix randomly selected proteins (Doenecke and McCarthy, 1975). When treated preparations were centrifuged in cesium chloride gradient, radiolabeled-T_3 sedimented with the DNA. Further, DeGroot and associates (1974) and Bernal et al. (1978) found that T_3-receptor complexes bind to chromatin.

The microenvironment of the loci of nuclear receptors has also been examined with the use of nucleases. Jump and Oppenheimer (1980) treated hepatic nuclei from euthyroid rats injected with $[^{125}I]T_3$ by limited digestion with deoxyribonuclease I and micrococcal nuclease. This treatment released a discrete $[^{125}I]T_3$-labeled chromatin fragment (5.8S) which is larger than the T_3 nuclear receptor (3.5S). Gardner (1978) presented

results that the thyroid hormone receptors are preferentially associated with nucleosome oligomers. Gottesfeld et al. (1974, 1976) have obtained evidence that DNAase II treatment of chromatin releases a fragment that is enriched in actively transcribed gene sequences. But both Levy and Baxter (1976) and Samuels et al.(1977) showed that most of the receptors are not located in the transcriptively active region. To reconcile this with the concept that T_3-receptor complexes initiate a good number of physiological processes, it is possible that only a small portion of receptors bound with T_3 is needed to induce some kind of general and widespread change in the chromatin and most of the receptors remain to be "spare" receptors. These findings are consistent with the viewpoint that the nuclear receptors bind to DNA in the internucleosomal spacer region.

On the other hand, X-ray diffraction studies of TBPA structure by Blake and coworkers (1974, 1977, 1978) have found a putative DNA binding site formed by symmetrical β-pleated sheets containing Trp-41, Trp-41' residues and other ionic amino acid residues. It was hypothesized that the tryptophans could act as intercalators between the stacked base pairs of the B-form DNA and that this binding interaction could receive further contributions from the electrostatic attraction between the charged amino acids and the phosphates on the backbone of the DNA.

Secondly, TBPA has a molecular weight of 55,000 daltons, determined by polyacrylamide gel electrophoresis and equilibrium sedimentation (Branch et al., 1971) and molecular sieving gel chromatography (Rask et al., 1971). Further, this was confirmed from the studies on amino acid analysis and sequencing (Kanda et al., 1974). The subunit size and analysis by gel chromatography for equilibrium sedimentation (Branch et al., 1971;

Rask et al., 1971) gave values of 14,000 ~ 15,500. These results suggest

that TBPA consists of four subunits. Similar conclusions have been reached

by Alper et al. (1969), based on hybridyzation studies of the genetic

polymorphism of TBPA in rhesus monkeys. Moreover, peptide mapping of the

trypsin digest and the NH_2-terminal analysis indicate that the four sub-

units are identical. The study of TBPA structure culminated in the X-ray

structure determination which verified previously accumulated results.

In contrast, Sephadex G-100 and QAE-Sephadex gel chromatography of nuclear

receptor-containing extract display one major included peak with $[^{125}I]T_3$

binding capacity (Surks, et al., 1973; Latham et al., 1976). The mole-

cular weight was calculated to be 50,500 daltons.

In the preceeding Part I, we have used TBPA as a model system to show

that negative cooperativity in the binding of thyroxine to TBPA can be

demonstrated by the ligand dissociation enhancement, and this inhibitory

allosteric interaction is probably a result from subunit-subunit inter-

action communicated in the message of a conformational change. Therefore,

studies of ligand dissociation kinetics which is a highly sensitive metho-

dology by the use of radiolabeled hormone can gain further information on

the subunit structure of receptors. In view of the limited quantity of

nuclear receptors in cells and the difficulties in the purification, as

mentioned before, we want to present evidence which indicates the lack of

negative cooperativity in the case of the nuclear receptor and discuss

implications on the quarternary structure of the receptor.

II. Methods

Effect of unlabeled L-T_3 on the dissociation kinetics   Solubilized

nuclear receptors were used. The preparation has been described in detail

in Chapter Two. Measurement of dissociation at 22±1°C was carried out

by 100-fold dilution of preequilibrated (4 hours at 25°C) nuclear receptor-$[^{125}I]T_3$ complexes (60 µl) in 5940 µl of the phosphate buffer as described in Chapter Two, alone or containing various amounts of unlabeled L-T_3 ($1x10^{-8}$M ~ $1x10^{-6}$M). After indicated periods, aliquots (0.4 ml) were filtered to separate the bound from the free $[^{125}I]T_3$ as mentioned in Chapter Two. Nonspecific binding was determined by the remaining bound radioactivity of the sample with $1x10^{-5}$M unlabeled L-T_3, and was corrected as described in Chapter Two.

III. Results and Discussion

The dissociation curves for bound $[^{125}I]T_3$ are depicted in Fig.4-11 (individual fitting) and Fig.4-12 (overall fitting). The dissociation rate constants are fitted by the PROPHET computer system (Raub, 1974) as described earlier. The values of the dissociation rate constants and the squares of correlation coefficients (r^2) are presented in Table 4-2.

Statistical analysis indicated that the data obtained in three different concentrations of unlabeled L-T_3 can be included into a single dissociation curve within statistical significance level (as judged by the F-distribution test in analysis of covariance). Therefore, there is no dissociation enhancement in the case of $[^{125}I]T_3$ bound to the nuclear receptors; in turn, the "negative cooperativity" phenomenon present in TBPA is not characteristic of the receptor system.

As discussed before, one of the requirements to be an intermolecular allosteric protein is to be composed of multiple subunits (>1). Since this dissociation study indicated no negative cooperative interaction for the nuclear receptor system, alternatives for quarternary structure

LOG(%BOUND)
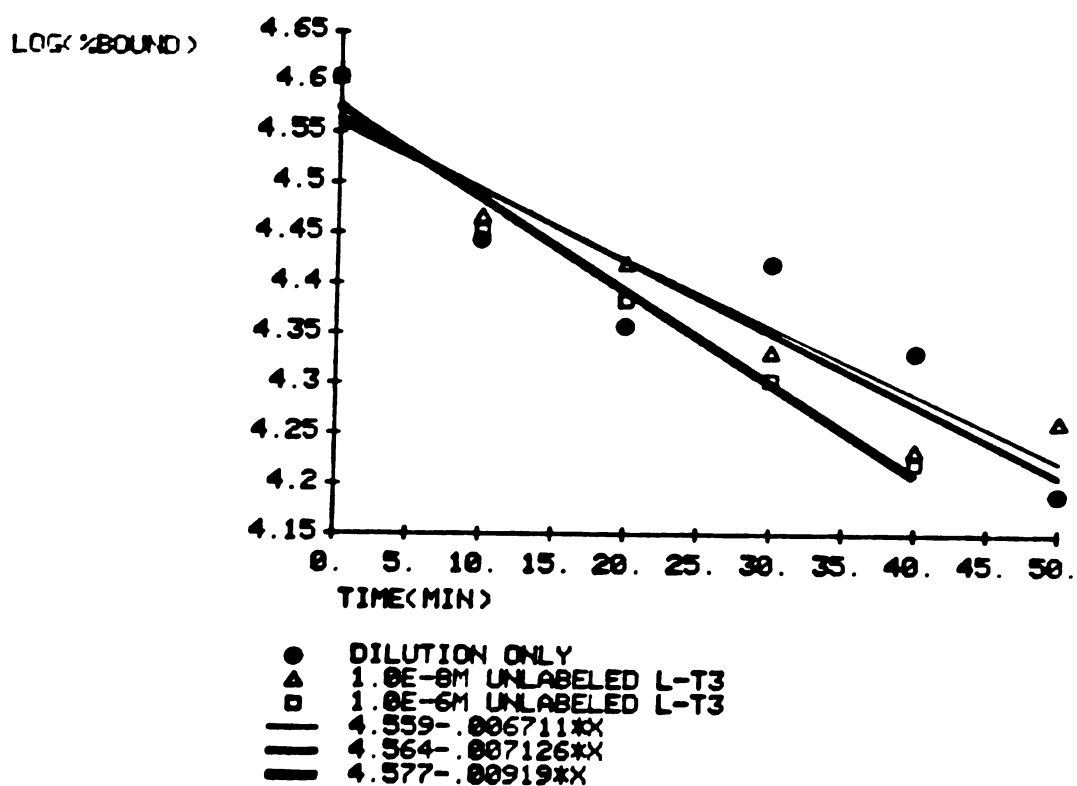
Fig. 4-11. Individual fittings of dissociation curves for
[^{125}I]T$_3$ bound to the nuclear receptor at 22°C.
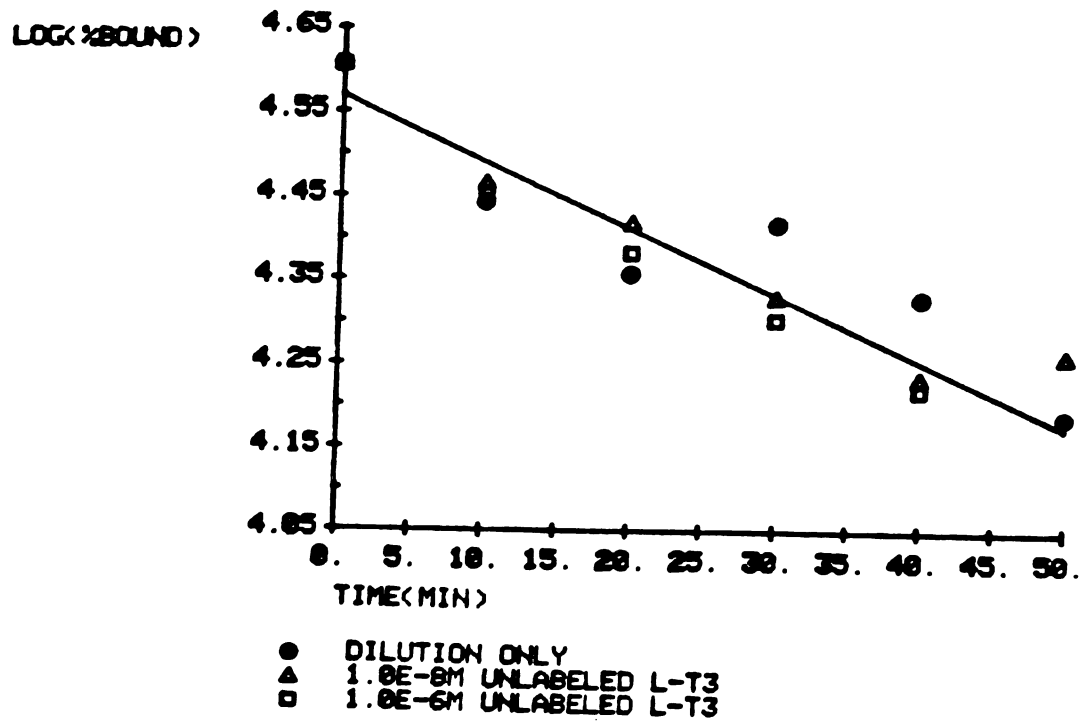
Fig. 4-12. Overall fitting of dissociation curves for [^{125}I]T$_3$ bound to the nuclear receptor at 22°C.

TABLE 4-2. Dissociation Rate Constants, Correlation Coefficients
and Half-Life of L-T$_3$-Nuclear Receptor Complex

Concentration of L-T$_3$(M)	Dissociation Rate Constant(min^{-1})	r^2	t½(min)
0	0.00671	0.835	103
1.0 x 10^{-8}	0.00712	0.919	97
1.0 x 10^{-6}	0.0092	0.977	75
Total	0.0073	0.88	95

in the nuclear receptor are limited to:

(1) a multi-meric protein, yet lacking negative cooperativity; or

(2) a monomeric protein that cannot have negative cooperativity.

Recently , the nuclear receptor has been reported to be covalently labeled in a specific manner with the N-bromoacetyl derivatives of $L-T_4$ and $L-T_3$ (Nikodem et al., 1980). N-bromoacetyl-T_3 competes for the same binding site with $L-T_3$ or T_4. High pressure liquid chromatography (HPLC) shows identical position for nuclear receptors whether bound with T_3, T_4 or bromoacetyl derivatives. SDS (sodium dodecyl sulfate) polyacrylamide gel electrophoresis gives one major radiolabeled component with a molecular weight of 56,000. Moreover, identical mobility is found for the nuclear receptor either bound to $[^{125}I]T_3$ or covalently labeled with N-bromoacetyl-$[^{125}I]T_3$ in the absence of SDS. It is suggested that the nuclear protein is a single-chain polypeptide.

In view of this new development, if verified, our dissociation data would tend to be in favor of a monomeric structure for the nuclear receptor. Thus, there may be a substantial difference in the quarternary structure between the nuclear receptor and TBPA. Since these two proteins have very similar molecular weight, and both have been postulated to have DNA-associated properties, in addition to some similar thyroid hormone-binding characteristics, it will be interesting to find out whether or not they are evolutionally related, and how. Pre-transcriptively by gene splitting, or post-transcriptively by m-RNA processing? Or could it be the kind of relationship between proinsulin and insulin, with removals of small segments of peptide in between subunits post-translationally to account for the difference in subunit structure? This will be further discussed in Chapter Six.

CHAPTER FIVE : INVESTIGATION OF BINDING CHARACTERISTICS OF ALTERED THYROID HORMONE RECEPTORS

I. Introduction

Biochemical studies by Eberhardt and coworkers (1979 ab) have suggested that the thyroid-hormone receptor, an acidic non-histone nuclear protein, exists as a "holo"-complex. This holo-complex consists of a "core"-subunit which has thyroid-hormone binding sites and a regulatory subunit, possibly histone components. The "holo"-receptor binds thyroid hormone analogs according to their thyromimetic potencies (e.g. $L-T_3$ > $L-T_4$ etc.). The core-receptor, however, binds $L-T_4$ more strongly than $L-T_3$. This is similar to the binding mode of TBG and TBPA.

The core-receptor can be generated from the holo-receptor by several means; including: (i) brief heating at 50°C for 10 min , (ii) dilution of the holo-receptor preparation, (iii) acidification from pH 7.6 to pH 6.0. All the three preparation cause a loss of binding capacity for $L-T_3$, but not for $L-T_4$ (Eberhardt et al., 1979 ab). Furthermore, the binding affinity of $L-T_3$ in the latter two cases is not changed, while there is a 200-fold decrease in $L-T_3$ binding affinity in the former case.

Reconstitution experiments of the core-receptor have been carried out with histones or heated choromatin extracts (Eberhardt et al., 1979 ab). The high-affinity of $L-T_3$ binding to the holo-receptors can be restored by addition of core-histones (H_2A, H_2B, H_3, H_4) and, to a less extent, by certain H_1 subfractions. However, this type of interconversion between the holo- and the core-receptor has been demonstrated with dilution- or acidification-converted preparations. There is only limited success for

spontaneous reversion to holo-receptors in the case of heat-converted preparation (Baxter et al., 1979) The relationship among these three preparations is not clear. From now on, unless stated otherwise, the nuclear receptor is designated as the holo-receptor, whereas the heat-treated receptor as the altered receptor. The recent finding prompted us to perform further binding studies to characterize the altered receptor with thirty thyroid-hormone analogs. It is hoped that our systematic approach by utilizing analogs can shed some light on the functional inter-relationship among these binding proteins.

In this study, we employed heat-treated altered receptors to examine:

(1) pH Profile of $L-T_4$ and $L-T_3$ binding.

(2) Temperature dependence of binding affinity of $L-T_4$.

(3) The binding specificities of thyroid-hormone analogs to the altered receptor with structural variations, not only on the outer-ring, but also on the inner-ring and the side chain (including DL optical isomers and desamino analogs).

(4) Contributions to binding free-energies by individual substituents.

(5) Comparisons of the binding data of the altered receptor with those of TBPA and TBG.

II. Preparation of Heat-Treated Nuclear Extract

Five to ten ml of the crude nuclear extract were incubated in a water bath at 50 ± 1°C for 10 min with intermittent shaking. After heating, this extract was removed and chilled immediately on ice, and centrifuged at 15,000 xg for 10 min at 4°C in a RC-5B Sorvall centrifuge to eliminate precipitated materials.

III. Elution Profile of $[^{125}I]T_4$ by Sephadex G-25 Gel Filtration

This buffer used in this chapter was made of 50 mM sodium phosphate (pH 7.6), 0.2 M $(NH_4)_2SO_4$, 1 mM EDTA, 0.2 mM dithiothreitol, and 5% glycerol. The elution profile of the binding assay mixture, drawn from data obtained by Sephadex G-25 (medium) minicolumn gel chromatography at $25\pm1°C$, is shown in Fig. 5-1. The triangles represented a standard incubation containing heat-treated thyroid-hormone receptors and $[^{125}I]T_4$. The circles represented a parallel incubation, except a 1000-fold excess of unlabeled $L-T_4(1x10^{-6}M)$ was added. The first peak to emerge from the column was contained in the excluded volume (1.4 ml) and was the altered receptor-- $[^{125}I]T_4$ complex. The second peak at 2.3 ml was $[^{125}I]$ iodide. The third peak of $[^{125}I]T_4$ was not eluted until between 5.0 and 8.5 ml of elution volume, in which the elution buffer must be switched to 0.25 M NaOH. When comparison between the elution profiles of holo-receptor (Bolger and Jorgensen, 1980) and altered receptor binding was made, their similarity could be clearly seen. This could be explained on the basis of exclusion due to high molecular weight.

IV. Incubation Kinetics

Optimal incubation time was determined at different temperatures (4°C and 15°C), as illustrated in Fig. 5-2. Unless stated otherwise, experimental procedures were the same as described previously. At indicated time points, duplicate aliquots of 0.4 ml of the incubation mixture in parallel sets of tubes at each temperature were drawn to determine total bound radioactivity and nonspecifically bound radioactivity, respectively. It was found that 4-6 hours were needed to reach equilibrium for the altered receptor preparation.
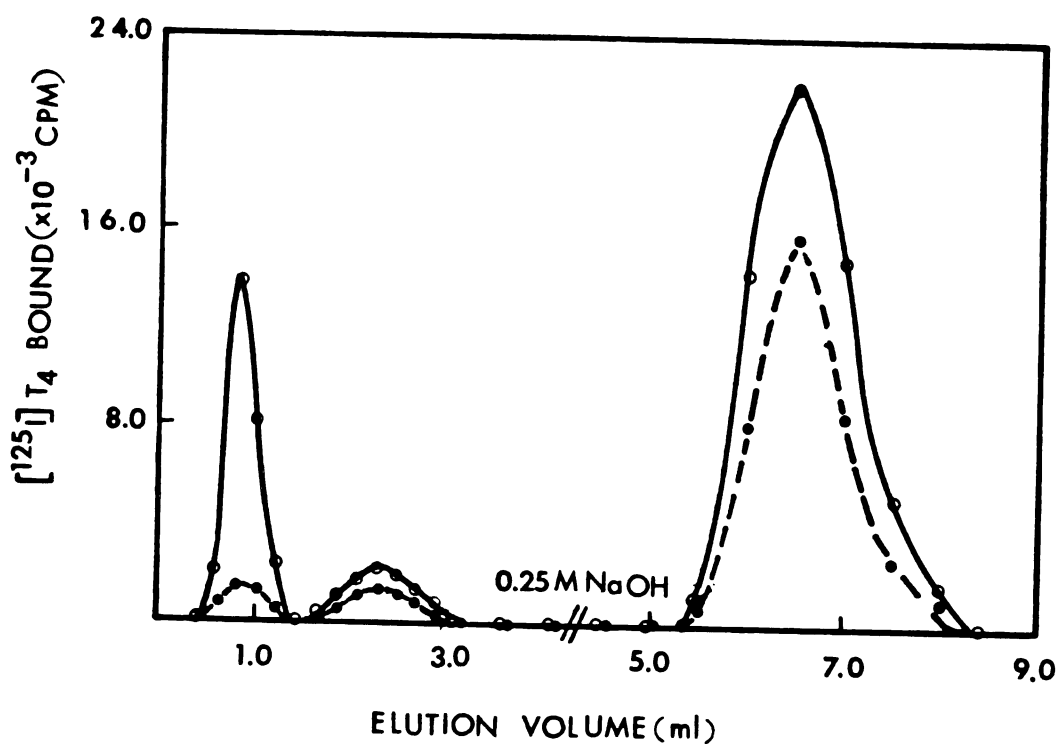
Fig. 5-1. Elution profile of $[^{125}I]T_4$ at 4°C. The incubation
mixture (0.5 ml) contained $[^{125}I]T_4$(0.3 ml), and the altered
receptor preparation (0.1 ml) in the absence (—O—) or
presence (--●--) of unlabeled L-T$_4$(1 μM), and was eluted with
the incubation buffer (50 mM sodium phosphate, pH 7.6, 0.2 M
ammonium sulfate, 1.0 mM dithiothreitol, 5% glycerol) through
Sephadex G-25 minicolumns (bed volume=2.0 ml). The incubation
was performed at 25°C for 4-6 hours. The first peak from the
left represented L-T$_4$ bound to the altered receptor. The
second and third represented iodide and free L-T$_4$ respectively.
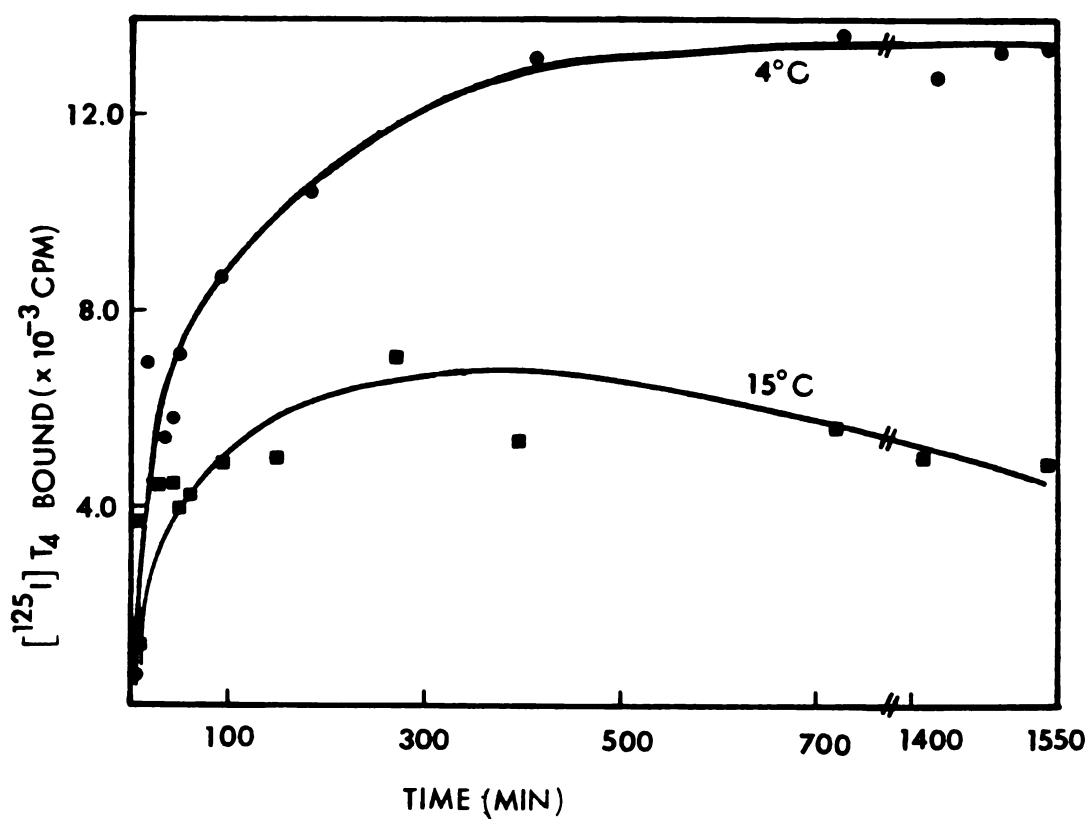
Fig. 5-2. Kinetics of incubation. Samples of altered receptor preparations were incubated for various length of time at 4°C and 15°C in the incubation buffer as described in Fig. 5-1. Incubations were terminated by chilling (5 min in an ice bath) and were assayed for specific binding radioactivity as described under "Incubation Kinetics".

V. pH Dependence of Altered Receptor Binding to [^{125}I]T$_4$ and [^{125}I]T$_3$

Standard 0.5 ml incubation mixture contained heated receptor (50 µl, 70 ug of protein), 2 nM of [^{125}I]triiodothyronine, or [^{125}I]thyroxine, and various buffers: 25 mM citric acid /50 mM NaH$_2$PO$_4$(pH 4.8 ~ 5.4), 0.1 m NaH$_2$PO$_4$ (pH 5.5 ~ 7.5), 50 mM Tris (pH 7.6 ~ 8.4), or 0.1 m Na$_2$CO$_3$/ NaHCO$_3$ (pH 8.5 ~ 10.3). In all the buffers (NH$_4$)$_2$SO$_4$ was used to standardize ionic strength to 0.6 to match that of the incubation buffer. Assay mixtures were incubated at 25±1°C for 4-6 hours. Parallel incubations containing excess unlabeled L-T$_4$ (1x10^{-6}M) were used as described under "Methods" in Chapter Two to determine nonspecific binding at each pH. The profiles illustrated in Fig. 5-3, 5-4 included pH-dependence data of holo-receptor binding by Latham et al. (1976) for the purpose of contrast. As seen in Fig. 5-3, there was virtually no difference between these two receptor forms with regard to L-T$_4$ binding. In contrast, Fig. 5-4 showed a sharply decreased binding for L-T$_3$ at all the pH's examined. In addition, the optimal pH was also shifted to 8.4 (the pKa of L-T$_3$). It appeared that ionization of the phenolic hydroxyl group could facilitate the binding interaction. As discussed in Chapter Two, this feature was in common with serum binding proteins (TBPA, TBG, and HSA).

VI. Thermodynamic and Kinetic Properties of L-T$_4$ Binding to Altered Nuclear Receptors

Scatchard plots for L-T$_4$ at various temperatures (4°C, 11°C, 15°C, 25°C) were drawn in Fig. 5-5 from data obtained by using procedures as described in Chapter Two. Initially, the curvature of this plot looked as if there were two classes of binding sites. Subsequently an experiment of

Fig. 5-3.

Fig. 5-4.

PH
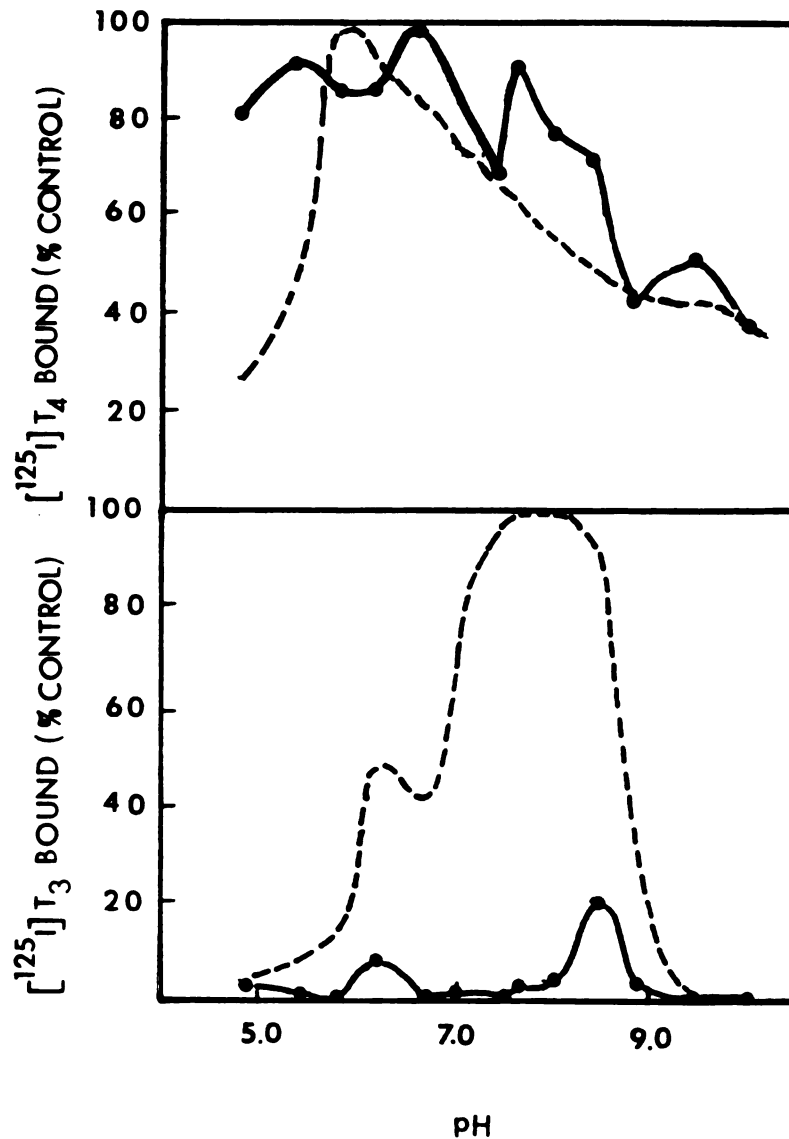
Figs.5-3 and 5-4. pH dependence of [^{125}I]T$_4$ and [^{125}I]T$_3$ to altered receptors. Standard incubation mixtures contained altered receptors (50 μl), 5 mM[^{125}I]T$_4$ (above) or [^{125}I]T$_3$ (below) were incubated with various buffers as stated under "pH Dependence of Altered Receptor Binding to [^{125}I]T$_4$ and [^{125}I]T$_3$". Reaction mixtures were incubated at 25℃ for 4½ hours. In both plots solid line (—●—) represented the altered receptor binding from our study, while dotted line (– – – –) represented the holo-receptor binding extracted from the results of Latham and associates(1976). The curve for L-T$_3$ binding to the altered receptor was enlarged 66-fold.
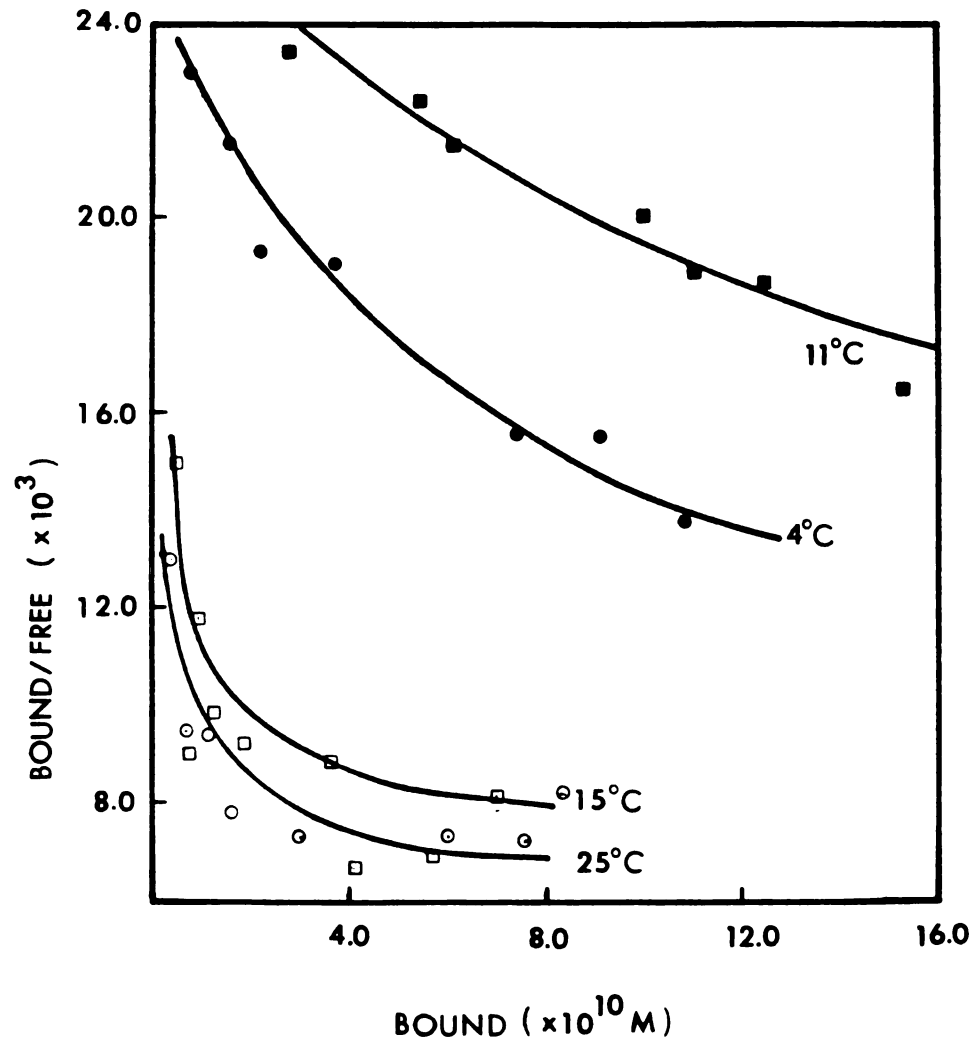
Fig. 5-5. Scatchard analysis of $[^{125}I]T_4$ binding to altered
receptors at various temperature : 4°C(●), 11°C(■),
15°C(□) and 25°C(○).

dissociation kinetics, as described in Chapter Four, was performed at 4°C. The result indicated that the second class of sites was likely due to nonspecific binding, because negligible enhancement of dissociation in the presence of 1×10^{-9}M unlabeled L-T$_4$ was observed in Fig. 5-6. This was interpreted to mean that no negative cooperative interactions were present. Then these second sites must be due to either (i) independent sites; or (ii) nonspecifically bound sites. An independent class of second sites was also considered less likely, since the holo-receptor constitutes only single class of binding sites (see Chapters Two and Four).

The data of these equilibrium association constants for the specific binding site, obtained from the Scatchard plot, are listed in Table 5-1.

TABLE 5-1. Binding Association Constants of L-T$_4$ to the Altered Receptor at Various Temperatures

Temperature(C°)	Ka($\times10^{-7}$ M^{-1})
4	2.55
11	3.10
15	4.46
25	4.82
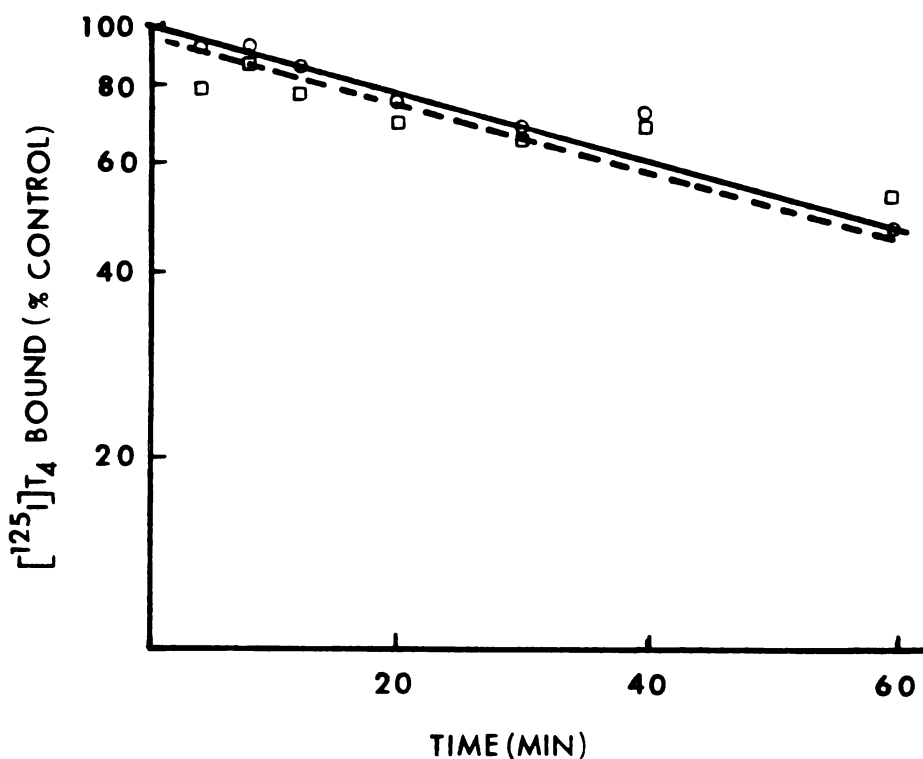
Fig. 5-6. Dissociation kinetics of $[^{125}I]T_4$ bound to the altered receptors at 4°C. The altered receptor was incubated to equilibrium with $[^{125}I]T_4(3\times10^{-9}M)$ at 4°C for 6 hours. 250 μl of the $[^{125}I]T_4$-altered receptor complex were transferred to 4750 μl of phosphate buffer at pH 7.6 in the absence(—O—) or presence (—□—) of unlabeled L-T4(1 nM). Nonspecific binding was measured by addition of 10μM unlabeled L-T4. At indicated intervals duplicates from incubation tubes were assayed as described in Fig. 5-1. After correction for nonspecific binding, the radioactivity in the eluted fraction at indicated time intervals, expressed as percentage of the radioactivity at 0 min, was plotted as a function of time.

Temperature-dependence studies of the equilibrium binding constant can be utilized to determine binding enthalpy change (ΔH), and binding entrapy change (ΔS). The former is used to measure change in energy content during bond-breaking and bond-making process, while the latter to measure change in orderliness of the system plus the surrounding in the binding interaction. This measurement is based on the well-known van't Hoff equation, assuming that ΔH and ΔS are independent of temperature:

$$\ln K_a = \frac{-\Delta H}{RT} + \frac{\Delta S}{R}$$

where Ka is the equilibrium affinity constant of the ligand

R is the gas constant (= 1.987 cal/mole deg)

T is the temperature in degrees of Kelvin (°K)

In Fig. 5-7, the equilibrium affinity constant Ka of the specific binding site of the altered receptor, obtained from the Scatchard analysis, was plotted as the logarithm of Ka versus the reciprocal of the temperature ($1/T$). As seen in the equation of van't Hoff, ΔH can be determined from the slope, and ΔS from the Y-intercept of the plot. These data, together with earlier data reported for TBG by Korcek and Tabachnick (1976), TBPA by Nilsson and Peterson (1971),and for holo-receptor by Bolger (1977) are listed in Table 5-2. It can be seen that the altered receptor possessed an endothermic ΔH (+5.0 Kcal/mol), different from the exothermic ΔH's of TBG, TBPA , and the holo-receptor. However, a marked ΔS (+51.8e.u.) resembled these of TBG and TBPA, although it was also very different from that of the holo-receptor. The large increase in ΔS clearly portrays a more hydrophobic binding site for the heated receptor, since one of the possible consequences by heat treatment is first to expose hydrophobic
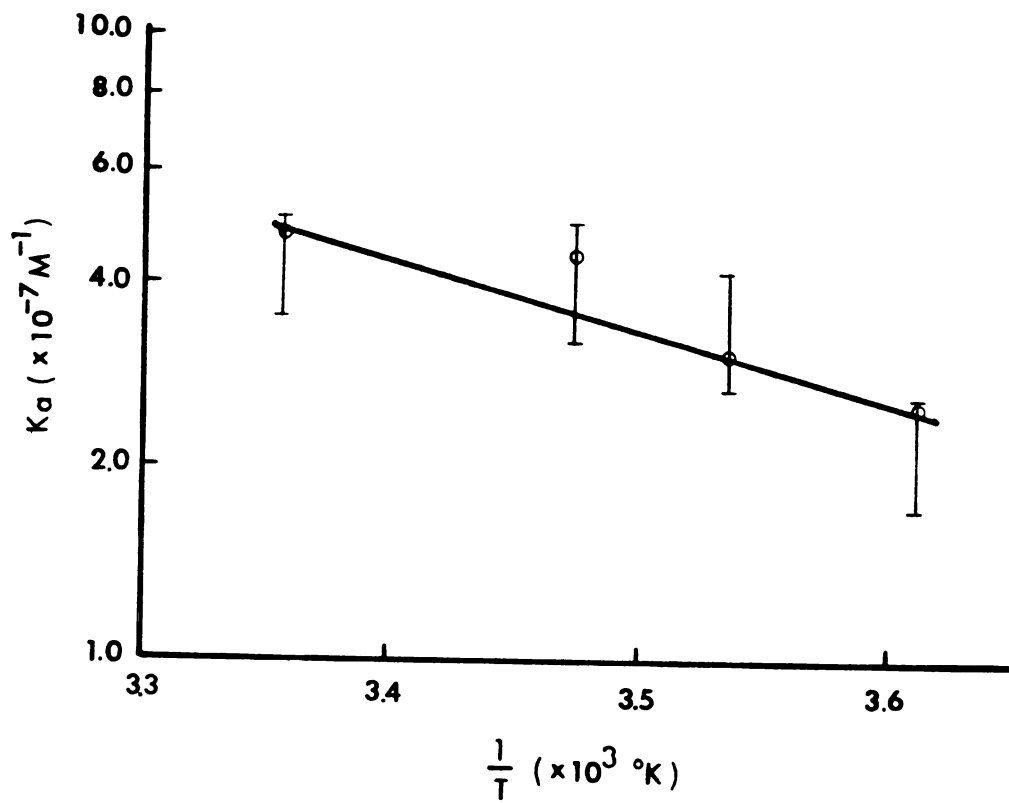
Fig. 5-7. Temperature dependence of L-T$_4$ binding affinity to the altered receptor. The data shown in Table 5-1 were used in this van't Hoff plot. The ordinate was in the logarithmic scale.

TABLE 5-2. Thermodynamic Parameters: ΔH(Enthalpy), ΔS(Entropy) and ΔG(Free Energy) of TBG, TBPA, Altered-Receptor and Holo-Receptor Binding

Ligand	Binding Protein	ΔH° (Kcal/mol)	ΔS° (cal/mol-deg)	ΔG°(298°K) (Kcal/mol)
	Altered-Receptor	+5.0	+51.8	-10.5
L-T$_4$	TBG[a]	-9.0	+16.7	-13.9
	TBPA[b]	-5.3	+14.3	-9.6
L-T$_3$	Holo-Receptor[c]	-11.0	+5.7	-12.8

[a] Data from Korcek and Tabachnick (1976)

[b] Data from Nilsson and Peterson (1971)

[c] Data from Bolger (1977)

domains to predominantly aqueous surroundings and then for them to fold collectively inward to form hormone-binding regions by hydrophobic bonding. In order to judge the accuracy of data resulted from the binding assay by the method of gel filtration, it is important to demonstrate that dissociation of ligand·receptor complexes is minimal during the separation process. So we measured the dissociation rate constant at various temperatures. Incubation mixture (6 ml) contained the altered receptor (500 μl), 0.5 nM $[^{125}I]T_4$ and incubation buffer, and was incubated for 6 hours at each temperature (4°C, 9°C and 15°C). Parallel incubation in the presence of 20 μM unlabeled L-T_4 was added to measure nonspecific binding. Then a chasing dose of 200 μl of 5×10^{-4}M unlabeled L-T_4 was added to the former (final concentration=20 μM) to induce dissociation, whereas the same volume of incubation buffer was added to the latter. The remaining procedures of separation at indicated intervals of time were the same as described in Chapter Four. The data were analysed according to the first-order kinetics, as stated previously. The results are shown in Table 5-3.

TABLE 5-3. Dissociation Rate Constants of L-T_4 Binding to the
Altered Receptor at Various Temperatures

Temperature(°C)	k_{-1}(min^{-1})	$t_{\frac{1}{2}}$(min)
4	7.06×10^{-3}	98
9	1.41×10^{-2}	49
15	1.69×10^{-1}	4

As indicated in Table 5-3, the dissociation rate constant for the altered receptor at 4°C was 7.06×10^{-3} min^{-1}, equivalent to a half life of 98 min. Since it took 30 sec to complete the separation of bound and free [^{125}I]T$_4$, the accuracy of the binding data in this chapter was assured. In addition, it is noted that the dissociation rate constant for the altered receptor was, in general, much greater than that for the holo-receptor (see Part II of Chapter Four). This is in support for the idea that the binding site of the altered receptor is more accommodative than that of the holo-receptor so that the ligand could move out more easily.

Table 5-4 presents the apparent binding association constants (relative to L-T$_4$=100%) of thirty thyroid hormone analogs, including those measured in this (altered receptor) binding study as well as those from holo-receptor binding studies (Bolger and Jorgensen, 1980), TBPA binding studies (Andrea et al., 1980), and TBG binding studies (Hao and Tabachnik, 1971; Snyder et al., 1976). The analogs contain a wide spectrum of variations in the thyronine nucleus. The substituents include (i) 3,5,3', and 5'-iodine atoms (ii) other 3,5,3', and 5' substituents (iii) side-chain optical isomers (iv) desamino analogs. Table 5-5 presents the incremental contributions to binding free energy, calculated from reference compounds as described . The results are further illustrated in Fig. 5-8 (altered receptor vs. TBPA), and Fig. 5-9 (altered receptor vs. holo-receptor). Interestingly, these binding proteins or receptors are from various tissues and species. TBPA and TBG are from human serum, while holo- and altered nuclear receptors are from rat liver. Nevertheless, it has been reported that : (i) Human and rat receptors have comparable binding modes among a number of analogs (Schuster et al., 1979) (ii) Different responsive tissues of the same species, e.g. liver, kidney, pituitary, etc., also

TABLE 5-4. Thyroid Hormone Analogs: Identification, Affinities to Altered-Receptor, TBPA, TBG, and Holo-Receptor
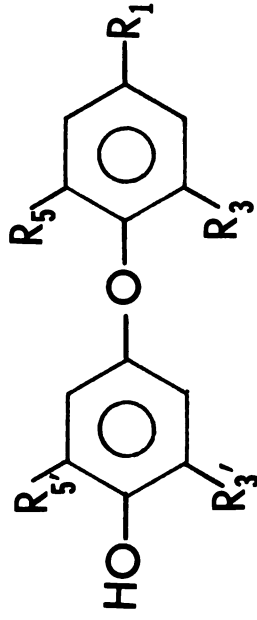
No.	Compound (X)	DL	R_1	R_3	R_5	$R_{3'}$	$R_{5'}$	Altered Receptor[a]	TBPA[b]	TBG[c]	Holo-Receptor[d]
1	L-T_4	L	Ala	I	I	I	I	100	100	100	100
2	L-T_3	L	Ala	I	I	I	H	3.7(±2.3)	9.2	3.3	694
3	R-T_3	L	Ala	I	H	I	I	18.4(±6.1)	32.9	38	1.3
4	3',5',T_2	DL	Ala	H	H	I	I	4.6(±1.8)	3.3	.1	.005
5	3,3',-T_2	L	Ala	I	H	I	H	1.73(±1.0)	.64	1.3	5.2
6	3,5-T_2	L	Ala	I	I	H	H	0.81(±0.42)	.32	.07	.62
7	3'-T_1	DL	Ala	H	H	I	H	0.021(±0.014)	.068	.023	.0027
8	3-T_1	L	Ala	I	H	H	H	0.012(±0.007)	.014	.05	.029
9	T0	L	Ala	H	H	H	H	0.0048(±0.0021)	.002	.1	0

[a] $K_{L-T_4}=5.07 \times 10^7$ M^{-1}

[b] $K_{L-T_4}=4.91 \times 10^7$ M^{-1} (Andrea et al., 1980)

[c] $K_{L-T_4}=2.5 \times 10^9$ M^{-1} (Hao and Tabachnick, 1971; Snyder et al., 1976)

[d] $K_{L-T_4}=1.71 \times 10^8$ M^{-1} (Bolger and Jorgensen, 1980)

[e] The abbreviations used were: Ala=alanine; Form=formic; Ac=acetic; Prop=propionic; Bu=butyric.

TABLE 5-4. (Continue)

Other 3,5,3',5', Substituted Thyronines

	DL	R_1	R_3	R_5	$R_{3'}$	$R_{5'}$	Core-[a] Receptor	TBPA[b]	TBG[c]	Holo-[d] Receptor
10 $3,5-(Me)_2-3'-I$	L	Ala	Me	Me	I	H	0.47(±0.23)	.84		3.75
11 $3,5-(Me)_2-3',5'-I_2$	L	Ala	Me	Me	I	I	1.0(±0.53)	.78		.12
12 $3-Me-5,3'-I_2$	DL	Ala	Me	I	I	H	0.67(±0.61)	2.1		8.47
13 $3-Me-5,3',5'-I_2$	DL	Ala	Me	I	I	I	9.9(±9.0)	8.0		8.15
14 $3,5-(Et)_2-3'-I$	DL	Ala	Et	Et	I	H	0.69(±0.41)	.49		4.51
15 $3,5-(Et)_2-3',5'-I_2$	DL	Ala	Et	Et	I	I	0.99(±0.53)	2.5		.69
16 $3,5-(iPr)_2-3'-I$	DL	Ala	iPr	iPr	I	H	0.78(±0.42)	.25		.96
17 $3,5-(sBu)_2-3',I$	DL	Ala	sBu	sBu	I	H	0.36(±0.25)			0.26
18 $3,5-I_2-3'-iPr$	L	Ala	I	I	iPr	H	0.25(±0.16)	.83	3.53	646
19 $3,5-I_2-3'-iPr-5'-I$	L	Ala	I	I	iPr	I	10.2(±5.1)			9.35
20 $3,5-I_2-3'-iPr-5'-Br$	L	Ala	I	I	iPr	Br	19.6(±7.0)			165
21 $3,5-I_2-3'-iPr-5'-Cl$	L	Ala	I	I	iPr	Cl	19.1(±7.3)			396
22 $3,5-Br_2-3'-I$	L	Ala	Br	Br	I	H	10.2(±9.0)			
23 $3,5-Br_2-3',5'-I_2$	L	Ala	Br	Br	I	I	113(±68.0)	82	161	4.82

D-isomers

	DL	R_1	R_3	R_5	$R_{3'}$	$R_{5'}$	Core-[a] Receptor	TBPA[b]	TBG[c]	Holo-[d] Receptor
24 $D-T_4$	D	Ala	I	I	I	I	21.4(±17.6)	3.7	38	13.2
25 $D-T_3$	D	Ala	I	I	I	H	.63(±0.34)	.25	.80	436

Acidic Side-Chain Analogs

		R_1	R_3	R_5	$R_{3'}$	$R_{5'}$	Core-[a] Receptor	TBPA[b]	TBG[c]	Holo-[d] Receptor
26 T_4-Form		Form	I	I	I	I	1.1(±0.3)	186		68.3
27 T_4-Ac		Ac	I	I	I	I	1.4(±0.2)	676	1.7	580
28 T_4-Prop		Prop	I	I	I	I	6.0(±0.8)	298	3.6	401
29 $T_4-Butyr$		Bu	I	I	I	I	2.7(±0.3)	267		682
30 T_3-Ac		Ac	I	I	I	H	0.38(±0.10)	19.7	0.3	1962

TABLE 5-5. Comparison of Free Energy Contributions of Halogen Atoms
in Binding to Altered-Receptor, TBPA, TBG, and Holo-Receptor

Substituent P	Parent Analogs[a]		Altered-Receptor[b]	TBPA[c]	TBG[d]	Holo-Receptor[e]
	XPH	XHH	$\Delta\Delta G^1(P)^f$ (Kcal/mol)			
3-I	$3,3',5'-T_3$	$3',5'-T_2$	-0.84(±0.10)	-1.41	-3.52	-3.29
	$3,3'-T_2$	$3'-T_1$	-2.62(±0.50)	-1.38	-2.38	-1.20
	$3-T_1$	T_0	-0.54(±0.30)	-1.11	-1.91	
	$L-T_4$	$3,3',5'-T_3$	-0.97(±0.17)	-0.69	-0.57	-2.56
5-I	$3,5,3'-T_3$	$3,3'-T_2$	-0.41(±0.43)	-1.64	-0.54	-2.90
	$3,5-T_2$	$3-T_1$	-2.51(±0.36)	-1.94	-0.20	-1.82
	$3,5,3'-T_3$	$3,5-T_2$	-0.85(±0.39)	-2.08	-2.28	-4.16
3'-I	$3,3'-T_2$	$3-T_1$	-2.95(±0.41)	-2.38	-1.93	-3.08
	$3'-T_1$	T_0	-0.87(±0.38)	-2.11	-1.60	
	$L-T_4$	$3,5,3'-T_3$	-1.96(±0.37)	-1.47	-2.01	+1.15
	$3,3',5'-T_3$	$3,3'-T_2$	-1.40(±0.49)	-2.42	-1.99	+0.80
	$3',5'-T_2$	$3'-T_1$	-3.19(±0.36)	-2.39	-0.87	-0.37
5'-I	Me_2I_2	Me_2I	-0.46(±0.37)	+0.05		+2.05
	$MeII_2$	MeIIH	-1.60(±0.58)	-0.84		+0.02
	Et_2I_2	Et_2I	-0.22(±0.28)	-1.00		+1.11
	Br_2I_2	Br_2I	-1.42(±0.67)			
	$I_2-iPr-I$	$I_2-iPr-H$	-2.38(±0.46)			+1.14
5'-Br	$I_2-iPr-Br$	$I_2-iPr-H$	-2.77(±0.36)			+0.80
5'-Cl	$I_2-iPr-Cl$	$I_2-iPr-H$	-2.75(±0.38)			+0.29

a
Parent analogs were defined in Table 5-2

b
Calculated from the data of Table 5-2

c
Calculated from the data of Andrea, et al. (1980)

d
Calculated from the data of Snyder, et al. (1976)

e
Calculated from the data of Bolger and Jorgensen (1980)

f
$\Delta\Delta G^1(P) = \Delta G° (XPH) - \Delta G° (XHH) = 0.5912 \ln \frac{K(XHH)}{K(XPH)}$

TABLE 5-5a. Comparison of Free Energy Contributions of 3,5-Substituents in Binding to Altered Receptor, TBPA, TBG, and Holo-Receptor

Substituent P	Parent Analogs[a] XPQ	Altered Receptor[b] XII	TBPA[c]	TBG[d]	Holo-Receptor[e]
			ΔΔG[f](PQ)		
$3,5\text{-Me}_2$	$3,5\text{-(Me)}_2\text{-}3'\text{-I}$	$3,5,3'\text{-T}_3$	+1.21	+1.42	+3.09
	$3,5\text{-(Me)}_2\text{-}3',5'\text{-I}_2$	$3,5,3',5'\text{-T}_4$	+2.72	+2.87	+3.98
$3,5\text{-Me-I}$	$3\text{-Me-}5,3'\text{-I}_2$	$3,5,3'\text{-T}_3$	+1.00	+0.87	+2.60
	$3\text{-Me-}5,3',5'\text{-I}_3$	$3,5,3',5'\text{-T}_4$	+1.37	+1.87	+2.63
$3,5\text{-Et}_2$	$3,5\text{-Et}_2\text{-}3'\text{-I}$	$3,5,3'\text{-T}_3$	+0.99	+1.73	+2.98
	$3,5\text{-Et}_2\text{-}3',5'\text{-I}_2$	$3,5,3',5'\text{-T}_4$	+2.73	+2.37	+2.94
$3,5\text{-iPr}_2$	$3,5\text{-iPr}_2\text{-}3'\text{-I}$	$3,5,3'\text{-T}_3$	+0.92	+2.13	+3.89
$3,5\text{-}3\text{-Bu}_2$	$3,5\text{-}3\text{Bu-}3'\text{-I}$	$3,5,3'\text{-T}_3$	+1.38		+6.02
$3,5\text{-Br}_2$	$3,5\text{-Br}_2\text{-}3'\text{-I}$	$3,5,3'\text{-T}_3$	-0.60		
	$3,5\text{-Br-}3',5'\text{-I}_2$	$3,5,3',5'\text{-T}_4$	-0.07	+0.12	+1.79
$3,5\text{-H}_2$	$3'\text{-I}$	$3,5,3'\text{-T}_3$	+3.06	+2.90	+7.36
	$3',5'\text{-I}$	$3,5,3',5'\text{-T}_4$	+1.82	+0.61	+5.85

Footnotes a,b,c,d,e were defined in Table 5-5.

$$^{f}\ \Delta\Delta G^{1}(PQ) = \Delta G(XPQ) - \Delta G(XII) = 0.5912 \ \ln \frac{K(XII)}{K(XPQ)} \qquad \text{at } T = 298^{\circ}K$$

exhibit similar binding specificities (Oppenheimer, 1979) (iii) Human

TBPA and rat TBPA have closely resembling binding affinity for L-T_4

$(1-3.5x10^8M^{-1})$, in addition to nearly identical UV absorption spectrum,

and high resistance to subunit dissociation reagents (Navab et al., 1977).

(iv) There are only four different amino acids out of the first thirty

residues from the NH_2-terminal of both human TBPA and rat TBPA (Navab

et al., 1977). Thus the comparative analysis used in this chapter for

various binding proteins should be more or less valid.

VII. Comparisons of Free-Energy Contributions of 3, 5, 3', and 5' Iodine Atoms to Binding with the Altered Receptor, Holo-Receptor, TBPA and TBG

The relative binding activities to the altered receptor of L-T_4, T_0

and of other various tri-, di-, and mono-iodo thyronines are shown in

Table 5-4 (Nos. 1-9). It can be seen that analogs with 3',5' outer-ring

iodine atoms bound more strongly than did those without to the altered receptor.

For example, L-T_4>L-T_3, R-T_3>L-T_3, 3',5'-T_2>3,3'-T_2>3,5-T_2. In contrast,

the relative order in holo-receptor binding was: L-T_3>L-T_4>R-T_3, 3,5-T_2>

3,3'-T_2>3',5'-T_2. Furthermore, the binding to altered receptors was also

stereospecific, namely, L-T_4>D-T_4, and L-T_3>D-T_3.

Strikingly, the binding data of R-T_3 (No. 3) and 3',5'-T_2(No. 4)

showed that while the compounds had very little affinity to the holo-

receptor (1.3% and 0.005%, respectively), they did have remarkable affinity

to the altered receptor (18.4% and 4.6%), TBPA (32.9% and 3.3%), and TBG

(38% and 0.1%). This indicated that the structural requirements for

binding to the altered receptor were more like those of the plasma binding

proteins than those of the holo-receptor, despite the same preference of

L-T_4 to L-T_3 (or D-T_4 to D-T_3).

The main features of Table 5-5 are the following: (a) The 3-iodine added to the inner-ring was energetically favorable in all proteins. (Altered receptor -0.5 ~-2.6 Kcal/mol; holo-receptor -1.2 ~-3.3 Kcal/mol; TBPA -1.1 ~-1.4 Kcal/mol; TBG -1.9 ~-3.5 Kcal/mol). (b) The 5-iodine atom was also energetically favorable (altered receptor -0.4 ~-2.5 Kcal/mol; holo-receptor -1.8 ~-2.9 Kcal/mol; TBPA -0.7 ~-1.9 Kcal/mol; TBG -0.2 ~-0.6 Kcal/mol). At this position, the altered receptor binding resembled more TBPA binding than TBG binding. (c) The 3'-iodine, in general, contributed favorably to the binding, (altered receptor -0.9 ~-3.0 Kcal/mol; holo-receptor -3.1 ~-4.2 Kcal/mol; TBPA -2.1 ~-2.4 Kcal/mol; TBG -1.6 ~-2.3 Kcal/mol). The greater contribution by the 3'-iodine in holo-receptor binding might attribute to its close proximity to the receptor (Andrea et al., 1979). (d) The 5'-iodine contribution was a marker to distinguish the altered receptor and binding proteins from the holo-receptor. It was energetically favorable to the altered receptor (-0.2 ~-3.2 Kcal/mol), TBPA (-0.0 ~-2.4 Kcal/mol), and TBG (-0.9 ~-2.0 Kcal/mol). However, it is repulsive in nature to the holo receptor (0.0 ~ 2.0 Kcal/mol).

As in TBPA (Andrea et al., 1980), Fig. 5-8 showed that an additional outer-ring iodine (3'-I or 5'-I) to each thyronine yielded more incremental change in binding free energy to the altered receptor than did an additional inner-ring iodine (3-I or 5-I) to the same nucleus. Similarly, removal of an outer-ring iodine resulted in a greater loss in binding free energy than did removal of an inner-ring iodine.

In contrast, Fig. 5-9 demonstrated that addition of 3'-I was more (or at least equally) desired in the holo- than in the altered receptor. While addition of 5'-I was not favored in terms of binding energetics in the holo-receptor, the opposite was true in the case of altered receptors.

T_4

$5'I$ -1.96 $5I$
(-1.41)
-0.91
(0.69)

$3,5,3'-T_3$ $3,3',5-T_3$

$3'I$ $5I$ $5'I$ $3I$
-0.91 -0.91 -1.90 -0.91
(-2.09) (-1.69) (-2.09) (-1.69)

$3,5-T_2$ $3,3'-T_2$ $3',5-T_2$

$5I$ $3'I$ $3I$ $5'I$
-2.51 -2.95 -2.01 -3.19
(-1.90) (-2.39) (-1.39) (-2.39)

$3-T_1$ $3'-T_1$

$3I$ $3'I$
-0.96 -0.97
(-1.19) (-2.19)

T_0
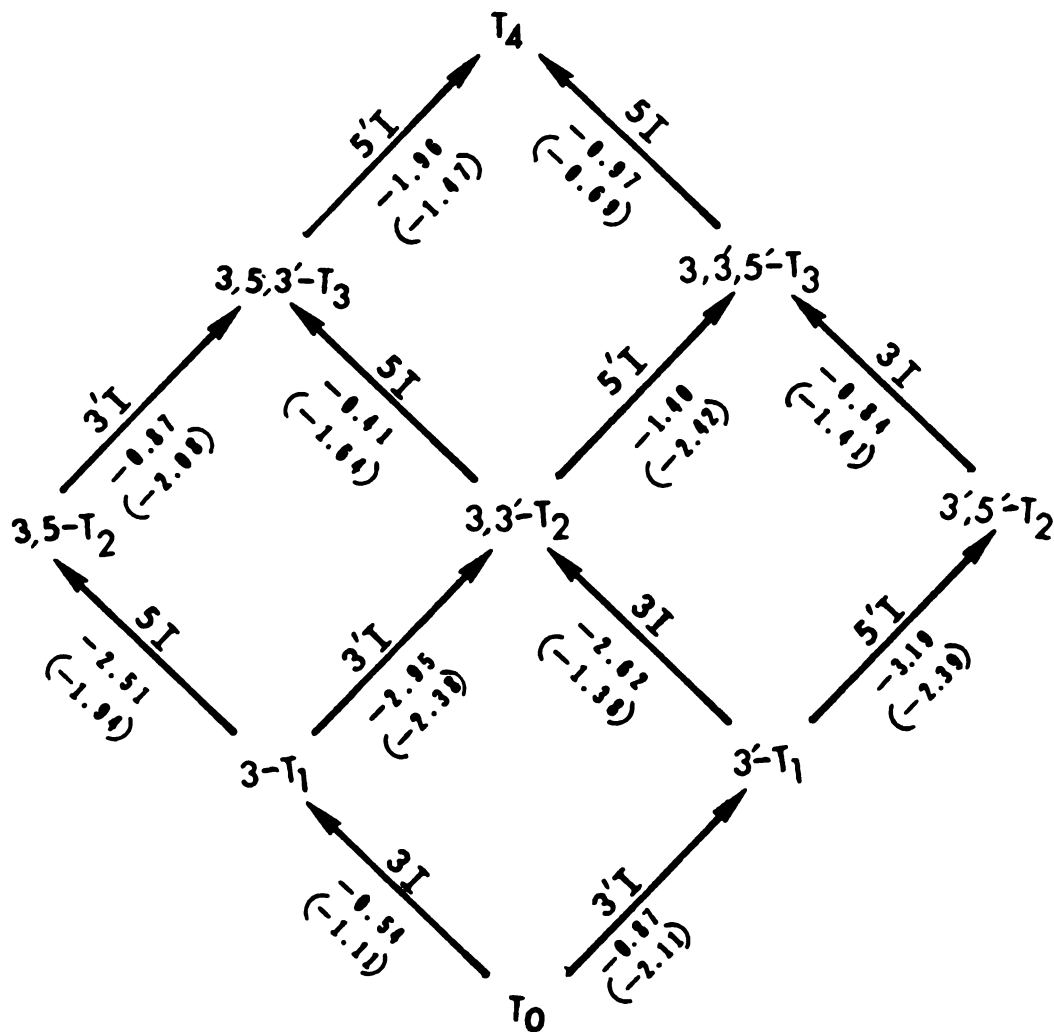
Figs. 5-8 & 5-9 Schematic diagram of contribution to the free energy
of binding with the altered receptor, generated by sequential
addition of iodine atoms to the thyronine nucleus. The figure
under each arrow corresponded to the change in binding free
energy when the designated iodine atom replaced the corres-
ponding hydrogen atom. The parallel figure in parentheses
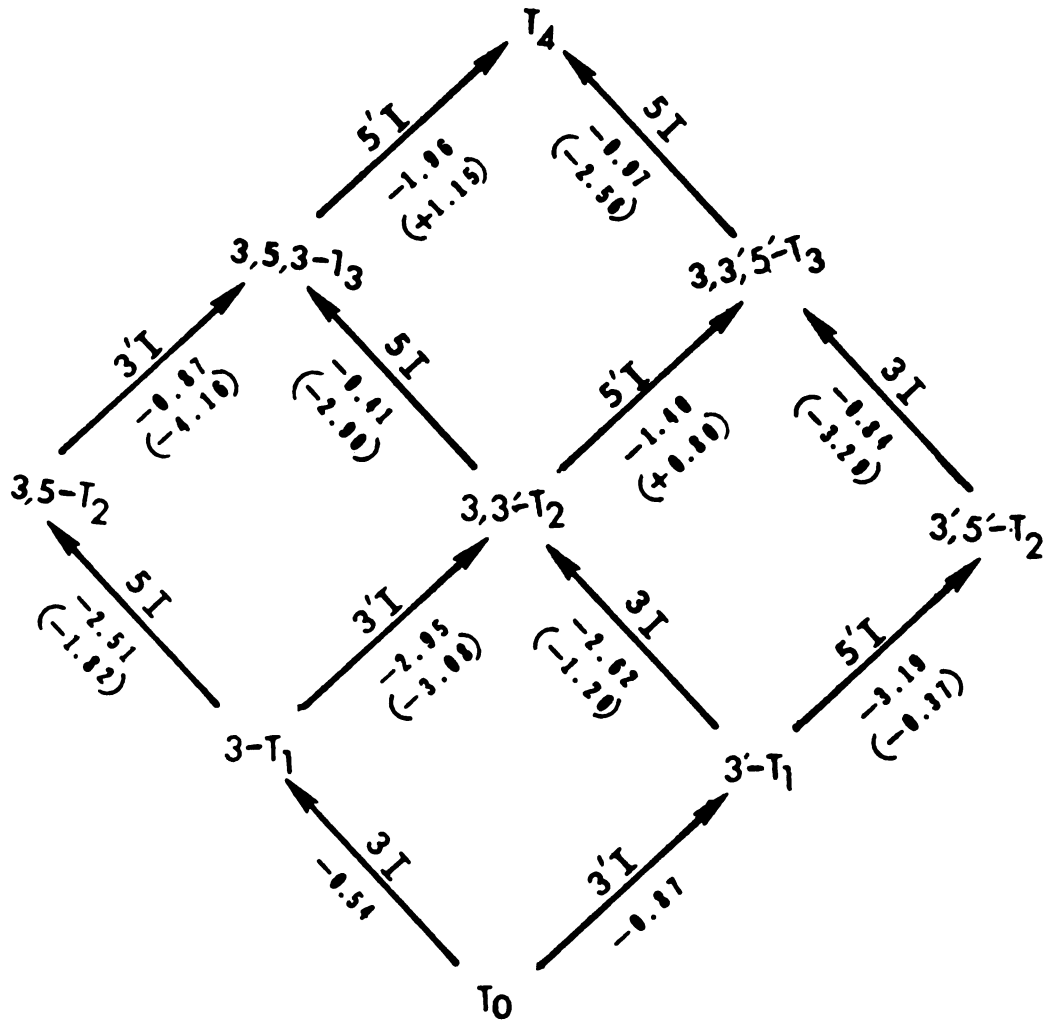corresponded to that for TBPA in Fig. 5-8, and for the holo-
receptor in Fig. 5-9.

Fig. 5-9.

Fig. 5-9 also showed that 3- and 5-iodines added favorably incremental affinity to both forms of nuclear receptors. In other words, the binding mode of the altered receptor resembled more closely that of TBG (or TBPA) than that of the holo-receptor.

VIII. Comparisons of Free-Energy Contributions of 3,5,3'5', Substituents Other Than Iodine

The relative binding affinities of other 3-, 5-, 3'-, 5'- substituted analogs are shown in Table 5-4 (Nos. 12-25). (a) Although in the nuclear holo-receptor binding, 3'-isopropyl-3,5-T_2 (No. 18) (646%)was found to be as good as L-T_3 (694%), where 3'-position was occupied by iodine (Bolger and Jorgensen, 1980), the former had only an affinity of 2.47% in the altered receptor binding, 0.83% in the TBPA binding, and 3.53% in the TBG binding. An additional 5'-I or -Br or -Cl (Nos. 19, 20, 21) increased the binding affinity to the altered receptor by 4-fold. Among these 5'-halogen analogs the binding activity peaked at the bromine atom, which might be due to the combined effects of hydrophobic interaction, steric effect and ionization effect as mentioned below. In contrast, the nuclear holo-receptor binding peaked at the chlorine atom which is of the smallest size among Cl, Br, and I, again due to the repulsiveness in the holo-receptor described earlier.

It has been suggested that while the primary contribution of the 3' group to the holo-receptor binding is by hydrophobic interaction, it is by charge-transfer, and inductive effects besides hydrophobic bonding to TBPA binding (Andrea et al., 1980) The possible interactions between the 3'- or 5'-iodine atom and the nearby residues are hydrogen-bonding with the proton of peptide hydroxyl groups or formation of charge-transfer complexes

with the lone-pair orbitals of peptide oxygen atoms (Andrea et al., 1980).

These interactions could not exist in the case of 3'-isopropyl substitution.

In addition, the inductive effect of the 3'-iodine (or 5'-iodine) to inc-

rease the ionization of the neighboring 4'-hydroxyl was absent. The same

inductive effect operated in the 5'-I, -Br, or -Cl analogs (Nos. 19, 20, 21).

This effect can be reflected in the lower pKa of the 4'-OH of L-T_4 (6.73),

compared with that of L-T_3 (8.45) and that of L-T_2 (~ 10). In TBPA this

would strengthen the interaction of the 4'- oxygen with the hydroxyls of

Ser-117 and Thr-119, via a well-defined H_2O molecule (Blake and Oatley, 1977).

This finding of similar electrostatic and steric requirements for sub-

stituents at 3' and 5' positions other than iodine has provided further

support to the idea that the binding site of the altered receptor shares

more similarity to that of TBG (or TBPA) than to that of the holo-receptor.

(b) To examine the effects of various inner-ring substitutions on binding,

eleven analogs (Nos. 4,7,10-11,22,23, 11-19,24-25) were used. In Fig. 10

a,b,c's, the ΔΔG's of 3,5-substituents were plotted versus the sum of their

Hansch parameter π (Fujita et al., 1964). ΔΔG defined as the free energy

contribution by 3,5-substituents, is the difference between ΔG's of 3,5-

disubstituted analog and its corresponding 3,5-diiodo analog. The physico-

chemical significance of π has been described in Chapter Three. The data

used in Fig. 10 a,b,c were shown in Table 5-3a. It is unfortunate that no

parallel data of TBG binding are available thus far.

ΔΔG's of alkyl substituents in the altered receptor or TBPA binding

varied in range of 0.87 ~ 3.06 Kcal/mol. In contrast, they varied between

2.60 ~ 7.36 Kcal/mol in the holo-receptor binding. Therefore, the binding

site of the altered receptor resembled more closely that of TBPA than that

of the holo-receptor based on smaller variation in ΔΔG. As can be seen in
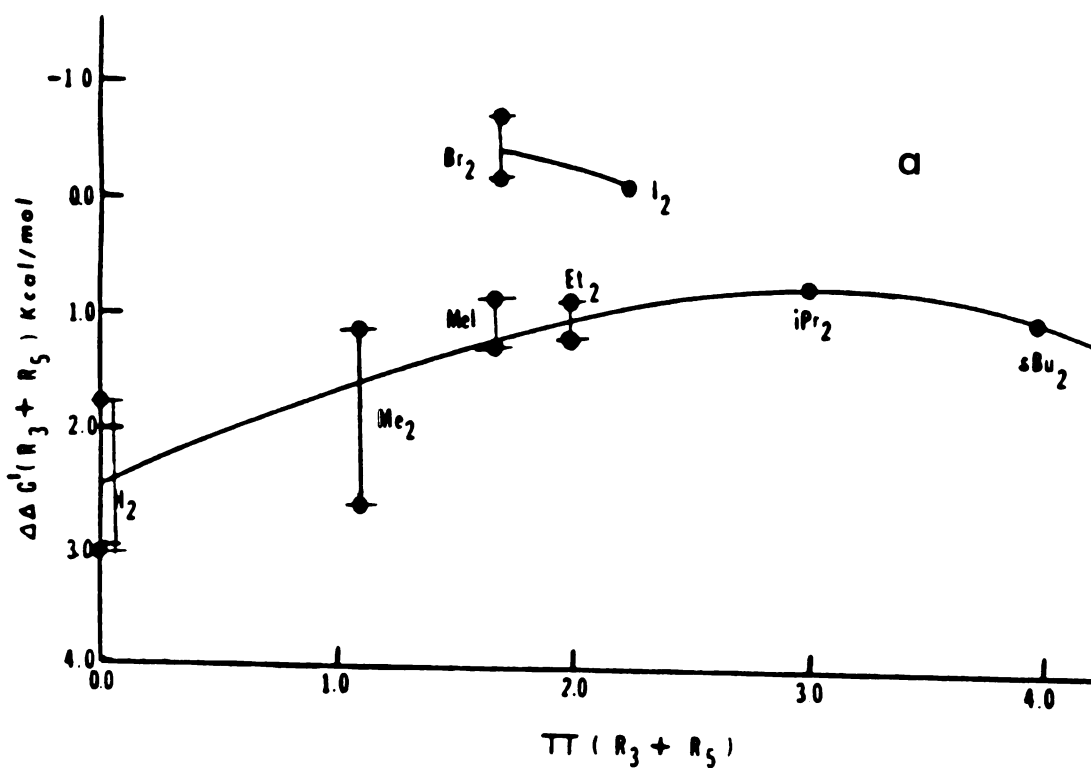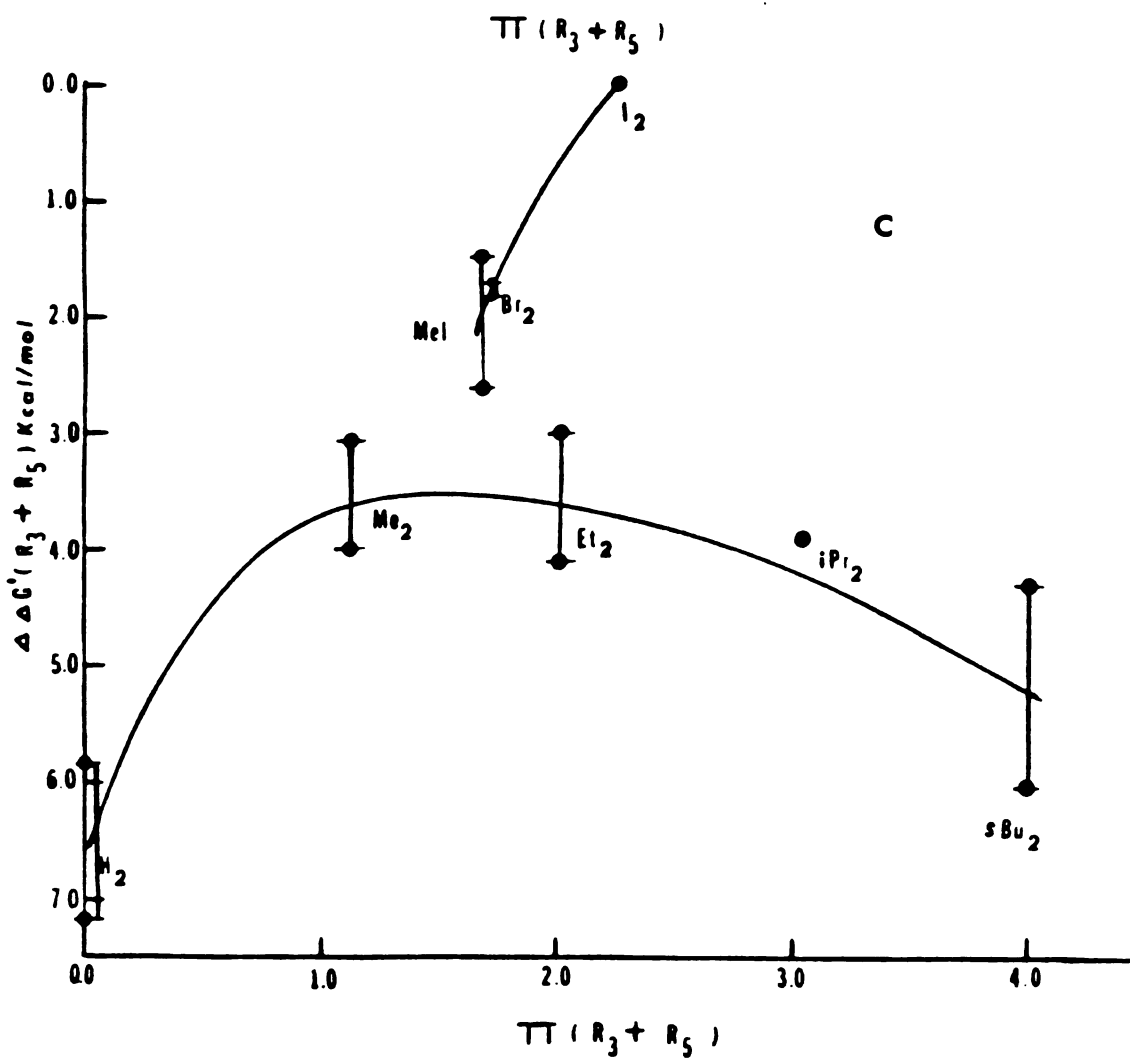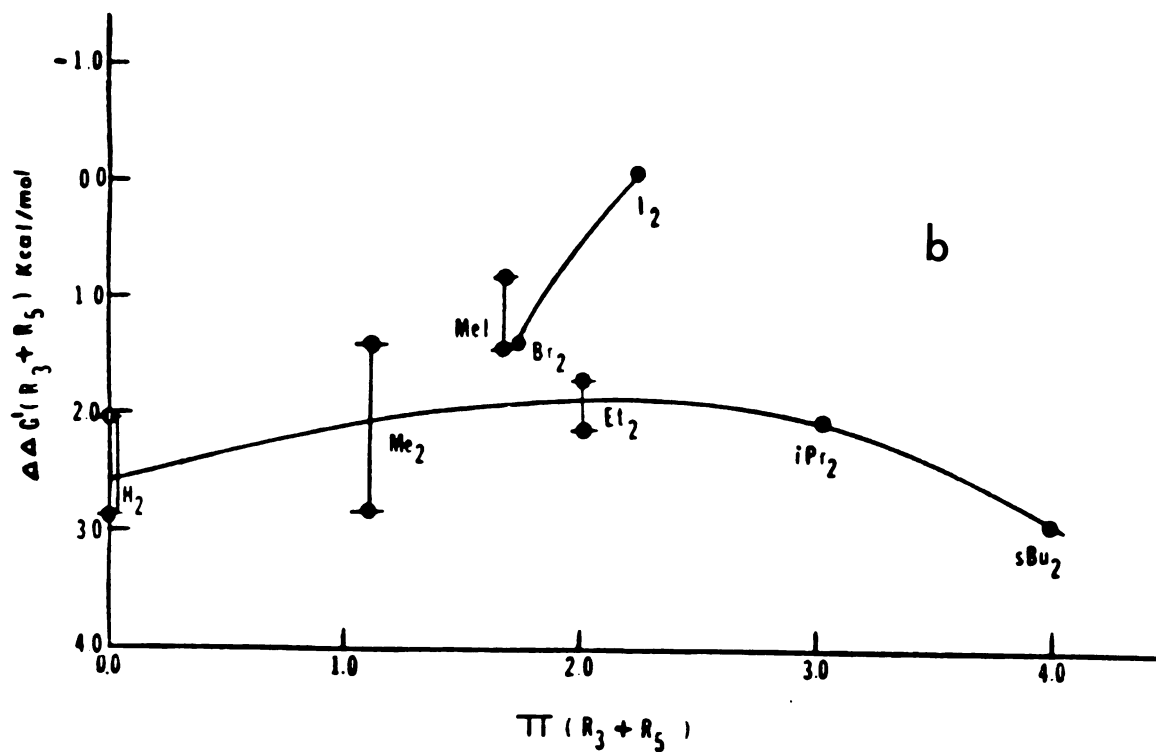
Fig. 5-10. a,b,c, Binding of 3,5- substituted thyronines to the
altered receptor (a), to TBPA (b) and to the holo-receptor (c).
The contributions of 3,5 substituents were plotted against the
sum of their Hansch π parameters. The data used were shown in
Table 5-5a.

b

c

Fig's 10a and 10b, the binding activity to the altered receptor was peaked with 3,5-diisopropyl substitution, while to it is to TBPA with 3,5- diethyl substitution. As compared with the relatively rigid structure of the holo-receptor in which the binding activity is peaked with 3,5-dimethyl substitution, it can still be suggested that the altered receptor and TBPA might have larger pocket-like space to accommodate bulky groups, owing to the fact that there is a good correlation between hydrophobicity parameter π and Van der Waals volume (Bondi, 1964).

It is noteworthy that the 3,5-diiodo and 3,5-dibromo analogs (Nos. 19-23) played a very unique role which could not be duplicated by alkyl groups (Nos. 11, 13, 15) with similar hydrophobicity. For example, 3',5'-diiodo-3,5-dibromo analog (No. 23) showed marked binding activity to the altered receptor (113%), TBPA (82%) and TBG (161%). In contrast, the relative binding affinity of 3',5'-diiodo-3,5-diethyl analog (No. 15) was 0.99% to the altered receptor, 2.5% to TBPA and 0.69% to TBG.

IX. Stereo-Specificity of Altered Receptor Binding

In ligand-receptor interactions, one of the important criteria to identify receptors is stereo-specificity. In this study, four optical isomers ($L-T_4$, $D-T_4$, $L-T_3$, $D-T_3$) were examined. The results revealed that $L-T_4$ bound five times more avidly than did $D-T_4$, and that $L-T_3$ bound six times more firmly than did $D-T_3$. This kind of L-isomer preference has also been observed in both TBPA and TBG binding. As mentioned in Chapter Two, X-ray crystallographic studies of TBPA (Blake and Oatley, 1977) have indicated that the hormone's $-COO^-$ and $-NH_3^+$ groups interact with Lys-15 Glu-54, respectively. Based on the X-ray data, Andrea et al. (1978) postulated from simple theoretical calculations that this stereo-specificity is resulted from an interplay of electrostatic and sterically repulsive

interactions. From our binding data alone, it can not be concluded whether or not analogous molecular events are responsible for the stereo-specificity.

X. Contributions of the Acidic Side Chain in Desamino Analogs

As indicated in Table 5-4, 3,5,3',5' -tetraiodo acidic (Nos. 26-29) and 3,5,3' -triiodoacetic (No. 30) analogs in which the side chains have only a negative charge, had lower binding affinities than did L-T$_4$ (No. 1) in which the side chain has a net charge of zero. The relative order of binding strength was: tetraprop > tetrac > tetrabutyr > tetraform > triac. The addition of 5'-I to 3,5,3'-triac (No. 30) resulted in a binding free energy contribution of 0.78($\pm$0.30) Kcal/mol, a reasonable value when compared with other 5'-I incremental change as listed in Table 5-5. Consistently, 3,5,3'-triac (No. 30) had a lower binding potency than did 3,5,3',5'-tetrac (No. 27).

It is striking to note that triac binding affinity (0.38% of L-T$_4$) was even poorer than was L-T$_3$ binding affinity (3.7% of L-T$_4$). On the other hand, thyroxine binding globulin (TBG), a major thyroid hormone transport protein, also shows comparably low affinities with the same analogs. Therefore, when contrasted with the very high binding affinities of these acidic analogs to TBPA, it goes without saying that the altered receptor binding had much more resemblance to TBG binding in this aspect among acidic side-chain analogs (Hao and Tabachnick, 1971; Snyder et al., 1976). These results indicated that the altered receptor and TBG use both the ammonium and carboxylate ions of the alanine side chain in the binding process, and that this region of the binding site of the altered receptor and TBG contain a predominance of negative charge.

So far, it has been demonstrated that the altered receptor has a binding mode resembling that of TBG and TBPA, concerning structural requirements at outer- and inner-ring substituents. More importantly, the altered receptor as well as TBG exhibited very low binding activity to acidic side-chain analogs in the same relative order. It is thus conceivable that the altered receptor could be the "missing-link" between the holo-receptor at one end, and TBG plus TBPA at the other, in the time course of evolution. As discussed previously, the following facts are known: (1) TBPA and TBG are serum thyroid-hormone binding proteins. (2) The nuclear holo-receptor is a DNA-associated protein. (3) X-ray crystollography shows that TBPA has two hypothetical DNA binding sites, whereas no DNA-binding properties have been ascribed to TBG of which X-ray structure has not been done. Therefore, there are good reasons to think that these proteins might be structurally related. Our results as presented in this chapter further supported this concept. Moreover, as can be seen in Table 5-6, their physicochemical properties including molecular weight, resistance to protein denaturants are quite similar, too. Nonetheless, to date there is no unambiguous analogy in subunit structure. The structure of TBPA has been well established to be tetrameric (Robbins et al., 1978). By contrast, there is still no consensus view on TBG structure. Nilsson and coworkers (1975) claimed that TBG is a stable tetramer of four identical subunits, very much like TBPA. However, Robbins et el.(1978) found that their results are compatible with a single chain or a polymer of nonrepeating subunits, but not with four identical subunits. Further, the structure of the nuclear holo-receptor has been indicated to be a monomer (Nikodem et al., 1980). Post-translational processing as in the case of proinsulin and insulin might be one of the possibilities to account for the difference in subunit

TABLE 5-6. Physicochemical Properties of Holo-Receptor,
            TBPA and TBG

	TBPA[a]	TBG[b]	Holo-receptor[c]
Molecular Weight	54,980	54,000	50,500
Number of Subunits	4	1[d] or 4[e]	1[f]
Resistence to Denaturants	strong	strong	strong[g]

[a] Data from Branch et al . (1971), Nilsson and Peterson (1971)
and Kanda et al . (1974)

[b] Data from Nilsson et al . (1975) and Robbins et al . (1978)

[c] Data from Latham et al . (1976)

[d] Data from Robbins et al . (1978)

[e] Data from Nillson et al . (1975)

[f] Data from Nikodem et al . (1980)

[g] Unpublished data from this laboratory by Kuo

structure among these proteins.

It seems probable that these three thyroid hormone-binding proteins could be related by divergent or convergent evolution. In divergent evolution, proteins from the same origin have taken up minor variations in amino acid composition ,sequence, and three-dimensional structure, yet retains most of functional similarity. By contrast, in convergent evolution proteins from different origins converge to share analogous active sites in genetically unrelated proteins. With current knowledge, it is not clear which of the two possibilities is more pertinent to these proteins.

In summary, this study has provided more insight into the binding characteristics to an altered form of the nuclear holo-receptor with 30 thyroid-hormone analogs. It has been shown:

(1) Like TBG and TBPA, analogs with 3',5' outer-ring iodine atoms bind more strongly to the nuclear altered receptor than do those without. Thus $L-T_4 > L-T_3$, $R-T_3 > L-T_3$, $3',5'-T_2 > 3$, $3'-T_2 > 3,5-T_2$. A combination of inductive effect, hydrophobic bonding and charge-transfer complexation by the 3',5'-iodine atoms, and electrostatic attractive forces and hydrogen-bonding by the 4'-hydroxyl group may be responsible for this binding behavior. By contrast, 3'-isopropyl substituent did not enhance binding as did 3'- or 5'- halogen sub-stituents. Presumably, this is due to a lack of the effects as described above.

(2) Like TBG, the nuclear altered receptor has a very low affinity to side-chain acidic analogs. By contrast, TBPA and the nuclear holo-receptor have high affinity to the same analogs. The nuclear altered receptor might serve as "missing link" between the holo-receptor and the thyroid hormone binding proteins.

(3) The binding to the nuclear altered receptor is also stereo-specific, i.e. $L-T_4 > D-T_4$, and $L-T_3 > D-T_3$.

(4) Variations of 3,5 substituents show that halogens exert stronger interactions with the altered receptor than do alkyl groups. Halogens appear to play a particular role which can not be duplicated by alkyl groups. When the relative free-energy contributions of 3,5 substituents to the altered receptor, TBPA, TBG and holo-receptor are compared, it can be seen that the former three proteins are more accommodative to bulky substituents than is the holo-receptor.

(5) It seems likely that thyroid hormone receptors and binding proteins belong to the same evolutionary family.

CHAPTER SIX : EPILOGUE AND PROSPECTS

In this dissertation, three protein molecules - the nuclear receptor, TBPA and the altered receptor - have been studied by using synthetic thyroid-hormone analogs. As shown in Chapter Two, the nuclear receptor and TBPA have shared some similarities in accommodation of bulky outer-ring groups, decreased binding with analogs containing the hydroxyl group at positions other than 4', and stereospecificity at the α-carbon of the side chain. In Chapter Five, the altered receptor has exhibited resemblances with TBPA in binding specificities, except that the former has had much lower binding affinity to acidic side-chain analogs.

In Chapters Two, Three and Four of this dissertation, the data also depicts a picture that indicates substantial differences between the nuclear receptor and the serum binding protein, TBPA , in : (1) the compactness of the binding site (2) the phenomenon of negative cooperativity of hormonal binding (3) the requirement of ionization state of 4'-hydroxyl group on the phenolic outer ring of thyroid hormone and perhaps (4) subunit structure. However, a broadened picture emerges due to the observation that the binding requirements of various analogs to the altered receptor and TBG are similar in Chapter Five. This raises a grand possibility that the nuclear receptor, TBPA and TBG may be related by divergent or convergent evolution in terms of function and structure. It is based on the information that:

(a) The binding characteristics (i.e. thermodynamics, kinetics and binding modes) of the heat-treated (altered) nuclear receptor resembles those of TBG, and to a lesser extent, TBPA, but not the nuclear receptor at all.

(b) They share a common function as thyroid-hormone binding proteins.

(c) Their strong resistance to protein-denaturing reagents has been demonstrated.

(d) The molecular weights of these proteins are similar.

(e) The nuclear receptor and TBPA may be DNA-binding proteins.

Very recently, Matthews and coworkers (1981) have proposed a common precursor for lysozymes of hen-egg-white (HEWL) and bacteriophage T4(T4L). On the basis of similarities in their modes of binding substrates, in the conformation of their backbones and in their modes of catalytic action, they concluded that these two enzymes are products of divergent evolution. Although their amino-acid sequences are nonhomologous, analysis of gene DNA coding for HEWL by Jung et al. (1980) has indicated a close agreement between the exons of HEWL and structural homology with T4L, with the exception of exon 4 (Note: exons are genetic regions which will be expressed). Exon 1 is absent from T4L and the major region of overlap corresponds to exons 2 and 3. Furthermore, these comparison of T4L and HEWL is fully consonant with the view that recombination within introns (Note: introns are genetic regions that will be lost from mature messenger RNA) can rearrange functional exonic regions into new orders in new gene products (Gilbert, 1978). This type of recombination can provide a mechanism for divergence of evolution. Therefore, a similar scenario may have been played in the case of thyroid-hormone binding proteins.

In the future, in order to further clarify the relationship among these thyroid-hormone binding proteins, several lines of studies are suggested below.

In view of the interesting binding data of the altered receptor, further characterization of its molecular weight by biophysical methods

is desirable. This can be performed by density gradient centrifugation with the altered receptor-L-T_4 complex at low temperatures to minimize to dissociation.

Synthesis of photoaffinity-labelling analogs can be extremely useful to many purification and characterization studies. So far, only an approach by modification of the amino group in the side chain has been undertaken. However, this method often leads to analogs with much diminished binding affinity (<1%). Based on the binding data of 3,5-diiodo-naphthyronines in Chapter Two, it seems reasonable to prepare 3'-azido-4'-hydroxyl-1'-(3,5-diiodo)-naphthyroacetic acid. This is owing to the similiarities in π, α, molecular refraction (MR) between N_3 and Br. Moreover, the acidic side chain can not only increase binding affinity several-fold, but also avoid the destruction of the azido group in the step of acidification to remove protection groups of the amino group.

The possible DNA-binding properties of TBPA or TBG are worth further investigations. Although DNA-binding sites for TBPA were previously proposed by Blake and Oatley (1977), only one study with plasma (MacLeod and Baxter, 1975) has been carried out to date. Under their experimental conditions, $[^{125}I]T_3$ may have bound to a component protein other than TBPA. Moreover, since the plasma binding proteins used in that study were not purified, low affinity DNA binding might have been left unnoticed. Therefore, in future attempts, other fast-seperating techniques, such as nitrocellulose filter adsorption assay (Hughes et al., 1981) with purified TBPA or TBG should be employed . In this type of study, either $[^{125}I]T_4$ or $[^{32}P]DNA$ can be used. Hughes et al. (1981) have recently reported that the progesterone receptor has high-affinity binding to DNA (Kd = 10^{-10}M), and single-stranded DNA containing limited nicks is preferred. Thus, it is

of interest to use DNA's of various tertiary structures. Further, it is

interesting to note that the gene of growth hormone (Martial et al., 1977a)

known to be regulated by thyroid hormone may be used in these studies.

Furthermore,electronmicroscopic studies by using purified proteins and

DNA will be interesting.

APPENDIX 1

```
COMPBIND:PROCEDURE;
     /* THIS PROCEDURE PRINTS A TABLE OF COMPETITIVE BINDING ASSAY
DATA AFTER RECEIVING INPUT OF:  FINAL COLD ANALOG CONCN.
TOT. COUNTS ADDED, % I- CORRECTION,
NUNSPECIFICALLY BOUND 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 TAI 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 TAI;
SCAN TAI COLS 1,5 TO 9;
IF %YESANSWER('TYPE YES TO CONTINUE') THEN EA;
T = %FITLINE( TAI,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 '

     SPACE HERE


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 GTAI FROM TAI AS COL 1 VS COL 8;
ADD CURVE TO GTAI FROM TAI AS COL 1 VS T.
EA0 GTAI;
END;
```
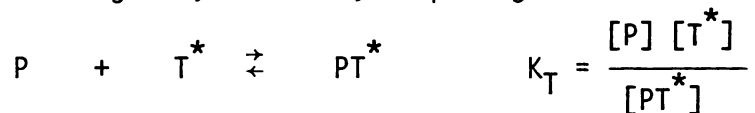
TABLE USED IN PROCEDURE COMBIND

DATAIN2 OR X 13C

1 CONCN. ANALOG ADDED (M) <NUMBER>	2 TOT. COUNTS ADDED <NUMBER>	3 CPM BOUND T3 PT3X <NUMBER>	4 (D) TOT COUNTS CORRECTED FOR IODIDE <NUMBER> CELL('TOT. COUNTS ADDED')-(5. /100 XCELL('TOT. COUNTS ADDED')

5 (D) PT3X- NON-SPEC. <NUMBER> CELL('CPM BOUND T3 PT3X')-2553	6 (D) 4/5 TOT COUNTS <NUMBER> (4 /5)XCELL('TOT COUNTS CORRECTED FOR IODIDE')	7 (D) T3X FREE CPM <NUMBER> CELL('4/5 TOT COUNTS')-CELL('CPM BOUND T3 PT3X')

8 (D) T3X/PT3X <NUMBER> CELL('T3X FREE CPM')/CELL('PT3X- NON-SPEC.')	9 (D) PT3X/T3X <NUMBER> 1 /CELL('T3X/PT3X')

10 (D)
CONCN. OF
T3 FREE (M)
<NUMBER>
(2.82E-15 XCELL('T3X
FREE CPM'))-(.12 X(2.82E-15 XCELL('T3X
FREE CPM')))

11 (D)
COLD T3 ADDED
/1+PT3X/T3X
<NUMBER>
CELL('CONCN.
ANALOG
ADDED (M)')/(1 +CELL('PT3X/T3X'))

12 (D)
(T3X/PT3X)XPT-
T3
<NUMBER>
(CELL('T3X/PT3X'X1.92E-15)-CELL('T3X/PT3X')

13 (D)
FREE ANALOG
CONCN (M/L)
<NUMBER>
CELL('CONCN.
ANALOG
ADDED (M)')-(5.4E-18 -(((CELL('PT3X/T3X')/1.19C9)-
(CELL('PT3X/T3X')XCELL('CONCN. OF
T3 FREE (M)')))))

Appendix II. <u>Derivation of Equation Used in Competition Binding Analysis</u>

Given two ligands, T^* and X, competing for one binding site,

$$P \quad + \quad T^* \; \rightleftarrows \; PT^* \qquad\qquad K_T = \frac{[P]\,[T^*]}{[PT^*]}$$

$$+$$

$$X$$

$$\downarrow\uparrow$$

$$PX$$

$$K_X = \frac{[P]\,[X]}{[PX]}$$

where $[T^*]$ = concentration of $[^{125}I]$ thyroid hormone

 $[X]$ = concentration of unlabeled competing analog

 K_T = dissociation constant for T^*

 K_X = dissociation constant for X

 $[P]$ = concentration of free binding sites

 $[PT^*]$= concentration of binding sites occupied by T^*

 $[PX]$ = concentration of binding sites occupied by X

 $[P_0]$ = total concentration of binding sites in the portein

In our studies we can measure PT, assuming that $[T^*]$, $[P_0]$ are fixed and that $[X]$ is varied.

(1) First define protein forms:

$$[P_0] = [PX] + [P] + [PT^*]$$

(2) Redefine in terms of constants, and measurable entities using K_T and K_X,

$$[P_0] = \frac{[P]\,[X]}{[PX]} \quad + \quad \frac{[K_T]\,[PT^*]}{[T^*]} \quad + \quad [PT^*]$$

Since $[P] = \dfrac{K_T}{[T^*]} [PT^*]$, substitute in the first term:

$$[P_0] = \frac{K_T \; [X]}{K_X \; [T^*]} [PT^*] \;+\; \frac{K_T}{[T^*]} [PT^*] + [PT^*]$$

$$[P_0] = [PT^*] \left(\frac{K_T}{K_X} \frac{[X]}{[T^*]} \;+\; \frac{K_T}{[T^*]} \;+\; 1 \right)$$

$$= \frac{[PT^*]}{[T^*]} \left(\frac{K_T}{K_X} \;+\; K_T \;+\; [T^*] \right)$$

Rearrange:

$$\frac{[T^*]}{[PT^*]} = \frac{1}{[P_0]} \frac{K_T}{K_X} [X] \;+\; \frac{K_T + [T^*]}{[P_0]}$$

Assume:

(1) The concentration of radiolabeled hormone free is much smaller than K_T(the equilibrium dissociation constant). Therefore, $[^{125}I]$-labeled hormone bound is neglible as compared with $[P_0]$.

(2) The added concentration of analog X is a good estimate of the concentration of free X.

Therefore: $[T^*] + K_T \cong K_T$, and $\dfrac{[T^*]}{[PT^*]} = \dfrac{K_T}{[P_0] \, K_X} [X] + \dfrac{K_T}{[P_0]}$

The ratio of the slope to the Y-intercept of the last equation is then the

reciprocal of K_X , namely, the equilbrium association constant of analog X.

Appendix III. <u>Derivation of Equation for Dissociation Kinetics of</u>
<u>a Two-Binding-Site Protein</u>

Assume that the dissociation of species P_{II} and P_I, as described in Chapter Four, Part I, is of the first order. Then, at any time t after the process has been initiated, the amount of radioactivity $(B(t))$ due to bound radio-ligand for species P_{II} and P_I, where P_{II} as well as P_I are determined by the mass action law at equilibrium prior to the dissociation process,

are:

$$B_{II}(t) = P_{II} e^{-k_{-1}^{II} t} \qquad \text{------------------------------(1)}$$

and

$$B_I(t) = P_I^{Total} e^{-k_{-1}^{I} t} \qquad \text{------------------------------(2)}$$

Therefore,

$$B^{Total}(t) = B_{II}(t) + B_I(t) \qquad \text{--------------------------(3)}$$

But

$$P_I^{Total} = P_I + P_{I'} \quad , \text{ where } P_{I'} \text{ is the species generated}$$

after one of the bound ligands is dissociated, and thus

$$P_{I'} = P_{II} (1 - e^{-k_{-1}^{II} t}) \qquad \text{--------------------------(4)}$$

Substitute (1), (2), (4) into (3)

$$B^{Total}(t) = P_{II} e^{-k_{-1}^{II} t} + [P_{II}(1-e^{-k_{-1}^{II} t}) + P_I] e^{-k_{-1}^{I} t}$$

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