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Decreased Triiodothyronine Binding to the Hepatic Nuclear Thyroid Hormone Receptor in the Diabetic Mouse

Thomas J. DeWind

Abstract

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The diabetic C57BL/KsJ-*db m* mouse has abnormal thyroid hormone levels and indications of thyroid hormone resistance. To investigate the basis of these abnormalities, the hepatic nuclear thyroid hormone receptor was extracted with 0.4 M KCl, 1.1 mM $MgCl_2$, 20 mM Tris/HCl, pH 7.9 from hepatocyte nuclei of normal C57BL/KsJ, heterozygous C57BL/KsJ-*db m* (*db/m*), and diabetic C57BL/KsJ-*db m* (*db/db*) mice. Normal and heterozygous mice were grouped together as the controls. Triiodothyronine (T_3) binding studies at 4°C using nitrocellulose filtration to separate free T_3 from receptor bound T_3 demonstrated an apparent dissociation constant of $(1.3 \pm 0.8) \times 10^{-10}$ M for controls which was significantly less than that of $(8.7 \pm 10.4) \times 10^{-10}$ M for diabetic mice ($p<0.01$, one tailed *t*-test). However, the maximum binding capacities were not significantly different at $(4.6 \pm 3.3) \times 10^{-13}$ moles/mg DNA for controls and $(3.2 \pm 4.6) \times 10^{-13}$ moles/mg DNA for diabetic mice. Triiodothyronine-receptor dissociation rates also

demonstrated a significantly greater dissociation of the diabetic T_3-receptor complex ($p<0.05$, one-tailed *t*-test). The half-life for dissociation was 101 ± 22 hours for controls versus 70 ± 21 hours for diabetics. Although equilibrium binding conditions were not achieved, the use of Scatchard analysis to compare the controls and diabetics is justified by the similar conclusion from dissociation experiments that T_3 binding is significantly decreased in the diabetic mouse. Triiodothyronine was found to interact freely with the receptor, binding 98 ± 2% of that predicted to be bound if T_3 were free in solution, even though 70 ± 2% of the T_3 is initially adsorbed to the glassware. However, adsorption to glassware did explain the negative values for specific binding above 10^{-6} M T_3 as calculated from the difference between total and nonspecific binding. Isoelectric focusing and sedimentation velocity of the receptor preparation did not demonstrate any differences between control and diabetic mice. Comparison of the level of saturation of the nuclear T_3 receptor in the diabetic mouse to other obese syndromes suggests that the decreased T_3 binding reported here has a significant impact on the obesity of the diabetic mouse.

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Each person whose signature appears below certifies that this dissertation in his opinion is adequate, in scope and quality, as a dissertation for the degree Doctor of Philosophy.

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Table of Abbreviations

ATP	adenosine triphosphate
BAT	brown adipose tissue
BCA	bicinchoninic acid
Bis	N,N'-methylene-bis-acrylamide
C.L.	confidence limit
Ci	curie, 3.7×10^{10} disintegrations per second
C_m	concentration in the mixing chamber for isokinetic gradient sedimentation
cpm	counts per minute
C_r	concentration in the reservoir for isokinetic gradient sedimentation
D-T_3	3,3',5-triiodo-D-thyronine
DNA	deoxyribonucleic acid
dps	disintegrations per second
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
GH	growth hormone, somatotropin
GH_1	a growth hormone producing cell line
GH_3	a growth hormone producing cell line
I_2	molecular iodine
K_a	association constant
K_d	dissociation constant
Kda	kilodaltons

L	ligand
L-T_3	3,3',5-triiodo-L-thyronine
LR	ligand-receptor complex
M	molar (moles/liter)
MBC	maximum binding capacity
MW	molecular weight
n	number of samples
pI	isoelectric pH
R	receptor
RNA	ribonucleic acid
rT_3	3,3',5'-triiodothyronine
T_3	3,3',5-triiodothyronine
T_4	thyroxine, 3,3',5,5'-tetraiodothyronine
TBG	thyroxine-binding globulin
TBPA	thyroxine-binding prealbumin
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	Tris(hydroxymethyl)aminomethane, $(HOCH_2)_3CNH_2$
TSH	thyroid-stimulating hormone, thyrotropin
ν	moles of ligand bound
V_m	volume of the mixing chamber for isokinetic gradient sedimentation
V_r	initial volume in the reservoir for isokinetic gradient sedimentation

1. Introduction

1.1 Thyroid Hormones

1.1.1 History

Thyroid hormones were isolated in 1914 by E. C. Kendall. Although he was able to crystalize them, their chemical nature was unknown. In 1927 Harington and Barger synthesized thyroxine (T_4) and showed it to be the major component released from the thyroid gland. In 1952 triiodothyronine (T_3) was synthesized and shown to be another important molecule mediating thyroid effects (Gross & Pitt-Rivers, 1953). The conversion of T_4 to T_3 in peripheral tissue was demonstrated by Sterling in 1970.

In spite of decades of study, many aspects of thyroid hormone action remain unknown. Although thyroid physiology is generally well established, the mechanism of hormone transport to its receptor, the relative importance of T_4 versus T_3, the cellular location of the receptor, and the control of the hormonal response all still remain to be fully elucidated.

1.1.2 Thyroid Gland Physiology

The thyroid gland contains the majority of the iodine found in the body. Iodide from the diet is actively concentrated in the thyroid gland where it is oxidized to elemental iodine by thyroperoxidase. Free radicals of the tyrosine residues in thyroglobulin molecules and of the

iodine atom then combine at the active site of thyroperoxidase to form monoiodotyrosine and diiodotyrosine. Thyroperoxidase is also involved in the coupling of monoiodotyrosine and diiodotyrosine to form T_3 and T_4 dipeptide derivatives in thyroglobulin. These iodinated residues in thyroglobulin are stored in follicles in an aggregation called colloid until thyroid hormone is required. Endocytosis of colloid followed by fusion with lysosomes leads to proteolytic enzymatic cleavage of thyroglobulin yielding the active forms of thyroid hormones which are then released into the blood stream.

Circulating thyroxine-binding globulin (TBG) binds more than three-fourths of the thyroid hormone released. Thyroxine-binding prealbumin (TBPA) and serum albumin also bind thyroid hormone so that only 0.3% of T_3 and 0.03% of T_4 remains unbound and freely diffusible.

The half-life of T_4 in plasma is 6 days while that of T_3 is less than 1 day. The liver is the major site of thyroid hormone inactivation. There T_3 and T_4 combine with glucuronic acid and the conjugates formed are excreted in the bile. In the intestine, bacteria cleave the conjugates to release the hormones which may then be reabsorbed into the blood stream. Thus, within the intestinal lumen 30% of the T_4 and 75% of the T_3 pools are

present in a form that is available to buffer plasma hormone levels (DiStefano et al., 1986). Inactivation of thyroid hormones also occurs through extrathyroidal deiodination and deamination.

1.1.3 The Effects of Thyroid Hormone

Thyroid hormones have a wide range of effects on vertebrates. Triiodothyronine stimulates cell replication, differentiation, development, thermogenesis, and O_2 consumption. Triiodothyronine regulates some enzyme levels and α_{2u}-globulin production. It also induces catecholamine receptor synthesis, stimulates mammary gland function, stimulates growth hormone production, and inhibits production and secretion of thyrotropin (TSH) and prolactin (Samuels, 1983). The effects of thyroid hormones on growth appear to be mediated through their effects on increasing growth hormone secretion and effectiveness (Schwartz, 1983).

Some of the effects of thyroid hormones are species or even tissue specific. Amphibians require thyroid hormones to undergo metamorphosis (Galton, 1983). During tadpole metamorphosis, the maximum binding capacity (MBC) for T_3 increases in erythrocytes, although not in hepatocyte nuclei. This increase is speculated to be involved in controlling tissue specific cell destruction (Galton & Germain, 1985). Thus, in lower vertebrates

thyroid hormones seem to be involved primarily in development (Weirich et al., 1987).

1.1.3.1 Development and Maturation

Although thyroid hormones are present in the fetus, they are not required prior to birth (Schwartz, 1983). Even after birth they are not required to maintain life; however, they are required for normal post-natal development. Hamburgh et al. (1971) proposed that thyroid hormones act to switch cells from a state of proliferation to one of differentiation. Consistent with this hypothesis, thyroid hormones are required for skeletal maturation and for brain development.

Although thyroid hormones are not necessary prior to birth, they must be present immediately after birth to have proper development. T_3 treatment at 2 months of age is too late to restore completely normal development (Schwartz, 1983). Replacement T_3 is needed in the rat within 10 days after birth to avoid permanent developmental damage.

Respiratory distress syndrome may also be related to low thyroid hormone levels. Since both T_3 and glucocorticoids appear to initiate the differentiation of lung, it may be necessary that both be diminished to have respiratory distress syndrome (Schwartz, 1983; Ikegami et al., 1987). In general, if an animal is hypothyroid at

the time of birth, a critical post natal period of hormone-dependent biochemical maturation may be missed.

1.1.3.2 Ribonucleic Acid (RNA)

One of the most widely reported effects of T_3 is that of influencing the production of specific RNAs. Dozin et al. (1985c) showed an 11 fold increase in the messenger RNA (mRNA) for malic enzyme after injecting rats with saturating levels of T_3. Fatty acid synthetase, acetyl-CoA carboxylase, ATP-citrate lyase, glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, growth hormone, α_{2u}-globulin, and carbamoyl-phosphate synthetase I mRNAs are also known to be increased by increased T_3 levels (Bismuth et al., 1985; Dozin et al., 1985c).

Triiodothyronine alters the levels of 19 of the 230 rat hepatic mRNAs that can be followed by a mRNA dependent translation system. This system generates ^{35}S-labeled proteins which can be separated by two-dimensional gel electrophoresis. The levels of 11 mRNAs are increased in parallel with T_3, 7 are decreased, and 1 is biphasic. These effects may be direct or indirect. For example, the T_3 stimulation of growth hormone production explains the effects seen on 6 of the mRNAs (Towle, 1983). Magnuson et al. (1985) strengthened the correlation between T_3 levels and mRNA levels by reporting that growth hormone mRNA levels are proportional to the nuclear T_3 receptor

occupancy. On the other hand, α-glycerophosphate dehydrogenase and hepatic malic enzyme mRNAs do not demonstrate a linear relationship.

In addition to increased RNA levels, all classes of RNA polymerase have increased activity after exposure to T_3. Hepatocyte nuclear RNA polymerase activity is elevated within 3-6 hours after T_3 administration (Barsano & DeGroot, 1983). This increase in activity is due to an increased amount of polymerase.

1.1.3.3 Heat Production

One of the first theories for the mechanism of stimulating cellular heat production proposed that thyroid hormones uncouple oxidative phosphorylation. However, this hypothesis has been abandoned due to several inconsistencies found in later experimental work. First, biologically inactive iodinated compounds also cause uncoupling. Second, other uncouplers could not reproduce the effects of T_3. Finally, with physiological doses of T_3 the ratio of inorganic phosphate incorporated per atom of oxygen consumed remained normal (Guernsey & Edelman, 1983).

The thermogenic response now appears to be primarily due to energy expended on increased sodium and potassium transport. This increase in ion transport in turn is due to an increased biosynthesis of the Na^+-K^+-ATPase

(Guernsey & Edelman, 1983; Towle, 1983). To a lesser extent, thermogenesis is the result of an increase in futile cycling where both biosynthetic and degradative pathways are stimulated by T_3.

1.1.3.4 Adrenergic-Type Effects

Some effects of T_3 mimic or supplement those of β-adrenergic stimulation. T_3 influences the adrenergic modulation of lipolysis (Williams & Lefkowitz, 1983). Conversely, blocking β-adrenergic events reduces the symptoms of thyrotoxicosis.

The heart demonstrates significant ties between T_3 and adrenergic-type effects. Triiodothyronine administration leads to sinus tachycardia and increased contractility in the same way as does sympathetic stimulation. In addition, T_3 increases the number of cardiac β-adrenergic receptors while decreasing the number of α-adrenergic receptors (Williams & Lefkowitz, 1983; Towle, 1983). Although some of the effects of T_3 and adrenergic stimulation are identical, T_3 does not have its effect at the β-adrenergic receptor. Neither does adrenergic stimulation lead to hyperthyroidism. Thus, the link between thyroid hormones and adrenergic stimulation remains to be elucidated.

1.1.3.5 Endoplasmic Reticulum Effects

Triiodothyronine is required to maintain normal hepatic endoplasmic reticulum (Roy, 1983). Growth hormone can partially normalize α_{2u}-globulin mRNA levels but T_3 is required to fully restore the level of α_{2u}-globulin protein to normal (α_{2u}-globulin is the major protein in the urine of male rats). Thus, one proposed effect of T_3 is to keep endoplasmic reticulum functioning normally, thereby allowing α_{2u}-globulin to be exported before it is degraded.

1.1.4 Deiodination of Thyroxine

In man 83% of T_3 is derived from non-thyroidal deiodination of T_4 (Oppenheimer, 1983). This conversion is important in that T_3 has 3-5 times greater biological activity than T_4. Since there is 3-5 times less T_3 than T_4, both exert an equal effect.

1.1.4.1 Deiodinase Types

There are two 5'-monodeiodination enzymes (McNabb et al., 1986). The type I 5'-monodeiodinase has a K_m for T_4 of 5 μM and is present in liver, kidney, and many other organs. The type II deiodinase is important in supplying intracellular T_3 (Tanaka et al., 1987). It has a K_m for T_4 of 3 nM and is located in brain, anterior pituitary, placenta, and brown adipose tissue. In brown adipose tissue the intracellular conversion of T_4 to T_3 is

required for optimal thermogenic function (Blanco & Silva, 1987).

1.1.4.2 The Significance of the T_3/T_4 Ratio

Wilkin and Isles (1984) studied serum T_3 and T_4 levels in euthyroid man and found the natural variations in T_3 levels to be independent of T_4 levels. However, the T_3/T_4 ratio seemed to buffer the variation in T_4. They concluded that a relatively constant level of thyroid hormone effects is achieved over a wide range of T_4 values by alterations in the T_3/T_4 ratio.

1.1.4.3 Factors Affecting the Deiodination of T_4

T_4 to T_3 conversion is increased by the availability of carbohydrate in the diet (Mariash & Oppenheimer, 1983; Schalch & Cree, 1985). Conversion is also increased by elevated levels of reduced sulfhydryls (Smith & Eastman, 1980). The sex hormones have some effects as well. Female rats approaching puberty show a decrease in T_3 synthesis not seen in males (Harris et al., 1979). In the pineal gland, the T_4 deiodinase follows a circadian rhythm defined by β-adrenergic stimulation (Tanaka et al., 1987). Thyroid hormones are also responsive to cold exposure with the T_3/T_4 ratio increasing more than 2-fold in rats exposed to cold. This adaptive change is mediated through the sympathetic nervous system (Guernsey & Edelman, 1983; Rudas & Pethes, 1986). Genetically obese mice will die in

the cold due to an inability to stimulate their 5'-deiodinase, thus illustrating the significance of this adaptation.

1.1.5 T_3 Transport

1.1.5.1 Serum T_3 Transport

Over 99% of the T_3 and T_4 are transported in the blood bound to plasma proteins. The level of free T_3 is thought to be about 6×10^{-12} M in euthyroid animals with a serum binding K_a of 1.6×10^{11} M^{-1}. Ordinarily it is the free fraction of a hormone that is thought to be biologically active; however, the concept of free hormone may not apply to thyroid hormones. Instead, there is a "collisional transfer theory" for thyroid hormones in which T_3 is directly transferred between proteins in a manner that mimics the existence of a small free fraction in solution (Oppenheimer, 1983). According to this theory, a large number of low affinity sites would bind most of the thyroid hormone and act to transport and buffer the supply. A few high affinity sites would then pick up T_3 from the low affinity sites and lead to the effects of thyroid hormone. In support of this theory, no lowering of the "free" hormone concentration resulted from the dilution of serum (Samuels, 1983), although with dilution, protein bound T_3 would be expected to buffer the free T_3 level.

1.1.5.2 Cytoplasmic T_3 Transport

Originally, the uptake of T_3 into cells was thought to be entirely due to diffusion (Surks et al., 1975). However, the levels of T_3 binding in the cell indicate that either free T_3 has been underestimated by equilibrium dialysis, or the cytoplasmic T_3 levels are 10-fold higher than that in plasma (Oppenheimer, 1983).

Active uptake of T_3 into cells seems reasonable in light of the presence of T_3 binding sites in the plasma membrane (Horiuchi et al., 1982b; Cheng, 1983; Casanova et al., 1984; Dozin et al., 1985b; Pontecorvi et al., 1987). Horiuchi et al. (1982a) found that the addition of monodansylcadaverine (which blocks receptor mediated endocytosis) blocks the uptake of T_3 and its accumulation in the nucleus.

Intracellular binding sites for T_3 have been described by Defer et al. (1975). Barsano and DeGroot (1983) described a group of cytosolic T_3 binding proteins having 170 times the binding capacity of the nuclear binding proteins, but which are only 1% occupied. Lennon et al. (1983) saw similar high affinity binding sites in the cytosol of brain cells in culture. Both high and low affinity sites were seen having K_a's for T_3 of 1.2 - 3.7 x 10^9 M^{-1} and 0.8 - 1.4 x 10^8 M^{-1}. The low affinity sites are thought to be involved in the storage and transport of

T_3, but the role of the high affinity sites is uncertain. In contrast to the above findings, Samuels and Tsai (1973) in an earlier examination of GH_1 cells did not find any evidence of cytosolic binding sites.

Further evidence for the involvement of some type of transport into the cell can be seen in the study of Halpern and Hinkle (1982) and Hashizume et al. (1987). Several drugs studied could slow T_3 uptake into whole cells, but not into isolated nuclei. Cytochalasin b (a microfilament disrupter), chloroquine (a lysosomal inhibitor), and dansylcadaverine (an inhibitor of transglutaminase) all inhibit the uptake of T_3 into whole cells.

1.1.5.3 Nuclear T_3 Transport

Surks et al. (1975) demonstrated that neither a cytoplasmic carrier nor energy metabolism was required to move T_3 into the nucleus. However, they did note that the apparent affinity of the purified receptor for T_3 *in vitro* was 1000-fold less than for cells *in vivo*. Oppenheimer (1985) later showed that there in fact is a stereospecific (D-L) energy dependent transport system that translocates T_3 from the cytosol to the nucleus, although antimycin A blocks the nuclear uptake of T_3 by a mechanism other than by inhibition of ATP synthesis (Valdivielso & Bernal, 1987). The presence of this transport system would

explain why less D-T_3 is found in the nucleus than L-T_3 even though D-T_3 has a higher affinity for the nuclear receptor.

Venkatraman and Lefebvre (1985) described two classes of binding sites in rat liver nuclear membranes which might be involved in T_3 translocation. One has a dissociation constant (K_d) of 1.8 nM and a maximum binding capacity (MBC) of 14.5 pmol/mg protein, the other has a K_d of 152.1 nM and a MBC of 346.8 pmol/mg protein. Evidence that these sites are capable of modulating T_3 delivery can be inferred from the finding that the specific binding of T_3 at these sites is altered by changing the composition of dietary fat (Venkatraman et al., 1986).

1.1.6 Cellular Location of the T_3 Receptor

Determining the subcellular location of receptors for a hormone with effects as diverse as those of thyroid hormones is likely to be difficult. Conclusive proof of the identification of the T_3 receptor requires adding isolated T_3-receptor complexes to an *in vitro* system and showing that duplication of the known effects of T_3 are dependent on these T_3-receptor complexes. Candidates in initial attempts to elucidate its site of action included cellular components known to bind T_3 and those thought to mediate cellular effects of thyroid hormones.

As previously stated, the effect of T_3 in elevating enzyme levels is correlated with mRNA levels. This suggests that one site of action might be the transcriptional complex. Effects on thermogenesis and respiration suggest that another possible site might be the mitochondrion. Dozin et al. (1985b) used photoaffinity labeling to label T_4 binding proteins in the plasma membrane, cytosol, and the nucleus of rat hepatocytes. Although they demonstrated the existence of multiple binding sites, they could not assign any function to these sites.

1.1.6.1 Mitochondria

Since one of the effects of T_3 is to increase respiration in the mitochondria, the mitochondrion would be expected to have a T_3 receptor. Accordingly, the inner mitochondrial membrane has been demonstrated to have T_3 binding sites which are associated with stimulation of mitochondrial RNA synthesis (Barsano & DeGroot, 1983). This mitochondrial T_3 receptor has a K_a 100 fold higher than the nuclear T_3 binding site and a molecular weight of 150,000. Hashizume et al. (1986) also found binding sites in the outer mitochondrial membrane; however, these sites were thought to be carrier proteins functioning merely to transfer T_3 to binding sites in the inner mitochondrial membrane. Even though the inner mitochondrial membrane

binding site has a high affinity for T_3, this site is not thought to be the primary initiator of the effects of thyroid hormones. In the absence of any known link between the mitochondria and the production of nuclear mRNA and with the early discovery of the nuclear binding site, this mitochondrial receptor has been largely ignored.

1.1.6.2 Nuclear

DeGroot and Torresani (1975) demonstrated T_3 binding to isolated nuclei; formaldehyde can cross-link a T_3 binding protein to chromatin (Spindler et al., 1975). Together these findings suggest that the T_3 receptor is in close proximity to DNA. The receptor can also be extracted from chromatin with 0.4 M KCl. It now appears to be non-randomly distributed on DNA and enriched on actively transcribing DNA (Apriletti et al., 1983; Gardner, 1975). The receptor is also preferentially found in the inter-nucleosomal spacer region.

Further evidence for receptor-chromatin interaction is seen in the studies of Anselmet et al. (1983). They demonstrated a rapid decrease in T_3 binding to purified receptor preparations during storage for 24-48 hours at 0°C. This loss of affinity was accelerated by the addition of exogenous arginine-rich histones (H3 & H4), but reversed by other histone fractions (especially H1)

and DNA. Addition of histones alone increased the number of binding sites remaining over time but decreased the apparent binding affinity. Addition of histones with DNA restored both the affinity and the number of receptors. Bismuth et al. (1985) also protected the nuclear T_3 receptor from degradation during isolation with histones, DNA, and a non-histone protein.

Additional evidence that the nuclear T_3 binding site is actually the T_3 receptor comes from T_3 analogs which show that relative affinities for this nuclear protein are comparable to their biological activity. Tissues which are responsive to T_3 also contain this protein in amounts proportional to their response to T_3 (Barsano & DeGroot, 1983).

Studies with T_3 responsive genes have shown that T_3 induces alterations in chromatin structure as indicated by demethylation and increased DNase I sensitivity (Jump et al., 1987; Nyborg & Spindler, 1986). Later studies on the 5'-flanking region of the growth hormone gene have demonstrated that thyroid hormones require the sequences between -254 and -241 in order to act as an enhancer of growth hormone transcription (Wight et al., 1987; Barlow et al., 1986; Koenig et al., 1987). The sequences between -46 and -21 are required for an inhibitory effect by thyroid hormones. These sites suggest sequence specific

binding by the DNA binding domain of the thyroid hormone receptor. However, the fact that T_3 modulates the growth hormone promoter in only a few cell types indicates that cell specific factors are also involved (Crew & Spindler, 1986). Also, short-lived proteins are required for almost all the effects of T_3 on RNA synthesis (Santos et al., 1987; Jacoby et al., 1987). By blocking protein synthesis as little as 30 minutes before T_3 addition, its ability to increase the transcription of growth hormone RNA is abolished. Thus, either there are labile transcriptional elements involved (but general RNA synthesis is not blocked), or else the T_3-receptor complex interacts with DNA-protein complexes.

1.1.7 Physical Parameters of the T_3 Receptor

The known physical parameters of T_3 receptors are summarized in Table 1. Table 2 summarizes the T_3 binding constants observed. Seelig et al. (1981) examined the kinetics of T_3-receptor association and dissociation, noting a discrepancy between the half-life for dissociation calculated from the two sets of data. They found that the addition of further aliquots of T_3 receptor to a previously incubated mixture of T_3 receptor containing an excess of T_3 did not increase specific T_3 binding. The combination of these two factors caused them to suggest that T_3 becomes associated with nonspecific

Table 1:
Physical parameters of thyroid hormone receptors including sedimentation coefficient, stokes radius, molecular weight, density, and isoelectric pH (pI).

Tissue Type/ Cell Compartment	Sediment. Coeff. (S)	Stokes Radius (nm)	MW (Kda)	Density (g/ml)	pI	Reference
Rat Liver/Nuclei			55-65			Bolger & Jorgensen, 1980
			59			Gruol & Kempner, 1982
	3.4-4.5		50.5			Barsano & DeGroot, 1983
			47		6.2	David-Inouye et al., 1982
			56, 45			Dozin et al., 1985a
	3.8					Samuels et al., 1980
			50-70		5.8	Apriletti et al., 1983
					7.8	Apriletti et al., 1983
	3.5	3.5				Latham et al., 1976
		3.4	49			Ichikawa & DeGroot, 1986
Rat GH_1/Nuclei	3.8	3.3	54	1.36		Perlman et al., 1982
			57, 47			Casanova et al., 1984
Rat GH_1/Intact cells			57, 47			Pascual et al., 1982

f/f_0 is 1.4 (Ichikawa & DeGroot, 1986; Latham et al., 1976) and $f/f_{0\ shape}$ is 1.212 (Perlman et al., 1982; Ichikawa & DeGroot, 1986).

Table 2:
Binding constants of thyroid hormone receptors.

Tissue Type/ Cell Compartment	10^{10} x K_d (M)	MBC (pmol/mg DNA)	Reference
Rat Liver/Nuclei	1.9	1	Spindler et al., 1975
Rat Liver/Nuclei	3.3	0.3	Gullo et al., 1987
Rat Liver/Nuclei	0.002-0.2		Barsano & DeGroot, 1983
Rat Liver/Nuclei	1		Dozin et al., 1985b
Rat BAT/Nuclei	3.2	0.4	Burgi & Burgi-Saville, 1986b
Rat Neuron/Nuclei	3.8	1.2	Gullo et al., 1987
Rat Glial/Nuclei	3.4	0.2	Gullo et al., 1987
Rat GH_1/Intact Cell	1.6		Samuels, 1983
Rat GH_3/Plasma Membrane	18		Horiuchi et al., 1982b
Low affinity site	2600		Horiuchi et al., 1982b
Beef Heart/Mitochondria	0.1		Backer et al., 1986
Mouse Liver/Nuclei (Fed)	2	0.7	Burgi et al., 1986
Mouse Liver/Nuclei (Fasted)	2	0.5	Burgi et al., 1986
Porcine Granulosa/Nuclei	55	1	Wakim et al., 1987
Human Lymphocyte/Nuclei	11.6		DeNayer et al., 1987
No cortisol	25		DeNayer et al., 1987
Human Lymphocyte/Nuclei	1.1	4.4	Bernal et al., 1978
Human Leukocytes/Nuclei	3.4	1.2	Burman et al., 1980
Human Adipose/Nuclei	0.13	2.2	Cronrath et al., 1988
1/2 Max. Malic Enzyme Induction	0.4		Towle, 1983
1/2 Max. GH stimulation	2.2		Towle, 1983

sites from which it is only slowly released. Previous studies utilized a T_3 binding resin to determine the level of free T_3. Seelig et al. (1981) determined that the resin removes T_3 from some of the nonspecific sites giving a false, high value for free T_3. They called these sites "inapparent, nonspecific sites" since they had not been recognized previously.

The presence of nonspecific sites with essentially irreversible binding over the time span of normal binding assays brings the assumptions for Scatchard plots into question. After considering the kinetics of association and dissociation, Seelig et al. (1981) proposed a model which could explain both the kinetic and Scatchard plot data. This model accounts for the normal appearance of Scatchard plots by proposing mimicry of equilibrium binding by a kinetic phenomenon. Thus, as suggested in a previous section, T_3 may not be completely free, but instead may be reversibly bound to nonspecific binding sites.

Yagura and Walfish (1982) disagree with the conclusion of Seelig et al. (1981) that the inapparent sites invalidate binding assays. They feel that prewarming the binding assay at 30°C for 40 minutes will allow equilibrium to be reached and by accounting for endogenous T_3, Scatchard plots will give meaningful

results. In an apparent concession to the widespread usage of Scatchard plots, Seelig and Oppenheimer (1982) have continued to report data analyzed by Scatchard plots and have agreed that the equilibrium constant depends markedly on temperature.

The results of binding studies have also been questioned because they may overlook the biological significance of 3,3',5'-triiodothyronine (rT_3) binding. Smith and Eastman (1980) demonstrated separate binding sites for T_3 and rT_3 and suggested that rT_3 may have a physiological role. Dithiothreitol (DTT) is added to most binding assays to maintain T_3 binding due to its effects on cysteine residues in the receptor; however, reduction decreases the amount of rT_3 binding to the point that it is insignificant, thus rT_3 binding is often overlooked. Reduced sulfhydryls may regulate nuclear T_3 and rT_3 binding, but current assay methods will mask this phenomenon.

Attempts in this study to utilize the DEAE-cellulose filtration method of Heintz et al. (1984) were unsuccessful. Personal communications with this laboratory confirmed the sensitive nature of this assay method. The use of G-25 Sephadex gel filtration, although time consuming, is suitable for binding studies on large amounts of crude receptor preparations. However, with

smaller amounts of more highly purified receptor, protein adsorption to Sephadex results in a complete loss of the receptor protein (Latham et al., 1981). Thus, binding studies completed without the use of nitrocellulose filtration (Inoue et al., 1983) had additional technical difficulties to overcome. The separation of receptor bound T_3 from free T_3 was generally difficult to carry out with good reproducibility.

1.1.8 Mechanism of Action of Thyroid Hormone

In light of the diverse effects of thyroid hormones, one can see why it has been difficult to determine their cellular site of action and their complete mechanism of action. Nevertheless, a number of theories have been proposed on the basis of assumed sites of action and supportive data have been presented for some of them. Proof of a mechanism would involve selective blockage of steps in the process to show that they are required for the transduction of the effect or conversely, the addition of components in an artificial system to recreate the effect. In the absence of requisite steps one relies on temporal sequences to suggest the path of action. The final products may also give clues to the identity of important steps. Since many of the effects of thyroid hormones result in alterations in the levels of proteins,

a major area of focus has been investigation of the steps involved in protein synthesis.

One mechanism whereby T_3 might influence the level of expression of a protein is to regulate the activity of RNA polymerase. The rate of synthesis of RNA is stimulated by T_3 and cytoplasmic RNA content is increased by 50-70% over that of the thyroidectomized rat following T_3 administration. This would support the hypothesis that RNA polymerase might be involved. Increases in RNA polymerase have also been seen in isolated nuclei incubated with T_3 (Towle, 1983). Similarly, Nakamura et al. (1987) reported that RNA polymerase I activity is increased by T_3 in mature rat brain. On the other hand, *in vivo* no differences in RNA polymerase activity were seen after T_3 administration by Shields and Tata (1976).

Savouret et al. (1985) found that thyroid hormone receptors interact preferentially with the SV40 minichromosome. Ten times more T_3 receptor was associated with the replicating viral DNA than with cellular DNA. This suggested a preference for transcriptionally active chromatin. Oppenheimer (1985) agrees with this observation and further suggests that T_3-receptor binding to chromatin might cause increased transcription. These speculations have been greatly bolstered by the previously mentioned studies (Wight et al., 1987; Koenig et al.,

1987; Barlow et al., 1986) which demonstrated a requirement for specific DNA regions 5' of the growth hormone gene in order to have T_3 stimulation of growth hormone transcription.

Since the amount of growth hormone is increased by T_3 and both have direct effects on RNA expression, their effects need to be separated. Up to 40% of the RNA stimulation associated with T_3 administration is directly related to the T_3 dependent increase in growth hormone. Growth hormone and T_3 effects can be separated since growth hormone effects on RNA polymerase peak at 3 hours; whereas, those of T_3 peak at 24 hours.

Another process that could mediate T_3 effects is phosphorylation. Changes in the phosphorylation of nuclear proteins have been observed as a result of administration or withdrawal of T_3; however, no mechanism has been proposed that requires phosphorylation. Knopp and Brtko (1984) were able to show a correlation between phosphorylation of nuclear non-histone proteins and changes in RNA synthesis resulting from the addition of T_3 or T_4. Additionally, the phosphorous content of ribosomes is lower in the thyroidectomized rat. This may lead to an altered ribosomal configuration and decreased translational activity.

Biro (1983) proposed a model for thyroid hormone action in which the two aromatic rings of T_3 are used to link two macromolecules. In this model, each of the rings of T_3 binds a macromolecule. The amino moiety of T_3 might also be incorporated into proteins which would further facilitate the linking of macromolecules. However, at high T_3 concentrations every binding site would be filled with a separate T_3 molecule, thus preventing linking. This model would then provide a simple explanation of why the elements of oxidative phosphorylation dissociate in thyrotoxicosis.

In addition to possible effects on macromolecular linking, phosphorylation, or effects on transcription, T_3 may have other means to assert its actions. The plasma membranes of cardiomyocytes appear to be a target for a direct action of thyroid hormone. T_3 effects are also tissue specific even though the nuclear receptors appear to be identical. Thus, there may be cell-specific factors which mediate the effect of T_3. In any event, since there appear to be multiple binding sites, there may be multiple mechanisms leading to the final T_3 dependent response.

1.1.9 Control of Thyroid Hormone Receptor Number

The nuclear receptor has a 5 hour half-life and about 95% of the receptor is replaced in 24 hours. This extensive renewal of receptor allows the number of binding

sites to be easily altered by changes in the rate of receptor synthesis. Addition of T_3 to GH_1 cells decreases the rate of synthesis of receptor by 60% (Samuels et al., 1983; Raaka & Samuels, 1981; Samuels et al., 1976). Later, it increases the rate of degradation by 30% so that receptor half-life decreases to 3.3 hours. In contrast, Nakamura et al. (1979) found that T_3 administration increases the number of rat liver nuclear T_3 receptors in hypothyroid rats. The anterior pituitary also demonstrates an increase in the number of T_3 receptors in response to T_3 (Lemarchand-Beraud et al., 1987).

Many conditions yield altered numbers of nuclear receptors. Burgi et al. (1986) summarized numerous reports indicating that a decreased MBC can result from fasting, thyroidectomy, hypophysectomy, adrenalectomy, partial hepatectomy, glucagon treatment, T_3 treatment, cholera toxin treatment, the presence of liver tumors, and uremia. Overfeeding increased the hepatic MBC, while cold-exposure and diabetes did not have an effect (Burgi & Burgi-Saville, 1986; Las & Surks, 1981). Again, conflicting results have been obtained from other tissues. Burman et al. (1980) found the T_3 receptor number in human leukocytes to increase with fasting and in hypothyroidism in contrast to the decreases reported in rats.

1.1.10 A Thyroid Hormone Receptor Gene

Weinberger et al. (1986) described a human c-erb-A gene located on chromosome 17 that encodes a T_3 binding protein. This gene has been sequenced and encodes a polypeptide of 456 amino acids with a molecular weight of 52,000 which is thought to be the T_3 receptor. Sap et al. (1986) have likewise isolated the c-erb-A gene from chickens and predicted a molecular weight of 46,000 for the gene product. The product of this gene is thought to be the thyroid hormone receptor because its affinity for T_3 analogs matches the known biological effects of these analogs. There may be several variants of the T_3 receptor since the genome contains a number of c-erb-A related genes and the c-erb-A gene encodes two mRNAs.

Comparison of steroid hormone receptor genes and the newly reported retinoic acid receptor gene (Giguere et al., 1987) to the thyroid hormone receptor gene has revealed a common structure. The receptor genes have 5 domains. The central domain expresses a highly conserved metal binding "finger" region which forms the DNA binding portion of the receptor. The fingers are formed by cys-X_2-cys repeats. A hinge region in the gene connects the DNA binding region to the specific ligand binding domain.

1.2 Triiodothyronine Resistance in Man

Thyroid hormone resistance is a state in which the effects of thyroid hormones are not expressed due to a failure to transduce the presence of ligand into its normal effects. High thyroid hormone levels exacerbate diabetes, therefore one might expect individuals with thyroid hormone resistance to display increased symptoms of diabetes. Patients with thyroid hormone resistance display deaf mutism, delayed bone maturation, stippled epiphyses, goiter, and high circulating thyroid hormone levels (Refetoff et al., 1972; Bernal et al., 1978). However, no increase in the number of patients showing diabetic symptoms was reported in those with thyroid hormone resistance. In general, the patients appeared to be euthyroid and had normal TSH levels even though T_3 and T_4 were high (Refetoff, 1982).

1.3 Thyroid Hormones and Diabetes

1.3.1 The Effects of Thyroid Hormones on Diabetes

In spite of the lack of a causal relationship between high thyroid hormone levels and diabetes in human studies, there are a number of interactions between them. The effects of thyroid hormones on insulin depend on the level of T_3 and T_4 present. Malaisse et al. (1967) found thyroid hormones to increase insulin levels in the hypothyroid rat, but to decrease insulin levels at high T_3

levels. Thus, thyroid hormones have a biphasic effect on islet function. High T_3 elevates blood sugar, increases the rate of glucose absorption, increases hepatic gluconeogenesis, and decreases hepatic glycogen content. With severe hyperthyroidism one sees diminished insulin secretion. Administration of T_4 to obese mice inhibits glucose-induced insulin secretion, as well as decreasing overall pancreatic insulin content (Lenzen & Kloppel, 1978).

In adipocytes in cell culture, low thyroid hormone levels are associated with greater conversion of glucose to carbon dioxide and lipids than at normal thyroid hormone levels (Mariash & Oppenheimer, 1983). Yet most studies report non-responsiveness to insulin in hypothyroid animals. This may be due to low growth hormone levels since addition of growth hormone restores responsiveness to insulin. Administration of T_3 to levels seen in hyperthyroid rats causes them to make more ketone bodies and less triglyceride than euthyroid rats (Olubadewo & Heimberg, 1985). Glycerol addition in cell culture reduces ketone body production and increases triglyceride synthesis suggesting that glycerol 3-phosphate acyltransferase is involved.

Tahiliani and McNeill (1985) observed that T_3 treatment normalizes the depression of a cardiac myosin

ATPase seen in the diabetic rat. Methyl palmoxirate is a fatty acid analog which inhibits fatty acid oxidation, thereby forcing carbohydrate utilization and preventing the depression of calcium uptake in sarcoplasmic reticulum in the diabetic rat. Triiodothyronine in combination with methyl palmoxirate can normalize diabetic cardiac function to the level of controls. Triiodothyronine administration also normalizes pulmonary lipogenic enzyme activity otherwise decreased in streptozotocin-induced diabetic rats.

1.3.2 The Effects of Diabetes on Thyroid Hormones

Das and Ganguly (1981) found the T_3 binding capacity in diabetic rat lung nuclei to be decreased. Diabetes, hypophysectomy, and thyroidectomy all lowered serum T_3 and T_4 as well as the nuclear MBC (Wiersinga et al., 1982). On the other hand, Las and Surks (1981) saw decreased serum T_3 and T_4 but saw no change in hepatic nuclear T_3 binding capacity or serum thyroid-stimulating hormone (TSH) levels in diabetics. Glucagon lowers the MBC and could be a mechanism in diabetes which might affect the MBC (Dillmann et al., 1978). Glucose increases serum T_3 and also the T_4 to T_3 conversion (Gavin et al., 1986). Conversely, insulin and steroid hormones decrease the T_4 to T_3 conversion (Hidal & Kaplan, 1986). In contrast, insulin is required for normal liver monodeiodinase

activity in LA/N-*cp* rats (Tulp & McKee, 1986) and insulin deficiency reduces T_3 levels. The clearance rate of T_3 and T_4 in the diabetic rat is also lower, but can be normalized by insulin (Jolin & Ortiz-Caro, 1985).

It should not be surprising that the effects of changing insulin levels in diabetes would also have effects on products considered to be primarily regulated by T_3. Glucocorticoids, insulin, epidermal growth factor (EGF), and T_3 are all involved in the control of growth hormone secretion. Glucocorticoid acts synergistically with T_3 to increase growth hormone mRNA, increasing the number of RNA polymerase molecules synthesizing growth hormone RNA. Insulin inhibits the glucocorticoid response and EGF interferes with T_3 effects. Conversely, malic enzyme is increased by insulin and T_3, while glucagon counters the T_3 effect (Roy, 1983).

Studies on the leukocytes of human diabetics have been conducted, although it is difficult to obtain reproducible data from leukocytes. Kvetny (1983) found in human mononuclear cells that the only statistically significant difference in thyroid hormone binding parameters was in the MBC between type I diabetics and normals. He saw no binding at 3°C after 18 hours, almost the same conditions used for binding in this study. The T_3 K_a values he observed for diabetics were slightly below

those of controls, but the differences were not significant.

Studies examining T_3 and T_4 levels in diabetic C57BL/KsJ-*db m* mice found total serum T_3 to increase slightly and T_4 to decrease slightly at puberty, which is also the time of onset of diabetic symptoms (Fehn, 1983). The T_3/T_4 ratio was significantly altered but fasting could normalize the ratio. Triiodothyronine seems to feedback on the thyroid, while T_4 inhibits the pituitary (Erfurth & Hedner, 1988; Larsen & Silva, 1983; Pascual et al., 1987). The thyroid gland was hypoactive in spite of normal TSH levels. This is probably due to the increased T_3. Although total serum T_3 is increased, there is increased serum binding of T_3 so that free T_3 is normal or only slightly elevated (Richard Fehn, personal communication).

1.3.3 Carbohydrate, Diabetes, and Thyroid Hormone

Studies comparing the induction of malic enzyme with the level of nuclear T_3 receptor saturation suggested that the T_3 signal is highly amplified (Oppenheimer, 1978). The biological response curve is also shifted to the left of the nuclear receptor occupancy curve implying a difference in stoichiometry (Samuels, 1983). Further investigation suggested that T_3 is acting to multiply a carbohydrate-derived signal, since carbohydrate increases

induction of lipogenic enzymes even in the absence of T_3 (Mariash & Oppenheimer, 1983). In the diabetic rat the response of malic enzyme to T_3 is diminished, but fructose restores T_3 induction of malic enzyme. This is significant because fructose, unlike glucose, can be utilized by diabetic animals. It also is consistent with the hypothesis that a product of carbohydrate metabolism is interacting with the T_3 signal.

Triiodothyronine levels may also be directly affected by the composition of the diet. Overfeeding in general increases T_3 levels in man, although T_3 levels increase only slightly in the rat (Mariash & Oppenheimer, 1983). The conversion of T_4 to T_3 is also partly dependent on the presence of carbohydrate. In opposition to Mariash & Oppenheimer, Yang et al. (1987) found serum T_3 to not be changed by a carbohydrate restricted diet if fat intake was not reduced even though calories were lowered. In two previous studies, they reported a change in T_3 with general caloric restriction.

1.3.4 Obesity and Thyroid Hormones

Tulp and McKee (1986) examined LA/N-*cp* rats which develop obesity. They found that these animals have lower serum T_3 and resting metabolic rates than lean animals. They also failed to show an elevation of serum T_3 with cold exposure, cafeteria feeding, and low protein diets.

In addition, they reported that these animals were refractory to the effects of injections of T_4, but not to T_3. They also have less *in vitro* conversion of T_4 to T_3. This decreased deiodinase activity was considered to be contributory if not causative in the development of obesity. Similarly, Khan et al. (1986a) found a decrease in T_3 receptors in nuclei from obese mouse liver which preceded the onset of obesity. They proposed that the decrease might be the primary determinant in the etiology of this condition.

On the other hand, only 50% of the Zucker fatty rats showed decreased T_3 receptor concentrations (Khan et al., 1986b). Since not all Zucker rats had reduced T_3 receptor levels, additional factors may be involved in their cold sensitivity, hypothermia, and obesity. Hillgartner and Ramsos (1987) examined obese mice and found the same nuclear hepatic T_3 MBC and K_d as normals. Their results conflicted with the results of Guernsey and Morishige (1979), but Hillgartner and Ramsos explain the results of Guernsey and Morishige as being an artifact of detergent treatment of the nuclei. Hillgartner and Ramsos did find that the nuclear occupancy of T_3 receptors was lower in obese mice than in normals. The obese animals had a decreased Na^+-K^+-ATPase activity and cold intolerance that was partially reversed by T_3 administration. They

proposed that lower nuclear occupancy in spite of normal serum T_3 levels results from reduced transport of T_3 from plasma to the nucleus. This position is consistent with a proposed decrease in membrane fluidity in obese mice. Decreased fluidity might be expected to affect membrane proteins and accordingly they have shown decreased transport of labeled T_3 across the plasma membrane.

1.4 Ligand Binding

In order to understand a ligand binding phenomenon, a model is useful as the framework on which to build that understanding. The simplest model is one in which one type of ligand (L) binds to a receptor (R) having only one binding site to form the ligand-receptor complex (LR).

$$L + R \rightarrow LR$$

This model is also the most popular because of its simplicity. All of the biological responses resulting from the presence of the ligand are considered to be due to the presence of the ligand-receptor complex. In order to compare the tendency of various ligands and receptors to associate or dissociate, a constant K_d is defined to be

$$K_d = [L][R]/[LR]$$

Another quantity called the maximum binding capacity is useful in understanding the ligand-receptor system. The maximum amount of ligand that can be bound if there is only one site per receptor is equal to the amount of

receptor present. Thus, in this model, the MBC is equal to the amount of receptor present. A third symbol often used is ν which is defined as the moles of ligand bound to the receptor ([LR]).

To experimentally determine the binding constants, the following scheme is often used. Generally, the receptor is purified to minimize interferences and background binding. A ligand which can be detected is incubated with the receptor preparation to allow binding. Separation of the free ligand from that bound to the receptor gives the equilibrium concentration of bound and free ligand for the receptor concentration used. By incubating the receptor with a range of ligand concentrations, a curve can be constructed and the K_d estimated. The amount of receptor with no ligand bound is then MBC - ν. Substitution into the definition of K_d yields

$$K_d = [L](MBC - \nu)/\nu$$

Since one determines the amount of bound ligand and free ligand, this equation is frequently rearranged to

$$\nu/[L] = MBC/K_d - \nu/K_d$$

From this Scatchard (1949) pointed out that a plot of $\nu/[L]$ versus ν will have a slope of $-1/K_d$ and an X axis intercept of MBC.

This type of analysis is now routine for studies on ligand-receptor interactions. It is not without potential faults however, especially in the case of thyroid hormones. Endogenous ligand remaining bound to the receptor after the isolation procedure must be accounted for in order to get the true K_d and MBC. A second problem is the determination of the amount of unlabeled ligand to add. In almost all binding assays there is a significant amount of binding to assay components that are not receptors. This "nonspecific" binding must be quantified and subtracted from the total amount of ligand binding in order to determine how much ligand is binding to the receptor. To determine the extent of nonspecific binding, unlabeled ligand is added with the assumption that an excess of unlabeled ligand will effectively compete against the labeled ligand binding to the receptor and leave only nonsaturable, nonspecific binding. But again, how much unlabeled ligand is the appropriate excess to add? Levitzki (1980) proposes that 10-fold more unlabeled than labeled ligand determines the level of nonspecific binding. More commonly 100 to 1000 times the level of labeled ligand is added. However, large excesses of unlabeled ligand may alter nonspecific binding, decrease binding by noncompetitive inhibition, alter equilibrium

conditions, or increase binding by competition with a second component (Clark & Peck, 1979).

As binding is examined over a range of ligand concentrations one must remember that equilibrium conditions with saturating ligand levels are not equilibrium conditions for subsaturating levels of ligand. The duration of the incubation period may have to be lengthened or the temperature may have to be increased to achieve equilibrium.

Even after completing an assay, potential problems remain in interpreting the data. The common practice of extrapolating from Scatchard plots to the X axis to determine maximum binding capacity has been questioned by Klotz (1982). He suggests plotting the moles of ligand bound versus the logarithm of the free ligand concentration to allow a better determination of the MBC. He further suggests that one must at least measure binding to the inflection point of such a plot to have any accuracy in determining the MBC.

Munson and Rodbard (1983) agree that maximum binding capacity cannot be proven, but they disagree with Klotz that alternative methods of plotting binding data are helpful. Alternative plotting schemes do not change the data contained in them, but only how they are presented. Instead, they recommend determining the shape of the error

range for the plot type chosen in order to understand the extent of error in any extrapolations made. They further recommend the use of nonlinear least squares fitting weighted according to the error distribution (Munson & Rodbard, 1980).

In a later paper, Munson (1984) suggests that linear regression of Scatchard plots is only useful for rough estimates of binding parameters. One should not regard nonspecific binding as fixed, but instead treat it as another parameter to be determined by fitting the data to the appropriate model. To accurately determine bound radioactive ligand, one needs to know label specific activity, tracer purity, counting efficiency, counter background, and the efficiency in separating bound and free ligand (Bonifacino & Paladini, 1981).

In the case of thyroid hormones, the intrinsic stickiness of T_3 caused researchers to examine how to apply the concept of free ligand. The question of availability of T_3 for interaction with the nuclear thyroid hormone receptor was examined by Seelig et al. (1981). As previously summarized, addition of excess receptor to a previously incubated mix of labeled T_3 and receptor did not cause the transfer of any T_3 from the nonspecific sites to the newly added receptor. This indicates that the nonspecifically-bound T_3 is not

available to participate in maintaining a binding equilibrium, since it did not transfer to the higher affinity receptor. They referred to the nature of this binding as "inapparent sites", which although most workers considered as reversibly bound to T_3, the T_3 was not available to interact with the receptor. If the measured binding does not reflect equilibrium conditions, Scatchard plots must be used with caution.

Yagura and Walfish (1982) disagree with Seelig et al. (1981) in that they could remove 90% of added T_3 by dialysis after incubation with a nuclear extract. Thus, according to Yagura the "inapparent sites" do not exist or are weak enough to allow a substantial amount of T_3 to be released and cross the dialysis barrier. Hillgartner and Romsos (1987) also refute the proposal of Seelig et al. (1981) in that their experiments showed resin adsorbable T_3 to be only 10% of the total T_3 after *in vivo* injection and isolation of nuclear extract.

Finally, Levitzki (1984) points out that not every high affinity site is a receptor. One must demonstrate ligand displaceability, saturability, and binding must correlate with a biochemical activity. The biological effectiveness of hormone analogs will depend on the amount that binds the receptor, the intrinsic efficacy in

eliciting a response, and the duration of binding to the sites.

1.5 The C57BL/KsJ-*db m* Mouse

In 1966, Hummel et al. described a spontaneous mutation which developed in C57BL/KsJ mice. Mice were studied which experienced an unusual increase in weight. These animals were found to have high blood sugar (200 mg/100 ml serum) and thus by definition are diabetic. Coleman and Hummel (1967) in defining this mutation found that it was transmitted on chromosome 4 as a unit autosomal recessive gene with full penetrance. Autosomal recessive mutations generally are the result of a single nucleic acid base change, which if true in this case, would mean that a change in a single gene product should explain all the alterations in this diabetic state. Diabetic animals are infertile, while heterozygotes can be distinguished from normals only by their offspring. Chick et al. (1970) disagreed that heterozygotes are the same as normals because they observed slight increases in blood glucose in males. Three stages are discernable in the development of the diabetic state (Basabe et al., 1983). In the period after birth from 10 to 30 days there is hyperinsulinemia and hyperglucagonemia. At 4 to 12 weeks of age, hyperglycemia and obesity develop. From 12 to 24 weeks, insulin resistance and β cell necrosis emerge with

premature death at 24-32 weeks. The diabetic defect may be due to the hypersecretion of glucagon leading to high hepatic glucose output with increased beta cell stimulation, thereby giving rise to high insulin levels and later insulin resistance until there is islet exhaustion (Bray & York, 1979). Increased lipogenesis leads to hypertriacylglycerolemia explaining the weight gain of diabetic animals. Diabetic animals also lack the T_3 induction of the Na^+-K^+-ATPase which would make them tend towards obesity and suggests thyroid hormone resistance.

Wyse and Dulin in 1970 reported that limiting food intake could stabilize blood sugar and insulin, although these parameters are still elevated relative to normals. In young animals, elevated insulin and increased glucose oxidation by adipose tissue maintain the glucose concentration at normal levels. With increasing age, elevated food intake, depressed glucose utilization, and continuous output of glucose by the liver, the pancreatic beta cells become exhausted.

The ability to produce colonies of this diabetic strain of mice has been enhanced by the inclusion, via crossbreeding, of a misty coat gene (C57BL/KsJ-*db m*) which excludes the diabetic gene due to repulsion (Chick & Like, 1970). Thus, with the development of fur, the animals

which did not receive the diabetic gene from their heterozygous parents could be easily distinguished by their dark gray coat from the black coated heterozygotes and diabetics. Offspring with black coats would either be valuable as breeding pairs if heterozygous, or would develop symptoms of diabetes upon maturation. Misty coat animals which do not carry the diabetic gene can be discarded without having invested any significant effort in rearing them.

1.6 Objectives of This Study

Since the diabetic mouse had indications of altered serum T_3 and T_4, yet the kinetics parameters of the 5'-monodeiodinase were not altered (Richard Fehn, personal communication), we proposed to study the T_3 binding and physical characteristics of the hepatic nuclear thyroid hormone receptor in these mice. Thus, we hoped to better understand why the differences in T_3 and T_4 levels between diabetic and heterozygous animals exist, to search for the gene whose alteration leads to the diabetic syndrome, and to further elucidate factors influencing the course of their diabetes.

2. Materials and Methods

2.1 Animals for the Study

A colony of C57BL/KsJ mice was developed by breeding sibling animals obtained from Jackson Laboratory (Bar Harbor, Maine). The mice were maintained on a diet of Purina rodent laboratory chow 5001 (Purina Mills, Inc., St. Louis, MO) and water *ad libitum* at 22 ± 1°C with light from 6 am to 6 pm in addition to partial window lighting. Livers from C57BL/KsJ-*db m* mice heterozygous (*db*+/+*m*) and homozygous (*db*+/+*db*) for the diabetic mutation were generously supplied by Dr. Richard Fehn, Department of Biology, California State University, San Bernardino, CA. The diabetic and heterozygous mice were maintained on a diet of Purina rodent chow and water *ad libitum* at 23°C with light from 6 am to 8 pm. One of the four sets used in the binding studies was fasted 24 hours prior to termination; however, no significant differences were seen in their K_d or MBC compared to nonfasting animals. Diabetics were distinguished from heterozygotes by the fact that diabetics become fat while heterozygotes remain lean. Offspring homozygous for the misty coat color gene (+*m*/+*m*) were discarded on the basis of their coat color (dark gray), since diabetics and heterozygotes are black.

To control for circadian rhythms, the animals were killed between 8 am and 10 am. One set of livers was

frozen on a -80°C metal plate, but more generally livers were frozen in liquid nitrogen. Once frozen, livers were kept at -70°C until used in the isolation of thyroid hormone receptor.

2.2 Buffers

All buffers were made in ultra-filtered deionized water (resistance >15 megohms-cm, Sybron/Barnstead Nanopure, Boston, MA). Unless specified otherwise, all chemicals are reagent grade.

Buffer 1 contained 0.25 M sucrose (Schwarz/Mann Ultra Pure, Cambridge, MA), 2 mM $MgCl_2$, and 20 mM Tris/HCl (Schwarz/Mann Ultra Pure, Cambridge, MA), pH 7.6 at 4°C.

Buffer 2 contained 50 mM NaCl, 10% (v/v) glycerol, 2 mM ethylenediaminetetraacetic acid (EDTA), 5 mM 2-mercaptoethanol, and 20 mM Tris/HCl, pH 7.6 at 4°C. The pH titration of buffer 2 was difficult because the available calomel combination electrodes gave unstable readings and available silver chloride combination electrodes would be damaged by the mercaptoethanol. Buffer 2 was therefore adjusted to approximately pH 7.6 as determined by colorpHast pH indicator strips for pH 6.5-10.0 (EM Science, Cherry Hill, NJ). Since the batch to batch variation was significant in its effect on receptor binding, all diabetic/non-diabetic comparisons were done in paired assays utilizing the same batch of buffer.

Buffer 3 contained 0.4 M KCl, 1.1 mM $MgCl_2$, and 20 mM Tris/HCl, pH 7.9 at 4°C.

2.3 Hepatic Thyroid Hormone Nuclear Receptor Isolation

Unless noted otherwise, all pellet resuspensions were in 10 ml of buffer per gram of original wet liver. Following the procedure of Inoue et al. (1983), the nuclear T_3 receptor was dissociated from chromatin and solubilized. Normal C57BL/KsJ mice were killed by cervical dislocation (Cervical Dislocators Inc., Wausau, WI) at 10-80 weeks of age. Livers were immediately surgically removed from the animals and frozen in liquid nitrogen. Diabetic animals 10-15 weeks old were used and heterozygotes ranged in age from 10 to 22 weeks old. However, most of the animals in all groups were approximately 11 weeks old. Altered T_3 binding with age was only suspected in the diabetic mice.

As needed, livers were thawed in buffer 1 at 4°C. A minimum of 3 livers were minced with scissors and homogenized on ice in 60 ml of buffer 1 with 12 strokes of a glass-teflon homogenizer (Thomas C24915, Philadelphia, PA) driven at 1200 rpm. The homogenate was filtered through 4 layers of cheese cloth and centrifuged at 1,500 x g for 12 minutes at 4°C (DuPont Sorvall RC-5B Refrigerated Superspeed Centrifuge, SS-34 rotor, Newtown, CT). The supernatant was discarded and the pellet was

Figure 1:
Flowchart of the isolation procedure for the nuclear T_3 receptor. Bold numbers are the step numbers referred to in the text.

Kill by cervical dislocation
Surgically remove liver
Freeze liver in liquid nitrogen

Use immediately | Store at -70°C for later use

Weigh liver
Thaw in Buffer 1 (4°C)
Mince with scissors
1 Homogenize in a glass-teflon homogenizer
Filter through cheesecloth
Centrifuge 12 min., 1,500 x g

Decant & Discard Supernatant | Resuspend Pellet in Buffer 1
Centrifuge 10 min., 3,000 x g

Decant & Discard Supernatant | Resuspend Pellet in Buffer 1 + 0.5% Triton X-100
Centrifuge 10 min., 3,000 x g

Decant & Discard Supernatant | Resuspend Pellet in Buffer 1 + 0.5% Triton X-100
Centrifuge 10 min., 3,000 x g

Decant & Discard Supernatant | Resuspend Pellet in Buffer 2
Centrifuge 10 min., 3,000 x g

Decant & Discard Supernatant | Resuspend Pellet in Buffer 3
Extract receptor for 30 min.
Centrifuge 20 min., 35,000 x g

2
Put supernatant in dialysis bag | Retain pellet of DNA
Dialyze overnight
Centrifuge 10 min., 3,000 x g

3
Measure volume of supernatant | Discard pellet
Aliquot into Eppendorf tubes
Freeze in liquid nitrogen
Store at -70°C until needed

resuspended in the homogenizer with 3 strokes in buffer 1. This suspension was centrifuged 10 minutes at 3,000 x g and the supernatant discarded. The pellet was resuspended with vortexing in buffer 1 which had 0.5% Triton X-100 added. After centrifugation for 10 minutes at 3,000 x g, the supernatant was discarded and the pellet was again resuspended in buffer 1 containing 0.5% (v/v) Triton X-100. The suspension was then centrifuged 10 minutes at 3,000 x g. The supernatant was discarded and the pellet was resuspended in buffer 2. After centrifugation for 10 minutes at 3,000 x g, the supernatant was discarded and the pellet resuspended by stirring for 30 minutes at 4°C in 1 ml of buffer 3 per g liver. This served to remove the receptor from the chromatin and solubilized it. After removal of the stir bar, centrifugation at 35,000 x g for 20 minutes yielded a pellet containing the DNA which was later assayed to determine the DNA content of the pooled liver tissue.

The supernatant was dialyzed at 4°C against buffer 2 in Spectrapor membrane tubing overnight (cut off molecular weight 3500, Spectrum Medical Industries, Inc., Los Angeles, CA). Following dialysis, the solution was clarified by centrifugation at 3,000 x g for 10 minutes. The pellet was discarded and the supernatant was used as the receptor preparation.

2.4 DNA Assay

Total liver DNA was measured by the method of Patterson (1979) which utilizes a diphenylamine color reaction. The pellet containing DNA from the 35,000 x g centrifugation in the receptor isolation procedure (Figure 1, step 2) was suspended in 2 ml of 0.5 M perchloric acid and heated in a water bath at 90°C for 15 minutes, then centrifuged at 3,000 x g for 10 minutes. The supernatant was saved and the pellet washed with 2 ml of 0.5 M perchloric acid and the centrifugation repeated. This supernatant was combined with the previous supernatant and the total volume brought to 10 ml with 0.5 M perchloric acid. A 10 µl aliquot was diluted to 1 ml with 0.5 M perchloric acid. A diphenylamine working solution containing 0.75 g diphenylamine dissolved in a mixture of 50 ml of glacial acetic acid and 0.75 ml of concentrated sulfuric acid was prepared just prior to use. An acetaldehyde solution containing 0.5 ml of acetaldehyde in 24.5 ml of water was stored at 4°C. Two ml of Burton's reagent (Burton, 1956) consisting of 0.1 ml of acetaldehyde solution and 20 ml of diphenylamine solution was added to the 1 ml of diluted sample. The mixture was incubated 16-20 hours at room temperature and the absorbance determined at 600 nm with a Beckman DB-GT spectrophotometer (Fullerton, CA). The absorbance was

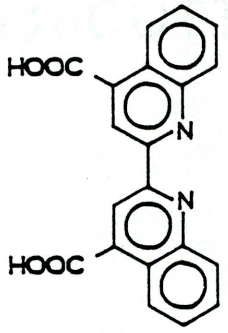
converted to mg DNA using a linear standard curve derived from stock solutions of calf thymus DNA (0 to 0.04 mg DNA/ml) dissolved in 5 mM NaOH.

2.5 Protein Assay

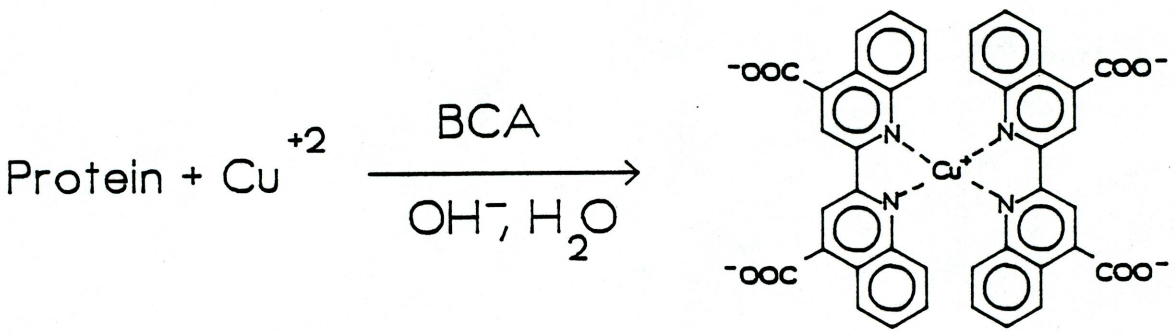
The Pierce Chemical Company BCA protein assay kit (Rockford, IL) was used to determine protein concentration. This assay is based on the ability of protein to form Cu^{+1} ions from Cu^{+2} ions in alkaline solutions. When in the presence of bicinchoninic acid (BCA, Figure 2), the Cu^{+1} ion interacts with two BCA molecules to form a BCA-Cu^{+1} complex which absorbs strongly at 562 nm.

Fifty ml of reagent A (BCA in a buffered basic aqueous solution) was combined with 1 ml of reagent B (4% aqueous copper sulfate solution) to form the working reagent. Fifty µl of the receptor preparation was diluted with 350 µl buffer 2. Bovine serum albumin was prepared as a standard (0 to 0.25 mg/ml) in buffer 2. A 0.15 ml aliquot of the sample was combined with 3 ml of working reagent and incubated at 60°C in a temperature-regulated water bath (Precision Scientific Company, No. 66648, Chicago, IL) for 30 minutes. Samples were cooled to room temperature in about 30 minutes and the absorbance was determined at 562 nm.

Figure 2:
The structure of bicinchoninic acid (BCA) and its usage in assaying protein concentration.



Bicinchoninic Acid (BCA)



2.6 [^{125}I]T_3-Receptor Binding Assay on Nitrocellulose

Receptor binding of T_3 was measured by the method of Inoue et al. (1983). A 0.1 ml aliquot of the receptor preparation was combined with 0.01-0.1 pmoles [^{125}I]T_3 in buffer 2 to a total volume of 0.5 ml in 12x75 mm borosilicate glass tubes (VWR Scientific Inc., San Francisco, CA). Nonspecific binding was determined by including 500-fold more unlabeled than labeled T_3. The tubes were covered with parafilm (American Can Co., Greenwich, CT) and incubated for 18-25 hours at 4°C. Nitrocellulose membrane disks (Schleicher & Schuell 25 mm, 0.45 micron, Keene, NH) were placed on a Millipore glass fritted filtration apparatus (Bedford, MA). All of the solutions were held at 4°C but for convenience, the filtration apparatus was not cooled except by the cold solutions as they passed through it. The membranes were moistened by aspiration with 3.5 ml of buffer 2. The samples were diluted with 3.5 ml of buffer 2 and filtered through the nitrocellulose at 12 ml/minute. Each incubation tube was rinsed by vortexing with 3.5 ml of buffer 2 which was then used to rinse the filtration apparatus including the nitrocellulose membrane. A second 3.5 ml aliquot of buffer 2 was used to rinse the incubation tube, filtration apparatus, and the nitrocellulose membrane. The membrane was washed twice

more with 7 ml of buffer 2, then membrane-bound ^{125}I was determined by counting the membrane for one minute in polypropylene tubes using a Nuclear Chicago gamma counter (model 1185, Des Plaines, IL). Counting efficiency was 85.3 ± 0.1%.

2.7 Determination of $^{125}I^-$ in Stock [^{125}I]T_3 Solutions

Iodide was separated from T_3 by the method of Green (1972). A 1 x 5 cm G-25 superfine Sephadex column (Pharmacia Fine Chemicals Inc., Piscataway, NJ) was equilibrated with 0.015 M NaOH, 0.5 M NaCl at room temperature. Ten µl of the [^{125}I]T_3 solution to be assayed was added and 1 ml fractions collected. The column was washed with 5 ml of 0.015 M NaOH, 0.5 M NaCl to elute the iodide peak near the total column volume. Under these conditions T_3 is weakly adsorbed to Sephadex partly due to interaction between the hydroxy-ether groups in Sephadex and the hydroxyl on the terminal aromatic ring of T_3. At low ionic strength, the carboxyl groups of Sephadex act as a weak cation exchanger to bind T_3. Elution can then be effected by increasing the pH which diminishes the interaction between Sephadex and T_3.

Elution of T_3 and T_4 was accomplished by washing with 10 ml of 0.1 M NaOH, 0.005 M NaCl. The fractions were counted to quantify ^{125}I, and the $^{125}I^-$ to [^{125}I]T_3 ratio in the stock solution was determined by comparing the

areas under the peaks. This ratio then allowed the calculation of the percent of the radioactivity that is due to $[^{125}I]T_3$.

2.8 Calculating the Amount of $[^{125}I]T_3$ in Stock Solutions

The use of low specific activity ligands labeled with short half-life nuclides requires a correction in the calculation of the amount of labeled ligand present which is rarely addressed in the literature. Bolger and Jorgensen (1980) in an appendix to their paper have dealt with this problem. Radioactive decay follows the equation

$$N_t = N_0 e^{-kt}$$

where t is time, N_t is the amount of radionuclide at time t, N_0 is the amount of radionuclide at time zero, and k is the decay rate constant equal to the natural logarithm of 2 divided by the nuclide decay half-life. If the ligand is labeled with only one radioactive nuclide per ligand molecule, the total number of disintegrations will equal the loss of labeled ligand molecules.

Since only the radiolabeled ligand decays, the specific activity changes with time and the calibration constant for converting counts to moles of ligand changes. In order to correct for the loss of labeled ligand one must know the amount of both radiolabeled ligand and unlabeled ligand. The amount of labeled ligand can be determined from the theoretical maximum specific activity

if all the ligand was labeled. This maximum specific activity is determined by the nuclide half-life. Since with decay the amount of nuclide remaining to decay decreases, the calculated maximum specific activity will also decrease as the time period over which it is calculated is increased. As an example, at the end of one half-life, 1/2 of the original nuclide will have decayed over that time period; similarly, calculated over 2 half-lives 3/4 of the original nuclide will have decayed, but since there has been twice the amount of time, the activity per time period drops from 1/2 to 3/8.

The decay from one mole of ^{125}I over the first one second time interval is $N_0 - N_1$ or $6.022 \times 10^{23}(1-e^{-\ln 2/5,184,000})$ for ^{125}I which has a half-life of 60 days (5,184,000 seconds). Expressed in microcuries (μCi) per μg of $[^{125}I]T_3$ this equals 3353 μCi/μg. Dividing the total μCi of $[^{125}I]T_3$ present by 3353 would then give the micrograms of labeled T_3 present. Dividing the total μCi of $[^{125}I]T_3$ present by the specific activity would give the total micrograms of T_3 present. From the amounts of total T_3 and labeled T_3 one can calculate the unlabeled T_3 present by taking the difference (total-labeled = unlabeled). When utilized at a date after the calibration date, only the labeled ligand will have been destroyed by the recoil energy of decay.

From the decay equation, the amount of remaining labeled ligand can be calculated. The current specific activity (μCi/μg) can then be calculated by dividing the total activity present (μCi) by the sum of the unlabeled ligand and the remaining labeled ligand (μg). Total activity divided by the current specific activity gives the total amount of T_3 present: Total activity (μCi)/current specific activity (μCi/μg) = T_3 (μg). Converting the counts per minute (cpm) to activity present (cpm/counting efficiency (cpm/dps) x 3.7 x 10^{10}dps/Ci) = activity (Ci)) and using the current specific activity allows one to convert the cpm observed to the amount of T_3 present.

2.9 Association/Dissociation Kinetics

A number of 0.1 ml aliquots of the receptor preparation were incubated at 4°C in 1-6 pM [^{125}I]T_3 in borosilicate glass tubes. The total volume was adjusted to 0.5 ml by the addition of buffer 2. Four hours after initiating the incubation, 18 μl of 18 μM unlabeled T_3 dissolved in 0.02 M NaOH was added to 9 tubes. Unlabeled T_3 was added to another set of 9 tubes at 24 hours and to a third set of 9 tubes at 48 hours. The loss of bound, labeled T_3 with time was followed utilizing the nitrocellulose assay. Reassociation of the labeled ligand and receptor is prevented by the excess unlabeled ligand present. As shown by Seelig et al. (1981), a bimolecular,

reversible association between T_3 and its receptor has a rate equation of

$$d(T_3R)/dt = k_{+1}[T_0 - T_3R][R] - k_{-1}[T_3R]$$

where T_0 is the total T_3 added, k_{+1} is the association rate constant, and k_{-1} is the dissociation rate constant. Assuming T_3 is in the tracer range, the concentration of unbound receptor will not change significantly and $R = R_0$. Since the integral of $dX/(A + BX)$ is $1/B \times \ln|A + BX|$, integration of the rate equation yields

$$\frac{1}{-(k_{+1}[R_0] + k_{-1})} \ln|k_{+1}T_0[R_0] - T_3R(k_{+1}[R_0] + k_{-1})| -$$

$$\frac{1}{-(k_{+1}[R_0] + k_{-1})} \ln|k_{+1}T_0[R_0]| = t - 0$$

After rearranging

$$\frac{T_3R}{T_0} = \frac{k_{+1}[R_0]}{k_{+1}[R_0] + k_{-1}} \left[1 - e^{-(k_{+1}[R_0] + k_{-1})t}\right]$$

The maximum amount of T_3 will be bound when t is infinite. Thus, the maximum T_3R/T_0 (y_{max}) is

$$k_{+1}[R_0]/(k_{+1}[R_0] + k_{-1})$$

If the percent of T_3 bound to receptor is y, and λ is $k_{+1}[R_0] + k_{-1}$, then the equation above simplifies to

$$y_{max} - y = y_{max}e^{-\lambda t}$$

The time required for half of the maximum amount of ligand that will eventually bind to the receptor to become bound to the receptor can be calculated from the binding versus

time curve assuming that the amount of dissociation of ligand from the receptor is negligible in the time required for association. A plot of the natural logarithm of (the maximum percent of added ligand bound at any time minus the current percent of added ligand bound) versus time will give a linear plot:

$$\ln(y_{max} - y) = -\lambda t + \ln(y_{max})$$

with λ = -slope. By substituting 0.5 x y_{max} for y, one can see that the time for half maximal association ($T_{\frac{1}{2}asso}$) is $\ln(0.5)/-\lambda$. The value of k_{-1} can be calculated from the definition of λ and y_{max} to be $\lambda(1-y_{max})$. The expected dissociation half-life is $T_{\frac{1}{2}diss} = \ln(0.5)/-k_{-1}$. The value of k_{+1} is $(-\lambda - k_{-1})/R_0$, where R_0 is the MBC estimated from a Scatchard plot. The predicted K_d is then k_{-1}/k_{+1}.

When looking only at the T_3-receptor dissociation, the rate equation becomes:

$$d(T_3R)/dt = k_{-1}[T_3R]$$

Integration yields:

$$(1/k_{-1}) \times \ln|k_{-1}[T_3R]| - (1/k_{-1}) \times \ln|k_{-1}[T_3R_0]| = t - 0$$

where $[T_3R_0]$ is the level of T_3-receptor complex when the excess cold T_3 is added to begin the examination of dissociation only. Rearranging gives:

$$[T_3R] = ([T_3R_0]/k_{-1})e^{-k_{-1}t}$$

A plot of the natural logarithm of the percent of the added ligand remaining bound versus time yields a linear plot of the dissociation of the bound, radiolabeled ligand:

$$\ln(T_3R/T_0) = -k_{-1}t + \ln(T_3R_0/k_{-1}T_0)$$

The observed half-life for the dissociation of the T_3-receptor complex is ln(0.5)/slope.

2.10 Scatchard Binding Analysis

A 0.1 ml aliquot of the receptor preparation was incubated overnight at 4°C in 10^{-11} to 10^{-8} M [^{125}I]T_3 in buffer 2 in a total volume of 0.5 ml. Nonspecific binding was quantified in the presence of a 500 fold excess of unlabeled T_3 over labeled T_3. The bound [^{125}I]T_3 was determined using the nitrocellulose assay. As described by Scatchard (1949), a simple model for ligand-receptor interaction can be linearized by plotting bound ligand/[free ligand] versus bound ligand. This can be seen from the equation

$$\nu/[L] = MBC/K_d - \nu/K_d$$

where ν is the concentration of bound ligand, [L] is the concentration of free ligand, MBC is the maximum binding capacity, and K_d is the dissociation constant. Scatchard plots of moles of bound T_3 per mg protein/[free T_3] (Y axis) versus moles of bound T_3 per mg protein (X axis) were constructed. Linear regression of the transformed

data gave the K_d (-1/slope) and maximum binding capacity (X axis intercept).

2.11 Isokinetic Sucrose Gradient Sedimentation Velocity

A 0.1 ml aliquot of the receptor preparation was incubated overnight at 4°C in 1 nM [^{125}I]T_3. The free ligand was separated from protein bound ligand by gel filtration with a 0.6 x 7 cm G-25 coarse Sephadex column (Pharmacia Fine Chemicals, Piscataway, NJ) at room temperature. The column was equilibrated with 20 mM Tris/HCl, pH 7.6 at 22°C, 20 mM NaCl, 0.1% (v/v) Triton X-100, then the incubated sample was added and the void volume collected. The void volume was predetermined by the elution of blue dextran 2000 (Pharmacia Fine Chemicals Inc., Piscataway, NJ). The column was washed between samples with 0.1 M NaOH, 0.005 M NaCl to remove residual free T_3. The void volume fraction was concentrated approximately 50% by evaporation under a dry nitrogen stream. This void volume concentrate was layered on an isokinetic sucrose gradient in polyallomer tubes for a Beckman SW60 rotor (Palo Alto, CA).

To form isokinetic gradients, the concentration in the mixing chamber (C_m) was 5% sucrose in 10 mM KCl, 10 mM Tris/HCl, pH 7.4 at 22°C, 1 mM EDTA, 20 Units Trasylol/ml. The concentration in the reservoir (C_r) was 24.1% sucrose in the same buffer. The volume of fluid in the mixing

chamber (V_m) was held at 4.86 ml while the gradient was formed and the initial volume in the reservoir (V_r) was 5 ml (McCarty et al., 1974; Perlman et al., 1982). Centrifuge tubes were filled to within 0.5 cm of the top. Samples were centrifuged at 300,000 x g (54,000 rpm in an SW60 rotor) for 20 hours at 2°C in a L2-75B ultracentrifuge (Beckman, Palo Alto, CA). The centrifuge was allowed to coast to a stop without braking.

The tubes were fractionated with an ISCO 182 gradient fractionator (Lincoln, NE) removing the solution from the bottom for radioactivity determinations. Two drop fractions were collected and counted in a gamma counter for ^{125}I. For the protein sedimentation standard (20 µl of 5 mg ovalbumin/ml) the solution was removed from the top of the tube through a quartz windowed flow cell following the absorbance at 280 nm (ISCO UA-4 absorbance monitor, dual beam optical unit type 4, Lincoln, NE). The distance sedimented in isokinetic gradients is proportional to the sedimentation coefficient so that the ratio of the distance traveled is also the ratio of the sedimentation coefficients between the standard and sample.

2.12 Immobiline Isoelectric Focusing Gels

A 0.1 ml aliquot of the receptor preparation was incubated overnight at 4°C in 1 nM [^{125}I]T_3. The free

ligand was separated from protein bound ligand by gel filtration with a 0.6 x 7 cm G-25 coarse Sephadex column. The T_3-receptor complex was eluted in the void volume. The column was equilibrated with and the protein bound T_3 eluted with 20 mM Tris/HCl, pH 7.6 at 22°C, 20 mM NaCl, 0.1% (v/v) Triton X-100. Washing the column between runs with 0.1 M NaOH, 0.005 M NaCl removed free T_3 which was discarded. The void volume fraction was concentrated by evaporation under a dry nitrogen stream. Ten µl of glycerol was added to the void volume concentrate.

Aqueous stock solutions of 10% ammonium persulfate and 10% N,N,N',N'-tetramethylethylenediamine (TEMED) were prepared just prior to use. The acrylamide-bis stock solution contained 2.91 g acrylamide and 0.09 g N,N'-methylene-bis-acrylamide (bis) in water to a total volume of 10 ml. The pH of the acidic (dense) gel solution was taken when it consisted of 207.5 µl of pK 6.2 immobiline, 163.2 µl of pK 3.6 immobiline, and 1.7 ml of water. Then 692 µl of acrylamide-bis stock solution, 1.01 ml glycerol, and 372 µl of water were added. The pH of the basic (light) gel solution was taken when it consisted of 207.5 µl of pK 6.2 immobiline, 44.3 µl of pK 3.6 immobiline, and 1.82 ml of water. Then 692 µl of acrylamide-bis stock solution and 1.38 ml of water were added. The acidic gel solution was placed into the mixing chamber of a linear

gradient maker while the basic gel solution was placed in the other chamber. Addition of 11.1 µl of 10% ammonium persulfate and 18 µl of 10% TEMED to each chamber initiated polymerization. Using the gradient maker, a co-linear density and pH gradient was formed in a 12 ml syringe inserted into the gel apparatus (0.35 mm x 14 cm x 16 cm). Once the gradient was formed, the syringe plunger was used to quickly expel the gel solution into the gel apparatus. A 20 gauge needle was used instead of the 30 gauge needle suggested by the gel apparatus manufacturer since the 30 gauge needle clogged too quickly. Initially the gel apparatus was tipped on its side so that the gel solution would run down the side to avoid trapping air bubbles. As the gel solution was poured into the apparatus, it was gradually leveled. Heating for one hour at 50°C completed polymerization of the gel. The sample wells were washed with water, then filled with anolyte (0.02 M NaOH). The catholyte was 0.02 M acetic acid.

The void volume concentrate was loaded onto the gel and run at 20 watts constant power for 3 hours in a Hoefer 600 vertical slab gel apparatus (San Francisco, CA) connected to a Bio-Rad 3000/300 power supply (Richmond, CA). The voltage was limited to a maximum of 2500 V. The apparatus was cooled to 4°C with a refrigerated water recirculator (Lauda K-2/R, Brinkmann Instruments, West

Germany). After electrophoresis, the lanes were cut out and each lane was sliced into 2 mm sections. The sections were counted for ^{125}I in a gamma counter.

2.13 Estimation of Free [^{125}I]T_3 by Binding to Subsequent Receptor Additions

A 0.1 ml aliquot of the receptor preparation was combined with 0.13 pmoles [^{125}I]T_3 in buffer 2 to a total volume of 0.5 ml in 12x75 mm borosilicate glass tubes. Nonspecific binding was determined by including 380 pmoles unlabeled T_3 in an otherwise matched set of tubes. The tubes were covered with parafilm and incubated at 4°C. After incubating for 24 hours, a second aliquot of 0.1 ml of the receptor preparation was added to half of the total and nonspecific binding tubes. The incubation was then continued for all tubes at 4°C for another 24 hours. The amount of [^{125}I]T_3 bound was determined by nitrocellulose filtration as described previously in section 2.6 on the [^{125}I]T_3-receptor binding assay. The amount of free T_3 after 24 hours of incubation was calculated from the increase in specific T_3 binding when the second aliquot of receptor preparation was present, assuming that the equation

$$\nu/[L] = MBC/K_d - \nu/K_d$$

describes the proper model for T_3 binding.

2.14 Molecular Weight Determination by G-100 Sephadex

The molecular weight of the nuclear T_3 receptor was estimated by G-100 Sephadex chromatography (Pharmacia Fine Chemical, Piscataway, NJ). The gel was hydrated in and equilibrated to 20 mM Tris/HCl, pH 7.6 at 22°C, 20 mM NaCl, 0.1% (v/v) Triton X-100 in a boiling water bath for 5 hours. After cooling to room temperature, the gel was poured into a 13.2 x 0.7 cm column. Molecular weight standards of aldolase (158,000), chymotrypsin A (25,000), and ovalbumin (43,000) were prepared at 5 mg/ml in the same buffer. The column was run at room temperature monitoring the absorbance at 280 nm to detect the elution of the protein standards. Plotting the logarithm of the molecular weight against the fraction number for the half maximum absorbance on the peak front yielded the calibration curve.

A 0.1 ml aliquot of the receptor preparation was incubated in 2.6 nM [^{125}I]T_3 at room temperature for 4 hours. The incubated preparation was then run on the column and eluted with 20 mM Tris/HCl, pH 7.6 at 22°C, 20 mM NaCl, 0.1% (v/v) Triton X-100. Fractions were collected and the ^{125}I content determined to assess the location of the T_3-receptor complex. The molecular weight was determined by comparing the fraction number with the

half maximum counts per minute (cpm) in the peak front to the standard curve.

2.15 Statistical Methods

The slopes in the Scatchard plots for control and diabetic T_3-receptor binding were compared by a one-tailed *t*-test (Student, 1908) and by the method of Dixon and Massey (1969). See the Appendix for an explanation of the Dixon and Massey method.

For dissociation kinetics, Student's one tailed *t*-test was used to determine the probability that the dissociation half-life was greater in controls than in the diabetics. Since the dissociation rate should be the same in all replicates irregardless of the initial level of binding, a composite slope was determined for each group, control and diabetic, by forcing all the lines to have a common slope in a linear least squares fit. If one has j lines with n_i points in line i and with k specifying the points in line i where i is the line number (1 to j), the slope (m) for all the lines as a group is:

$$\frac{\sum_{i=1}^{j}\left[\sum_{k=1}^{n_i} X_{i_k} Y_{i_k}\right] - \sum_{i=1}^{j}\left[\frac{\sum_{k=1}^{n_i} X_{i_k} \sum_{k=1}^{n_i} Y_{i_k}}{n_i}\right]}{\sum_{i=1}^{j}\left[\sum_{k=1}^{n_i} X_{i_k}^{2}\right] - \sum_{i=1}^{j}\left[\frac{(\sum_{k=1}^{n_i} X_{i_k})^{2}}{n_i}\right]}$$

With intercepts (b_i) at

$$\frac{\sum_{k=1}^{n_i} Y_{i_k} - m \sum_{k=1}^{n_i} X_{i_k}}{n_i}$$

See the Appendix for the derivation of these equations and a sample calculation.

All experimental values given are the sample mean plus or minus the 95% confidence limits (C.L.). The 95% confidence limits are calculated as the critical *t*-statistic times the standard deviation of the mean. For calculated values, the 95% confidence limit is estimated by propagation of error as by Peters et al. (1974). See the Appendix for a brief explanation of this method.

3. Results

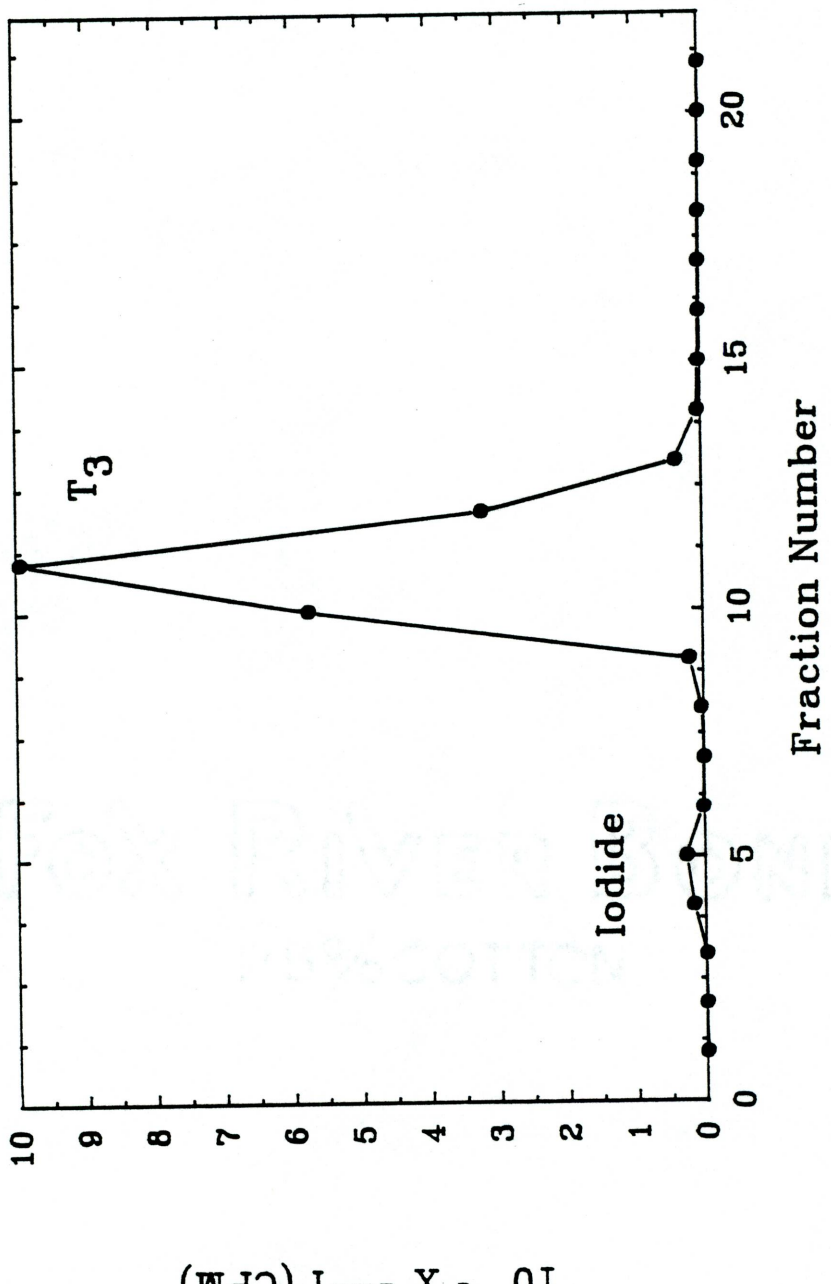
3.1 Isolation of the Hepatic Nuclear T_3 Receptor

The protein concentration in the nuclear T_3 receptor preparation (Figure 1, step 3) was 1.1 ± 0.3 mg/ml (n=10, mean ± 95% C.L.) for controls and 1.5 ± 0.4 mg/ml (n=7) for diabetics. The DNA content determined from the pellet in step 2 of Figure 1 was 1.4 ± 0.7 mg/g liver (n=8) for the controls and 1.5 ± 2.1 mg/g liver (n=3) for the diabetics. There was no significant difference between diabetic and control parameters for either measurement. There were significant differences ($p<0.001$) in the weights of the two liver types however. Control livers averaged 1.3 ± 0.2 g (n=32), while diabetics have larger livers averaging 2.5 ± 0.7 g (n=19). Thus, the average DNA per liver is higher in diabetics, 3.8 ± 5.4 mg/liver versus 1.8 ± 1.0 mg/liver for controls. However, the large spread in the distribution of the DNA values prevents this difference from being statistically significant at the 95% confidence level.

3.2 Separation of Iodide and T_3

Figure 3 illustrates the separation of iodide from T_3 by G-25 Sephadex chromatography. Commercial stock [^{125}I]T_3 solutions generally contain less than 2% iodide upon arrival. Spontaneous deiodination of T_3 increases

Figure 3:
Separation of iodide from T_3 in commercial stock [^{125}I]T_3 solutions by G-25 Sephadex chromatography. The column was equilibrated and iodide eluted with 0.015 M NaOH, 0.5 M NaCl. [^{125}I]T_3 was eluted with 0.1 M NaOH, 0.005 M NaCl.


10^{-4} X ^{125}I (CPM)
10
9
8
7
6
5
4
3
2
1
0
0
5
10
15
20
Fraction Number
T_3
Iodide

free iodide by about 1% per month. The [^{125}I]T_3 solutions used for binding assays had less than 5% iodide.

3.3 T_3 Adsorption During Manipulations

Triiodothyronine handling is difficult due to the tendency of T_3 to adsorb to most surfaces. With the use of polypropylene micropipettor tips and polypropylene Eppendorf tubes or even with siliconization of the equipment used in the preparation of stock T_3 solutions, there is a significant loss of T_3 to the tips and tubes. Only 70 ± 2% of the T_3 expected to be transferred is actually "free" to the extent that a subsequent aliquot can transfer it to another tube. Pipettor tips retain 5.2 ± 0.5% of the T_3 taken up into them, while Eppendorf tubes remove from solution 26 ± 2% of the T_3 placed in them, relatively independent of the volume. Therefore, the amount transferred in all serial dilutions of T_3 must be corrected to 70% of that expected for each transfer made.

An understanding of this phenomenon provides an explanation for the downward curvature in plots of saturation of binding as the level of T_3 is increased beyond the physiological range. At high levels of T_3, the cold T_3 added (10^{-6} to 10^{-4} M) to determine nonspecific binding begins to compete with nonspecific adsorption of T_3 to the incubation tube resulting in a significantly greater amount of "free" T_3 (both labeled and unlabeled).

This decrease in nonspecific adsorption of labeled ligand causes both the total and nonspecific binding to be increased. As T_3 levels are increased above 10^{-4} M, T_3 precipitates out of solution causing nonspecific binding to rapidly increase above the level of "total" binding, eventually yielding negative values for specific binding. If a 500-fold excess of cold T_3 is utilized in determining nonspecific binding, 5 nM [^{125}I]T_3 in the total binding tube will have a corresponding nonspecific binding tube with 2.5 μM unlabeled T_3, and thus may exhibit disproportionately high binding in the nonspecific tube. Therefore, analysis of the binding curve was examined only in the region below 5 nM T_3 (which spans the physiological range) and previously confusing results above that level can be properly ignored as an artifact.

3.4 Isokinetic Sucrose Gradient Sedimentation

Sedimentation of the T_3 receptor after incubation with [^{125}I]T_3 gave broad bands in both radioactivity and absorbance at 280 nm. The sedimentation coefficient of 1.9 ± 0.2 S obtained for both control (n=7) and diabetic (n=4) mice is at variance with that of 3.4-4.5 S reported for rat liver. A protease inhibitor (Trasylol) was used during the sedimentation procedure but not throughout the isolation procedure. Although Latham et al. (1976) and Perlman et al. (1982) used protease inhibitors in their

receptor isolation procedures, Barsano and DeGroot (1983) and Samuels et al. (1980) did not, yet obtained similar values. Thus, it is unlikely that the low sedimentation coefficient obtained in this study is due to degradation of the mouse receptor from a failure to inhibit proteolysis during isolation. Furthermore, the molecular weight determination on the mouse receptor did not indicate a reduction in the mass of the mouse receptor as compared to the rat.

Only one peak was present in each sample indicating that there is only one form of the receptor in the preparation. A *t*-test did not indicate a significant difference between controls and diabetics. Thus, any difference between the control and diabetic receptor is not great enough to cause a difference in the sedimentation coefficient. Figure 4 shows an isokinetic sucrose gradient sedimentation profile. The small upward trend in the control under the 3.6 S peak for ovalbumin was not invariably present in other profiles.

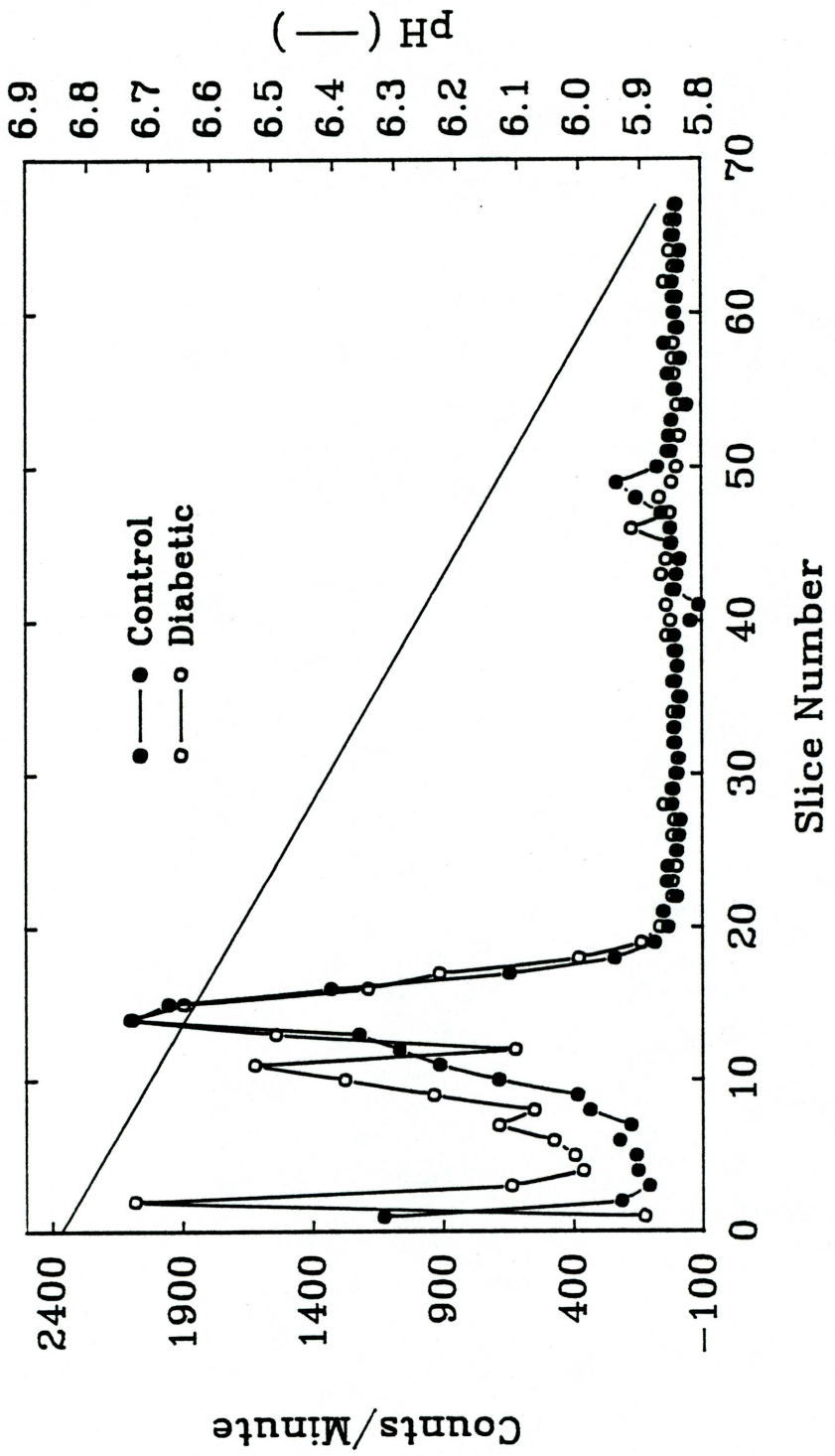
3.5 Isoelectric Focusing of the [^{125}I]T_3-Receptor Complex

Isoelectric focusing on Immobiline narrow range pH gradients resolved receptor bound T_3 from free T_3 at isoelectric pH's (pI's) of 6.64 ± 0.02 (n=5) for controls and 6.64 ± 0.05 (n=3) for diabetics (Figure 5). As might be expected from the observed values, a *t*-test indicated

Figure 4:
Sample profile from isokinetic sucrose gradient sedimentation of the [^{125}I]T_3-receptor complex. Fraction 1 is the top of the centrifuge tube and fraction 42 is the bottom of the tube. The absorbance at 280nm is of ovalbumin (3.6 S) run in a separate tube.

Counts/Minute
400
350
300
250
200
150
100
50
0
−50
0
5
10
15
20
25
30
35
40
45
Fraction Number
50
40
30
20
10
0
10^3 X Absorbance 280 nm (——)
Control
Diabetic

Figure 5:
A representative sample of isoelectric focusing of the $[^{125}I]T_3$-receptor complex on a linear pH gradient Immobiline gel. The pH range is 5.873 to 6.842. Only one major peak was found consistently in all the gels.


pH (——)
6.9
6.8
6.7
6.6
6.5
6.4
6.3
6.2
6.1
6.0
5.9
5.8
Control
Diabetic
2400
1900
1400
900
400
−100
Counts/Minute
0
10
20
30
40
50
60
70
Slice Number

no significant difference in the pI values for the T_3-receptor complex between the two groups. Immobiline gels are capable of resolving proteins differing in their pI by as little as 0.001 pH units indicating a high degree of similarity in pI for the control and diabetic receptor preparations.

3.6 Scatchard Analysis Binding Constants

Although Scatchard plots are not rigorously correct in a system which does not reach equilibrium, the constants obtained from such a treatment have value in making comparisons between similar groups. Figure 6 demonstrates the close correlation between the specific T_3 binding data and the curve calculated from Scatchard analysis of the data. It also shows that T_3 binding by the receptor preparation is saturable, supporting the conclusion that a T_3 receptor is present. Table 3 summarizes the constants found for normal C57BL/KsJ, heterozygous (*db/m*), and diabetic (*db/db*) mouse hepatic nuclear T_3 receptors. Figure 7 re-emphasizes a comparison between controls and diabetics. The average K_d of (1.3 ± 0.8) x 10^{-10} M (n=8) for controls is significantly less than that of (8.7 ± 10.4) x 10^{-10} M (n=4) for the diabetic animals. The statistical significance was determined by comparing the slope of the regression line in Scatchard plots using the method of Dixon and Massey (1969) as

Figure 6:
T_3 binding saturation curve for the control and diabetic hepatic nuclear T_3 receptor preparation. Quadruplicates were run at each T_3 concentration. The curves are the expected binding derived from the constants calculated by Scatchard analysis of the data. The difference in maximum binding capacity between the control and diabetic mice was not significant when normalized to binding per mg DNA.

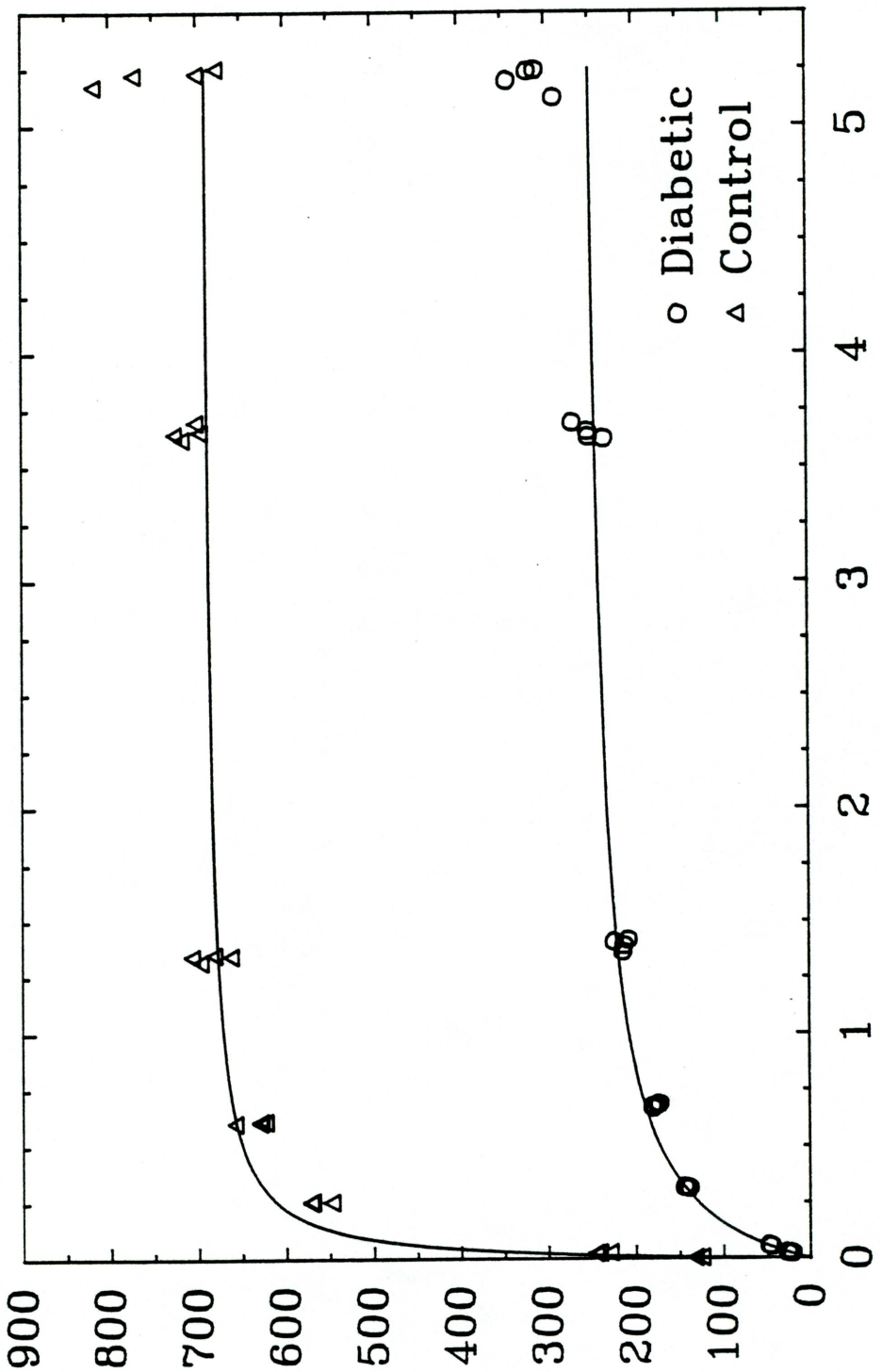

T_3 Bound (fmoles/mg Protein)
900
800
700
600
500
400
300
200
100
0
0
1
2
3
4
5
[Free T_3] (nM)
o Diabetic
Δ Control

Figure 7:
Scatchard plot of T_3 binding to the control and diabetic hepatic nuclear T_3 receptor preparation. The lines represent the best fit as determined by linear least squares regression assuming all error is in the Y axis. The difference in maximum binding capacity between the control and diabetic mice was not significant when normalized to binding per mg DNA.

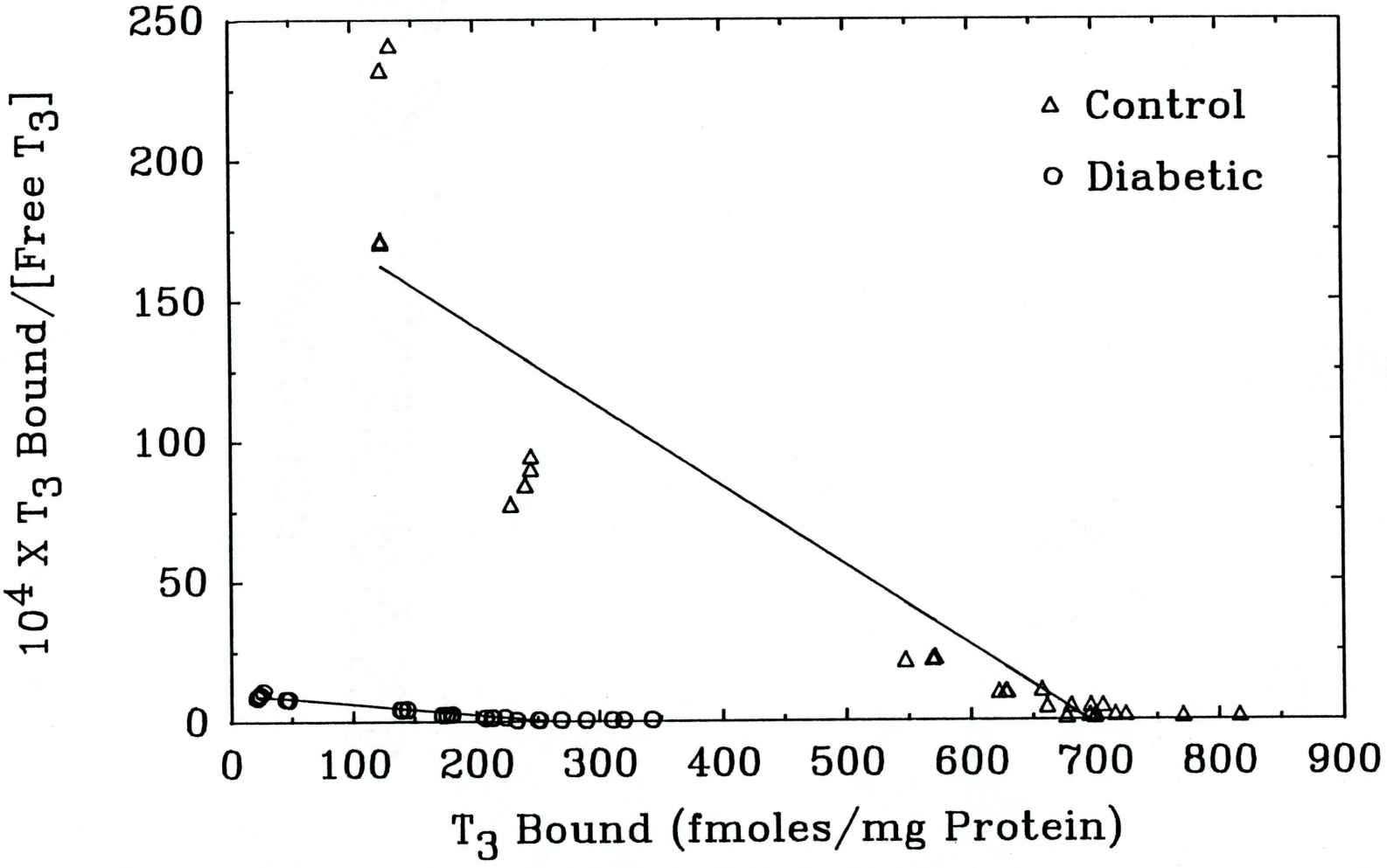

250
200
150
100
50
0
0
100
200
300
400
500
600
700
800
900
Control
Diabetic
10^4 X T_3 Bound/[Free T_3]
T_3 Bound (fmoles/mg Protein)

Table 3:
Scatchard plot binding constants for normal, heterozygous (*db/m*), and diabetic (*db/db*) mouse hepatic nuclear T_3 receptor preparations. Control and diabetic T_3 receptor preparations in the same row were assayed in pairs using the same buffer and stock solutions while each separate row used separate batches of buffers. MBC is moles/mg protein, Age is in weeks. Asterisk (*) signifies animals fasted for 24 hours prior to killing.

Values From Scatchard Plots

of Hepatic Nuclear T_3 Receptor T_3 Binding

	Control			Diabetic	
Control Animal Type	10^{10} x K_d (M)	10^{13} x MBC (moles/mg prot.)	Age	10^{10} x K_d (M)	10^{13} x MBC (moles/mg prot.)
Heterozygous	2.9*	2.3*	11	17*	2.7*
Normal	0.91	5.2	11	13	1.3
Heterozygous	0.35	6.9	15	3.0	2.9
Heterozygous	0.55	7.9	15	1.6	4.1
Normal	0.72	4.5	46-72		
Normal	2.3	5.5	46-72		
Normal	2.0	2.2	57-60		
Heterozygous	0.95	8.5	22		
Average	1.3 ± 0.8	5.4 ± 1.9		8.7 ± 10.4	2.7 ± 1.6

outlined in the Appendix. In each comparison, the probability of controls and diabetics being from the same distribution and differing only due to random variation was less than 5%. If both control and diabetic receptors were in fact the same, then each binding assay would be an independent sample of the population. The probability that one would see similar differences in the slope over many experiments due to randomness alone would then be the product of the probabilities obtained by the method of Dixon and Massey (1969) for each matched pair. In this manner, the overall probability of getting the above results is 2×10^{-8}, indicating that the two groups are not the same. The difference in the K_d values was also determined to be statistically significant by a one tailed *t*-test ($p<0.01$). Since nuclear T_3 levels are about 10 times greater than cytoplasmic T_3 levels which in turn are 10 times greater than plasma free T_3 levels, the nuclear T_3 level is about 10^{-10} M (Surks et al., 1975; Pardridge & Landaw, 1987). If the free T_3 concentration in the nucleus were 10^{-10} M, the above K_d values would predict 43% saturation of the receptor in heterozygotes but only 10% saturation of the T_3 receptor in diabetics.

Although the maximum binding capacity of $(5.4 \pm 1.9) \times 10^{-13}$ moles/mg protein in the assay for controls and $(2.7 \pm 1.6) \times 10^{-13}$ for diabetics seem to be dissimilar,

they must be normalized, prior to comparison, to the amount of nuclear material per animal since the T_3-receptor complex has its effects on the transcription of DNA. When the maximum binding capacity was scaled to the amount per liver, the controls demonstrated (7 ± 3) x 10^{-13} moles/liver which was not statistically different from (11 ± 8) x 10^{-13} in diabetics. Similarly, when maximum binding capacity was calculated on the basis of liver DNA content, no significant difference was found between the values of (4.6 ± 3.3) x 10^{-13} moles/mg DNA for controls and (3.2 ± 4.6) x 10^{-13} for diabetics.

3.7 Kinetics of Association and Dissociation

Although Scatchard analysis has been shown above to demonstrate a difference between the K_d for T_3 of controls and diabetics, it can not distinguish whether the difference is due to the receptor, or if the "inapparent, nonspecific binding" suggested by Seelig et al. (1981) is actually causing the difference. Another method to determine the extent of ligand-receptor dissociation is to observe the rate at which labeled ligand is lost from the receptor with time. This method is valuable in that it ignores any "inapparent, nonspecific binding" allowing the T_3-receptor dissociation to be examined independently.

Association half-lives of 8.5 hours for controls (n=3) and 6.2 hours (n=1) for diabetics are not

significantly different (compare the slopes in Figure 8). However, the half-life of labeled ligand binding in the presence of excess unlabeled ligand is significantly greater for controls, 101 ± 22 hours (n=10) versus 70 ± 21 hours for diabetics (n=5, $p<0.04$, see Table 4). Figure 9 illustrates the association of T_3 with the control receptor preparation as well as the dissociation of T_3 from the control receptor preparation, while Figure 10 illustrates the T_3 association and dissociation for the diabetic receptor preparation. Figure 11 is a combination of the dissociation data from Figures 9 and 10 plotted on the same scale to facilitate comparison of the slopes. The association data indicates that the K_d should be 2×10^{-10} M and the half-life for dissociation should be 6 hours. The predicted K_d agrees well with the observed K_d; however, the half-life for the dissociation of the T_3-receptor complex does not agree with the observed half-life. This indicates that the binding conditions are not equilibrium binding conditions.

3.8 Molecular Weight Estimation by G-100 Chromatography

The receptor molecular weight was estimated by G-100 Sephadex chromatography to be 65,000 ± 3,000. Denaturing conditions were not used in order to allow $[^{125}I]T_3$ binding to act as a label for the receptor during elution. Since the conditions were not designed to give a random

Table 4:
Half-lives for the dissociation of the hepatic nuclear T_3-receptor complex for normal, heterozygous (*db/m*), and diabetic (*db/db*) mice. The data for normals and heterozygotes were combined to form the control group.

Half-lives of the Hepatic Nuclear T_3-receptor Complex

from Normals, Heterozygotes, and Diabetics (Hours)

Normals	Heterozygotes	Diabetics
78	92	60
96	128	69
97	176	73
78	65	49
100		98
95		
91 ± 10	115 ± 67	70 ± 21

Control 101 ± 22 Diabetic 70 ± 21

Figure 8:
The initial portion of the association curve between T_3 and the hepatic nuclear T_3 receptor preparation transformed into a linear form. The Y axis is on a logarithmic scale. The lines represent the best fit as determined by linear least squares regression assuming all error is in the Y axis.

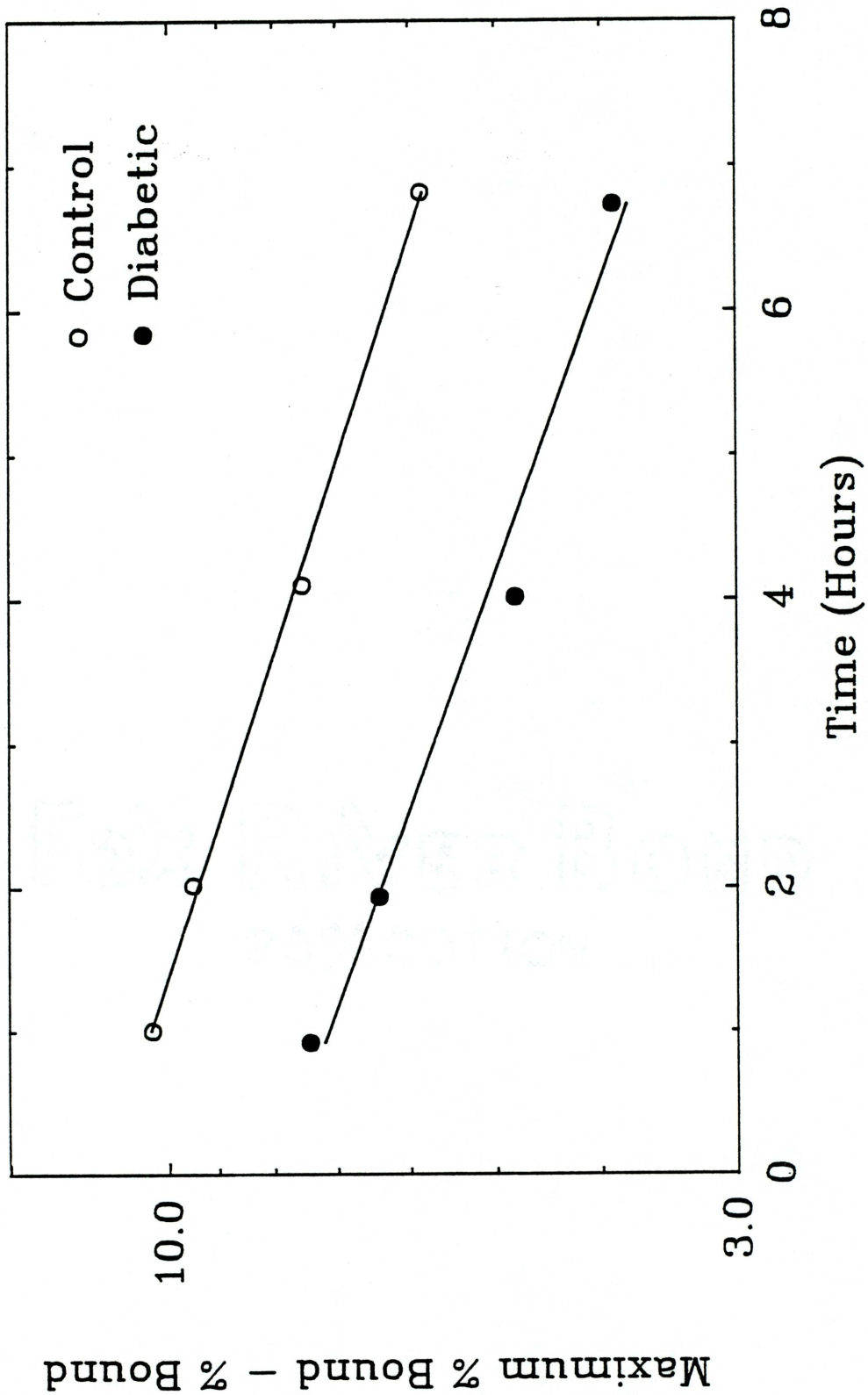

Maximum % Bound – % Bound
10.0
3.0
0
2
4
6
8
Time (Hours)
o Control
● Diabetic

Figure 9:
The association and dissociation curves for the control hepatic nuclear T_3 receptor preparation. Open circles give the association curve while open squares, triangles, and diamonds are the data points for the three dissociation curves. The Y axis plots on a logarithmic scale the percent of the tracer ligand added that became bound. The lines represent the best fit as determined by linear least squares regression assuming all error is in the Y axis.

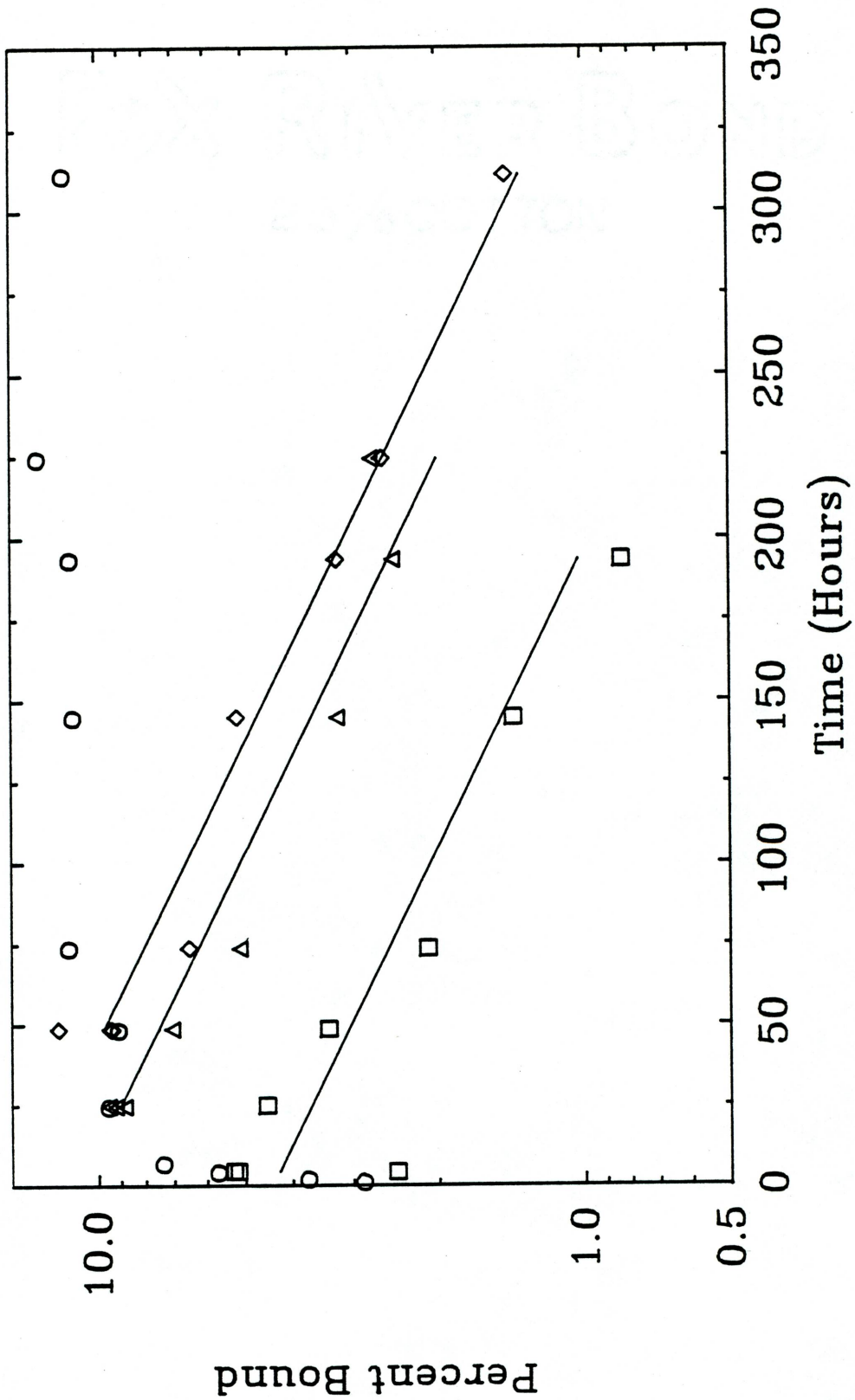

Percent Bound
10.0
1.0
0.5
0
50
100
150
200
250
300
350
Time (Hours)

Figure 10:
The association and dissociation curves for the diabetic hepatic nuclear T_3 receptor preparation. Open circles give the association curve while open squares, triangles, and diamonds are the data points for the three dissociation curves. The Y axis plots on a logarithmic scale the percent of the tracer ligand added that was bound. The lines represent the best fit as determined by linear least squares regression assuming all error is in the Y axis.

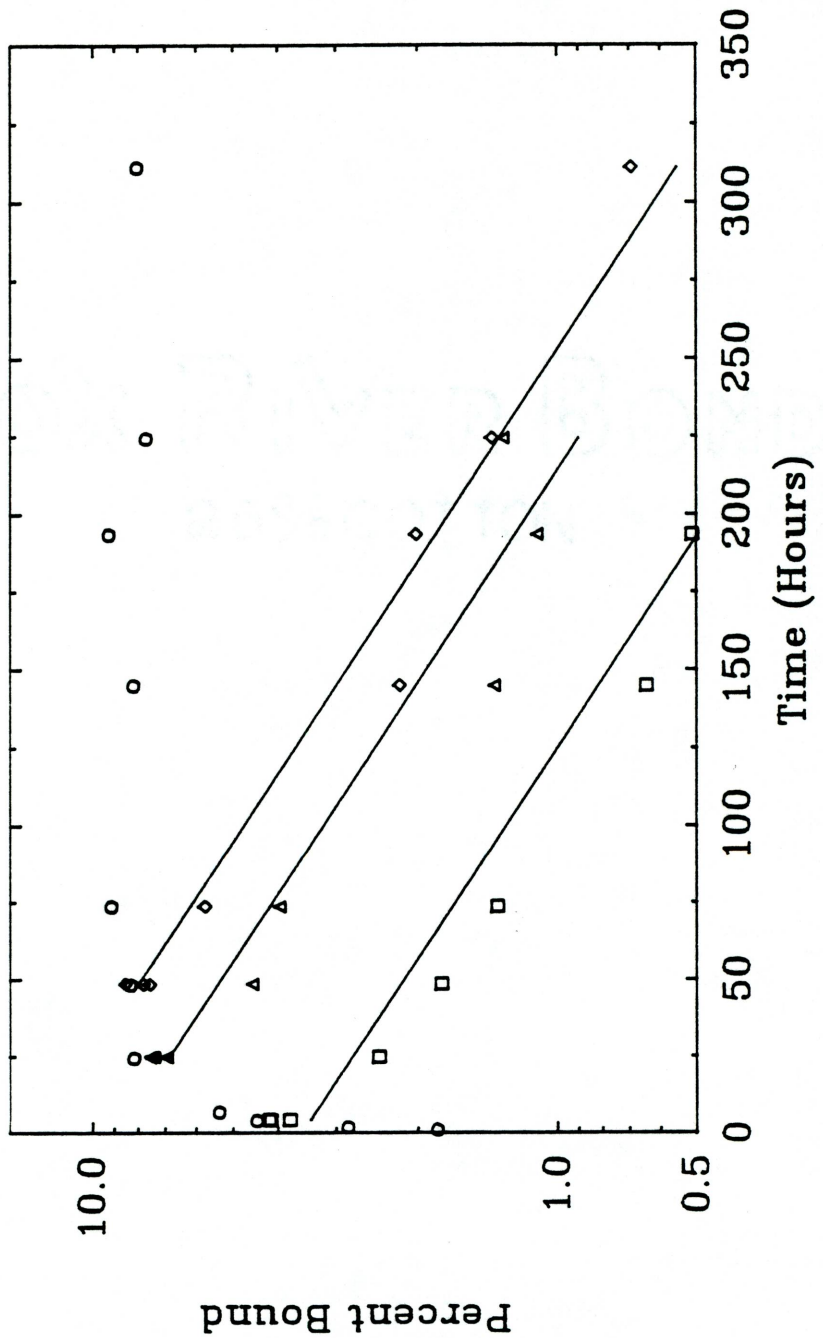
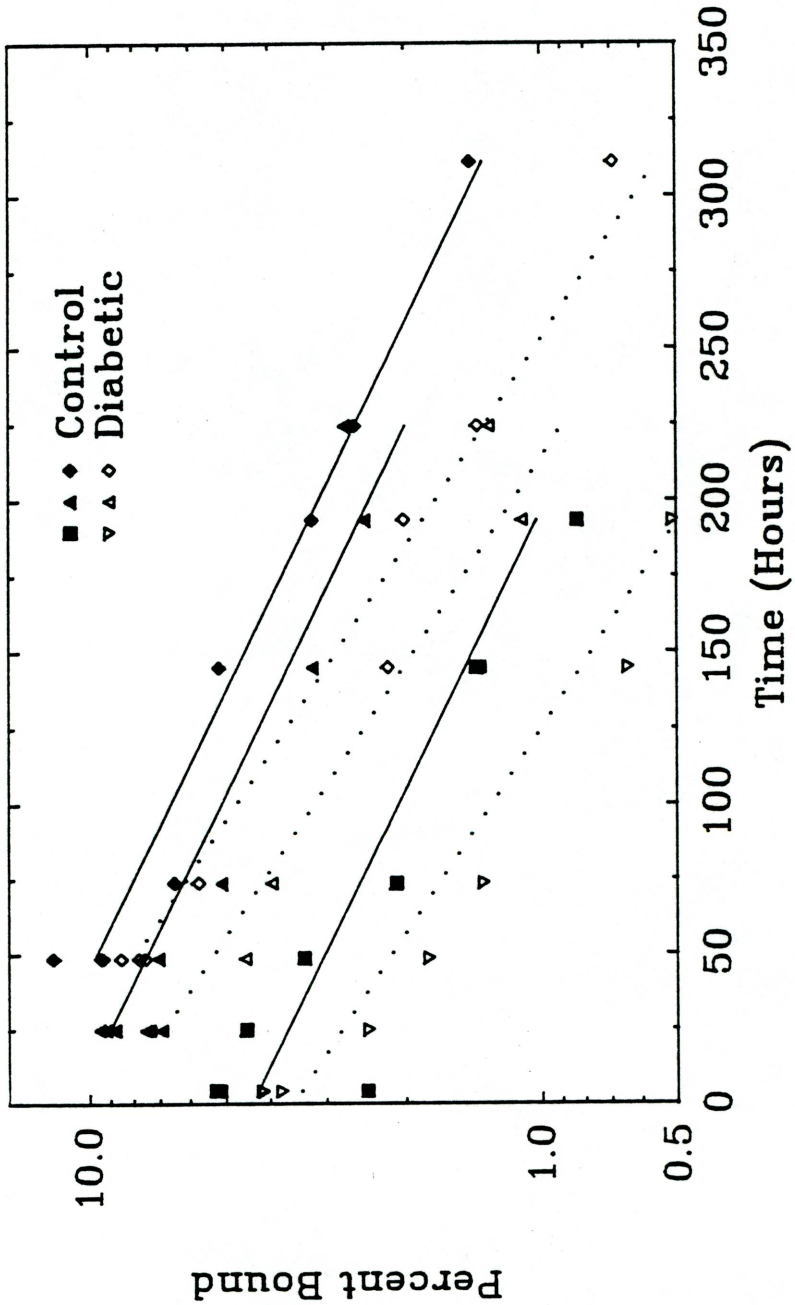

Percent Bound
10.0
1.0
0.5
0
50
100
150
200
250
300
350
Time (Hours)

Figure 11:
The dissociation curves for both control and diabetic hepatic nuclear T_3 receptor preparations. Filled symbols are the data points for controls while open symbols are the data points for diabetics. The lines represent the best fit as determined by linear least squares regression assuming all error is in the Y axis. The solid lines are the dissociation of the control T_3-receptor complex, the dotted lines are the dissociation of the diabetic T_3-receptor complex.


Control
Diabetic
Time (Hours)
0
50
100
150
200
250
300
350
10.0
1.0
0.5
Percent Bound

coil configuration, the conformation of the receptor molecule may have played a significant role in the value obtained. Thus, this value is tentative until the receptor can be detected in a denatured state in which the Stokes radius is known to relate more closely to molecular weight.

3.9 Estimation of Free [^{125}I]T_3 by Receptor Binding

After 48 hours of incubation with (1.29 ± 0.04) x 10^{-13} moles of T_3 at 4°C, the tubes with one aliquot of receptor specifically bound (2.91 ± 0.07) x 10^{-14} moles of [^{125}I]T_3 (n=5). The tubes with two aliquots of the receptor preparation, one added immediately and the other added after 24 hours of incubation, specifically bound (5.0 ± 0.1) x 10^{-14} moles of [^{125}I]T_3 (n=5). If T_3 were free to interact with the additional receptor aliquot, then the binding for the tubes with two aliquots should have been 5.14 x 10^{-14} moles when K_d is 1.3 x 10^{-10} M. Thus, 98 ± 2% of the T_3 was able to interact with the second receptor aliquot indicating that under these conditions, T_3 can be considered to be free in solution. The tubes with only one aliquot of the receptor preparation were used to determine the MBC. This value was doubled for the tubes containing two receptor preparation aliquots and used to calculate the expected T_3 binding in the tubes with two receptor preparation

aliquots. Free T_3 was calculated as the total T_3 added minus the specifically bound T_3 minus the nonspecifically bound T_3 minus the T_3 left on the walls of the incubation tube.

4. Discussion

4.1 The Use of Equilibrium Binding Analysis

Seelig et al. (1981) and all other workers have used Scatchard plots extensively. In order to make direct comparisons of the data in this study with the results reported in the literature, it is helpful to use the same method of analysis. However, the fact that many people adopt a method does not assure that the method is valid.

Equilibrium binding analysis such as that used by Scatchard (1949) is based on the assumption that binding is measured at equilibrium and is reversible. The conclusion of Seelig et al. (1981) that this assumption is not valid for T_3 at 0°C would appear to invalidate Scatchard plots as a method of describing the thyroid hormone receptor binding system. However, non-equilibrium conditions do not necessarily negate the significance of the results of equilibrium type analysis, especially when used to compare two similar groups of animals.

As mentioned in the Introduction and Results sections, Seelig et al. (1981) concluded that the interaction between T_3 and nonspecific binding sites over a 24 hour time period demonstrates essentially irreversible binding. Thus, after incubating excess T_3 with the T_3 receptor preparation, all the T_3 became associated with either the receptor or with nonspecific

binding sites. The addition of more T_3 receptor to the equilibrated system demonstrated the irreversibility of binding to nonspecific sites in that there was no increase in the specific T_3 binding. If a transfer of T_3 from the nonspecific sites to the added T_3 receptor protein was possible, the specific T_3 binding should have increased.

Although Seelig et al. (1981) continue to use Scatchard plots in spite of these concerns, a more reassuring fact to consider is the binding of T_3 analogs. As reviewed by Samuels (1983), there is an excellent correlation between the relative binding constants obtained through equilibrium binding analysis and the relative biological activity of T_3 analogs. The binding constants determined from Scatchard plots all demonstrate a direct relationship with the effects of ligand binding, thereby confirming the value of this method.

Another point in favor of utilizing equilibrium binding analysis to understand the binding of thyroid hormone is that the kinetic model proposed by Seelig et al. (1981) predicts essentially the same binding as predicted by Scatchard plots. Thus, even if Seelig et al. (1981) are correct in their conclusion that Scatchard plots are invalid, the method nevertheless accurately predicts the binding behavior. It is important to recognize that if Seelig et al. (1981) are correct, the

constants obtained are apparent equilibrium binding constants and may not reflect what binding would be under true equilibrium conditions. But, solely on the basis of fitting the data, the binding constants are useful for predicting and comparing ligand binding. Thus, the apparent K_d values of $(1.3 \pm 0.8) \times 10^{-10}$ M for controls and $(8.7 \pm 10.4) \times 10^{-10}$ M for diabetics can be used to demonstrate a significant difference in the level of T_3 binding to the nuclear T_3 receptor in these two groups under physiological T_3 concentrations.

One concern raised by Seelig et al. (1981) remains which must be considered when comparing binding constants for thyroid hormones. The proposed "inapparent, nonspecific sites" sequester T_3 making it unavailable to the receptor itself, yet this T_3 appeared in their assay as free T_3. If one were to compare two receptor preparations with identical receptor binding characteristics, but with different amounts of inapparent, nonspecific binding, the increased inapparent binding would lower the amount of free T_3 available to interact with other binding sites. This would lower the amount bound to the receptor and other nonspecific sites. Since the reason the sites are "inapparent" is that they do not show up in the assay as nonspecific binding, the effect would be to lower total binding, including both

nonspecific and specific binding. Thus, the binding curve would in effect be shifted to the right. Since nonspecific binding increases linearly with increasing ligand concentration, the effect would be equivalent to a linear transformation of the X axis of a saturation plot. The maximum binding capacity would not be affected, but K_d would be increased. A difference in the level of "inapparent, nonspecific binding" somewhat greater than the error in binding measurements would lead to the conclusion that the two groups differed in their K_d even though the receptors were identical.

Since this study used nitrocellulose filtration rather than a T_3 binding resin to separate bound from free T_3, there is probably less removal of T_3 from weakly bound nonspecific sites. Thus, the problems associated with "inapparent, nonspecific sites" would be diminished. However, as a safeguard against falsely stating the binding of controls to be different from that of diabetics, the dissociation of T_3 from the receptor was studied to further characterize any alterations in the interaction of the receptor with T_3.

Alterations in "inapparent, nonspecific binding" only affect the amount of T_3 available to interact with other nonspecific binding sites and with the receptor. By incubating labeled T_3 with the receptor preparation until

bound T_3 levels reach a plateau and then blocking reassociation of dissociating labeled T_3 by adding excess unlabeled T_3, the rate of the decrease of specific binding should not be affected by a lower initial level of binding due to "inapparent, nonspecific binding". Since the dissociation half-life for controls (101 ± 22 hours) was significantly greater than that for diabetics (70 ± 21 hours), one can conclude that the T_3-receptor interaction is weaker in diabetic liver. This reinforces the validity of the larger K_d for diabetics reported in this study.

Thus, although the basic assumptions for equilibrium binding analysis have not been demonstrated, Scatchard plots are used in this study with confidence that the binding constants obtained, in conjunction with the dissociation half-lives, can be used to compare receptor binding.

4.2 The Assumption of Free T_3

Although many workers studying thyroid hormones assume that T_3 is free in their binding assay solutions, this is only partially true. As previously mentioned, at 0°C Seelig et al. (1981) demonstrated that no free T_3 was available as determined by the lack of additional specific T_3 binding after addition of more T_3 receptor preparation. This indicates that all the T_3 interacted with the components in the original incubation mixture in such a

fashion that it was not available for binding to the added receptor. However, Yagura and Walfish (1982) demonstrated that after incubation at 4°C, 90% of the resin-absorbable T_3 could cross a dialysis membrane. This indicates that the T_3 is free in solution. They concluded that under their conditions for binding (4°C) T_3 would be expected to interact with additional receptor. This finding appears to contradict the results of Seelig et al. (1981). Yagura and Walfish (1982) stated this information as supported by unpublished data, but they did not clarify how this finding was determined. If they actually measured the ability of T_3 to equilibrate across a dialysis membrane after resin binding, it would not have any bearing on the availability of T_3 to bind to the receptor since the point made by Seelig et al. (1981) was that the resin strips T_3 off the "inapparent, nonspecific sites". It should be no surprise that when T_3 is removed from any interaction with binding sites it is then free to cross a dialysis membrane. With the data presented one cannot properly evaluate the results of Yagura and Walfish (1982) and can only tentatively conclude that the difference in binding conditions may have led to the contradictory results. This conclusion is supported by the results in this study demonstrating 98 ± 2% of the expected binding to a second

aliquot of the receptor preparation under conditions similar to those used by Yagura and Walfish (1982).

However, as stated in the results, T_3 was bound nonspecifically to all the surfaces used in the assay of T_3 binding including glass, siliconized glass, polystyrene, and polypropylene. In serial dilutions, free T_3 decreased to 70% of the expected value with each dilution step. Thus, the concentration of free T_3 is decreased by 30% due to binding to glassware alone. After correcting for T_3 adsorption to glassware, the level of nonspecific T_3 binding to the receptor preparation in the presence of excess unlabeled T_3 was only 3% of the remaining T_3. Since most of the incubated T_3 passes through the filter, it should be free in solution. This would also support the conclusion of Yagura and Walfish (1982) that significant amounts of T_3 are free.

The value of 98% of the expected increase in binding for the addition of a second aliquot of the receptor preparation was calculated without correcting for nonspecific adsorption to the incubation tube. It appears that the T_3 initially adsorbed to the incubation tube, which in a serial dilution would not be seen as free in solution, is nevertheless able to interact freely with the receptor. Thus, only in serial dilutions does the correction have to be made for adsorption to glassware.

The T_3 adsorbed to the incubation tube in receptor binding studies can be considered as free in solution.

4.3 Significance of the Maximum Binding Capacity Differences Between Controls and Diabetics

Although the difference between the T_3 MBC of (4.6 ± 3.3) x 10^{-13} moles/mg DNA for controls versus (3.2 ± 4.6) x 10^{-13} moles/mg DNA for diabetics did not achieve statistical significance, a difference might be expected on a theoretical basis. Glucagon administration to diabetic rats lowers the MBC (Dillmann et al., 1978) as well as lowering the serum T_3 in dogs (Kabadi & Dragstedt, 1987). Although basal circulating levels of glucagon are normal in the diabetic mouse, the control of glucagon secretion is altered (Bray & York, 1979). Arginine stimulates excessive glucagon release and glucose does not suppress glucagon release. Thus, periodic hypersecretion of glucagon could occur with a corresponding decrease in the MBC for T_3.

The failure to demonstrate a statistically significant difference does not mean the parameters compared are actually identical. An increased sample size may in the future demonstrate a difference in the MBC between controls and diabetics. In any case, the data presented here indicate that any difference found would be small. It appears that either a hypersecretion of

glucagon is not present or the levels are not high enough to significantly lower the MBC in the diabetic C57BL/KsJ mouse.

4.4 The Diabetic Mouse

4.4.1 Recessive Genes

Diabetes in the mouse is a recessively inherited disorder. Recessive diseases are generally the result of an alteration in a single DNA base pair. Thus, diabetes in the mouse could have resulted from a single base pair change. If so, the alteration to the protein encoded by that gene should explain all the symptoms of the syndrome.

4.4.2 The T_4 5'-monodeiodinase

If one altered protein is the root of the syndrome, then the symptoms should suggest its identity. One change observed in the diabetic mouse is an increase in serum T_3 and a decrease in serum T_4 (Fehn, 1983). Accordingly, the 5'-monodeiodinase was investigated in the hope that it might be the protein whose gene was altered. Utilizing the method of Chiraseveenuprapund et al. (1978), liver and kidney homogenates were assayed for the conversion of T_4 to T_3. However, no difference was found in the kinetic parameters of the 5'-monodeiodinase *in vitro* between diabetic and control mice (Richard Fehn, personal communication).

4.4.3 Obesity

Another consequence of the diabetic mutation is obesity. One means by which an obese state can be induced is to decrease the basal metabolic rate. Since the maintenance of ion gradients by the Na^+-K^+-ATPase pump comprises a significant portion of the energy expended on the basal metabolic rate, it has been investigated in a number of syndromes characterized by obesity.

Studies on the genetically obese *ob/ob* mouse utilizing ouabain to block the Na^+-K^+-ATPase pump have demonstrated that the obese mouse has a reduction in energy expenditure on Na^+-K^+ transport (Guernsey & Morishige, 1979). More relevant to T_3 receptor studies however is their finding that these mice demonstrate decreased Na^+-K^+-ATPase activity due in part to a decreased nuclear T_3 maximum binding capacity. Furthermore, Khan et al. (1986a) demonstrated a parallel progression in both obesity and the decrease in the number of T_3 receptors. They further suggest that the decrease in T_3 receptors is the primary cause of the obesity. The genetically obese Zucker fatty rat also shows a decrease in the MBC of T_3 as one progresses from normal to heterozygous to homozygous for the fatty gene (Goldberg et al., 1988).

A similar effect mediated by a different mechanism was proposed by Hillgartner and Romsos (1987). They did not report reduced numbers of T_3 receptors in obese mice, but still found decreased nuclear T_3 binding. They suggested that decreased nuclear T_3 binding was the result of decreased transport of plasma T_3 to the nucleus because of altered membrane fluidity. Another example of an alternative mechanism to achieve similar results is reported by Tulp and McKee (1986). They proposed that the cause of obesity in LA/N-cp rats is related to decreased T_4 to T_3 conversion. Thus, there is widespread support for a causal role of altered T_3 binding in obesity.

Nevertheless, there is not universal support for T_3 involvement in all obesities. Anselmet et al. (1984) did not find any alteration in T_3 binding parameters in cell lines derived from the obese mouse. In addition, other mechanisms aside from decreased T_3 binding may lead to obesity. In one study, the Zucker fatty rat demonstrated a reduced T_3 binding capacity in only 50% of the obese animals indicating that other factors could be involved (Khan et al., 1986b).

4.4.4 T_3 Binding in Obese and Diabetic Mice

Since the obesity related reductions in T_3 binding were proposed by some authors to be the cause of the obesity, a comparison to the diabetic mouse would indicate

whether the increase demonstrated in the K_d in the diabetic mouse is great enough to influence its obesity. The T_3-receptor complex is the form that leads to the effects of T_3 so the number of complexes per cell should be the parameter that is most closely related to obesity. To compare the various obese states, the saturation level for each class of obese animals was estimated from their T_3 binding constants assuming that the nuclear T_3 concentration was 10^{-10} M. The percent saturation was normalized by multiplying it by the number of binding sites per cell in C57BL/KsJ controls (~5000). The controls would have roughly 2000 T_3-receptor complexes per cell, the obese LA/N-cp rat would have 1500, the obese mouse would have 1000, and the diabetic mouse would have 500. Since the diabetic mouse would have the lowest relative number of T_3-receptor complexes, it would be more likely to display obesity due to reduced T_3 effects than would the obese mouse. Thus, the difference in the saturation level of the T_3-receptor complex of 10% in diabetics versus 43% in controls as reported in this study is a significant reduction in terms of its expected effect on the animal.

4.4.5 T_3 Resistance

The obesity, cold intolerance, and elevated serum T_3 seen in the diabetic animal are consistent with a

reduction in thyroid hormone effects. Having suggested that the obesity of the diabetic mouse may be directly related to the reduced level of T_3 binding to the nuclear T_3 receptor, it may be further suggested that all of the diabetic symptoms may be due to a change in the receptor and that the gene which was modified is that of the T_3 receptor. However, clinical syndromes which demonstrate thyroid hormone resistance do not display the symptoms of diabetes. As previously mentioned, Refetoff (1982) did not observe any diabetic symptoms in patients with thyroid hormone resistance. On the other hand, the lack of correlation between thyroid hormone resistance and diabetes in man does not exclude the possibility of a connection in the mouse. However, it does require that the correlation be solidly established if one is to use it to explain the aberrant physiology in mice.

4.4.6 Other Alterations in the Diabetic Mouse

Adipsin is a serine protease homolog secreted by adipose cells which may function as a regulatory molecule (Flier et al., 1987). Glucose and high insulin levels decrease the amount of adipsin while mild diabetes has the opposite effect. In the C57BL/KsJ (*db/db*) mouse adipsin levels are extremely low (Flier et al., 1987). Since adipsin may be a regulatory molecule and it has altered expression in the diabetic mouse, it is potentially a

modulator of the diabetic state as well. On the other hand, the low adipsin levels could be merely a reflection of the high levels of insulin which are transiently present in the diabetic mouse. Further research is warranted to establish adipsin's significance.

4.5 Proposed Model for the Diabetic Mouse

If the diabetic mouse has normal free T_3 levels and if an altered T_3 receptor gene is at the core of the diabetic mutation, then the following sequence of events, a modification of the events proposed by Bray and York (1979), are a reasonable model for the development of the diabetic state which is consistent with the currently available data. Decreased T_3 binding by the nuclear thyroid hormone receptor leads to decreased Na^+-K^+-ATPase activity. This in turn leads to hypersecretion of insulin in response to glucose stimuli. Decreased ion transport also leads to decreased re-uptake of norepinephrine in the hypothalamus, thereby altering food intake regulation and leading to hyperphagia. Hyperinsulinemia leads to hyperlipogenesis and eventually to down-regulation of insulin receptor number. As obesity develops due to increased food intake coupled with lower energy expenditure on the maintenance of ion gradients, decreased locomotion furthers the drive towards obesity. The liver continues to supply glucose by accelerating

gluconeogenesis, furthering the impetus for high insulin output, eventually leading to beta cell exhaustion. The 5'-monodeiodinase shifts more T_4 to T_3 in an attempt to overcome the thyroid hormone resistance leading to increased T_3 levels which inhibit thyroid function. Serum T_3 binding proteins then increase and achieve normalization of free T_3 levels.

This is not the only model possible nor does decreased binding of thyroid hormones need to be an initial event. It is quite possible that another factor released from the mutated gene could lead to a different cascade of events. Post-translational modifications of the receptor or alterations in binding cofactors, such as the histone fractions known to modify T_3-receptor binding could yield data such as that presented here yet avoid any requirement for the receptor *per se* to be altered. Recent advances in the genetics of the thyroid hormone receptor suggest that there may be multiple forms for the T_3 receptor which may have different T_3 affinities (Thompson et al., 1987). DNA probes for the thyroid hormone receptor gene indicate that as many as 5 thyroid hormone receptors may exist. Altered overall binding could be the result of altered levels of receptor subtypes which have varying affinities for T_3.

4.6 Indications of a Change in the T_3 Receptor Gene

If there were a mutation in the gene coding for the nuclear T_3 receptor one might see evidence in some physical parameter of the nuclear T_3 receptor which would be altered as well. Obviously, binding might be different, but if other characteristics were also changed one would be reasonably certain that significant alterations were made to the receptor. Therefore, the isoelectric point and sedimentation coefficient were determined in this study. However, neither parameter demonstrated a significant difference from controls indicating that the receptor was not severely altered. The receptor's molecular weight of 65,000 was higher than the 52,000 predicted by the sequence of the human c-erb-A gene which encodes the human thyroid hormone receptor (Weinberger et al., 1986). Gruol and Kempner (1982) used radiation inactivation and target theory to demonstrate that the effective mass of the receptor in rat is 59,000 daltons.

It has been shown that other molecules are present *in vivo* which are involved in determining the level of T_3 binding (Anselmet et al., 1983; Bismuth et al., 1985). Accordingly, the use of relatively crude receptor preparations may have advantages over more highly purified preparations in that these binding cofactors may be

retained with the receptor during partial purification as indicated by the large molecular weight determined in this study. By retaining the receptor complex in a state more like that seen *in vivo*, the binding constants obtained are more likely to reflect the level of binding in the cell.

4.7 Future studies

Determining the binding constants in diabetic mice as they develop, especially at ages from birth until sexual maturation, may reveal dynamic alterations in T_3 binding which, if they were to parallel the development of the diabetic symptoms, would suggest that altered T_3 binding might be closely involved. More extensive examination of the kinetics of ligand-receptor association might also reveal interesting findings, since the relative difference between the association half-lives of controls and diabetics is almost the same as the relative difference between the dissociation half-lives. In this study, more data were collected on the dissociation half-lives, which caused the difference between controls and diabetics to become statistically significant when in fact both aspects of ligand-receptor interaction may be affected.

Further purification of the receptor to homogeneity would aid in determining the molecular weight of the core receptor under denaturing conditions, as well as in the chemical characterization of the T_3 receptor to see if

there are any physical changes between controls and diabetics. Differences might be expected in the amino acid composition, level of glycosylation, or in the presence or composition of a binding cofactor. Since the sequence of a human thyroid hormone receptor is known, portions could be used to probe for the mouse receptor. With the mouse gene in hand, direct studies on the sequence of the diabetic and normal receptor gene would give a straightforward answer to the question of whether any mutation occurred in the receptor gene.

Although the nuclear T_3 receptor has been referred to extensively as the only true receptor, that has yet to be proven. Further research needs to be conducted to demonstrate conclusively that this protein is in fact a necessary link in the transduction of the T_3 signal and the expression of the diverse effects of thyroid hormones. The mechanism of action for thyroid hormones is also somewhat speculative at this time and could benefit from more investigation. A final goal would be the complete determination of all the mechanisms whereby T_3 effects the cell, including cells exhibiting perturbations such as diabetes.

5. Appendix

5.1 Derivation of the Equations for Multiple Lines With One Common Slope

The derivation of the equations for many lines with a single slope follows the concepts used in deriving the equation for simple linear least squares regression on one line. The theory of least squares regression generally assumes that all the error in experimental data is in the Y axis. It also assumes that the true relationship between the X and Y axis is indeed linear. Thus the true line that one wishes to calculate should run through the true mean Y for each X. The measured Y values should have a normal distribution scattered around the average Y due to random variation.

Least squares analysis simply means one minimizes the sum of the squares of the differences between the predicted Y values and the measured Y values. In the case of many lines with the same slope one is trying to determine:

(1) $Y_1 = mX_1 + b_1$

(2) $Y_2 = mX_2 + b_2$

(3) $Y_3 = mX_3 + b_3$

et cetera for each line

Thus one needs to determine the value of m, b_1, b_2, b_3, etc.

If one has j lines with n_i points in line i and with k specifying the points in line i where i is the line number (1 to j), the deviation of the measured Y_{ik} from the predicted Y is

(4) $Y_{ik} - (mX_{ik} + b_i)$ or $Y_{ik} - mX_{ik} - b_i$

The sum of the squares of the deviations for n_i points is then

(5) $\Sigma(Y_{ik} - mX_{ik} - b_i)^2$

To minimize the sum of the squares of the deviations from all the lines let

(6) $Q = \Sigma(Y_{1k} - mX_{1k} - b_1)^2 + \Sigma(Y_{2k} - mX_{2k} - b_2)^2 + \ldots$

To find the value of a variable at the maximum or minimum of a function one takes the partial derivative of the function with respect to that variable and sets it equal to zero. Solving for the variable under investigation will give the value of that variable when the function is at either its maximum or minimum. Thus one must evaluate $\delta Q/\delta m$, $\delta Q/\delta b_1$, $\delta Q/\delta b_2$, $\delta Q/\delta b_3$, etc. Re-writing the equation above

(7) $Q = (\Sigma Y_{1k}^2 - 2m\Sigma X_{1k}Y_{1k} - 2b_1\Sigma Y_{1k} + m^2\Sigma X_{1k}^2 + 2mb_1\Sigma X_{1k} + n_1b_1^2) + (\Sigma Y_{2k}^2 - 2m\Sigma X_{2k}Y_{2k} - \ldots$

The partial derivatives are then

(8) $\delta Q/\delta m = (-2\Sigma X_{1k}Y_{1k} + 2m\Sigma X_{1k}^2 + 2b_1\Sigma X_{1k}) + (-2\Sigma X_{2k}Y_{2k} + 2m\Sigma X_{2k}^2 + 2b_2\Sigma X_{2k}) + (\ldots$

(9) $\delta Q/\delta b_1 = -2\Sigma Y_{1k} + 2m\Sigma X_{1k} + 2n_1b_1$

(10) $\delta Q/\delta b_2 = -2\Sigma Y_{2k} + 2m\Sigma X_{2k} + 2n_2 b_2$

(11) $\delta Q/\delta b_3 = -2\Sigma Y_{3k} + 2m\Sigma X_{3k} + 2n_3 b_3$

et cetera

Setting the partial derivatives equal to zero and simplifying gives

(12) $0 = (-\Sigma X_{1k}Y_{1k} + m\Sigma X_{1k}^2 + b_1\Sigma X_{1k}) + (-\Sigma X_{2k}Y_{2k} + m\Sigma X_{2k}^2 + b_2\Sigma X_{2k}) + (. . .$

(13) $0 = -\Sigma Y_{1k} + m\Sigma X_{1k} + n_1 b_1$

(14) $0 = -\Sigma Y_{2k} + m\Sigma X_{2k} + n_2 b_2$

(15) $0 = -\Sigma Y_{3k} + m\Sigma X_{3k} + n_3 b_3$

et cetera

Solving equations 13-15 the for the intercepts b_1, b_2, b_3, etc. to get equations 16-18 and substituting into equation 12 yields equation 19.

(16) $b_1 = (\Sigma Y_{1k} - m\Sigma X_{1k})/n_1$

(17) $b_2 = (\Sigma Y_{2k} - m\Sigma X_{2k})/n_2$

(18) $b_3 = (\Sigma Y_{3k} - m\Sigma X_{3k})/n_3$

et cetera

(19) $0 = (-\Sigma X_{1k}Y_{1k} + m\Sigma X_{1k}^2 + \Sigma X_{1k}[\Sigma Y_{1k} - m\Sigma X_{1k}]/n_1) + (-\Sigma X_{2k}Y_{2k} + m\Sigma X_{2k}^2 + \Sigma X_{2k}[\Sigma Y_{2k} - m\Sigma X_{2k}]/n_2) + (. . .$

Rearranging

(20) $\Sigma X_{1k}Y_{1k} + \Sigma X_{2k}Y_{2k} + . . . = m\Sigma X_{1k}^2 + \Sigma X_{1k}\Sigma Y_{1k}/n_1 - m(\Sigma X_{1k})^2/n_1 + m\Sigma X_{2k}^2 + \Sigma X_{2k}\Sigma Y_{2k}/n_2 - m(\Sigma X_{2k})^2/n_2 + . . .$

Isolating the terms with the slope (m)

$$(21)\quad \Sigma X_{1k}Y_{1k} - \Sigma X_{1k}\Sigma Y_{1k}/n_1 + \Sigma X_{2k}Y_{2k} - \Sigma X_{2k}\Sigma Y_{2k}/n_2 + \ldots =$$
$$m\Sigma X_{1k}^2 - m(\Sigma X_{1k})^2/n_1 + m\Sigma X_{2k}^2 - m(\Sigma X_{2k})^2/n_2 + \ldots$$

Factoring out m

$$(22)\quad \Sigma X_{1k}Y_{1k} - \Sigma X_{1k}\Sigma Y_{1k}/n_1 + \Sigma X_{2k}Y_{2k} - \Sigma X_{2k}\Sigma Y_{2k}/n_2 + \ldots =$$
$$m(\Sigma X_{1k}^2 - (\Sigma X_{1k})^2/n_1 + \Sigma X_{2k}^2 - (\Sigma X_{2k})^2/n_2 + \ldots)$$

Solving for the slope (m) and combining the similar sums

$$(23)\quad m = \frac{\sum_{i=1}^{j}\left[\sum_{k=1}^{n_i} X_{i_k} Y_{i_k}\right] - \sum_{i=1}^{j}\left[\frac{\sum_{k=1}^{n_i} X_{i_k} \sum_{k=1}^{n_i} Y_{i_k}}{n_i}\right]}{\sum_{i=1}^{j}\left[\sum_{k=1}^{n_i} X_{i_k}^2\right] - \sum_{i=1}^{j}\left[\frac{(\sum_{k=1}^{n_i} X_{i_k})^2}{n_i}\right]}$$

With intercepts (b_i) at

$$(24)\quad \frac{\sum_{k=1}^{n_i} Y_{i_k} - m \sum_{k=1}^{n_i} X_{i_k}}{n_i}$$

5.2 Sample Calculation

Assume the following 3 data sets are the data points for 3 lines which should have the same slope.

X_1	Y_1
1	2
2	4
3	6
4	8
5	10

X_2	Y_2
1	3
2	5
3	7
4	9

X_3	Y_3
1	7
2	9
3	11

The following are calculated

X_1	Y_1	X^2	XY
1	2	1	2
2	4	4	8
3	6	9	18
4	8	16	32
5	10	25	50
Sums			
15	30	55	110
$(\Sigma X)^2$			
225			

X_2	Y_2	X^2	XY
1	3	1	3
2	5	4	10
3	7	9	21
4	9	16	36
10	24	30	70
100			

X_3	Y_3	X^2	XY
1	7	1	7
2	9	4	18
3	11	9	33
6	27	14	58
36			

Evaluating the first complex summation in equation 23

$$\sum_{i=1}^{j} \left[\sum_{k=1}^{n_i} X_{i_k} Y_{i_k} \right]$$

For i=1 the sum of XY for all the 5 points is 110, for i=2 the sum of XY for all the 4 points is 70, and for i=3 the sum of XY for the 3 points is 58. Thus the overall sum is 110 + 70 + 58 = 238. Evaluating the second complex summation in equation 23

$$\sum_{i=1}^{j} \left[\frac{\sum_{k=1}^{n_i} X_{i_k} \; \sum_{k=1}^{n_i} Y_{i_k}}{n_i} \right]$$

For i=1 the sum of X is 15, the sum of Y is 30, and n=5 thus the term for i=1 is 15x30/5 = 90. For i=2 one calculates 10x24/4 = 60; for i=3, 6x27/3 = 54. The sum for all values of i is then 90 + 60 + 54 = 204. Evaluating the last two complex summations in a similar fashion yields the following expression for the four complex summations:

$$(238 - 204)/(99 - 82) = 34/17 = 2$$

The intercept b_1 is then $(30 - [2 \times 15])/5 = 0$

$b_2 = (24 - [2 \times 10])/4 = 1$

$b_3 = (27 - [2 \times 6])/3 = 5$

Thus the 3 equations for the 3 data sets are

$Y = 2 \times X + 0$

$Y = 2 \times X + 1$

$Y = 2 \times X + 5$

5.3 Comparison of Two Slopes as Described by Dixon and Massey (1969)

Assuming the samples are linear, the slopes (m_1 and m_2) are calculated for the two samples by linear least squares regression. If s_{x1}^2 and s_{y1}^2 are the variances of the X's and Y's in the first sample and s_{x2}^2 and s_{y2}^2 are the variances of the X's and Y's in the second sample, then the unbiased

estimates of the variances about the population regression lines are

$$s^2_{y1.x} = (s_{y1}^2 - m_1^2 s_{x1}^2)(n_1 - 1)/(n_1 - 2)$$

and

$$s^2_{y2.x} = (s_{y2}^2 - m_2^2 s_{x2}^2)(n_2 - 1)/(n_2 - 2)$$

Assuming the population variances are equal, the pooled variance is

$$s^2_{y.x.p} = \frac{(n_1 - 2)s^2_{y1.x} + (n_2 - 2)s^2_{y2.x}}{n_1 + n_2 - 4}$$

And a *t* value can be calculated as

$$\frac{m_1 - m_2}{s_{y.x.p}\sqrt{\frac{1}{(n_1 - 1)s_{x1}^2} + \frac{1}{(n_2 - 1)s_{x2}^2}}}$$

If this *t* value is greater than the critical *t* from a table of *t* values, one would reject the null hypothesis and conclude that the two slopes are different at the probability level used in the critical *t*.

5.4 Propagation of Error

As described by Peters et al. (1974), assume one has three experimentally determined, independent parameters *x*, *y*, and *z*. If *w* is a function of these three parameters, the variance in *w* is approximately

$$(\partial w/\partial x)^2 V_x + (\partial w/\partial y)^2 V_y + (\partial w/\partial z)^2 V_z$$

If there are more parameters, the above equation can be expanded with similar terms, one for each parameter.

Since the 95% confidence limit is related to the square root of the variance, the 95% confidence limit of w can be estimated by the 95% confidence limits of x, y, and z.

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