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EFFECTS OF Ah-INDUCERS ON THE
ACTIVITY OF THYROID-REGULATED ENZYMES
AND CONTROL OF THYROID HORMONE METABOLISM

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EFFECTS OF Ah-INDUCERS ON THE ACTIVITY OF THYROID-REGULATED ENZYMES AND CONTROL OF THYROID HORMONE METABOLISM

By

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ABSTRACT

EFFECTS OF Ah-INDUCERS ON THE
ACTIVITY OF THYROID-REGULATED ENZYMES
AND CONTROL OF THYROID HORMONE METABOLISM

By

William Louis Roth

TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin) is the most toxic of a class of polycyclic aromatic hydrocarbons which cause the induction of enzymes associated with the Ah locus. Some of the effects of Ah inducers on metabolism appear to be explainable as secondary effects of the induction of the Ah locus enzymes mentioned above. Although these enzymes were discovered by virtue of their catalytic activity towards carcinogenic compounds such as benzo[a]-pyrene and 3-methylcholanthrene, they are known to hydroxylate and or conjugate fatty acids, steroids, and thyroxine (T4).

Since (T4) conjugation and excretion was known to be increased in the rat on treatment with Ah-inducers, we attempted to determine whether T3 levels dropped with T4 in plasma or liver. In our experiments T3 levels did not drop with T4.

In pharmacokinetic studies, T3 was produced from T4 in a pulse which lagged T4 uptake by 4 - 5 min. The relative size of this pulse in treated vs control rats was similar to the ratio of specific activities for T4 between the

respective groups. In light of reports on the passive and active uptake of T3 and T4, these data suggested that deiodination of T4 is regulated by a saturable uptake system which runs in parallel with a nonsaturable, diffusive uptake of T4, driven by its hydrophobicity.

While attempting to determine the thyroid status of Ah-inducer treated rats, we discovered that liver malic enzyme was increased by Ah-inducers in a thyroid hormone dependent manner. The induction of malic enzyme by glucocorticoids is thyroid hormone dependent, ie. thyroid hormones must be present for induction to occur. Certain symptoms of Ah-inducer toxicity, such as thymic involution, hyperlipidemia, and fatty liver development can also be produced by glucocorticoid administration, and might be explained if Ah-inducers had corticomimetic properties in addition to their interactions with the Ah receptor. Experiments designed to determine whether TCDD could act as a ligand for the glucocorticoid receptor demonstrated no affinity of TCDD for this receptor, but left open the possibility that the Ah receptor may be able to regulate some of the same genes as does the glucocorticoid receptor.

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DEFINITIONS AND ABBREVIATIONS

Cytochrome P-450	One of a set of terminal monooxygenases found in the endoplasmic reticulum. Binding of carbon monoxide to the heme iron of these enzymes shifts the normal absorption band (the Soret band), which occurs near 425 nm, to a position near 450 nm.
AHH	Aryl Hydrocarbon Hydroxylase. The name given to an oxidative activity which appears on induction of cytochrome P-450c.
Ah locus	A gene which has been shown to control the synthesis of cytochrome P-450c and other enzymes which appear on induction of AHH activity.
Ah receptor	A Cytosolic protein which specifically binds chemicals that induce AHH activity.
B[a]P	Benzo[a]pyrene, a common carcinogenic compound which induces AHH activity.
3-MC	3-methylcholanthrene, a carcinogenic compound which like B[a]P, induces AHH activity.
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin - the most potent inducer of AHH activity known.
T4	Thyroxine, precursor to the active thyroid hormone, 3,5,3'-triiodothyronine (T3).
T3	The active thyroid hormone, 3,5,3'-triiodothyronine.

Tetrac	Tetraiodothyroacetic acid - a minor metabolite of thyroxine.
Triac	Triiodothyroacetic acid - a minor metabolite of T3.

CHAPTER I

LITERATURE REVIEW

INTERACTIONS OF Ah INDUCERS WITH THE Ah LOCUS

Exposure of animals to xenobiotics frequently results in the induction of components of a class of monooxygenases called cytochrome P-450s. Certain isozymes of cytochrome P-450 are normally present at moderate levels, (apparently acting as catalysts in steroid metabolism) while others only appear on exposure to certain classes of chemicals which have shown to be responsible for the induction of those isozymes (54,55). Other enzymes which may be induced by each class of chemicals include glucuronosyl transferases (78), glutathione-S-transferases(5), and δ-aminolevulinic acid synthetase. Some enzymes required for synthesis of the porphyrin prosthetic groups of the P-450s are also induced by these compounds (127).

Two different types of cytochrome P-450 inducers have been studied extensively. For compounds typified by phenobarbital (PB), which are readily metabolized by the monooxygenases they induce (cytochromes P-450b and P-450e), induction lasts only a short period of time, and is directly proportional to the lifetime of the inducer before its catabolism (84,103). A family of aryl hydrocarbons which includes benzo[a]pyrene (B[a]P) 3-methylcholanthrene (3-MC), dibenz[a,h]anthracene, certain symmetric halogenated biphenyls, and halogenated dioxins, of which 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is the most potent, induce a second set of enzymes. Cytochrome P-450c

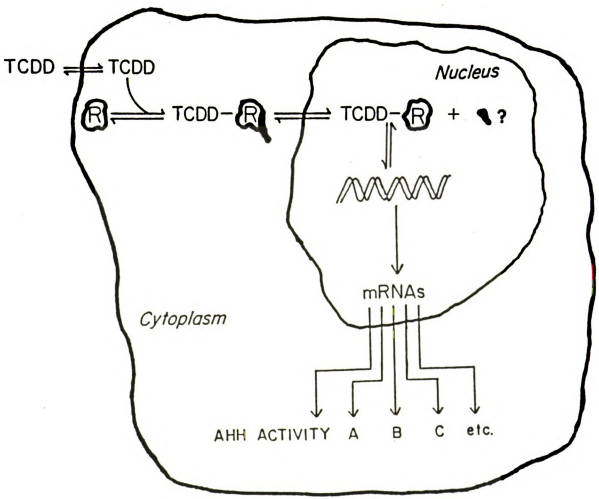
(sometimes called aryl hydrocarbon hydroxylase or AHH), epoxide hydratase, and 4-nitrophenol glucuronosyl transferase are the best studied of these aryl hydrocarbon inducible enzymes, and have been shown to be coordinately regulated by a receptor which binds to chromosomal enhancing sequences of the Ah-locus (20,53,67,74) as shown in figure 1. The concentration of a particular compound required to induce a certain level of P-450c is relatively constant across species having a demonstrable Ah receptor.

For TCDD, some induction can be observed at 0.1 nmol/Kg (32 ng/Kg or 32 parts per trillion). Fifty percent of maximal induction occurs at about 1 nmol/Kg (ED_{50}), and maximal induction occurs between 10 and 33 nmol/Kg (46,103,111). Halogenated cyclic aromatic compounds vary in their ability to bind to the Ah receptor. Most of these compounds are not good ligands for the receptor, but those that are have estimated binding constants in the nanomolar range (K_D = 0.1 - 20 nM), and are often refractory to metabolism by the monooxygenase activities they induce (7,84).

In the cases of 3-MC and B[a]P, induction of AHH results in rapid oxidation of 3-MC and B[a]P to products which have reduced affinity for the Ah receptor. Synthesis of cytochrome P-450s ceases as these compounds are metabolized. An unfortunate result of the oxidation by AHH is that these oxidation products can form adducts with DNA, creating potential sites for mutation (17,64).

Figure 1

Proposed mechanism for Ah-locus control and Ah-receptor action.


TCDD
TCDD
R
TCDD–R
TCDD–R
+ ?
Nucleus
mRNAs
Cytoplasm
AHH ACTIVITY
A
B
C
etc.

For three widespread environmental contaminants, polychlorinated biphenyls (PCB), polybrominated biphenyls (PBB), and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), the result of exposure is an intense and sustained induction of the aryl hydrocarbon hydroxylase system at 30-70 X the activity found in liver and other tissues of unexposed animals (85,110,111).

Chronic exposure to low levels of TCDD results in accumulation of this compound in adipose tissues. It has been suggested that mobilization of these residues may result in AHH induction and activation of common carcinogenic chemicals such as benzo[a] pyrene, that might otherwise be excreted or sequestered in lipids without activation (17,31,83,87).

Large doses of TCDD produce a distinctive set of symptoms including anorexia, hyperlipidemia, hyperkeratosis and thyroid hyperplasia (2,8,104). In some animals, such as the guinea pig, acutely toxic doses of Ah-inducers coincide with the ED50 for induction of cytochrome P-450c. However, many other species, such as the mouse and hamster, show no morbidity at doses of these compounds which are far in excess of those required for induction of Ah-locus enzymes.

At a biochemical level, alterations in the metabolism of thyroid hormones (8,104), glucocorticoids (6,37), and testosterone (85) have all been described following TCDD treatment, but clear biochemical cause -> effect relationships have not been established for these changes,

although linkages with cytochrome P-450 and glucuronosyl transferase induction have been suggested. These, and other metabolic alterations produced by Ah inducers such as hyperlipidemia, may be secondary or tertiary to induction of Ah-locus enzymes. Some may, alternatively, be the result of hormone-mimetic properties of Ah-inducers.

Rozman et al. (112) demonstrated that thyroidectomy was protective to animals treated with 100 ug/kg TCDD. Treatment of thyroidectomized rats with thyroxine restored sensitivity to TCDD. Later work has shown that thyroidectomy does not change the pattern of cytochrome P-450 induction, indicating that symptoms of acute toxicity are not the result of AHH induction alone (60,111,113).

Rickenbacker and McKinney (108) suggested that PCBs and TCDD have thyromimetic properties. Our work (111), and that of Osborne et al.(96) has shown that TCDD is not thyromimetic. Our work (Chapter 2) indicated that the induction of certain lipogenic enzymes (eg. malic enzyme) by Ah inducers is dependent on the presence of thyroid hormones, suggesting a biochemical explaination for Rozman's results in terms of the multihormonal regulation which has been established for lipogenic enzymes (12,49,135). We hypothesized, on the basis of the similarity between malic enzyme induction by TCDD and reports of a requirement for both thyroid hormones and glucocorticoids in the induction of malic enzyme, that TCDD might be corticomimetic. We have shown, as discussed in

chapter 3, that this is not the case. However, we have not been able to rule out the possiblity that the Ah receptor acts as an enhancer towards expression of these lipogenic enzymes, in the same fashion as does the glucocorticoid receptor.

The discussion here and in the chapters which follow requires an understanding of the similarities and differences between the Ah receptor, glucocorticoid receptor, and thyroid hormone receptors. The last chapter requires a similar familiarity with thyroid hormone metabolism. For these reasons, the remainder of this chapter reviews the properties of these receptors, and the metabolic pathways of thyroxine and triiodothyronine.

General Characteristics of the Ah Receptor

Several studies of the characteristics of the Ah receptor have been made to date. The identification of a discrete, low-capacity, high-affinity binding protein for 3-MC was first reported by Filler and Litwack (45) in 1974. This report was followed several years later by a study which identified a low-capacity, high affinity binding protein for TCDD (53). Studies by other authors in the last six years have shown that the 3-MC and TCDD binding fractions represent either a single protein, or very similar proteins which perform nearly identical functions (14,58,90,91).

The specific binding fraction in crude cytosol has been

consistently reported to sediment at about 9.0 S on 5-20 % sucrose density gradients (58,90), and 5.0 S on glycerol gradients(20). The estimated molecular weight has ranged from 130,000 to 245,000 (20,45,91), depending on the methods and assumptions used. The reported K_d of the Ah receptor for TCDD has ranged from 3.0 nM to < 1.0 nM, depending on both the assay method and animal species used (46,58,91). Claims have also been made for the existence of other distinct specific binding species in the 4-5 S region of sucrose density gradients or the 60-70,000 Mr fractions from gel filtration (64,128).

The mechanism by which the Ah receptor induces cytochrome P-450 synthesis has received considerable attention, but has been limited by the crude nature of the cytosolic and nuclear preparations available for its study. Experiments employing gel filtration, DNA-affinity chromatography, and proteolytic enzymes have shown that the Ah receptor has a single type of aryl hydrocarbon binding site, a DNA binding site, and an additional domain that apparently is required for transport across the nuclear membrane (20). The receptor cannot bind to DNA until it has bound an aryl hydrocarbon molecule. Once it has bound to DNA, it promotes transcription of at least one component of the Ah system, the mRNA for cytochrome P-450c (52,129).

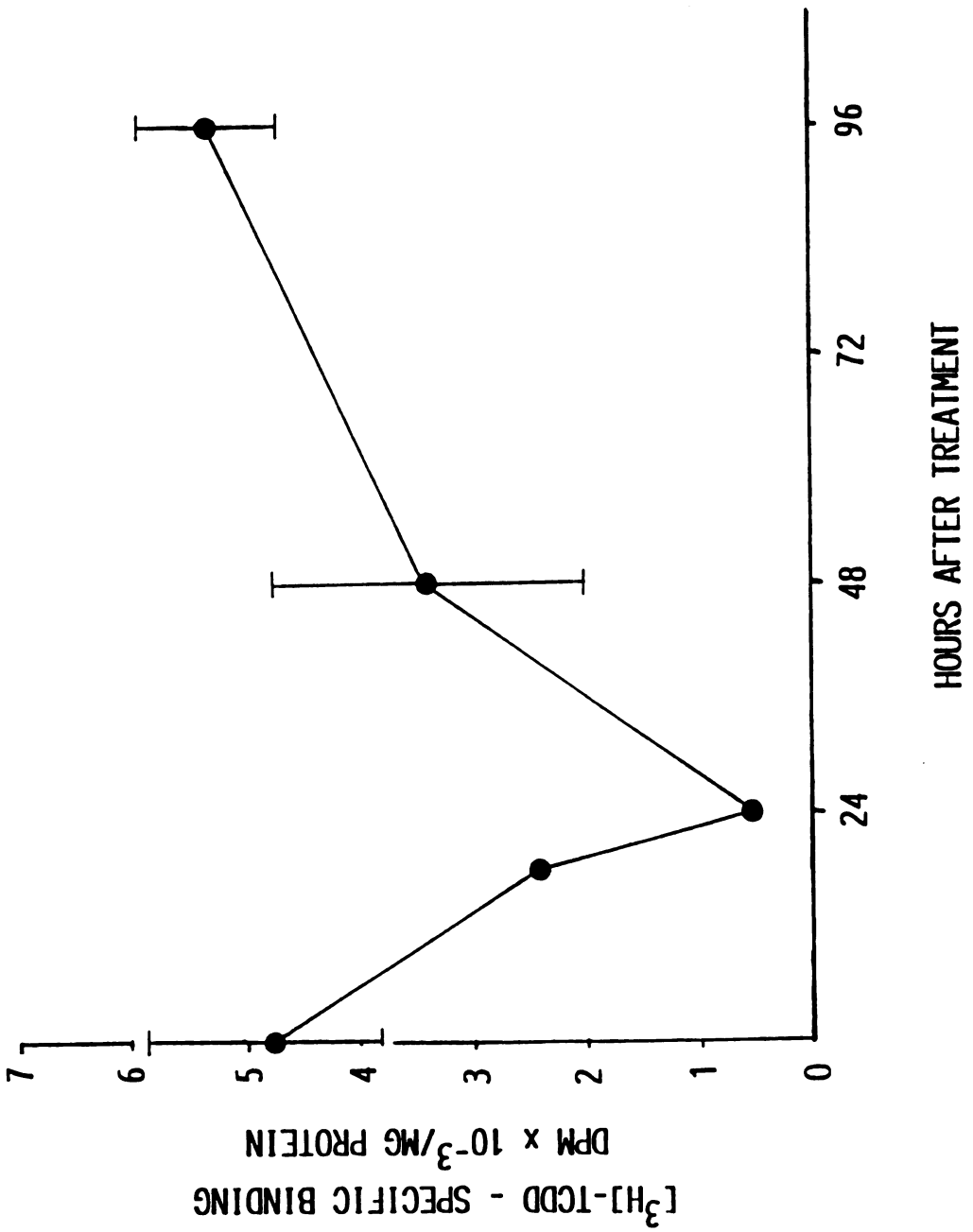
Movement of the receptor from the cell cytoplasm and its accumulation in the nucleus after aryl hydrocarbon exposure has been demonstrated by several authors (53,64,74,134).

From 4 to 12 hours post treatment with an Ah-inducer, the concentration of receptor in the cytoplasm rapidly declines, while nuclear concentrations rise. This drop in the specific binding of 2,3,7,8-[^{3}H]-TCDD in the cytosol following treatment of rats with the compound 3,4,5,3',4',5'-hexabromobiphenyl(HBB) is shown in figure 2. Note the reappearance of specific activity in the cytosol after 24 hours. Although the question remains as to whether or not this is newly synthesized protein, it is clear from our experiments that the concentration of receptor does not increase above pretreatment levels in the cytosol of HBB-treated animals. The induction of cytochrome P-450s and glucuronosyl transferases is detectable in the liver at about 12 hours, reaching a plateau at 48 hours. This process can be detected in cultured hepatocytes within 30 min. of exposure. Mutants have been identified in cultured hepatoma cells which are deficient in binding of the receptor to DNA. Mutants have also been found which cannot transport their ligand-receptor complexes from the cytosol to the nucleus (74).

Many structure-activity studies have been performed to determine the structural requirements for ligand binding to the Ah receptor (7,14,47,90). The relative affinity of the receptor for a particular ligand is determined by a competitive binding assay, which employs [^{3}H]-TCDD as the ligand with highest affinity. The ability of a 100-fold excess of 8 common Ah inducers to displace TCDD from the

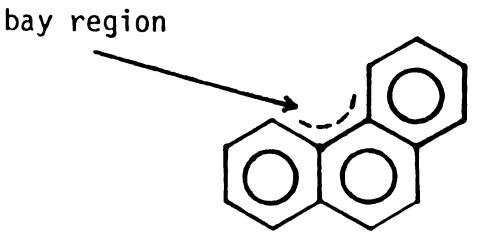
Figure 2

Specific binding of [^{3}H]-TCDD to liver cytosolic protein after treatment with 10 mg/Kg 3,4,5,3',4',5'-hexabromobiphenyl.


[3H]-TCDD - SPECIFIC BINDING
DPM x 10-3/MG PROTEIN
0
1
2
3
4
5
6
7
24
48
72
96
HOURS AFTER TREATMENT

receptor is shown in figure 3. The compounds which bind most tightly are those which are most hydrophobic and which are capable of assuming an energetically stable, planar configuration.

Although there appears to be some tendency for these compounds to be capable of forming a phenanthrene - like "bay region", as shown below, it is difficult to see how TCDD would assume such a configuration.



Steroid hormones have this type of phenanthrene structure, and were investigated as possible natural ligands for the receptor early on (90). None of the compounds tried were potent competitors when [^{3}H]-TCDD was used as the radio-ligand. However, studies of steroid hormone receptors have influenced the direction of Ah receptor studies.

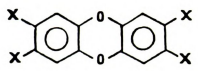
Steriod Hormone Receptors

Cytosolic receptors have been identified for glucocorticoid, mineralocorticoid, and sex steroids. All of these proteins have specific binding sites for a single "natural" steroid, and a binding site for DNA. As was the case for the Ah recptor, synthetic ligands, such as dexamethasone and

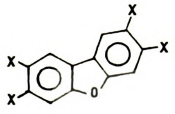
Figure 3

Structures of 8 aryl hydrocarbons which displace TCDD from the Ah receptor and the % displacement with 100 X excess competitor.*

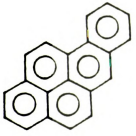
* from references 7,14,53.



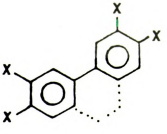
2,3,7,8-tetrahalo-dibenzo-p-dioxin (TCDD) 100



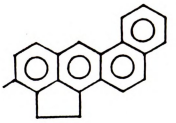
2,3,7,8-tetrahalo-dibenzofuran 98



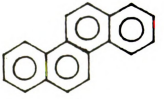
Benzo[a]pyrene 96



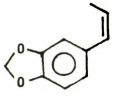
3,4,3',4'-tetrahalo-biphenyl 95



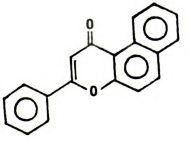
3-methylcholanthrene 95



Chrysene 82



Isosafrole 67



β-Napthoflavone 55

triamcinolone acetonide, are required as binding assay ligands, because the natural ligands bind less tightly and are rapidly metabolised in the crude cytosolic assay systems (21,139).

The molecular weights of steroid hormone receptors have been estimated to lie within the range of 67,000 - 89,000 , and have sedimentation coefficients of 8 - 9 S. As with the Ah-receptor, proteolytic fragments have been identified which bind steroid, but do not bind to DNA. Reports of such low molecular weight binding species created some controversy before definitive evidence of receptor proteolysis was shown, and methods to mitigate it were developed (1,136,140). Once a ligand molecule is bound, steroid hormone receptors undergo an activating transformation to a complex of lower molecular weight (4.5 - 5 S) and higher affinity for the ligand. This transformation process is temperature dependent, and can be inhibited by oxidation of sulfhydryl groups with molybdate. The activated form can be produced in vitro by incubating the ligand-receptor mixture with dithiothreitol at 25° to 30° C (21). The Ah receptor binds its ligands with equal efficiency at 0° C and 25° C, does not have a demonstrable "activated" form, and cannot be stabilized with molybdate (35).

Thyroid Hormone Receptor

Investigation of the mechanism of action of thyroid hormones has been pursued using significantly different strategies and with qualitatively different results from those obtained in glucocorticoid investigations.

The action of thyroid hormones has been suggested to be regulated in four steps. Our work (chapter 4), when viewed in light of other reports, suggests that step (c) may preceed (b) and be synchronized with step (a):

a) Circulating thyroxine is transported across the plasma membrane both via passive diffusion and by a process that involves association with membrane receptors and endocytosis into endosomes via "coated pits" (23,42,56).

b) Thyroxine is then specifically bound by a protein in the cytosol. This protein has been poorly characterized, although reports in the literature have shown it to be specific and distinct from serum binding proteins (32,57,121).

c) Thyroxine is deiodinated to 3',3,5-triodothyronine (T3), which binds specifically to another cytosolic protein which is not well-studied.

d) T3 is somehow transported to the nucleus, and binds to a nuclear receptor protein, which is stereospecific in its affinity for L-T3. This stereospecificity is reflected in studies of nuclear uptake of T3, in which L-T3 accumulated in the nucleus, but D-T3 remained in the cytosol (116).

The thyroid hormone receptor which actually binds to DNA is thought to be located exclusively in the cell nucleus, has a basic subunit size of 3.8 S, and molecular weight of 54,000 (95,98). This receptor appears to associate with both histone and non-histone proteins of chromatin, and protects about 36 base pairs of DNA from cleavage by DNase I (3). While it has been shown that thyroxine and T3 are transported by endocytosis into the cell from circulating plasma carrier proteins, and across the nuclear membrane to putative nuclear receptors, no careful study of the cytoplasmic or membrane vectors has been made. Intensive study has been made of one of the serum T4 binding proteins, transthyretin (prealbumin). This protein has been shown by X-ray crystallography to have a single symmetric binding site for thyroxine, and what appears to be a possible DNA binding site (15,16). Transthyretin also has four binding sites for retinol binding protein, which is a vector for transport of retinol, and is involved in the regulation of retinol concentrations in plasma and tissues (121). The presence of binding sites for both retinol binding protein and thyroxine suggests that this protein is part of a control mechanism for retinol metabolism that involves thyroid hormones(13,88,132).

In contrast with studies reviewed above for the thyroid hormone receptor system, no studies have been made of the association of the Ah receptor with chromatin proteins in vivo, or to determine whether identifiable sequences of DNA

are protected by Ah receptor binding. Jones et al. (67) have recently cloned the upstream controlling sequences of DNA coding for cytochrome P-450c from a mouse hepatoma line. Restriction endonuclease cleavage and deletion analysis of these isolated sequences indicated the existence of a TCDD-responsive enhancer region between nucleotides -1580 and -1310. An inhibitory domain was identified between nucleotides -1310 and -695. A promoter region which was not TCDD- responsive was found near the start of transcription, between nucleotides -45 and -8. Thus the gene may actually be regulated by three different proteins: a) the Ah receptor, (b) a repressor binding protein, and (c) a promoter binding protein.

THYROXINE AND TRIIODOTHYRONINE METABOLISM

Thyroxine Production

Thyroxine (T4) is the principal thyroid hormone synthesized by the thyroid gland or thyroid tissue of all species which have been studied. The available evidence suggests that the thyroid is an evolutionary outgrowth of the salivary glands, which still contain a small number of cells which are capable of concentrating iodide, and may produce small amounts of T4 following surgical thyroidectomy (33). Synthesis of T4 proceeds via iodination of the tyrosine residues of a high molecular weight globulin - thyroglobulin. Formation of the ether linkage of the

thyronine structure occurs after iodination. The complete biochemical mechanism of thyronine synthesis is not well understood, but both iodination and phenyl ether formation are known to be catalyzed by a peroxidase. A similar reaction is used to make iodothyronines synthetically, using t-butyl peroxide as the catalyst (119).

Thyroxine is transported via the plasma, principally associated with two different proteins - transthyretin, which normally carries 85 % of plasma T4, and thyroid binding globulin (TBG) which carries small amonts of plasma T4 (109). Transthyretin has a moderately strong affinity for T4, with a K_d of 10^8. TBG has a higher affinity for T4, with a K_d of about 10^{10}. During periods of food deprivation, synthesis of TBG is enhanced. Increasing the concentration of TBG reduces the amount of free T4 in plasma, and reduces the rate of T4 uptake and deiodination by tissues (66).

Thyroid Hormone Uptake by Tissues

Transport of T3 and T4 into tissues is thought to occur via two parallel mechanisms, one active and dependent on ATP, the other passive and driven primarily by the relative hydrophobic properties of T3 and T4 (42,44,59,72,73).

Thyroid hormones possess ionic (carboxyl and immonium groups), polar (ether and hydroxyl groups), and hydrophobic (iodophenyl rings) domains. As a result they are insoluble in both water and nonpolar organic solvents, but are

moderately soluble in polar organic solvents such as alcohols and water-soluble ethers. The partition coefficients of T4 measured in n-heptane/water, n-octanol/water (97), and lecithin/water (63) systems are 0.0004, 91.0, and 12,000, respectively, reflecting the polarity of the organic phase. T3 is less hydrophobic, and more water soluble than T4. When T3 and T4 move through a hydrophobic matrix, for example, a reverse-phase chromatography column, the T3 retention time is about 1/2 that of T4 (62). Bulk uptake rates for T3 and T4 from perfusion media follow the same pattern, and were initially thought to be entirely due to diffusion (59,97,106). However, experiments with primary hepatocyte cultures and blood cells have shown that a portion of both T3 and T4 uptake is active, ATP dependent, and saturable (42,44,56,72,73). Cheng et al.(23) have demonstrated what appears to be receptor-mediated endocytosis of tetramethyl rhodamine-T3 in hepatocytes. This evidence, and the inhibition of the saturable, high affinity uptake of T3 by colchicine, ouabain, and cyanide (56,73) suggest that at least a portion of T3 and T4 uptake proceed by receptor-mediated endocytosis from the plasma. The 5'-deiodinase, discussed below, has been shown to have a plasma membrane location. Leonard et al. (75) suggested that the plasma membrane location of the deiodinase "...may represent a physiological mechanism to allow efficient production of ... T3, from an intrinsically inactive precursor T4, without the obligatory penetration

by T4 of the intracellular space." Our results from pharmacokinetic studies of T4 metabolism (chapter 4) indicate that an association between the active uptake system and the deiodinase might serve to deliver a controlled flux of T4 to the deiodinase, while excluding the flux of T4 which passively diffuses into the cell. Such interactions are known for cholesterol metabolism and may be essential for control of the metabolism of hydrophobic compounds in general, since they cannot be excluded from the cell by the lipid membrane as are water soluble compounds such as amino acids.

Production of T3 from T4

Although the concentration of T4 in the plasma is approximately 50 X that of T3 (50 nM vs 1 nM), it is ineffective in stimulating thyroid hormone-dependent processes *in vivo* when its deiodination is totally inhibited. The active hormone, L-3',3,5-triiodothyronine (T3), is produced in tissues by deiodination of T4. Two thiol-dependent deiodinases have been identified which catalyze this reaction. Both can use glutathione as a substrate, but are thought to utilize a glutaredoxin for transfer of the reducing equivalents *in vivo* (51). Type I deiodinase, which is found in the plasma membrane of liver, and kidney, can be inhibited by several thiourea and thiopyrimidine compounds, the most potent of which is propylthiouracil (PTU) (26). Type II deiodinase, which is

found in the pituitary and nervous tissue in general, is not inhibited by PTU and has been found to contribute about 30 % of the T3 in the whole body pool (120,133). Several authors attributed physiological activity to T4 (prior to this discovery) which actually resulted from tissue T3 produced by type II deiodinase.

The thyroid hormone nuclear receptor, discussed earlier with the Ah and glucocorticoid recptors, does bind T4 in vitro. However, the combination of its low affinity for T4 and the transport kinetics for T4 into T3 producing tissues ensure that T4 is ineffective in the absence of deiodinase activity. Several studies have shown that tissue concentrations of T4 are much lower than in plasma. VanDoorn, et al. (130,131), using an isotopic equilibrium technique, found that T4 levels in the liver and kidney were about one half those in plasma. Conversely, T3 concentrations were higher in tissues than in plasma. Liver and kidney T3 concentrations were 5 - 10 X those in plasma.

Catabolism of T3 and T4

Both T4 and T3 are metabolized in the liver and/or simply excreted from the liver into the bile. It has been estimated that 30-50 % of T4 is conjugated with glucuronic acid via a UDP-glucuronosyl transferase in the liver (126). This conjugate appears in the bile, but apparently can be hydrolyzed by bacterial β-glucuronidase in the intestine, as little conjugated T4 appears in the feces (125,126).

Another 20 % of T4 is deiodinated to form either T3 or rT3, both of which are more rapidly metabolized and eliminated than T4 (40). About 30 % of T4 in the bile appears as free T4, which can be resorbed from the intestine(27). The catabolic products formed from T3 are similar to those formed from T4, but appear more rapidly (9,40). Two different diiodothyronines can be formed from T3 : 3,5-diiodothyronine and 3',3-diiodothyronine. Since these compounds are very difficult to separate chromatographically, they are often considered together as T2 (125). The total amount of T2 excreted in bile represents about 30 % of all T3 metabolites. T3-glucuronide constitutes another 40 %, while unconjugated T3 makes up the balance. Only 3-5 % of the total output of T3 is excreted in the urine. However, the kidneys eliminate approximately 90 % of the iodide which results from deiodination.

Ether link cleavage has long been identified as a minor pathway of thyroid hormone catabolism, but the source of the the iodothyroine metabolites has only recently been identified. Burger et al.(19) have shown that ring cleavage is catalysed by a peroxidase present in "activated" leucocytes. Unactivated leucocytes do not catalyze ring cleavage. This discovery may explain a long recognized decline in thyroid hormone levels which occurs in patients with certain infectious diseases. The metabolic pathways discussed above are summarized in figures 4 and 5.

Figure 4

Pathways and metabolites resulting from T4 metabolism.

MAJOR METABOLITES OF T4

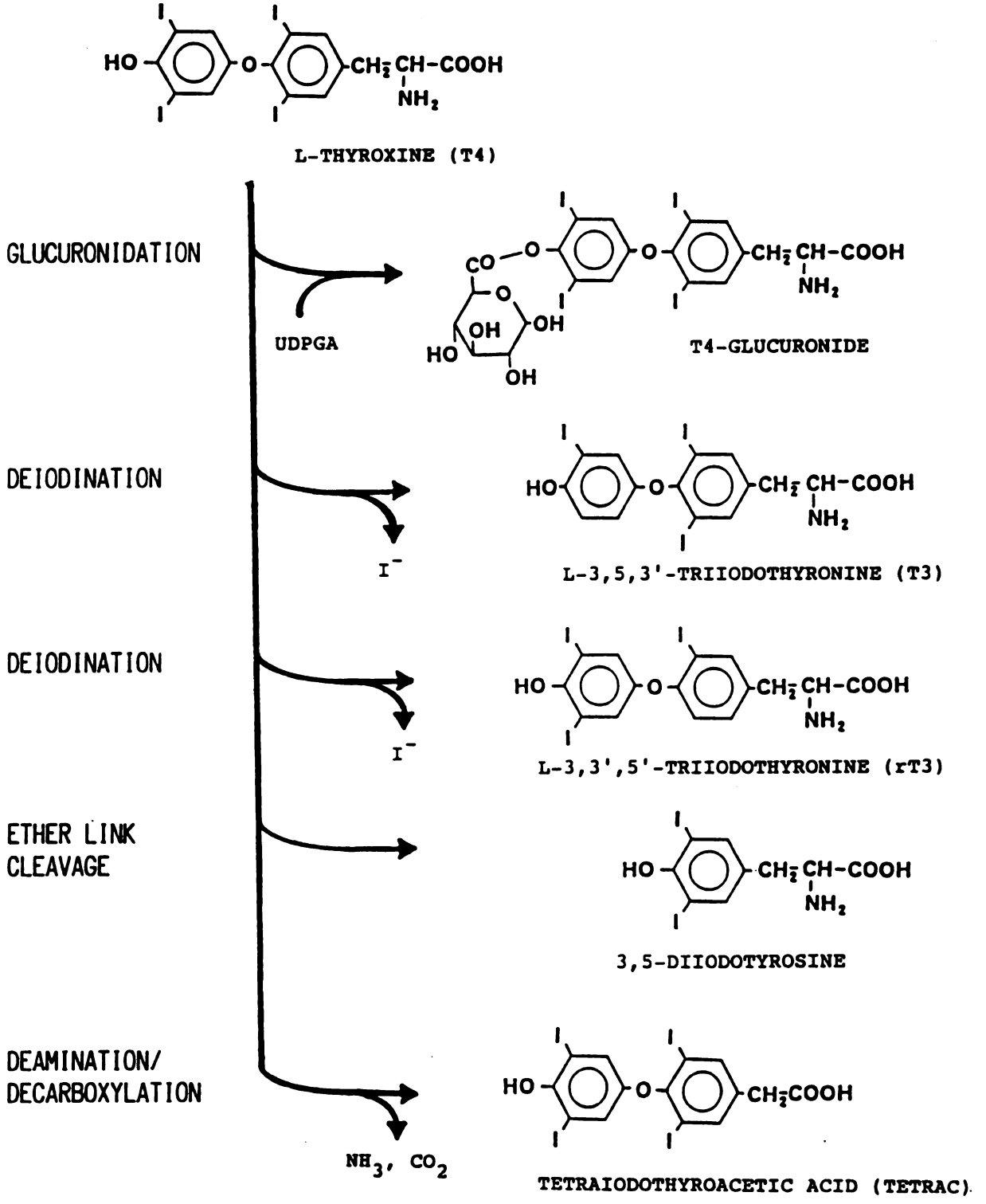
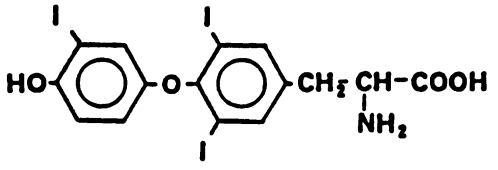


Figure 5

Pathways and metabolites resulting from T3 metabolism.

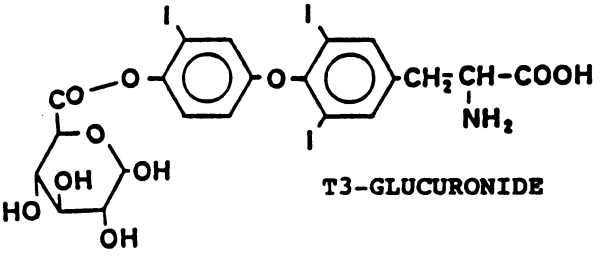
MAJOR METABOLITES OF T3



L-3,5,3'-TRIIODOTHYRONINE (T3)

GLUCURONIDATION

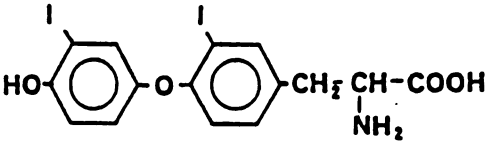
UDPGA



T3-GLUCURONIDE

DEIODINATION

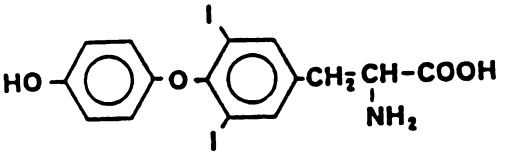
I^-



3,3'-DIIODOTHYRONINE (T2)

DEIODINATION

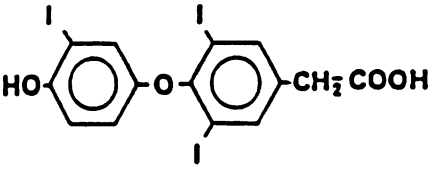
I^-



3,5-DIIODOTHYRONINE

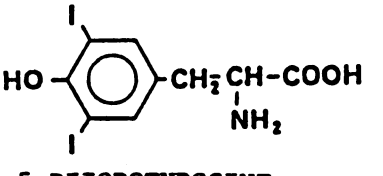
DEAMINATION/
DECARBOXYLATION

NH_3, CO_2



TRIIODOTHYROACETIC ACID (TRIAC)

ETHER LINK
CLEAVAGE



3,5-DIIODOTYROSINE

Ah INDUCERS AND THYROID STATUS

Several authors have noted decreased levels of T4 in the serum of animals treated with Ah inducers, accompanied by increased excretion of T4 and hyperplastic goiter (8,48,89). The hamster may be an exception to this rule, as Henry and Gasiewicz (61) reported increased levels of T4 in a comparative study of rats and hamsters, while confirming the decline in T4 seen by other investigators in rats (104). Reports of T3 levels have been contradictory, and were therefore considered to be unreliable.

Increased excretion of T4 has been associated with an increased activity of PNP-glucuronosyl transferase (PNPGT). Studies in our laboratory have shown that glucuronosyl transferase activity towards T4 (T4GT) increases in parallel with PNPGT (unpublished). Qualitatively similar findings have been reported by two other laboratories, but there is a large variance in results reported between laboratories (48,108). The change in T4 levels can be convincingly explained by the increased T4GT activity, but the increase, or lack of change in T3 levels reported in Ah inducer treated animals is paradoxical in view of the kinetics reported for T4 deiodinase in vitro. Several studies agree in reporting low catalytic rates and low K_m values for deiodination of T4 (26,28,69,133). As shown in appendix A , estimates of T3 concentrations derived using these binding and rate constants and known concentrations

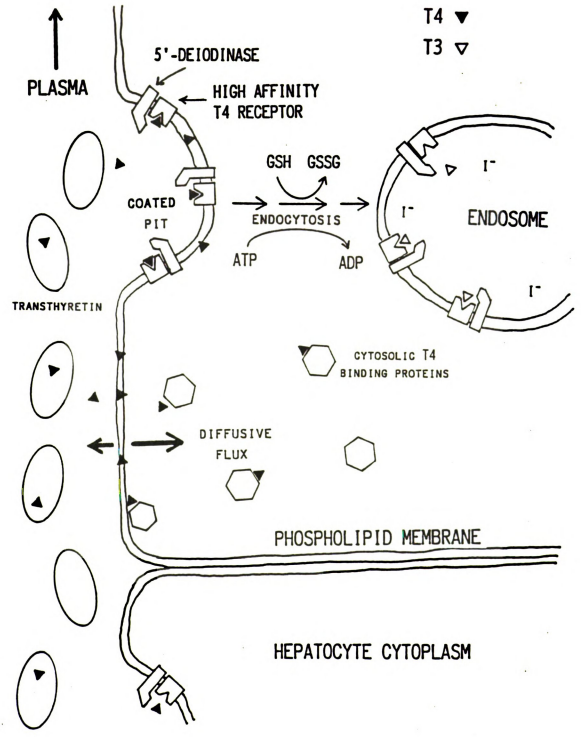
of T4 are directly proportional to these T4 levels. Since this relationship does not hold *in vivo*, in animals treated with Ah inducers , the *in vitro* measurements probably do not reflect the kinetics of the deiodination system *in vivo*.

A clue to the explaination of this phenomenon, which will be explored further in chapter 4, was the independent observation by several laboratories that the hepatic and kidney deiodinase activities were primarily associated with plasma membrane or lysosomal fractions (28,75,79).

Deiodinase activity correlated well with other markers for plasma membrane, such as the Na^+-K^+ ATPase. It is well known that the the reaction kinetics of enzymes associated with membranes are highly sensitive to the environment in which they are assayed, and that parameters measured *in vitro* are often only poorly representative of what occurs *in vivo*. Plasma membrane enzymes are a special case, in that most enzymes which are found in this membrane are involved in signal transmission and/or amplification, or are involved in the transport of substrates from the plasma into the cell. I believe that the discrepancy between the *in vitro* behavior of the deiodinase and the *in vivo* results can be explained by an association of the deiodinase with the high affinity uptake system which transports T4 from the plasma into the cell.

Figure 6

Proposed scheme for control of T3 production from T4 by controlling flux of T4 to the plasma membrane deiodinase.


T4 ▼
T3 ▽
PLASMA
5'-DEIODINASE
HIGH AFFINITY
T4 RECEPTOR
GSH GSSG
COATED
PIT
ENDOCYTOSIS
ATP
ADP
I⁻
I⁻
I⁻
ENDOSOME
TRANSTHYRETIN
CYTOSOLIC T4
BINDING PROTEINS
DIFFUSIVE
FLUX
PHOSPHOLIPID MEMBRANE
HEPATOCYTE CYTOPLASM

CHAPTER II

PLASMA THYROID HORMONE CONCENTRATIONS AND ACTIVITIES OF THYROID-REGULATED ENZYMES IN LIVER AFTER TREATMENT WITH TCDD

ABSTRACT

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) caused a depletion of serum thyroxine (T4), but paradoxically did not change T3 levels in serum of rats. The activities of the thyroid regulated enzymes α-glycerolphosphate dehydrogenase (GPD) and malic enzyme (ME) were determined in livers of normal and thyroidectomized (THX) rats treated with 0.1 to 100 nmol TCDD/kg body weight. Mitochondrial GPD activity did not change significantly as a function of TCDD dose in either normal or THX rats. ME activity was induced by TCDD in a dose-dependent fashion, but only in non-THX animals. The absence of ME induction in THX rats treated with TCDD indicates that TCDD is not intrinsically thyromimetic. The dependence of ME induction on thyroid hormones is much like the thyroid hormone dependent, multihormonal induction of ME by insulin and glucocorticoids. However, TCDD had no additive or synergistic effects on induction of ME activity in THX rats fed T3.

A 30% decrease in steady-state plasma T3 levels of T3-fed animals treated with TCDD relative to T3-fed controls suggested that T3 catabolism was more rapid in TCDD treated rats than controls. Thus a thyroid hormone dependent, multihormonal interaction is suggested as the basis for induction of ME by TCDD, but a strictly T3-dependent process has not been ruled out.

INTRODUCTION

Large doses of TCDD cause a distinctive set of pathological symptoms including anorexia, transient hyperlipidemia, hyperkeratosis, and thyroid hyperplasia (2,8,105), symptoms often associated with abnormal thyroid function. Rozman et al. (112) recently demonstrated that thyroidectomy was partially protective to animals treated with 100 ug/Kg TCDD. In these experiments, 70-80% of normal or euthyroid animals expired 45 days after treatment with TCDD, while none of the thyroidectomized animals died at this dose of TCDD. In the same experiment, treatment of thyroidectomized rats with thyroxine restored sensitivity to TCDD. TCDD also induces aryl hydrocarbon hydroxylase (cytochromes P-450c and P-450d) and other Ah-locus enzymes (110). Many studies have shown a correlation between an Ah-locus response and toxicity of TCDD (53,110). However, recent studies (60,113) have shown that cytochrome P-450 induction is unaffected by thyroid status, eliminating changes in P-450 expression as a possible explanation for the sparing effect of thyroidectomy. It has been hypothesized on the basis of computer modeling studies (107) that the effects resembling thyroid dysfunction are the result of intrinsic thyromimetic properties of Ah-inducers. It was therefore of interest to determine whether TCDD and other Ah-inducers have any intrinsic thyromimetic properties. Several symptoms of TCDD intoxication involve derangement of lipid

metabolism, including hyperlipidemia, fatty liver and elevated cholesterol levels (104,105). Since the liver is a major organ for lipid metabolism and thyroid hormone metabolism, in addition to being the most studied tissue with respect to biochemical effects of TCDD, it may be the most important tissue in which to determine the thyroid status of following TCDD exposure. The thyroid status of the liver was studied by measuring the activity of two hepatic enzymes which are inducible by thyroid hormones. Malic enzyme (ME), which can be induced by thyroid hormones, is a major source of NADPH used in fatty acid synthesis. Thyroid hormone induction of this enzyme is inhibited by glucagon (gluconeogenic conditions), and is stimulated by insulin (glucose sufficient, lipogenic conditions) and glucocorticoids (49,114,135). Mitochondrial α-glycerol-phosphate dehydrogenase(GPD) is a thyroid hormone inducible enzyme associated with the mitochondrial electron transport system. Induction of this enzyme is influenced by glucocorticoids, but is not generally affected by the glucagon/-insulin ratio (18). Our results show that TCDD has no thyromimetic properties, but that one of these enzymes can be induced by TCDD only in the presence of thyroid hormones.

MATERIALS AND METHODS

Chemicals

TCDD was a gift from Dr. Fumio Matsumura, Pesticide Research Center, Michigan State University. L-3,5,3'-Triiodo

thyronine (T3) was obtained from Chemical Dynamics Corporation, South Plainfield, NJ. Ethoxyresorufin was obtained from Pierce Chemical Co., Rockford,IL. 4-(2-Hydroxyethyl)-1-piperazine ethane sulfonic acid (HEPES,Ultrapure) was obtained from Mannheim-Boehringer Biochemicals, Indianapolis, IN. Fatty-acid free bovine serum albumin, malic acid, DL-α-glycerophosphate, and NADP were obtained from Sigma Chemical Co., St. Louis, MO. Methoxyflurane was obtained from Pitman-Moore, Inc., Washington Crossing, NJ. All other chemicals were reagent grade.

Animals

Male Sprague-Dawley rats weighing 150-175 g obtained from Charles River Laboratories were used in all experiments. Surgical thyroidectomy was performed on age and weight matched rats using methoxyflurane for anesthesia. Thyroidectomized rats were allowed 5-7 days recovery before further treatments. The success of thyroidectomy was assessed by measuring total plasma T4 and T3 by radioimmunoassay. All animals were housed in large (20"x16"x8") polycarbonate cages, 3 rats per cage, with hardwood bedding. Food (Wayne Lab Blox, Chicago, IL) and water were provided ad libitum, except as noted.

Treatments

All operations involving the use of TCDD were performed in isolation rooms under negative pressure. Solutions of TCDD

in corn oil were made up by mixing a concentrated solution of TCDD in benzene (1 ml) with an equal volume of corn oil. This mixture was warmed to 70° C in a water bath for 6-10 hr to remove the benzene. The remaining corn oil solution of TCDD was then diluted serially to obtain concentrations such that each rat received 1 ml corn oil/kg body weight. All rats were given a single dose of either corn oil alone, or TCDD in corn oil by gavage on day 0 of each experiment. In some experiments rats were treated with T3 in the diet by mixing powdered food (Wayne Lab Blox) with either ethanol alone (10 ml/kg diet) or appropriate concentrations of T3 in ethanol (10 ml/kg). Powdered food was weighed and presented to rats in small metal cans(100 gm capacity) which were placed in a larger metal dish to catch spilled food. Each morning, the contents of these two containers was sifted and weighed. The small can was refilled with fresh food and returned to the cage.

Buffers and Solutions

HEG buffer consisted of 25 mM HEPES, 1.5 mM EDTA, and 10% glycerol, adjusted to pH 7.4 with NaOH. HEDG buffer was made by adding dithiothreitol to HEG buffer to 1 mM immediately before use. HE (25 mM HEPES, 1.5 mM EDTA, pH 7.4) buffers containing glycerol, sucrose or dimethyl sulfoxide (DMSO) were used to prepare and store mitochondria and microsomes. Phosphate buffer, used in the α-glycerolphosphate dehydrogenase assay, contained 100 mM Na_2HPO_4, 5 mM EDTA, and 10 mM $MgCl_2$, pH 7.4. Ferricyanide

solution was 1.6 mM KCN and 1.6 mM $K_3Fe(CN)_6$. Solution A consisted of phosphate buffer plus ferricyanide solution, 5:4, with 162 mg Na_2 α-glycerolphosphate added per 10 ml immediately before use. Solution B (blank) consisted of phosphate buffer plus ferricyanide solution 5:4, without α-glycerolphosphate.

Tissue Preparation

Except where noted, rats were sacrificed by CO_2 asphyxiation and decapitation 72 hr after treatment with TCDD. Trunk blood was collected into EDTA-containing tubes (Vacutainer; Becton-Dickinson, Rutherford, NJ) on ice. Livers were perfused with ice-cold saline in situ using a blunt, plastic-tipped syringe, and then excised into cold saline on ice. Individual livers were blotted, weighed, and homogenized in a glass Potter-Elvehjem homogenizer with a Teflon pestle using 3 ml of cold HEDG buffer/g tissue for preparation of mitochondria, microsomes, and cytosol. Homogenates were centrifuged at 10,000 x g for 20 min to give a nuclear pellet, a crude mitochondrial layer, and a supernatant layer. The supernatant layer was carefully removed and centrifuged for 70 min at 105,000 x g to give "cytosol" (clear supernatant) and a crude microsomal pellet. Lipid was aspirated, and cytosol collected into polystyrene tubes which were frozen on dry ice immediately after collection. Crude microsomal pellets were resuspended in HE buffer containing 20 % glycerol, and stored at -80° C

for later processing. Crude microsomes were washed by resuspending in HEG buffer followed by centrifugation at 105,000 x g for 90 min. The resulting pellets were then resuspended in HE buffer containing 20% glycerol prior to cytochrome P-450 and ethoxyresorufin-O-deethylase (EROD) determinations. The crude mitochondrial layers from the 10,000 x g centrifugation were gently poured off the nuclear pellet into separate tubes, resuspended in 25 ml of 0.25 M sucrose in HE buffer, centrifuged at 10,000 x g for 20 min, and the supernatant discarded. The resulting mitochondrial pellets were resuspended in HE buffer containing 20% DMSO for storage at -80^{o}.

Standard Thyroid Hormone Radioimmunoassays

Aliquots of plasma or thyroid hormone standards made up in 10 mg/ml albumin were extracted by placing 400 ul of sample or standard in 1.5 ml microfuge tubes, followed by 1 ml methanol. The tubes were then centrifuged for 10 min at 8,000 rpm in a microcentrifuge, after which time 1 ml of the resulting supernatant was cooled to about -60^{o}C, and vacuum dried. In later experiments, a high pressure liquid chromatography (HPLC) separation was added between extraction and drying as described in the next section. The residue was dissolved in 200 ul of 10 mg/ml fatty acid-free bovine serum albumin. Total T4 and T3 concentrations were determined by radioimmunoassay (RIA). Samples (100 ul) were incubated with T4 or T3 tracer solutions in antibody -coated tubes (Becton-Dickinson) for

90 min, washed with water, and counted on an LKB 1271 gamma counter. Plasma concentrations were computed from the logit regression coefficients obtained from RIA of extracted T3 and T4 standards in albumin. Extraction efficiencies were determined by extracting spiked samples, and comparing extracted to unextracted standards. The efficiencies were 65 - 70% for T4, and 95-100% for T3. Stock solutions of T3 and T4 were prepared by sonicating about 10 umol of the sodium salt of each hormone in 10 ml of HPLC solvent, followed by dilution to a final volume of 100 ml with HPLC solvent. Standards of T3 and T4 in albumin were prepared from these stocks by serial dilution of stocks with a solution containing 10 mg/ml albumin and 1% NaCl and were frozen between uses. Periodically the purity of T3 and T4 stock solutions was checked by HPLC using the procedure described below.

HPLC Separation of Thyroid Hormones

Thyroid hormones were separated by HPLC using a method similar to one of the methods of Hearn et al. (62). The stationary phase used was an Econosphere C-18 reverse-phase column (Alltech/Applied Science, Deerfield, IL). The mobile phase consisted of methanol:water:glacial acetic acid, 575:452:1, with 5 ml of 5 N NaOH added for each 3 liters of solvent. A flow rate of 1.5 ml/min at 3300 psi was routinely used. Under these conditions T3 eluted at about 8 minutes, while T4 was retained for about 13 minutes.

Plasma T3 and T4 were separated as follows: methanol extracts of plasma (1 ml) were adjusted to the same water and acetate content as the HPLC solvent by adding an acetate buffer to the extract. One milliliter of adjusted extract was then loaded onto the HPLC column, and fractions collected for 20 min. Fractions containing T3, and T4, respectively, were pooled in separate scintillation vials, cooled to about -60^{o} C, and vacuum dried. The residues were then taken up in 200 ul of albumin solution and analysed as in the standard RIA procedure. During method development stages and for checking the purity of standards, a Bioanalytical Systems (W. Layfayette, IL) LC-4B amperometric detector set at 0.875 volts was used to detect thyroid hormones. The presence of electrochemically active contaminants in plasma extracts, and a maximum sensitivity of about 10 nM (10 pmol/ml) made this device unsuitable for direct measurement of plasma T3 and T4.

Malic Enzyme Determinations

ME activity was determined essentially according to Hsu and Lardy (65). In brief, 100 ul aliquots of cytosol were mixed with a 1 ml aliquot of either 20 mM Malate-Tris and 1 mM $MnCl_2$ (blank) or the same solution with 2 mM NADP (sample) added immediately before use. The difference in absorbance between the two mixtures was recorded on a Perkin-Elmer model 124 double beam spectrophotometer.

The rate of formation of NADPH was determined using an

extinction coefficient for NADPH of 6.23 X 103 O.D. units/molar-cm and specific activity expressed as nmol NADPH/min-mg protein, following determination of cytosolic protein by the method of Lowry et al. (77).

α-Glycerolphosphate Dehydrogenase Determination

Mitochondrial GPD activity was determined essentially by the method of Bottger et al. (18) which employs ferricyanide as an electron acceptor for the enzyme. Disappearance of the 420 nm absorbance of ferricyanide is proportional to the oxidation of glycerolphosphate. Frozen mitochondrial preparations were rapidly thawed and washed by adding 25 ml of 250 mM sucrose-HE buffer per 2 ml of suspension. These suspensions were centrifuged at 10,000 X g for 20 min. The resulting supernatants were poured off and the mitochondrial pellets resuspended in 2 ml sucrose-HE buffer. Activity was assayed by mixing 1 ml of solution A with 50-100 ul of the mitochondrial suspension, and recording the change in absorbance at 420 nm with 1 ml of phosphate buffer and an equal amount of mitochondrial suspension in the reference cell. Absorbance was generally recorded for 5 min, after which time a blank was run using solution B rather than solution A in the above procedure. Protein content was determined by the method of Lowry et al. (77). An extinction coefficient of 914 O.D. units/molar-cm for ferricyanide was used in activity calculations.

Cytochrome P-450 and Ethoxyresorufin-O-Deethylase

Cytochrome P-450 levels were determined by the method of Omura and Sato (93). EROD activity was determined by the method of Pohl and Fouts (102).

Data Presentation

All points represent the mean ± standard deviation of measurements from three individual animals, unless otherwise noted. Where appropriate, 95% confidence limits and statistical significance were determined using Student's distribution. Values which were significantly different from controls at the 95% confidence level are designated with asterisks (*) or are noted in the figure legends.

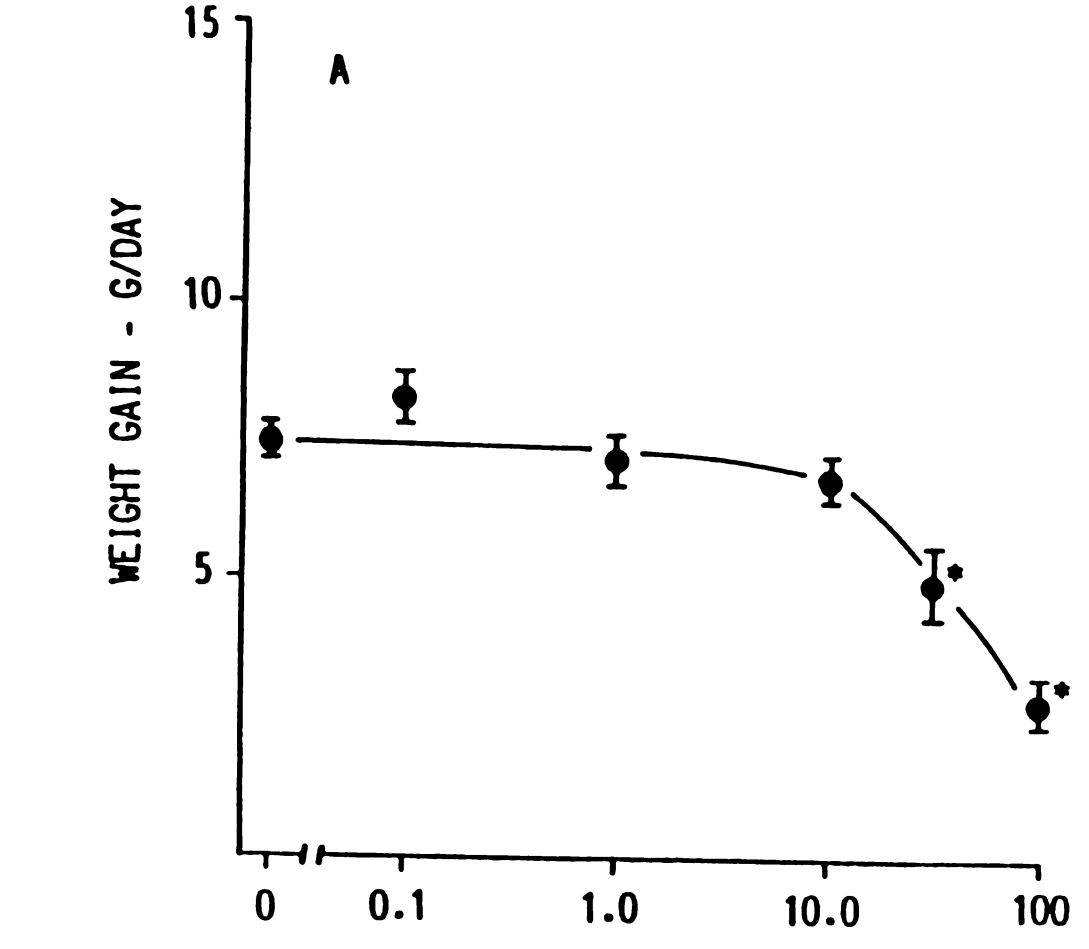
RESULTS

Figure 7 shows body weight gain and the induction of cytochrome P-450 in rat liver microsomes as a function of TCDD dose. It is important to note that almost full induction of cytochrome P-450 was achieved at a TCDD dose of 10 nmol/kg, while weight gain did not begin to decline until doses of 33 nmol TCDD/kg and above were reached. Figure 8 shows the induction of EROD activity in normal and thyroidectomized rats treated with TCDD. As reported by others (60,113), the pattern of induction of this cytochrome P-450 activity was not significantly affected by thyroid status.

Figure 7

a) Change in body weight of rats following treatment with TCDD. Each point represents the average daily weight gain of three rats as determined by linear regression analysis of of body weights between days 5 and 12 after dosing.

(b) Total liver microsomal cytochrome P-450 content 72 hr after TCDD treatment. All points corresponding to doses greater than 1 nmol/kg were significantly different from controls with $P<0.05$.


A
WEIGHT GAIN - G/DAY
15
10
5
0
0.1
1.0
10.0
100
TCDD DOSE (NMOL/KG BW)

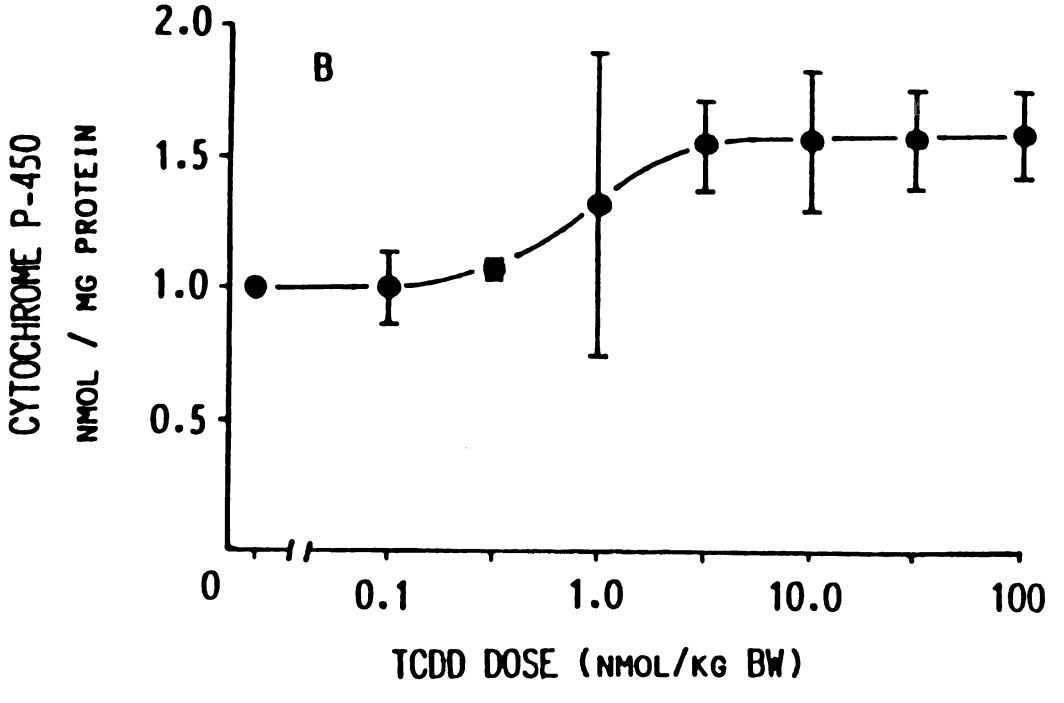
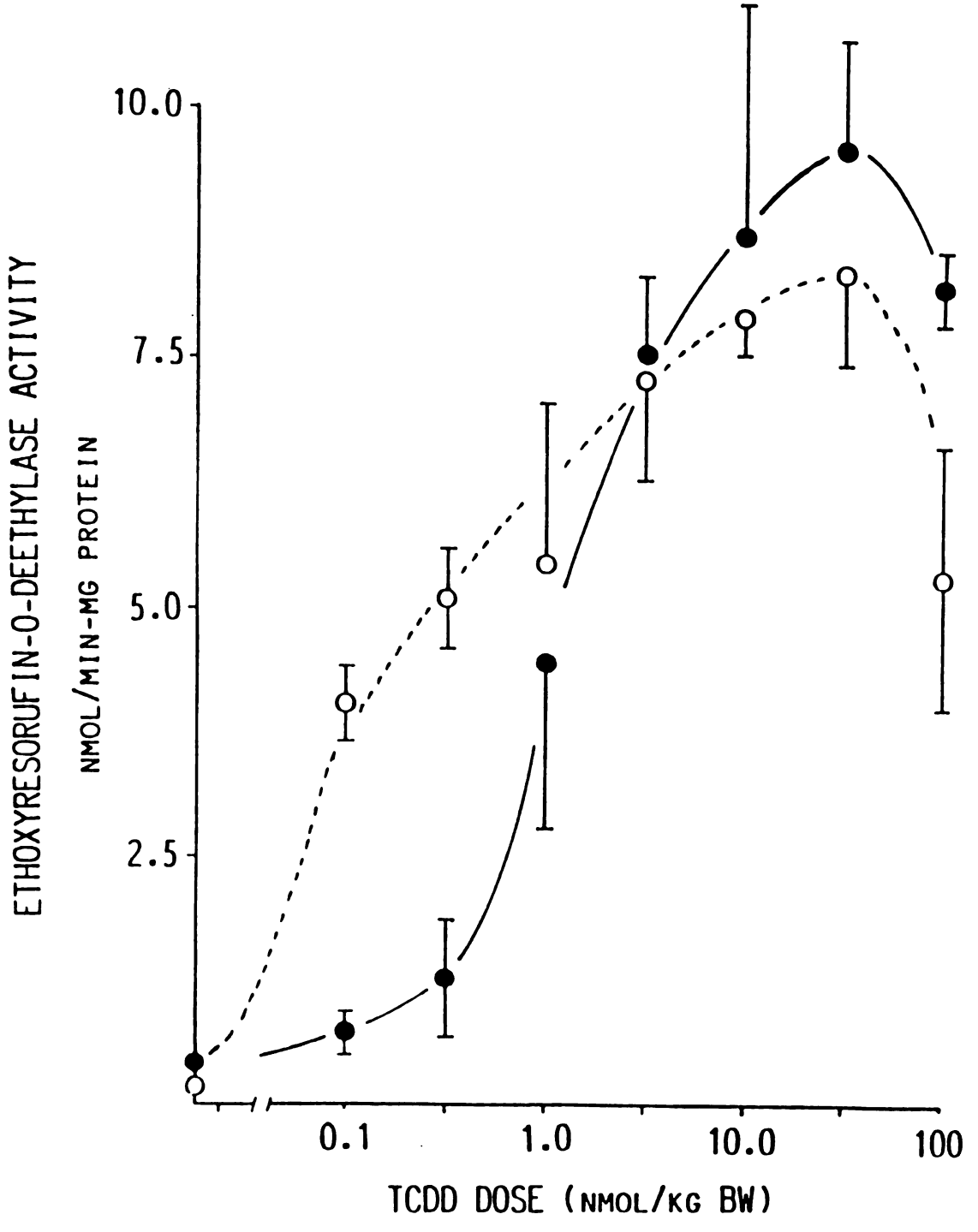

B
CYTOCHROME P-450
NMOL / MG PROTEIN
2.0
1.5
1.0
0.5
0
0.1
1.0
10.0
100
TCDD DOSE (NMOL/KG BW)

Figure 8

Ethoxyresorufin-o-deethylase activity of hepatic microsomes of normal (●—●) and thyroidectomized (○—○) rats 72 hr after TCDD treatment. All values of EROD activity greater than 2.5 nmol/min were significantly different from controls with $P<0.05$.


ETHOXYRESORUFIN-O-DEETHYLASE ACTIVITY
NMOL/MIN-MG PROTEIN
10.0
7.5
5.0
2.5
0.1
1.0
10.0
100
TCDD DOSE (NMOL/KG BW)

Thyroid hormone levels were initially measured by a standard RIA technique, but the accuracy of this method for total T3 was found to be unreliable because of antibody cross-reactivity from T4 (figure 9a). For this reason, extracts of plasma were subjected to HPLC prior to RIA to separate T3 from T4. This procedure did not change T4 measurements significantly (figure 9b), but changed T3 measurements dramatically (figure 9c). Total plasma T4 decreased following TCDD treatment in a dose-dependent fashion, with noticable changes occuring even at doses where no grossly observable symptoms of TCDD exposure were seen, ie. 1 nmol TCDD/kg body wt. (figure 9b). Almost half of the T3 measured by the standard RIA actually reflects cross-reactivity from T4, and the decrease noted using the standard assay was almost entirely due to loss of T4.

Despite drastically decreased plasma T4 levels, treatment of rats with TCDD was found to be correlated with increased activity of cytosolic ME activity in liver (figure 10). In general, ME activity followed the same trend as microsomal cytochrome P-450 and EROD activities, until the onset of anorexia at TCDD doses of 33 nmol/kg and above. However, induction of ME activity did not occur in THX rats at any of the TCDD doses tested. Starvation, hypoglycemia, and glucagon treatment have all been correlated with supression of ME synthesis(49,114,135), and may be responsible for the decline in ME activity observed at high TCDD doses. The activity of mitochondrial GPD was also measured in these

Figure 9

a) Cross-reactivity of T4 with the T3 antibody used for RIA. Concentrations are of T4 (■—■) and T3 (●—●) standards added to the T3 RIA tubes as described in methods. Lines drawn through the data points were determined from the expression

$$\text{Logit}(B/Bo) = \ln(B/(Bo-B)) = m*\ln[\text{ligand}] + b$$

where B = counts per min in sample, Bo= CPM in blank

m = slope of line, b = intercept.

b) Total extractable plasma T4 in rats 72 hr after treatment with TCDD. Standard RIA (●—●), HPLC-RIA (○—○) (see Methods). All points corresponding to doses greater than 1 nmol/kg were significantly different from controls with $P<0.05$.

c) Total extractable plasma T3 72 hr after treatment with TCDD, as measured by RIA. Standard RIA (●—●), HPLC-RIA (○—○) (see Methods).

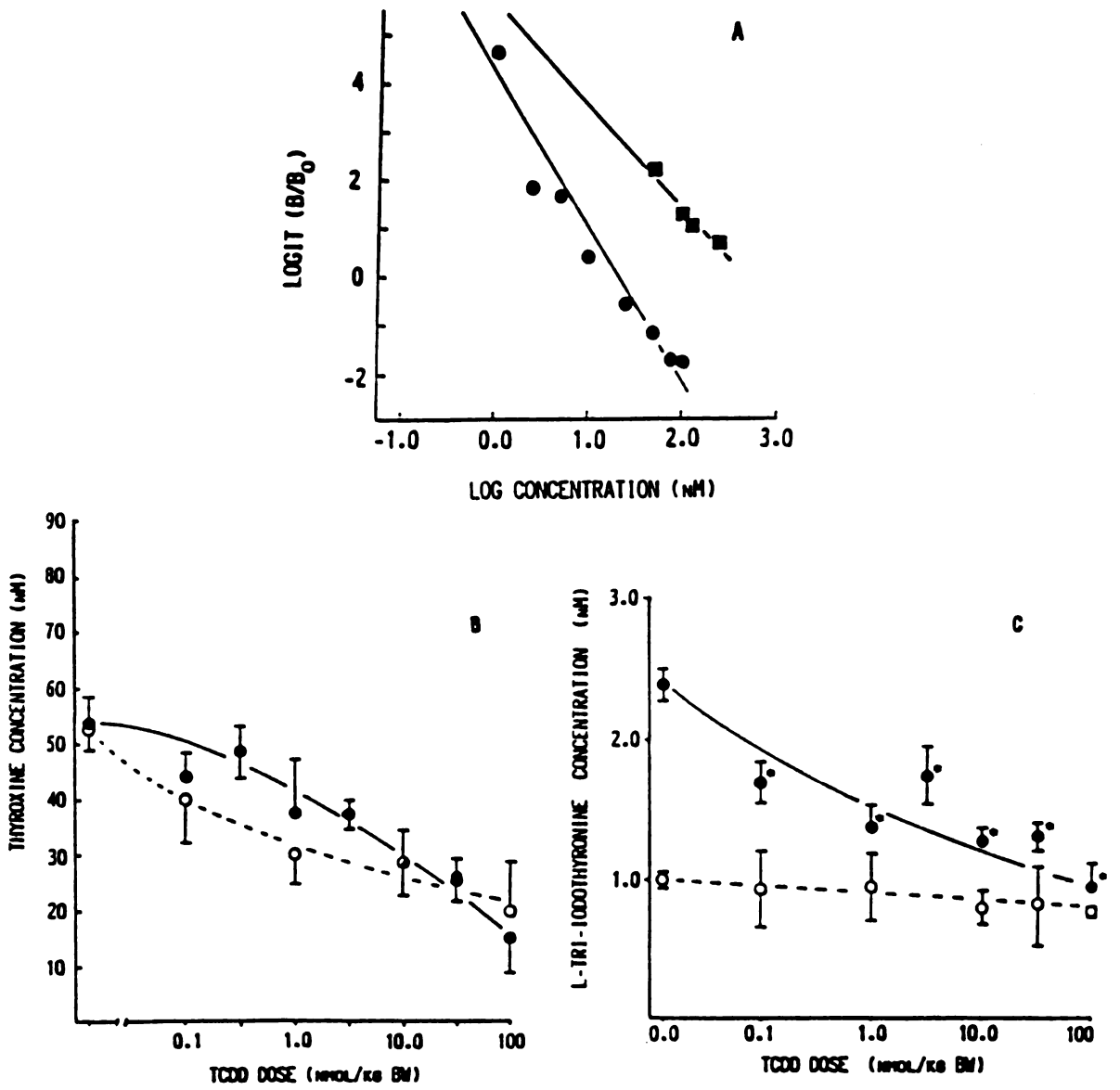

A
LOGIT (B/B0)
4
2
0
-2
-1.0
0.0
1.0
2.0
3.0
LOG CONCENTRATION (nM)
B
THYROXINE CONCENTRATION (nM)
90
80
70
60
50
40
30
20
10
0.1
1.0
10.0
100
TCDD DOSE (nmol/kg BW)
C
L-TRI-IODOTHYRONINE CONCENTRATION (nM)
3.0
2.0
1.0
0.0
0.1
1.0
10.0
100
TCDD DOSE (nmol/kg BW)

Figure 10

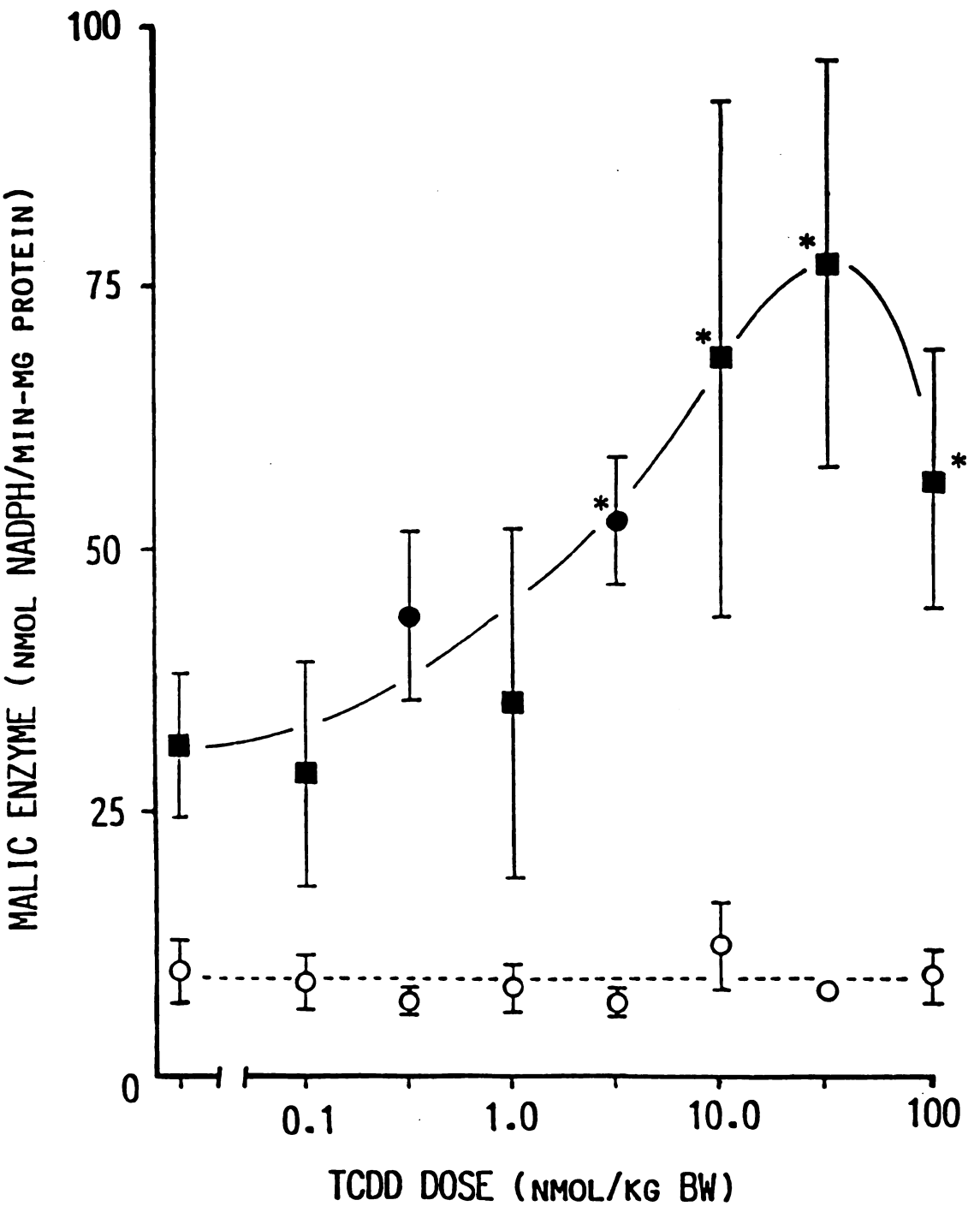
Malic enzyme activity in liver cytosol 72 hr after treatment with TCDD. For thyroidectomized rats (O—O), each point represents the average ± standard deviation (S.D.) for 3 animals. For normal rats, each data point represents either the average ± S.D. for 3 rats (●—●) or the pooled average (**u**) ± Sp for two separate experiments (■—■). Data were pooled using the formulas:

$$\mathbf{u} = (u_1 + u_2)/2 \qquad Sp = \frac{(n_1-1)S_1 + (n_2-1)S_2}{n_1 + n_2 - 2}$$

where u_i = mean of sample i,

n_i = number of values in sample i,

S_i = sample standard deviation.


100
75
50
25
0
MALIC ENZYME (NMOL NADPH/MIN-MG PROTEIN)
0.1
1.0
10.0
100
TCDD DOSE (NMOL/KG BW)
*
*
*
*

experiments. While this activity changed in a non-random fashion (figure 11), the changes observed were not statisti cally significant, nor were they characteristic of those produced by increased levels of T3 (18,114). There was a tendency for GPD activity to increase with TCDD dose in THX animals, however, only one dosage point had a value significantly different from controls (100 nmol TCDD/kg). The small sample (3 rats) and relatively small variation at this point make it possible that this is a statistical fluctuation rather than a significant change.

To investigate the thyroid hormone dependence of ME induction further, the dose response relationship for liver ME induction in THX rats as a function of dietary T3 was determined. THX rats treated with graded doses of T3 in the diet were compared with THX rats which received the same amounts of T3, but which were also treated with TCDD. Animals given 10 nmol TCDD/kg decreased their food consumption slightly, but the change was not significantly different from T3-fed controls (figures 12a & b). Rats receiving 33 nmol TCDD/kg decreased their food consumption by 40-50% (figures 12c & d) relative to T3-fed controls. No significant difference in ME activity was found between animals given T3 alone and those given T3 + TCDD when 10 nmol TCDD/kg was used (figure 13). Similar results were found with a dose of 33 nmol/Kg, but were difficult to interpret because hypophagia, and thus differences in the T3 dosages, were occuring at this dose of TCDD.

Figure 11

Liver mitochondrial α-glycerolphosphate dehydrogenase activity of normal (●—●) and thyroidectomized (○—○) rats 72 hr after treatment with TCDD.

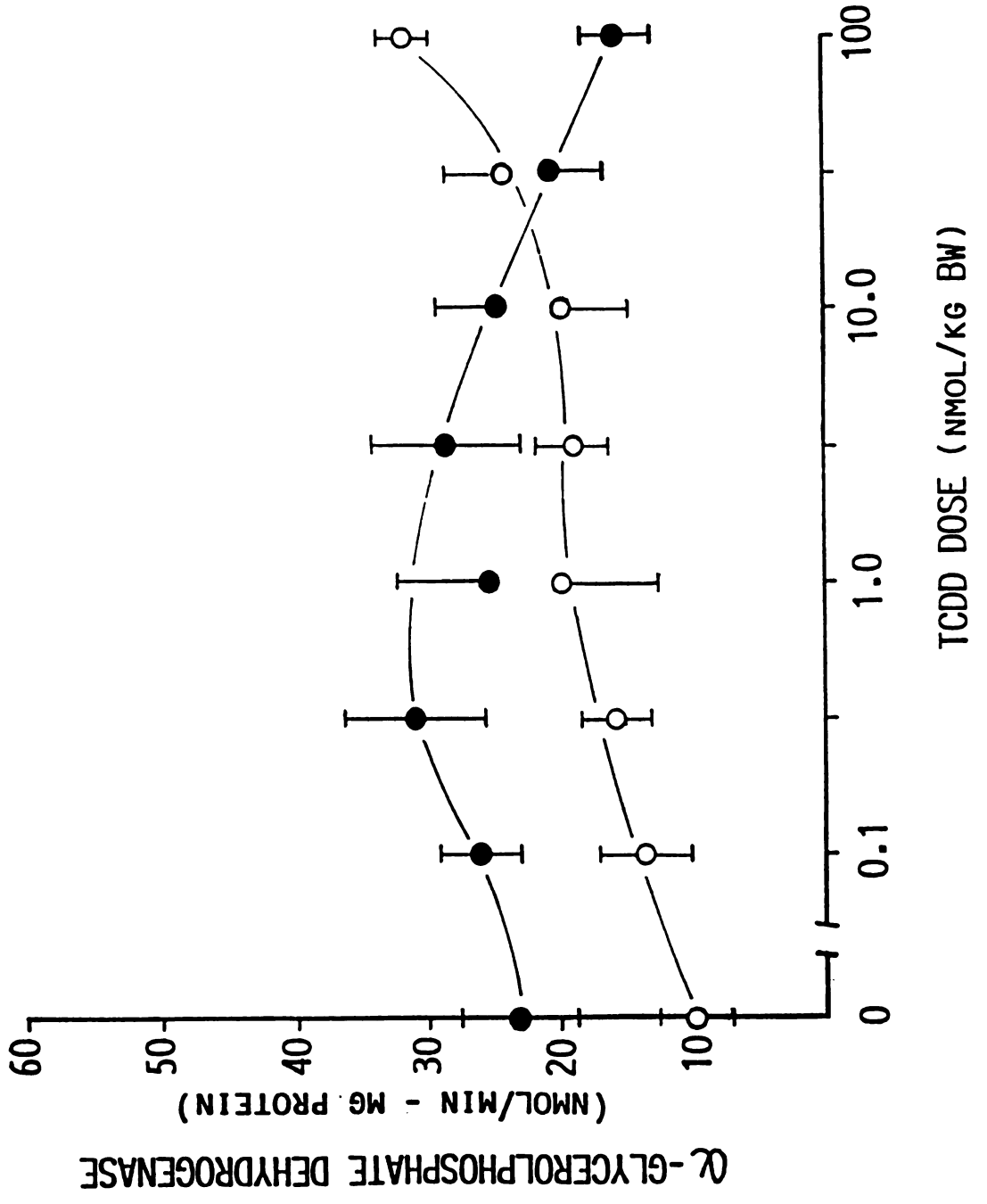

α-GLYCEROLPHOSPHATE DEHYDROGENASE
(NMOL/MIN - MG PROTEIN)
60
50
40
30
20
10
0
0.1
1.0
10.0
100
TCDD DOSE (NMOL/KG BW)

Figure 12

a & b) Food consumption of thyroidectomized rats given 0 (○—○), 50 (●—●), 100 (□—□), or 250 (■—■) ug T3 per kg-diet. Control animals (a) were treated with corn oil alone by gavage on day 0. TCDD-treated animals (b), were given an equal volume of corn oil containing TCDD. The final dose of TCDD was 10 nmol/kg-body wt. T3 diet was actually presented at day -3 (data not shown).

c & d) Food consumption of thyroidectomized rats given 0 (○—○), 25 (●—●), 50 (□—□) or 100 (■—■) ug T3 per kg diet. Control animals (c) were treated with corn oil alone by gavage on day 0. TCDD-treated animals (d) were given an equal volume of corn oil containing TCDD. The final dose of TCDD was 33 nmol/kg-body wt.

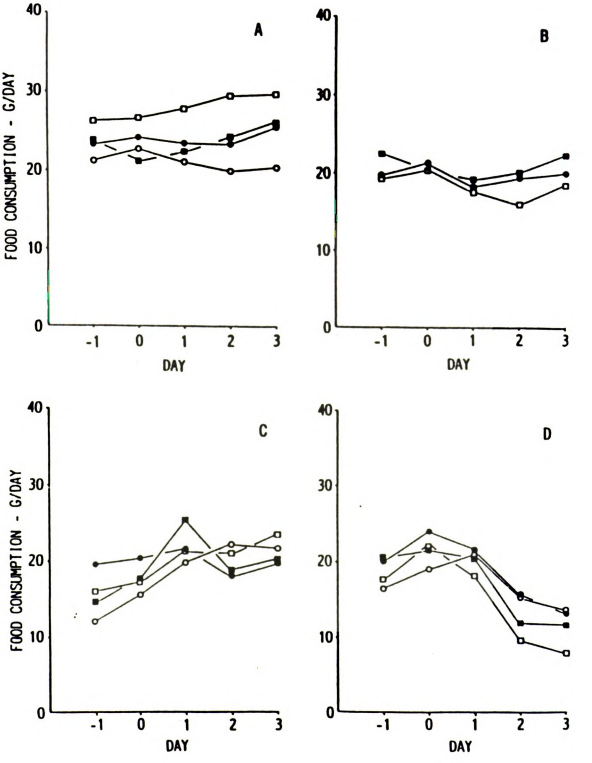
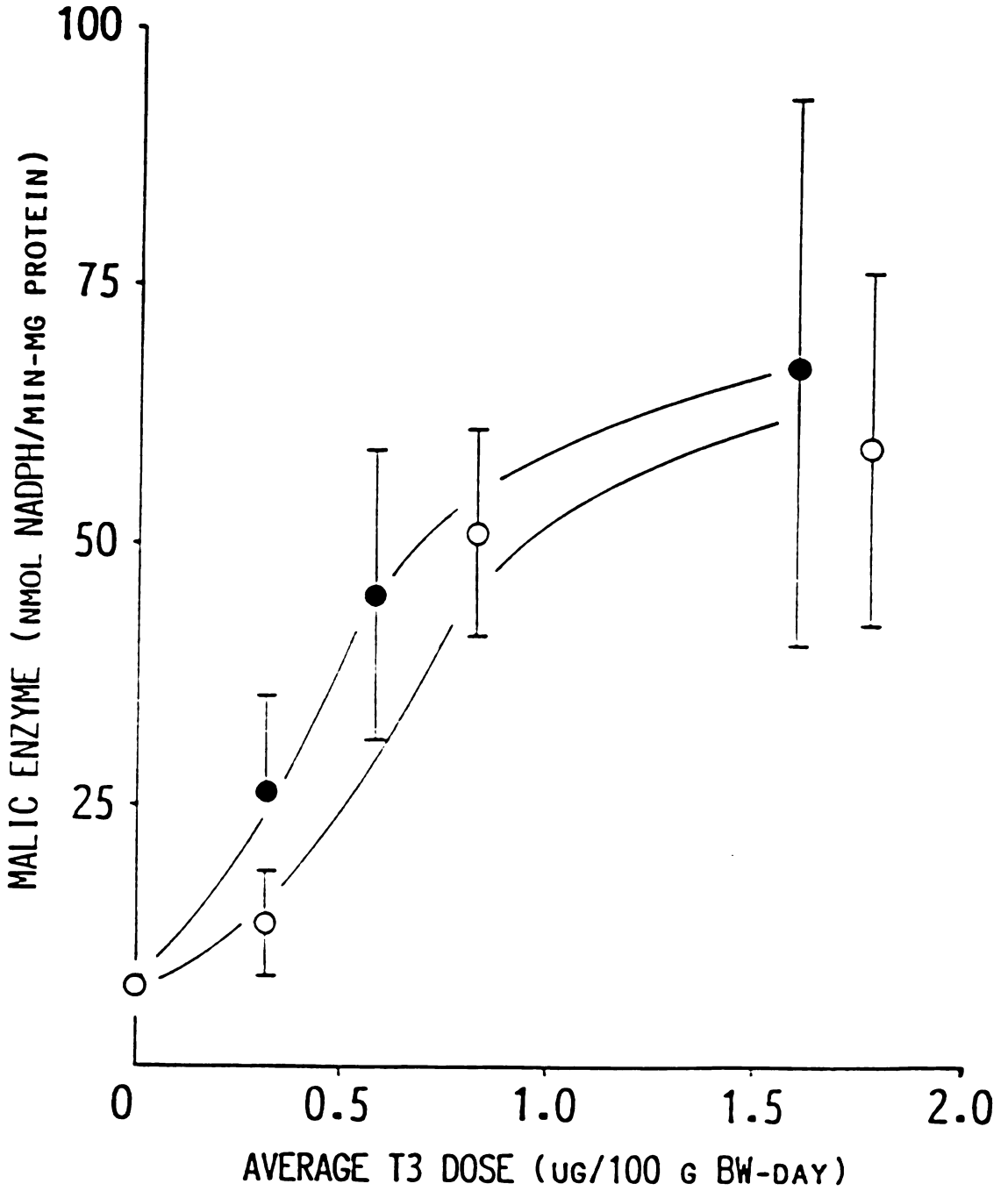

A
B
C
D
FOOD CONSUMPTION - G/DAY
DAY
0
10
20
30
40
-1
0
1
2
3

Figure 13

Malic enzyme activity in liver cytosol of thyroidectomized rats treated with dietary T3 alone (O—O) or dietary T3 + 10 nmol-TCDD/kg-body wt (●—●), as described in figure 6a. All animals were sacrificed on day 3 (72 hours).


100
75
50
25
0
0.5
1.0
1.5
2.0
MALIC ENZYME (NMOL NADPH/MIN-MG PROTEIN)
AVERAGE T3 DOSE (UG/100 G BW-DAY)

It is important to note that the steady-state plasma T3 level achieved in THX rats treated with TCDD was lower than those of rats fed diets containing the same amounts of T3 (figure 14). This was not the result of decreased food consumption since plasma T3 concentration plotted as a function of actual T3 dose/100 g body weight (food consumed X T3 concentration in food divided by body weight) gave different slopes for the two treatments.

DISCUSSION

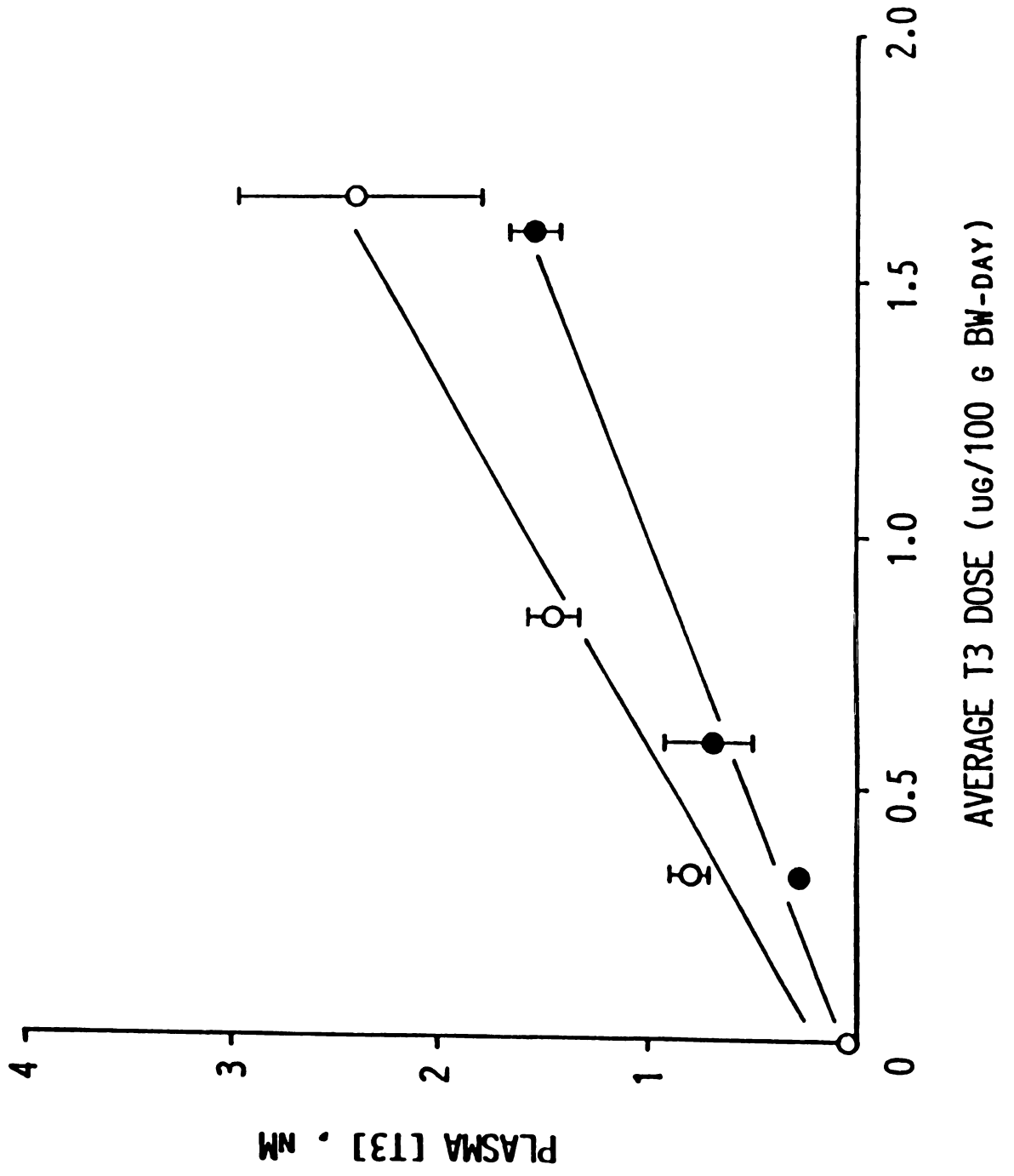
The thyroid status of TCDD treated animals and possible thyromimetic effects of TCDD and chlorinated biphenyls have been subjects of controversy in recent literature. Several investigators have proposed that animals become hyperthyroid following TCDD treatment, with some suggesting that TCDD and chlorinated biphenyls are thyromimetic, ie. T3 agonists (107). Others, observing the same changes we have seen in the thyroid status indicators ME and GPD (70) and anomalously high levels of T3 (105), insist that changes in these indicators are not a function of the thyroid status of treated animals but are rather the direct results of an effect of TCDD on a hypothalamic "body weight set-point". Our results indicate that neither of these interpretations are adequate to explain the metabolic changes observed after TCDD exposure.

The studies of Rozman et al. (112) suggest that toxicity is aggravated by the presence of thyroxine, which is already

Figure 14

Total extractable plasma T3 in thyroidectomized rats treated with dietary T3 alone (O—O) or dietary T3 plus 10 nmol TCDD/kg-body wt. (●—●). The change in plasma concentrations of T3 were linear functions of dose, with regression coefficients:

	T3 only	T3 + TCDD
Slope nM(day-100g/ug)	1.27 ± 0.17	0.89 ± 0.12
Intercept	0.21 ± 0.18 nM	0.02 ± 0.14 nM
	r^2 = 0.86	r^2 = 0.88


PLASMA [T3] , NM
4
3
2
1
0
0.5
1.0
1.5
2.0
AVERAGE T3 DOSE (UG/100 G BW-DAY)

greatly reduced in animals treated with TCDD.

We have extended the studies of Rozman et al. by showing that at least in the liver (a primary control point of fat metabolism and Ah-inducer action) changes in ME activity (which supplies NADPH for fatty acid synthesis) were dependent on T3 levels, and did not increase as the result of any intrinsic thyromimetic action of TCDD. However, this data does suggest that induction and suppression of malic enzyme may be explained by a thyroid-dependent, multi-hormonal mechanism. In the induction of thyroid-controlled proteins (malic enzyme, phosphoenolpyruvate carboxykinase, α2-macroglobulin, growth hormone) thyroid hormones often play a permissive role, ie. thyroid hormones enable a basal level of protein synthesis, while other hormones such as insulin, glucagon, and glucocorticoids are responsible for short-term regulation, but are inactive in the absence of T3 (86,92,118,123,135). While our studies show that factors other than the disturbance of thyroid status are involved in TCDD toxicity, it is also clear that separation of thyroid status from other factors is essential to understanding the total picture. Several investigators have shown decreased serum thyroxine levels in animals treated with Ah-inducers, including benzo[a]pyrene (48), methylcholanthrene (89), hexachlorobiphenyl, and TCDD (8,104,105). This decrease of T4 in serum has been shown to be associated with the induction of a hepatic glucuronyl transferase which can conjugate thyroxine (8,108). Plasma

T3 levels have previously been reported to be decreased (113), unchanged (104), or elevated (103) after treatment with TCDD. Our results show that plasma T3 levels do not change significantly, and show that some of the conflicting reports in the literature can be explained by cross-reactivity from T4. The failure of T3 to change when T4 levels are declining is a paradoxical situation if normal deiodination mechanisms are operative (25,28), i.e. T3 concentrations should be decreasing with those of T4. In experiments where we attempted to control T3 levels in THX rats, steady-state T3 concentrations in plasma suggested that T3 catabolism was increased in TCDD-treated animals. This finding further compounds the paradox of finding normal T3 levels in the presence of declining T4 .

It is important to note that endogenous T3 is produced from T4, primarily in the tissues where it acts (40,131); thus the concentrations of T3 in these tissues (as opposed to plasma concentrations) is of primary importance in under standing the induction of thyroid-controlled proteins such as ME. Recent studies in normal rats by Van Doorn et al. (131) have shown that T4 concentrations in liver are less than half that in plasma, while T3 levels were 5 - 10 X those found in plasma. Preliminary studies in our laboratory (data not shown) are largely in agreement with these results.

While measurement of plasma thyroid hormones has helped in understanding the influence of thyroid hormones on TCDD

toxicity, measurement of thyroid indices in tissue gives a different picture, suggesting that thyroid hormone concentrations and thyroid hormone metabolism must be measured in individual tissues to develop a complete understanding of the relationship between symptoms of TCDD exposure, effects of TCDD on thyroid status, and direct effects of TCDD which may combine with these changes in thyroid status to produce the observed symptoms.

CHAPTER III

INTERACTIONS BETWEEN LIVER THYROID HORMONES, TCDD AND THE GLUCOCORTICOID RECEPTOR IN THE INDUCTION OF MALIC ENZYME ACTIVITY.

ABSTRACT

Hepatic malic enzyme can be induced in rats in a dose-dependent fashion by exposure to TCDD. Although this process requires thyroid hormones, measurements of hepatic 3,5,3'-triiodothyronine (T3) levels indicated that malic enzyme induction does not result from local changes in T3 concentration. Similarities between this process and the induction of malic enzyme by glucococorticoids suggested that TCDD might be act by a corticomimetic mechanism. Competitive binding assays of TCDD with the synthetic glucocorticoid [6,7-^{3}H]-triamcinolone indicated that TCDD was not an effective ligand for the glucocorticoid receptor at toxicologically significant doses. Although it is possible that TCDD can enhance the synthesis of malic enzyme in the rat via a mechanism involving the Ah-receptor, no malic enzyme induction was observed when Ah-receptor sufficient (C57BL/6N) or deficient (DBA/2N) mice were treated with TCDD. We conclude that induction of malic enzyme by TCDD in the rat does not directly involve the glucocorticoid receptor, nor does it result from changes in hepatic T3 levels.

INTRODUCTION

TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin) is the most potent of a class of polycyclic aromatic hydrocarbons which cause the induction of cytochromes P-450c, P-450d (53) at least one glucuronyl transferase (78), glutathione-S-transferase (5), and other enzymes which have not been as well characterized. The induction of cytochrome P-450c has been shown to result from the binding of TCDD to an intracellular receptor, called the Ah-receptor, for which a specific enhancer region upstream of the cytochrome p-450c gene has been identified (67). Although the induction of cytochrome P-450s by TCDD and many other Ah-inducers is well understood, many symptoms of Ah-inducer exposure do not correlate well with cytochrome P-450 induction, or cannot be easily explained as secondary effects of the induction of Ah-locus enzymes. In particular, dosages of TCDD which cause acute toxicity do not correlate well with occupancy of the Ah receptor, as measured by cytochrome P-450 induction. The guinea pig begins to show morbidity at doses of TCDD of less than 1 nmol/kg body weight, which is approximately the ED50 for cytochrome P-450 induction (103). The Sprague Dawley rat shows no gross morbidity until doses of approximately 20 nmol/kg - a dose of TCDD at which cytochrome P-450 is maximally induced. Little mortality occurs in the Sprague Dawley rat until a dose of 100 nmol/kg is reached. Other strains of rats and mice likewise show no signs of acute toxicity at doses of TCDD

which are in excess of those required for cytochrome P-450 induction. Certain symptoms of Ah-inducer toxicity, such as thymic involution, hyperlipidemia, and fatty liver development, can also be produced by glucocorticoid administration, and might be explained if TCDD had corticomimetic properties.

We recently discovered that hepatic malic enzyme, which is normally regulated by thyroid hormones (114), insulin, glucagon, glucocorticoids (49,50), and dietary carbohydrate (80), was induced on exposure to either TCDD or 3,4,5,3′,4′,5′-hexachlorobiphenyl (HCB) (111). We have also shown that this induction is thyroid hormone dependent, ie. that it does not occur in thyroidectomized rats. In the experiments reported here, we have attempted to determine whether local tissue levels of T3 change enough to account for this induction, and if not, whether it can be explained by a corticomimetic property of TCDD.

MATERIALS AND METHODS

Reagents

[6,7-^{3}H]-Triamcinolone acetonide (^{3}H-TA), (43.6 Ci/mmol) was purchased from New England Nuclear, Boston, Mass. 2,3,7,8-Tetrachlorodibenzo-p-dioxin(TCDD) was a gift from Dr. Fumio Matsumura, Pesticide Research Center, Michigan State University. 3,4,5,3′,4′,5′-Hexachlorobiphenyl(HCB) was prepared by Pathfinder Laboratories Inc., St. Louis, MO. L-3,5,3′-Triiodothyronine (T3) was obtained from Chemical Dynamics Co., South Plainfield, N.J.

4-(2-Hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) was purchased from Boehringer Mannheim Biochemicals, Indianapolis,IN. Bovine serum albumin, dextran, DL-α-glycerolphosphate, NADP, L(-)-malic acid, hydrocortisone, dexamethasone, triamcinolone and L-thyroxine were all obtained from Sigma Chemical Co., St. Louis, Mo. All other chemicals were reagent grade.

Animals

Male, 275-300 g Sprague-Dawley rats, male, 25-30 g C57BL/6N and 25-30 g DBA/2N mice were obtained from Charles River Labs. Adrenalectomized male Sprague-Dawley rats were obtained from the same supplier. Rats were housed in large (20"x16"x8") polycarbonate cages with hardwood bedding, and were provided with food (Wayne Rodent Blox, Wayne Feeds, Chicago,IL) and water ad libitum, except as noted . Adrenalectomized rats were provided with normal saline in place of water to prevent sodium depletion.

Treatments

All operations involving the use of TCDD were performed in isolation as described in chapter 2. TCDD and HCB were given to animals by gavage in corn oil (1 ml/kg). In some experiments rats were treated with T3 in the diet by mixing powdered food with either ethanol alone (10 ml/kg diet) or appropriate concentrations of T3 in ethanol (10 ml/kg diet), followed by mixing for 10-15 min to ensure homogeneity and evaporation of ethanol.

Buffers and Solutions

HEG buffer consisted of 25 mM HEPES, 1.5 mM EDTA, and 10% glycerol, adjusted to pH 7.4 with NaOH. HEDG buffer was made 1 mM in dithiothreitol(DTT) by adding DTT to HEG buffer immediately before use. HE (25mM HEPES, 1.5mM EDTA, pH 7.4) buffers containing glycerol, sucrose, or dimethyl sulfoxide (DMSO) were used to prepare and store mitochondria and microsomes, as described under Tissue Preparation. Phosphate buffer, used in the α-glycerol-phosphate dehydrogenase assay, contained 100mM Na_2HPO_4, 5 mM EDTA, and 10 mM $MgCl_2$, pH 7.4 . Ferricyanide solution was 1.6 mM KCN and 1.6 mM $K_3Fe(CN)_6$. Solution A consisted of phosphate buffer plus ferricyanide solution, 5:4, with 162 mg Na_2 α-glycerolphosphate added per 10 ml immediately before use. Solution B (blank) consisted of phosphate buffer plus ferricyanide solution 5:4, without α-glycerolphosphate.

Tissue Preparation

All animals were sacrificed by CO_2 asphixiation and decapitation 72 hr after treatment with TCDD. Trunk blood was collected into EDTA-containing tubes (Vacutainer; Becton-Dickinson, Rutherford, NJ) on ice. Livers were perfused with ice-cold saline in situ using a blunt, plastic-tipped syringe, and then were excized into cold saline on ice. Individual livers were blotted, weighed and homogenized in a glass Potter-Elvehjem homogenizer with a

Teflon pestle using 3 ml of cold HEDG buffer/g tissue for preparation of mitochondria, microsomes and cytosol. For preparation of "stabilized" glucocorticoid receptor, the same procedure was used except that 20 mM molybdate was added, and 1 mM DTT deleted from the buffer.

Homogenates were centrifuged at 10,000 x g for 20 min to give a nuclear pellet, a crude mitochondrial layer, and a supernatant layer. The supernatant layer was carefully removed and centrifuged for 70 min at 105,000 x g to give "cytosol" (clear supernatant) and a crude microsomal pellet. Lipid was aspirated, and cytosol collected into polystyrene tubes which were frozen on dry ice immediately after collection. Crude microsomal pellets were resuspended in HE buffer containing 20 % glycerol, and stored at -80^{o} C for later processing. Crude microsomes were washed by resuspend ing in HEG buffer followed by centrifugation at 105,000 x g for 90 min., prior to ethoxyresorufin-O-deethylase (EROD) determinations. The crude mitochondrial layers from the 10,000 x g centrifugation were gently poured off the nuclear pellet into separate tubes, resuspended in 25 ml of 0.25 M sucrose in HE buffer, centrifuged at 10,000 x g for 20 min, and the supernatant discarded. The resulting mitochondrial pellets were resuspended in HE buffer containing 20 % DMSO for storage at -80^{o} c.

Thyroid Hormone Assays

Aliquots of plasma or thyroid hormone standards made up in 10 mg/ml albumin were extracted by placing 400 ul of sample or standard in 1.5 ml microfuge tubes, followed by 1 ml methanol. The tubes were then centrifuged for 5 min at 8,000 rpm in a microcentrifuge. Following centrifugation, 1 ml of supernatant was mixed with 280 ul buffer (0.03 M Na^+ acetate + 0.03 M acetic acid) to adjust the water and acetate content of the extract to match the HPLC solvent described in the next section.

Tissue thyroid hormones were extracted by homogenizing 1 to 5 grams of tissue with 5 volumes of ice-cold methanol in a Teflon-pestle homogenizer immediately after excision. Five milliliter aliquots of the methanol extracts were placed in 20 ml glass scintillation vials, cooled to -60° C, and vacuum dried. The residue was resuspended in 1.5 ml of HPLC solvent, centrifuged, and chromatographed as described in the next section.

Following HPLC separation, the T3 and T4 containing residues were dissolved in 200 ul of a 10 mg/ml fatty acid-free bovine serum albumin solution. Total T4 and T3 were then determined by radioimmunoassay(RIA). Samples (100 ul) were incubated with T4 or T3 tracer solutions in antibody-coated tubes (Becton-Dickinson) for 90 min, washed with water, and counted on an LKB 1271 gamma counter. Sample concentrations were computed from the logit regression coefficients obtained from RIA of extracted T3 and T4

standards in albumin.

HPLC Separation of Thyroid Hormones

Thyroid hormone-containing samples were separated by HPLC using an isocratic method of Hearn et al. (62).

The stationary phase used was an Econosphere C-18 reverse phase column (Alltech /Applied Science, Deerfield, IL). The mobile phase consisted of methanol:water:glacial acetic acid, 575:452:1, with 5 ml of 5 N NaOH added for each 3 liters of solvent. A flow rate of 1.5 ml/min at 3300 psi was routinely used. Under these conditions T3 eluted at about 8 minutes while T4 was retained for about 13 minutes. One milliliter of sample was loaded onto the column, and fractions collected for 20 min. Fractions containing T3 and T4, respectively, were pooled in separate scintillation vials, cooled to about -60° C, and vacuum dried. The residues were taken up in 200 ul of albumin solution and analysed as described in the RIA procedure above.

Glucocorticoid Receptor Assay

Unlabeled triamcinolone, hydrocortisone, and TCDD were dissolved and diluted in p-dioxane to yield solutions which were approximately 100 x the concentrations desired in the incubation mixtures. [6,7-^{3}H]-Triamcinolone acetonide (^{3}H-TA) tracer was made up as a 1 uM solution in p-dioxane so that 10 ul of this solution, when mixed with 1 ml of cytosol, gave a final ^{3}H-TA concentration of about 10 nM. Ligand tubes were made up by adding 10 ul of either the

desired cold ligand or 10 ul p-dioxane, followed by 10 ul of ^{3}H-TA.

All incubations were started by adding 1 ml of cytosol to each ligand tube and mixing before placing on ice or in water in a metabolic shaking incubator. Molybdate-stabilized cytosol preparations were incubated for two hours on ice. Dithiothreitol-containing cytosols were incubated for 30 min at 37^{o}C followed by 10 min on ice before stripping with charcoal (139). During incubation, charcoal tubes were prepared by pipeting 2 ml of a suspension of charcoal-dextran (10 mg charcoal, 1 mg dextran /ml) into 12 x 75 mm tubes, followed by centrifugation at 3500 rpm, and aspiration of the supernatant. Incubation was stopped by pipeting each incubation mixture into a charcoal tube, mixing, and placing on ice for 5 min. These tubes were then centrifuged, and the clear supernatant collected for analysis on sucrose density gradients.

Sucrose Density Gradient Centrifugation

Two different types of sucrose density gradients were used for the analysis of "stabilized", and "activated" glucocorticoid receptor, respectively. Linear 10-30 % sucrose gradients were made in 4.4 ml polyallomer tubes (Beckman Instruments) by mixing 1.7 ml of 10 % sucrose and 1.7 ml 30 % sucrose solutions in a gradient mixer. For analysis of the stabilized receptor, 20 nM molybdate was incorporated into both 10 and 30 % sucrose, made up in HEG buffer.

Analysis of the activated receptor was performed with sucrose solutions containing 300 mM KCl plus 1 mM DTT. Sucrose gradients were made up on the day of incubation, and kept at 4° C until use. A 300 ul aliquot of the incubation mixture was layered onto the sucrose gradient using a 500 ul Teflon-plunger Hamilton syringe.

The resulting gradients were spun for 16 hr at 48,000 rpm in a Beckman SW60Ti rotor at 4° C. Centrifuged gradient tubes were punctured at the bottom and drained into approximately 50 fractions. Scintillation fluid was then added to each fraction and the fractions counted for 1 min each.

Malic Enzyme and α-Glycerolphosphate Dehydrogenase Assays

These assays were performed as previously described in chapter 2, using modifications of the methods of Hsu and Lardy (65) and Bottger et al. (18), respectively.

Ethoxyresorufin-O-Deethylase(EROD) Assay

EROD activity (a specific assay for cytochrome P-450c) was determined by the method of Pohl and Fouts (102).

RESULTS

In experiments described in chapter 1 we demonstrated that liver cytosolic malic enzyme activity is induced by TCDD in normal rats, but not in thyroidectomized rats . We have obtained similar results with 3,4,5,3′,4′,5′-hexachlorobiphenyl, another potent Ah-inducer (data not shown). However, we could not be certain from these studies that

local concentrations of T3 in the liver (as opposed to plasma) were unchanged, and thus whether changes in T3, or a direct interaction with TCDD was responsible for this induction.

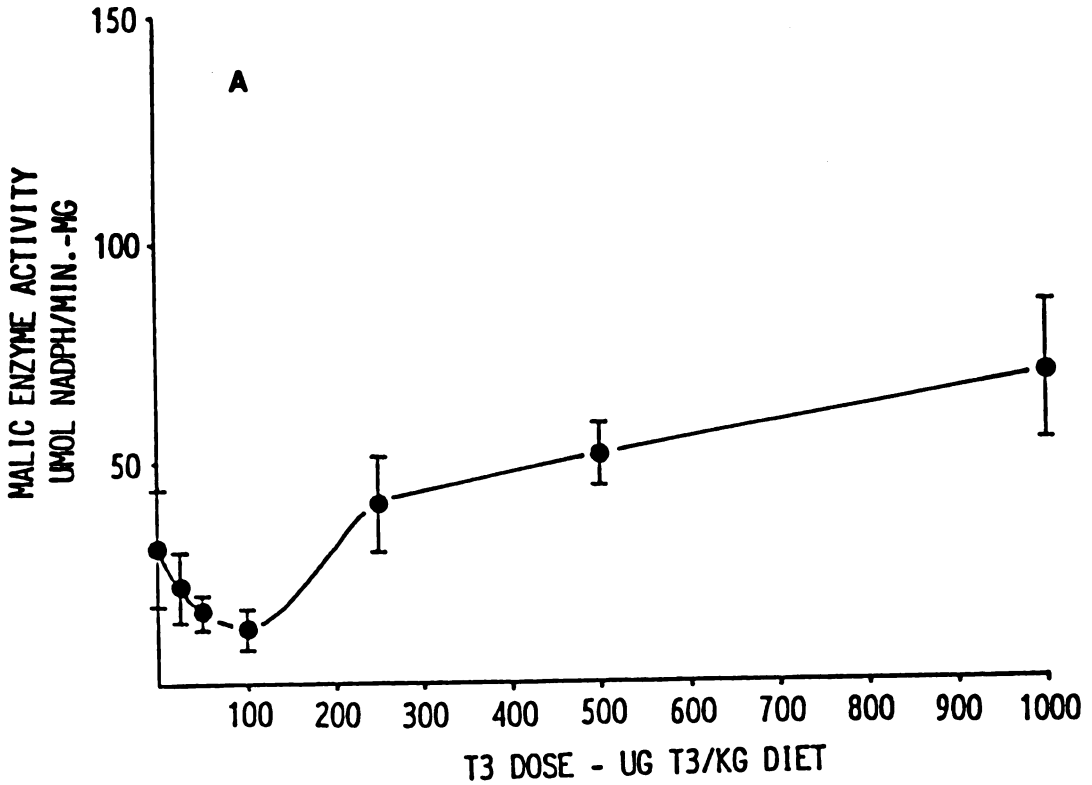
In this study we have clarified the situation with respect to tissue T3 levels. Figures 15 and 16 show that when rats are dosed with low levels of T3, hepatic malic enzyme and mitochondrial glycerolphosphate dehydrogenase follow tissue T3 concentrations, rather than those of plasma, and indicate that plasma and liver T3 concentrations do not necessarily change in parallel.

When the livers of TCDD treated rats were extracted and assayed for thyroid hormones, T3 remained unchanged relative to controls, while T4 levels dropped as in plasma (figure 17). Hepatic T3 concentrations were found to be about 4 x those in plasma, while liver T4 concentrations are about 1/2 that of plasma.

Since thyroid hormone levels and the pattern of enzyme induction indicated that the induction did not result from changes in thyroid hormone levels, but did resemble the thyroid hormone dependent induction of malic enzyme by glucocorticoids (135), we measured the ability of TCDD to act as a ligand for the glucocorticoid receptor. Figure 18 shows the sucrose density gradient profile of ^{3}H-TA binding to the molybdate-stabilized glucocorticoid receptor in the presence of excess hydrocortisone or triamcinolone. Triamcinolone is very tightly bound by this receptor, an

Figure 15

a) Hepatic malic enzyme activity after feeding T3 in the diet for 72 hours. (b) mitochondrial α-glycerolphosphate dehydrogenase activity following the same treatment as in (a). Points shown are the average ± S.D. of samples from 4 animals.


A
MALIC ENZYME ACTIVITY
UMOL NADPH/MIN.-MG
150
100
50
100
200
300
400
500
600
700
800
900
1000
T3 DOSE - UG T3/KG DIET

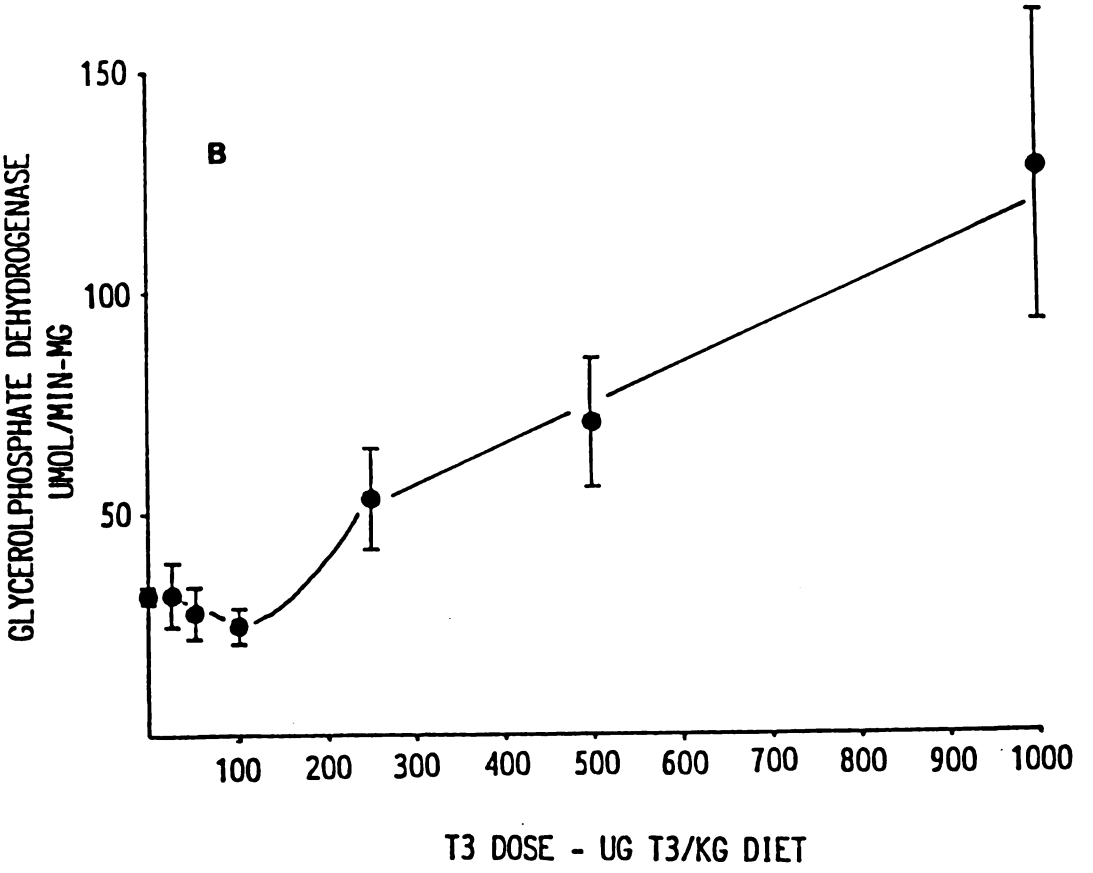

B
GLYCEROLPHOSPHATE DEHYDROGENASE
UMOL/MIN-MG
150
100
50
100
200
300
400
500
600
700
800
900
1000
T3 DOSE - UG T3/KG DIET

Figure 16

a) T3 concentrations in plasma (●—●) and liver (○—○) after dosing rats with T3 in the diet for 72 hours.

b) T4 concentrations in plasma (●—●) and liver (○—○) after treatment as in (a). Data points shown are the average of samples from four animals each ± S.E.M.

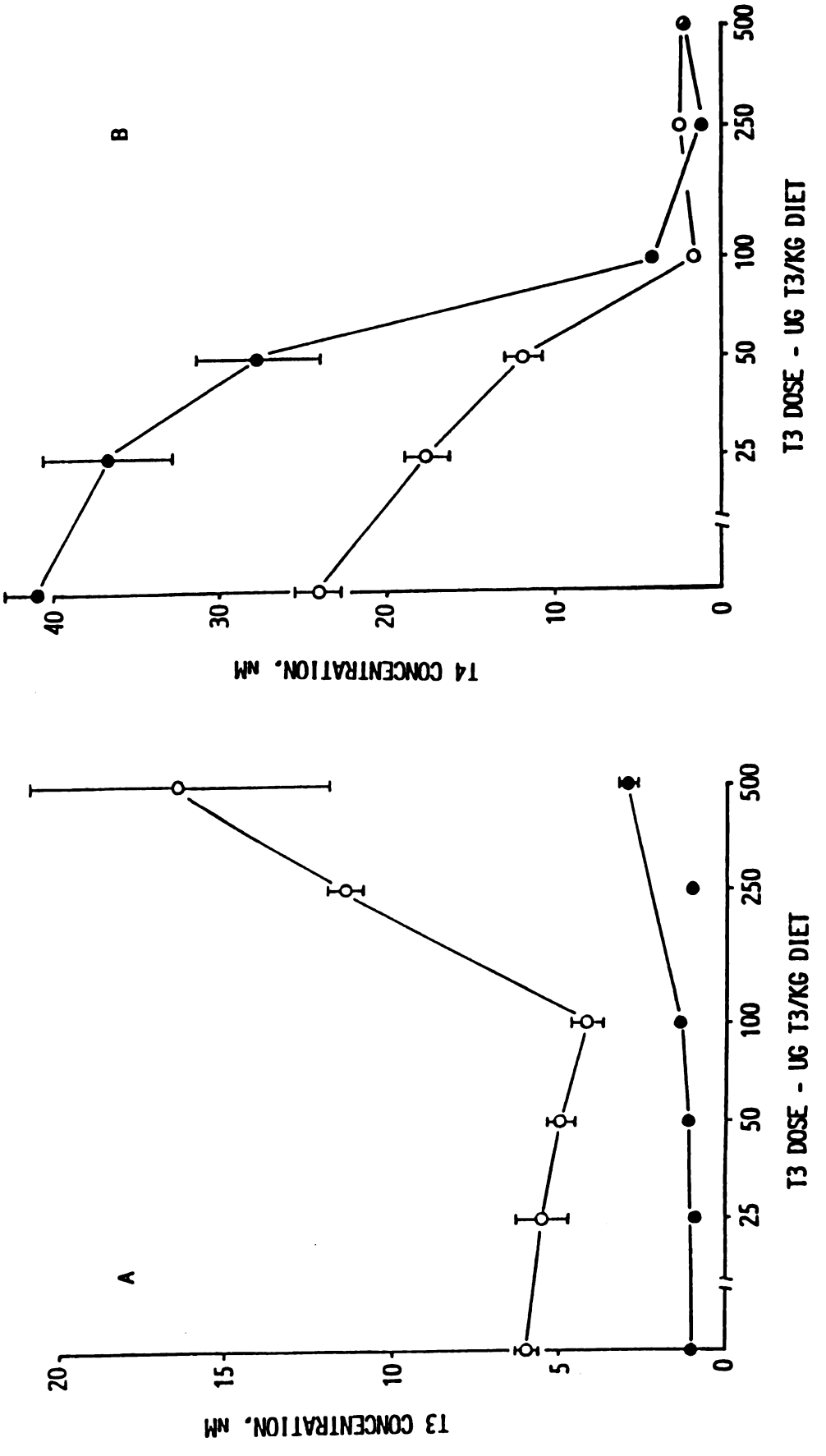

A
B
T3 CONCENTRATION, nM
T4 CONCENTRATION, nM
T3 DOSE - UG T3/KG DIET
T3 DOSE - UG T3/KG DIET
0
5
10
15
20
0
10
20
30
40
25
50
100
250
500

Figure 17

Hepatic T3 (●—●) and T4 (▲—▲) concentrations 72 hr after treatment with TCDD. Data points shown are the average of samples from three animals each ± S.D.

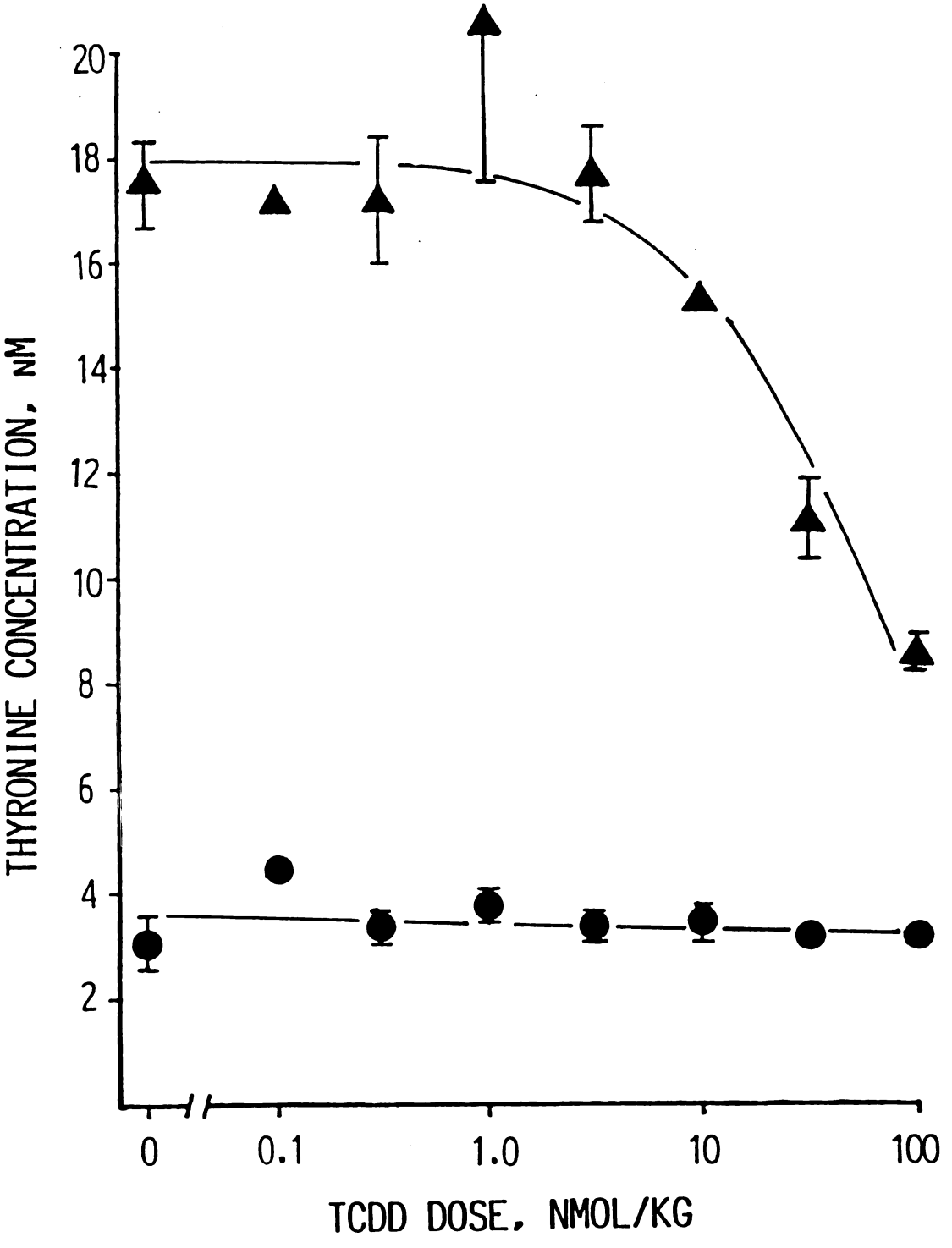
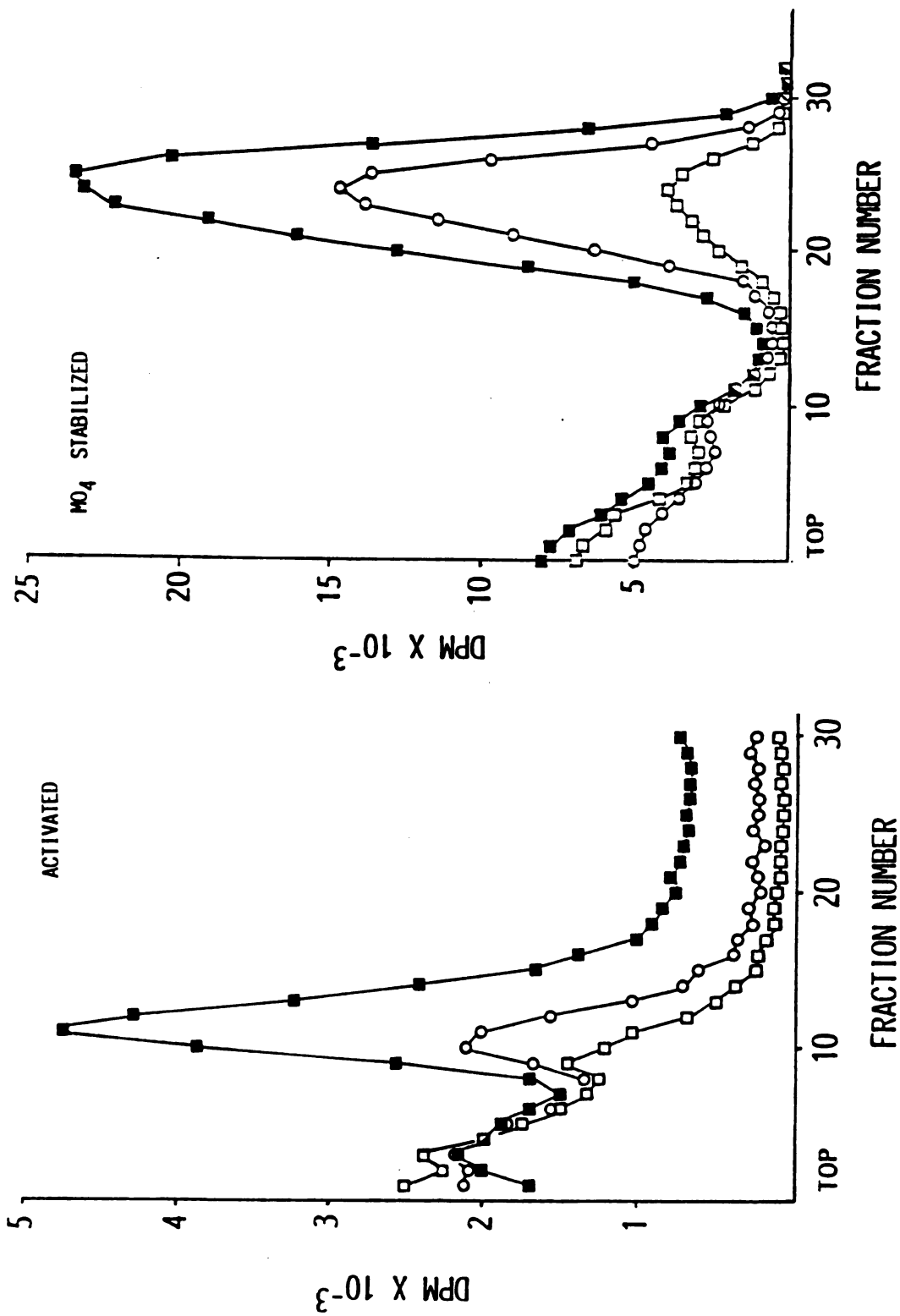

THYRONINE CONCENTRATION, nM
20
18
16
14
12
10
8
6
4
2
0
0.1
1.0
10
100
TCDD DOSE, NMOL/KG

Figure 18

^{3}H-triamcinolone binding to the MoO_4^- stabilized and activated glucocorticoid receptor with the following additions to the incubation mixtures:

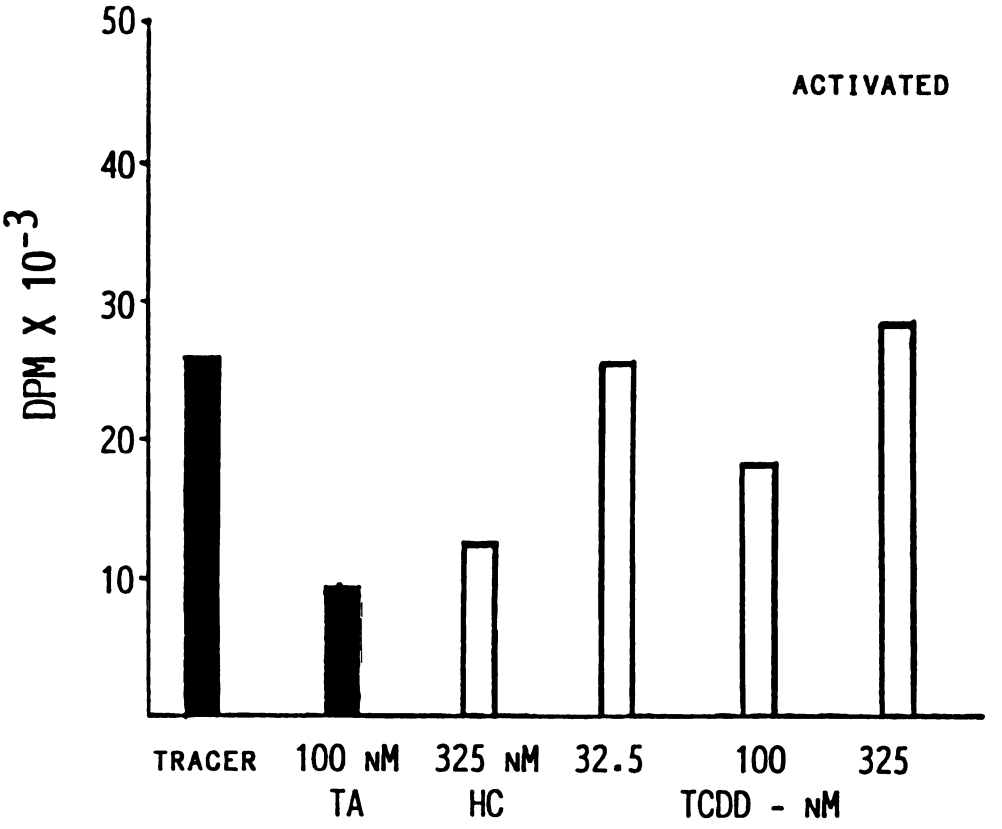
no competitor (■—■), 325 nM cortisol (○—○), or (□—□) 100 nM cold triamcinolone.


MO_4 STABILIZED
ACTIVATED
DPM X 10^{-3}
FRACTION NUMBER
TOP
25
20
15
10
5
4
3
2
1
30

order of magnitude more tightly than hydrocortisone (124). TCDD is at best a very weak ligand, showing no displacement of ^{3}H-TA at toxicologically significant concentrations (figure 19). These assays were repeated in the absence of molybdate, with a heat activation step (140) prior to density gradient centrifugation, on the theory that in the physiologically more relevant, nonequilibrium binding process, TCDD might bind to the receptor long enough to cause activation (figure 18), before it could be displaced by the more tightly bound triamcinolone. However, TCDD was found to be ineffective as a ligand for the "activated" receptor (figure 19). An additional postulate was that induction of malic enzyme by TCDD was mediated directly via the Ah-receptor. To test this possibility, two strains of mice, one with a functional Ah-receptor (C57BL/6N) and one with a non-functional Ah-receptor (DBA/2N) were treated with a range of TCDD doses from 0.1 nM to 1000 nM which resulted in cytochrome P-450c induction (measured by EROD activity) ranging from less than 10 % of maximum to maximal induction - with some induction occuring even in the DBA mouse (figure 20a). This range of doses overlaps all doses used in our studies with rats, as well as doses which are 10 x higher, to ensure coverage of the mouse LD50 dose. No induction of malic enzyme activity was observed at any of these doses of TCDD in either strain of mice (figure 20b).

Figure 19

Integrated peak areas (DPM from bound triamcinolone) of the activated and molybdate-stabilized glucocoticoid receptor with hydrocortisone (HC), triamcinolone (TA), or TCDD added to the incubation mixture as competitors.


ACTIVATED
DPM X 10^{-3}
50
40
30
20
10
TRACER
100 nM TA
325 nM HC
32.5
100
325
TCDD - nM

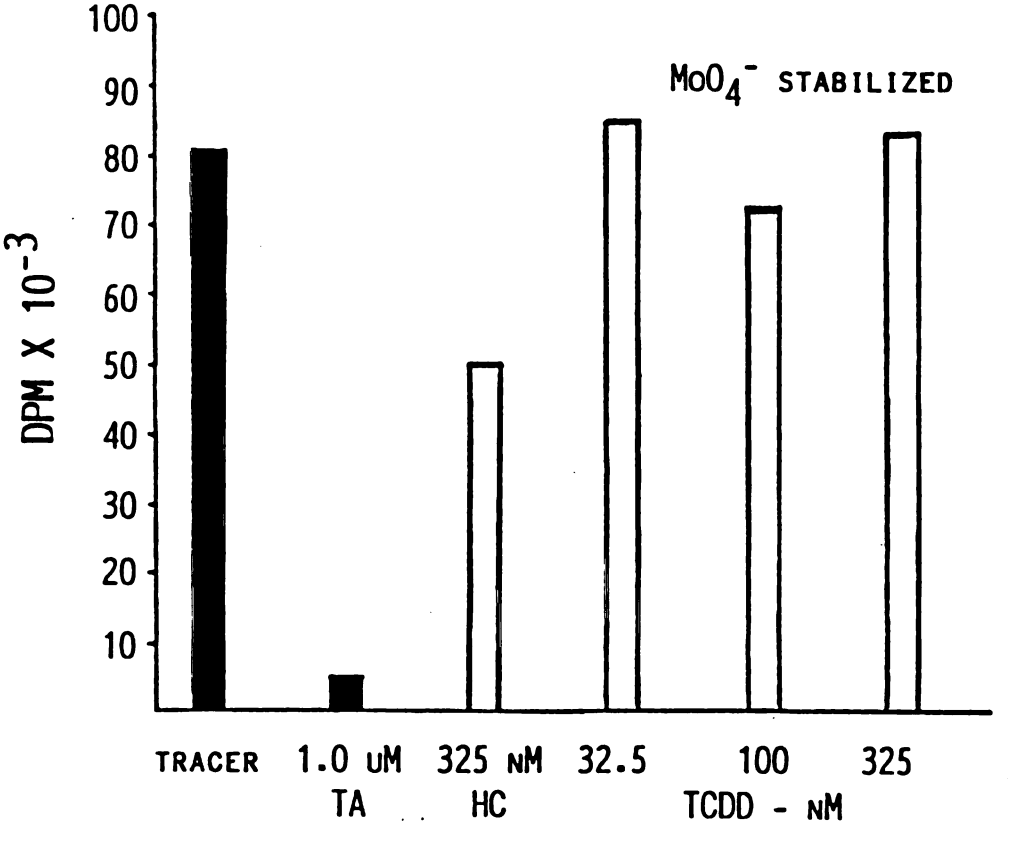
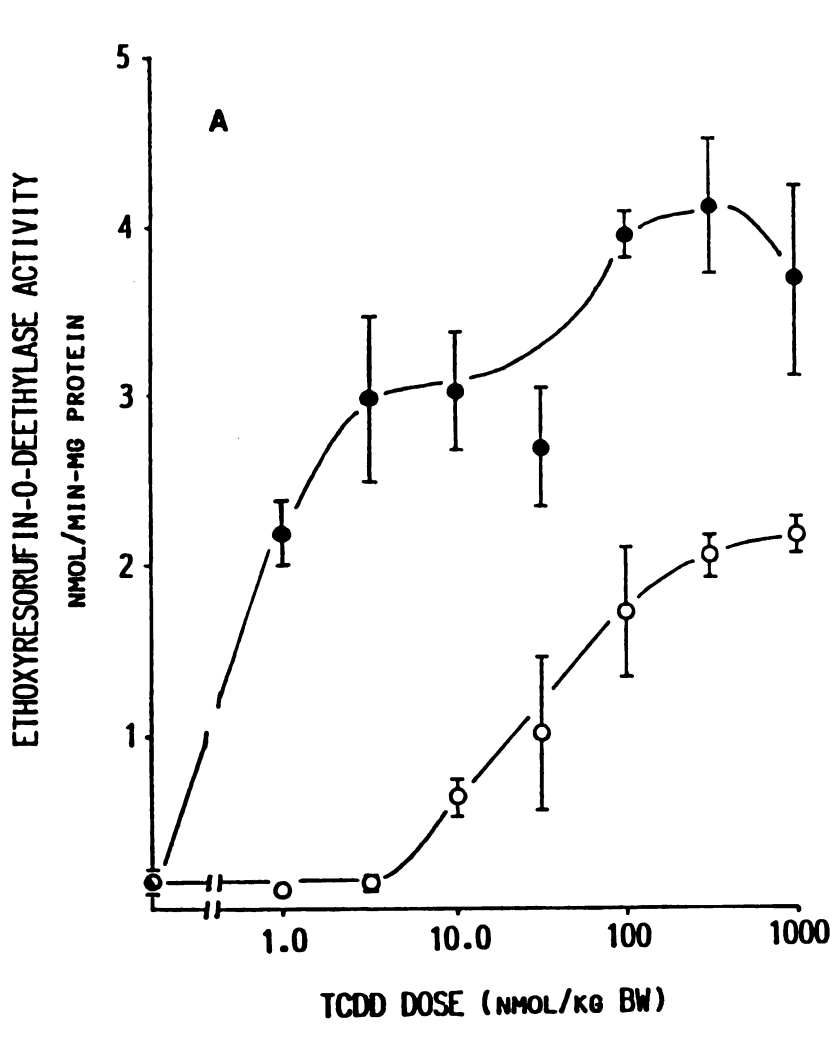
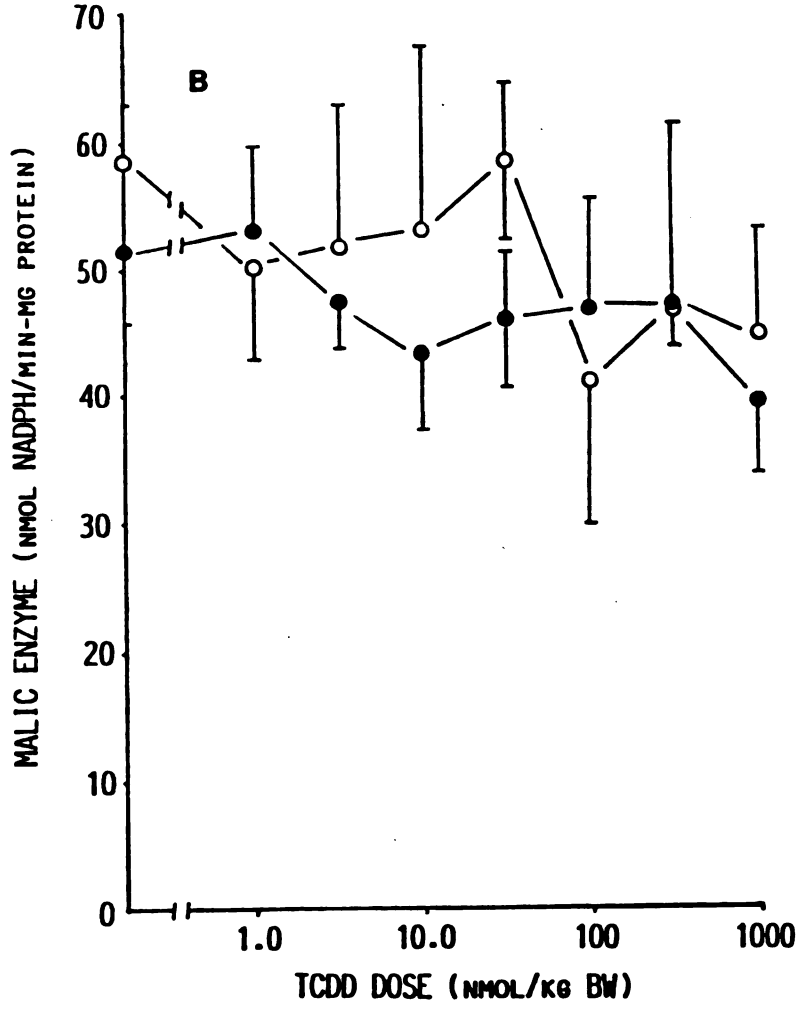

MoO_4^- STABILIZED
DPM X 10^{-3}
100
90
80
70
60
50
40
30
20
10
TRACER
1.0 uM TA
325 nM HC
32.5
100
325
TCDD - nM

Figure 20

a) Ethoxyresorufin-O-deethylase activity in liver microsomes of C57BL/6N (●—●), and DBA/2N (○—○) mice 72 hours after treatment with TCDD. All data points greater than 0.5 nmol/min-mg are significantly different from controls with $P<0.05$.

b) Liver malic enzyme activity in C57BL/6N (●—●) and DBA/2N(○—○) mice 72 hours after TCDD treatment. Each point shown represents the average of samples from 4 rats $\pm$ S.D.


A
ETHOXYRESORUFIN-O-DEETHYLASE ACTIVITY
NMOL/MIN-MG PROTEIN
5
4
3
2
1
1.0
10.0
100
1000
TCDD DOSE (NMOL/KG BW)


B
MALIC ENZYME (NMOL NADPH/MIN-MG PROTEIN)
70
60
50
40
30
20
10
0
1.0
10.0
100
1000
TCDD DOSE (NMOL/KG BW)

DISCUSSION

In recent years several authors have investigated the role of thyroid status and corticosteroid function in the toxicity of TCDD. Rozman et al. (112) have shown that thyroidectomy gives a degree of protection to rats treated with TCDD. Several studies, including our own, have shown that thyroid status does not alter the normal pattern of cytochrome P-450 induction, or change the physical characteristics of the Ah-receptor (60,111,113). We have shown (in chapter 2) that the induction of malic enzyme by TCDD is prevented by thyroidectomy. Malic enzyme synthesis is regulated by both dietary factors and hormones. Of these agents, only thyroid hormones are capable of inducing malic enzyme in the absence of the other agents. Induction of malic enzyme by insulin and glucocorticoids does not occur in the absence of thyroid hormones. In the present study, we have demonstrated that the induction of liver malic enzyme is not the result of alterations in local thyroid hormone concentrations.

Corticosteroid metabolism and glucocorticoid receptor function have been studied in TCDD-treated animals to determine whether TCDD produces corticosteroid-like effects by altering glucocorticoid metabolism. DiBartolomeis et al. (37) have discovered decreased diurnal peak plasma corticosterone levels and decreased production of corticosterone in rats treated with TCDD. Similar results were

obtained by Balk and Piper (6), who also discovered that TCDD treatment results in increased concentrations of an unusual steroid metabolite, 11-hydroxy progesterone. We were interested in the possibility that a specific biochemical effect, thyroid hormone - dependent induction of malic enzyme by TCDD, was mediated by a direct corticomimetic effect of TCDD, which would account for the fatty liver, depressed corticosteroid production, and the decreased binding capacity of the glucocorticoid receptor in TCDD treated rats (82,115). The results of our studies, when combined with those of DiBartolomeis et al., suggest that changes in corticosteroid production and glucocorticoid receptor binding are secondary or tertiary effects of TCDD, but certainly are not the result of direct corticomimetic properties of TCDD. Cuthill et al. (30) have noted the physical and functional similarities between the Ah- and glucocorticoid receptors. We attempted to determine whether the Ah-receptor was acting in a manner similar to that of the glucocorticoid receptor in malic enzyme induction, ie. that the Ah-receptor was mediating ME induction itself. The C57BL/6N and DBA/2N strains of mouse have been used in many studies of Ah-receptor function and in the study of the oncogenic consequences of cytochrome P-450c (aryl hydrocarbon hydroxylase) induction. In general, the lack of a functional Ah-receptor protects the DBA/2N mouse from chemical carcinogens which are activated by cytochrome P-450c. Although malic enzyme can be induced by

thyroid hormones in the mouse as in the rat (117), it is not induced by TCDD in either strain of mice. Other agents are known to induce or repress the synthesis of Malic Enzyme. Mariash and Oppenheimer (80) have shown that the induction of ME by glucose and fructose proceeds by a three or two carbon metabolite of the sugars, rather than the sugars themselves, and noted that dichloroacetate is an especially potent ME inducer . Dietary unsaturated fatty acids are effective suppressors of ME synthesis in mice, while both saturated and unsaturated fatty acids are effective suppressors of enzyme induction in rats .

Kelling et al.,(71) have shown that perfluorodecanoic acid (NDFDA) can increase ME activity, although their study suggests that this may be a direct effect on the enzyme rather than an inductive effect. Since NDFDA interferes with fatty acid synthesis, it could be acting by the same mechanism as dietary fats. Ayala et al. (4) have shown that malic enzyme activity can be induced by toxins which do not interact with the Ah-receptor. In these experiments, rats were treated with t-butyl hydroperoxide and or 1,3-bis-(chloroethyl)-1-nitroso-urea (BCNU). Malic enzyme induction occurred on treatment with t-butyl hydroperoxide, but not in the presence of BCNU. The authors suggest that BCNU reduces NADPH consumption by inhibiting glutathione reductase. They further suggest that NADPH has a repressive effect on malic enzyme synthesis, and that induction of enzymes which use NADPH cause its depletion and removal of

this putative repressor on ME synthesis. We note that BCNU (a) is known to elevate cAMP levels(36), which would suppress malic enzyme synthesis, and (b) is very similar in structure to known T4 5'-deiodinase inhibitors, and may inhibit deiodination of T4 to T3. Since BCNU inhibits glutathione reductase, and reduced glutathione is required for deiodination of T4 to T3, direct inhibition of the deiodinase would not be necessary for BCNU to produce a hypothyroid state, in which malic enzyme would be suppressed. Aside from these facts, we feel that a mechanism involving NADPH directly is unlikely, since none of the nicotine adenine dinucleotides have ever been shown to directly control gene expression.

Our failure to induce ME in the mouse underscores the complexity of hormonal interactions in the regulation of enzyme synthesis and intermediary metabolism. Hormonal effects that can be demonstrated in tissue culture often cannot be observed in the whole animal, and vice versa. Experimental results obtained in the rat or mouse are often thought of as generalized, cross-species phenomena, but may, as in the case of malic enzyme induction by TCDD, occur only in one or a few species. Compounds such as TCDD, which can change the metabolism of hormones by way of the enzymes they induce, change so many hormone dependent processes that the mechanism of any particular effect can only be determined if all hormones affecting that process are known and controlled.

CHAPTER IV

TRANSPORT AND METABOLISM OF THYROID HORMONES AND CONTROL OF T3 PRODUCTION FROM T4 IN THE RAT AFTER TREATMENT WITH TCDD.

ABSTRACT

Male Fischer rats treated with a single dose of 10 nmol TCDD/Kg body weight (3.23 ug/Kg) were injected intravenously with tracer doses of either [3,5-^{125}I]-thyroxine (T4) or [3'-^{125}I]-triiodothyronine (T3) 3 days later. Blood, liver, kidney, bile and urine samples were collected and extracted at time points ranging from 2 min to 15 hr post-tracer. HPLC separation of these extracts yielded data for tissue uptake, excretion, deiodination, and glucuronidation of T4, T3, and major metabolites in each compartment sampled. Transport and metabolism rates for a five compartment model were estimated and then optimized via the CONSAM analysis and modeling program. As predicted from the induction curve for cytochrome P-450c and associated glucuronosyl transferases by TCDD, glucuronidation of T4 was maximal at this dose of TCDD. However, steady-state T4 levels decrease only 40 %, compared with a 75 % drop which occurs at 77 nmol TCDD/Kg (25 ug/Kg). Tissue uptake and metabolism of T3 were not significantly changed between control and TCDD-treated groups. Our data for T3 production from T4 in the liver suggest that this deiodination reaction is controlled by the flux of T4 through the plasma membrane high affinity uptake system, rather than by the concentration of T4 in the cytoplasm of hepatocytes.

INTRODUCTION

A family of aryl hydrocarbons including benzo[a]pyrene, 3-methylcholanthrene, dibenz[a,h]anthracene, certain symmetric halogenated biphenyls, and halogenated dioxins, of which 2,3,7,8-tetrachlorodibenzo-p-dioxin(TCDD) is the most potent, induce a characteristic set of enzymes in the liver and other tissues of animals exposed to them.

Cytochrome P-450c (sometimes called aryl hydrocarbon hydroxylase or AHH), epoxide hydratase, and 4-nitrophenyl glucuronosyl transferase are the best studied of these aryl hydrocarbon-inducible enzymes, and have been shown to be coordinately regulated by a receptor which binds to chromosomal enhancing sequences of the Ah-locus (20,53,67).

The concentration of a particular compound required to induce a certain level of P-450c is relatively constant across species which have a demonstrable Ah receptor. For TCDD, some induction can be shown at 0.1 nmol/Kg (32 ng/Kg or 32 parts per trillion). Fifty percent of maximal induction occurs at about 1 nmol/Kg (the ED50), and maximal induction occurs between 10 and 33 nmol/Kg (46,103,111). In some animals, such as the guinea pig, acutely toxic doses of Ah-inducers coincide with doses near the ED50 for induction of these enzymes. However, many other species, such as the mouse and hamster, show no morbidity at doses of these compounds which are far in excess of those required for induction of Ah-locus enzymes. Several

metabolic changes associated with acute Ah-inducer exposure have been suggested to result from the induction of cytochrome P-450c and the glucuronosyl transferase. Increased biliary excretion and/or glucuronidation of thyroxine has been reported following treatment with various Ah-inducers, in association with severely depressed levels of thyroxine in the serum. Reports of T3 levels in these animals have been conflicting (8,105,112). Recent work in our laboratory indicated that cross-reactivity between T4 and the T3 antibody used in the radioimmunoassay could explain some of these results. When T3 and T4 were separated by HPLC prior to radioimmunoassay, it was found that T3 levls did not change significantly despite the drop in T4 levels (111). Reports of the relationship between T3 and T4 concentrations and T3 production from T4 in perfused liver, tissue homogenates, or microsomal preparations generally show linear increases in steady-state T3 concentration or deiodination rates with increasing T4 concentrations (25,66,97). Oppenheimer et al. (94) observed a conservation of T3 with dropping T4 levels following prolonged phenobarbital treatment, which was similar to that which we have found following Ah-inducer treatment. In that report, normal T3 levels were attributed to increased deiodination of T4.

Preliminary results in our laboratory, as well as those of Rickenbacher and McKinney(108), did not indicate any change in the in vitro (microsomal) deiodination rate

following TCDD treatment. Since the maintainance of T3 levels could conceivably result from alterations in uptake, production, or catabolism of T3, we began in vivo studies of the transport and metabolism of T3 and T4 in control and TCDD treated rats, using pharmacokinetic methods.

MATERIALS AND METHODS

Chemicals

TCDD was a gift from Dr. Fumio Matsumura, Pesticide Research Center, Michigan State University. [3,5-^{125}I]-Thyroxine (4400 Ci/mmol - inner ring labeled) and [3'-^{125}I]-3,5-Triiodothyronine (2200 Ci/mmol) were purchased from New England Nuclear (Boston, MA). L-3',5,3-Triiodothyronine (T3) was purchased from Chemical Dynamics Co. (South Plainfield, N.J.). 3',5',3-Triiodothyronine (rT3) was obtained from Calbiochem Inc. (LaJolla,CA). Thyroxine, 3,5-diiodothyronine (3,5-T2), diiodotyrosine, beta-glucuronidase (type IX), bovine serum albumin (BSA), heparin, and pentobarbital were obtained from Sigma Chemical Co.(St.Louis, MO). Methoxyflurane was from Pittman-Moore (Washington Crossing,N.J.), Aminophylline was from LyphoMed, Inc. (Melrose Park,IL). All solvents and other compounds were reagent grade.

Animals

Male Fischer rats weighing 250-300 grams were obtained from Charles River Labs (Portage,MI). Rats were given free access to water and food (Wayne Rodent Blox, Wayne Feeds,

Chicago,IL). For short (less than 1 hr) period tracer experiments, rats were kept in large (20"x 16"x 8") polycarbonate cages with hardwood bedding. Long period tracer experiments (2-16 hours) required the prior implantation of femoral catheters. Rats used in these studies were caged individually, in 14" X 12" X 7" polycarbonate cages. Silastic-to-Tygon catheters (.025 in OD) were implanted into the femoral vein under methoxyflurane anesthesia. Immediately after surgery, rats were given 2 mg aminophylline - i.p., to prevent laryngospasm that often follows surgery. These rats were allowed 3 days for recovery before further treatment.

Treatments

All operations involving the use of concentrated TCDD solutions were performed in isolation rooms under negative pressure, as previously described (111). All rats were given a single dose of either corn oil alone (1 ml/Kg) or 10 nmol TCDD/Kg by gavage on day 0 of each experiment. Tracer injections were started 3 days after TCDD dosing.

Tracer Injection and Sampling

^{125}I-Thyroxine and ^{125}I-T3 were purified by reverse phase HPLC (described below) and dried in the dark under a stream of argon 1 day before use. These compounds were resuspended in either a sterile-filtered, crude rat plasma transthyretin preparation (T4) or sterile-filtered rat plasma (T3), and were kept refrigerated overnight before use.

For short term (< 1 hr) experiments, rats were injected i.p. with 75 mg/Kg pentobarbital, and allowed 5 min for induction of anesthesia . The jugular vein was exposed above the left clavicle, and 100 ul (1-2 uCi) of tracer solution injected via a 250 ul luer lock, Teflon plunger, Hamilton syringe fitted with a 25 gauge needle. In long period experiments, 200 ul of blood was drawn from the catheters of conscious rats, after which the tracer was injected, followed by reinjection of the blood and 100 ul of sterile saline. At 10 min post-tracer, a single 1 ml sample of blood was withdrawn and treated as described below as a check on the catheter function and dose delivery. The long period rats were injected with 50mg/Kg pentobarbital via catheter 40 min before blood and tissue sampling, to allow insertion of a bile duct cannula, and collection of bile for 30 min before termination.

One minute prior to sampling, the rats were laparotomized, allowing removal of blood from the inferior vena cava followed by rapid excision of the liver and kidney at the appropriate sampling times. Each (1 ml) sample of blood was withdrawn with a 1 ml heparinized plasic syringe, injected into a pre-chilled(on dry ice) 1.5 ml Eppendorf vial, which was kept on ice (5 min) until it could be centrifuged to separate cells from plasma. A 400 ul sample of each resulting plasma was rapidly mixed with 1 ml methanol and refrigerated for later analysis. Urine was removed from the bladder using a plastic syringe with a 25 gauge needle,

after blood and tissue were collected.

Liver and kidney were perfused rapidly (for about 15 seconds) in situ with ice cold saline using a blunt-tipped 50 ml plastic syringe. These tissues were quickly weighed, and a sample (3-5 g of liver, one whole kidney) homogenized in 5 volumes of ice cold methanol with a Teflon-pestle Potter-Elvejehm homogenizer. The resulting homogenates were emptied into capped polyethyene centrifuge tubes (Corning) and refrigerated until further processing.

Processing of Plasma and Tissue Extracts

Methanol-plasma mixtures were centrifuged for 5 min, 24-48 hours after each experiment, to pellet the precipitated protein. One ml of the supernatant was removed and added to 280 ul of an acetate buffer (0.03 M Na acetate + 0.03 M acetic acid) to adjust the water and acetate content to that of the HPLC solvent (below). Liver and kidney homogenates were likewise centrifuged, and 5 or 4 ml samples, respectively, were chilled on dry ice in 20 ml glass scintillation vials, followed by vacuum drying (5 hr). The residues from this operation were resuspended in HPLC solvent, refrigerated, and centrifuged the following day. The clear supernatant was used for chromatographic analysis.

HPLC Separation of Plasma and Tissue Extracts

High pressure liquid chromatography was performed at a setting of 1.5 ml/min, and an average pre-column pressure

of 3300 ± 100 psi (with a 20 ml damping reservoir). The stationary phase used was an Econosphere C-18 reverse phase column (Alltech/Applied Science, Deerfield,IL). The mobile phase consisted of methanol:water:acetic acid 575:452:1, with 5 ml of 5 N NaOH added to each 3 liters of solvent. One ml samples were injected into a large sample loop, followed by collection of one sample every 30 sec for 20 min. Under these conditions iodide eluted in fractions 4-6 (2-3 min), T2 in fractions 9-13 (4-6 min) T3 in fractions 16-23 (8-11 min) and T4 from fraction 29-37 (15-19 min).

Peaks corresponding to rT3, triac and tetrac were often observed, but were either too small to quantitate (rT3 & tetrac) or overlapped other peaks (triac). Bile samples (100 ul) were mixed with either 300 ul of 10 mg/ml BSA or the same solution containing 100 units of β-glucronidase. Following incubation for 5 hours, these samples were extracted with methanol as for plasma, and chromatographed as above, but using 500:498:1 methanol:water:acetic acid as the solvent. T4 was flushed from the column during bile chromatography by injecting 1 ml methanol at fraction 32 (16 min), as its long retention time (33 min.) with isocratic elution doubled the number of fractions needed to complete the separation.

Data Reduction and Analysis

Extraction efficiencies for T3 and T4 were estimated by combining the results from non-radioactive, spiked and extracted samples (chapter 3) and counts of the supernatant

and precipitates of extracted radioactive livers (2 min post-tracer). Very little metabolism occurs in the 2 min samples, so we assumed that radioactivity in the precipitate was the same species as that in the supernatant, although this could not be confirmed by any techniques we know of. In general, extraction efficiency for T3 was 95 - 105 % on the basis of spiked samples, while that for T4 was about 60 $\pm$ 10 % (non-radioactive method) and 58 $\pm$ 2 % when estimated from counts of the supernatant and precipitate in extracts of livers at 2 min. Our procedure for determining the administered dose was to dilute the tracer solution with normal plasma, extract, chromatograph, and otherwise treat these dilutions as samples. Since the extraction efficiencies for plasma were very similar to those found for tissue extracts, no corrections were performed on data from tissue or plasma extracts.

Raw counts for fractions corresponding to the metabolites of interest were integrated and corrected for decay during analysis (generally 2 -> 7 days difference between 1st and last samples). The resulting values were used to compute either CPM/ml (plasma, bile, and urine), or CPM/g (liver & kidney). These values were then multiplied by the plasma volume, bile or urine flow/hr, or tissue weights to give whole organ values, after which they were divided by the dose determined from diluted tracer samples for each experiment to obtain results as % of dose. Crude transport and metabolism rates were determined for each compartment

using this data, and were then used as starting points for determination of refined rates and variance information by a nonlinear least-squares optimization and kinetic modeling program (CONSAM 29, references 10 and 11). A four compartment model comprized of plasma, liver, kidney, and the residual carcass was used initially for analysis of T3 and T4 tracer data. These models were later modified by the addition of separate hepatocyte membrane and cytoplasmic compartments to allow data fitting.

Computation of expected T3 production levels assuming dependence of the 5'-deiodinase on cytoplasmic or plasma membrane T4 concentrations was accomplished by integrating the five compartment models for T3 and T4 metabolism into a interconnected ten compartment model (Appendix A).

RESULTS

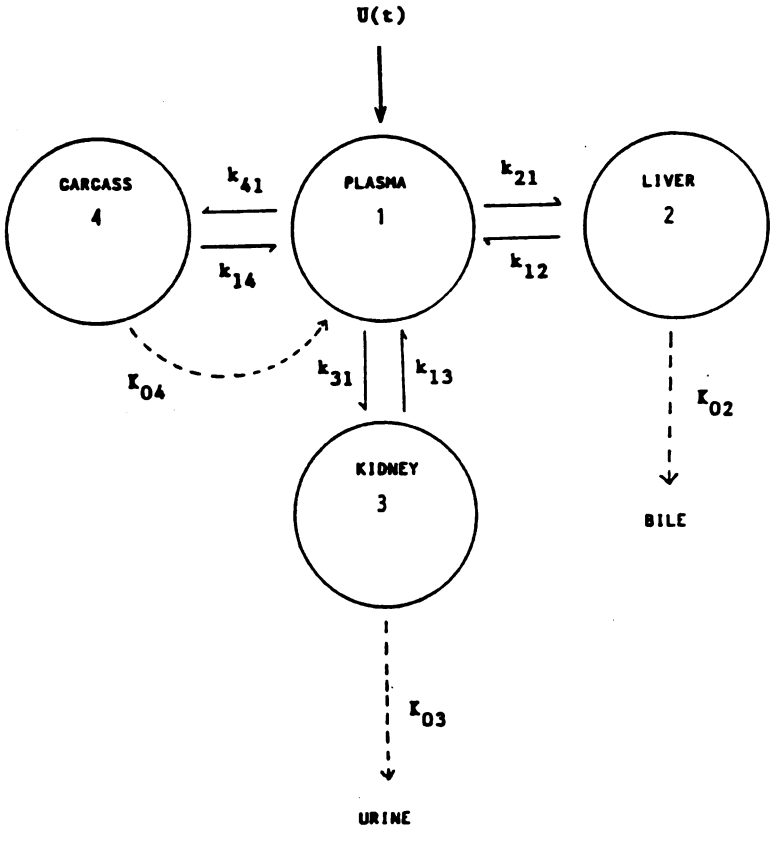
The overall model structure and rate constants determined for transport between and metabolism in its compartments are shown in figure 21, table 1, and table 2.

We initially attempted to fit our data to a four compartment model, illustated in figure 21, but found that the liver data could not be fit for either T3 or T4 unless the liver was divided into a retentive membrane compartment and a cytoplasmic compartment, as shown in figure 21. Our best fit results for each of these schemes are shown in figures 22 and 23, with the predicted masses in the individual and

Figure 21

Four and five compartment models for uptake and metabolism of T3 and T4. First order rate constants were fit to plasma, liver, and kidney data. The modified five compartment model of thyroid hormone metabolism contains separate membrane and cytoplasmic compartments in the liver.

FOUR COMPARTMENT MODEL OF THYROID HORMONE METABOLISM



FIVE COMPARTMENT MODEL FOR THYROID HORMONE METABOLISM

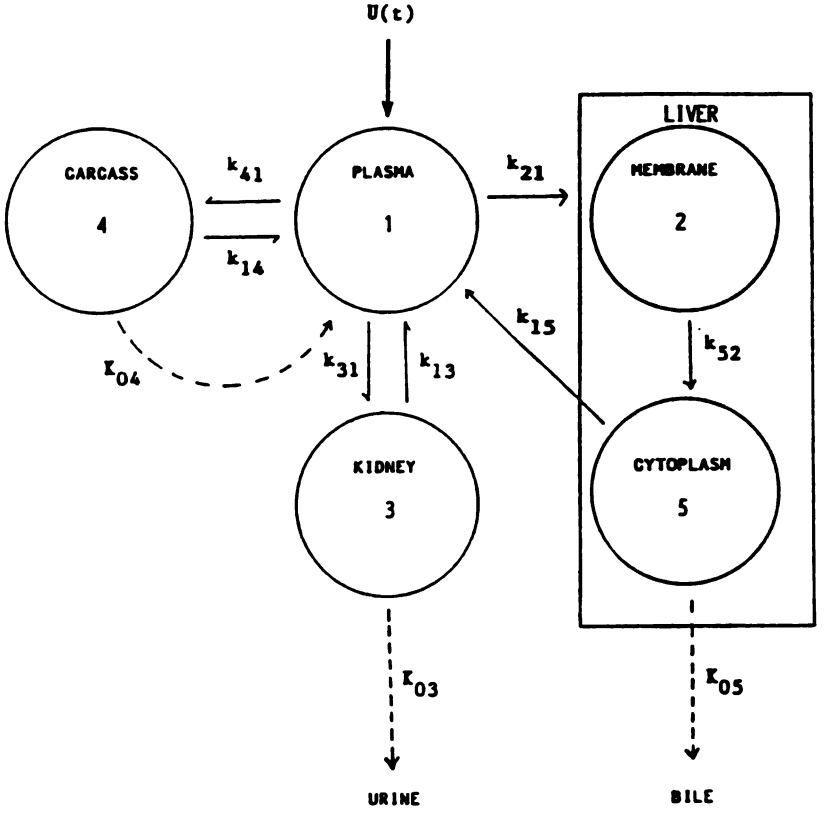
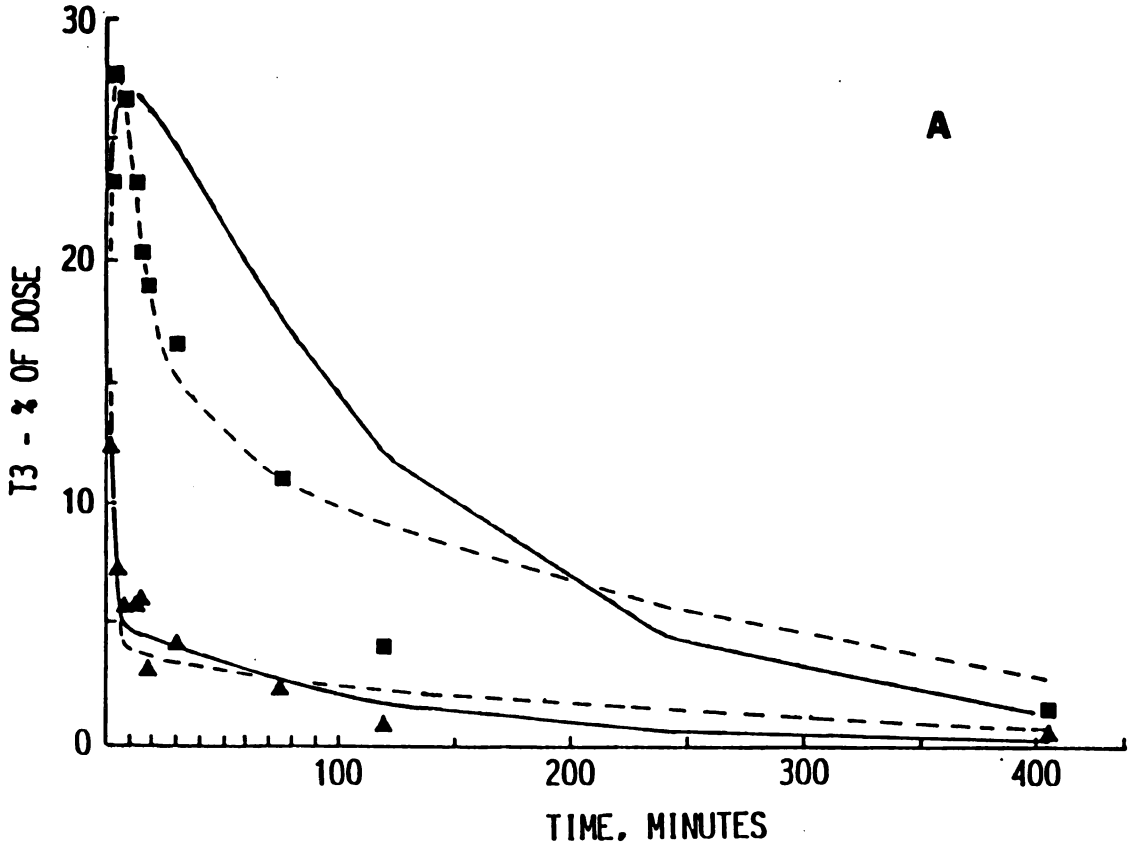


Figure 22

(a) Best fit for plasma (▲—▲), and liver (■—■) T3 tracer data in normal rats using a four (——) and modified five (----) compartment model. Rate constants determined for total uptake, recycling, and metabolism of T3 are given in table 1. (b) Predicted masses of T3 in the membrane, cytoplasmic and combined comparmtents of the liver. Note that the maximum occurs at 5-6 min.
Each point represents the average of three samples.


A
T3 - % OF DOSE
30
20
10
0
100
200
300
400
TIME, MINUTES

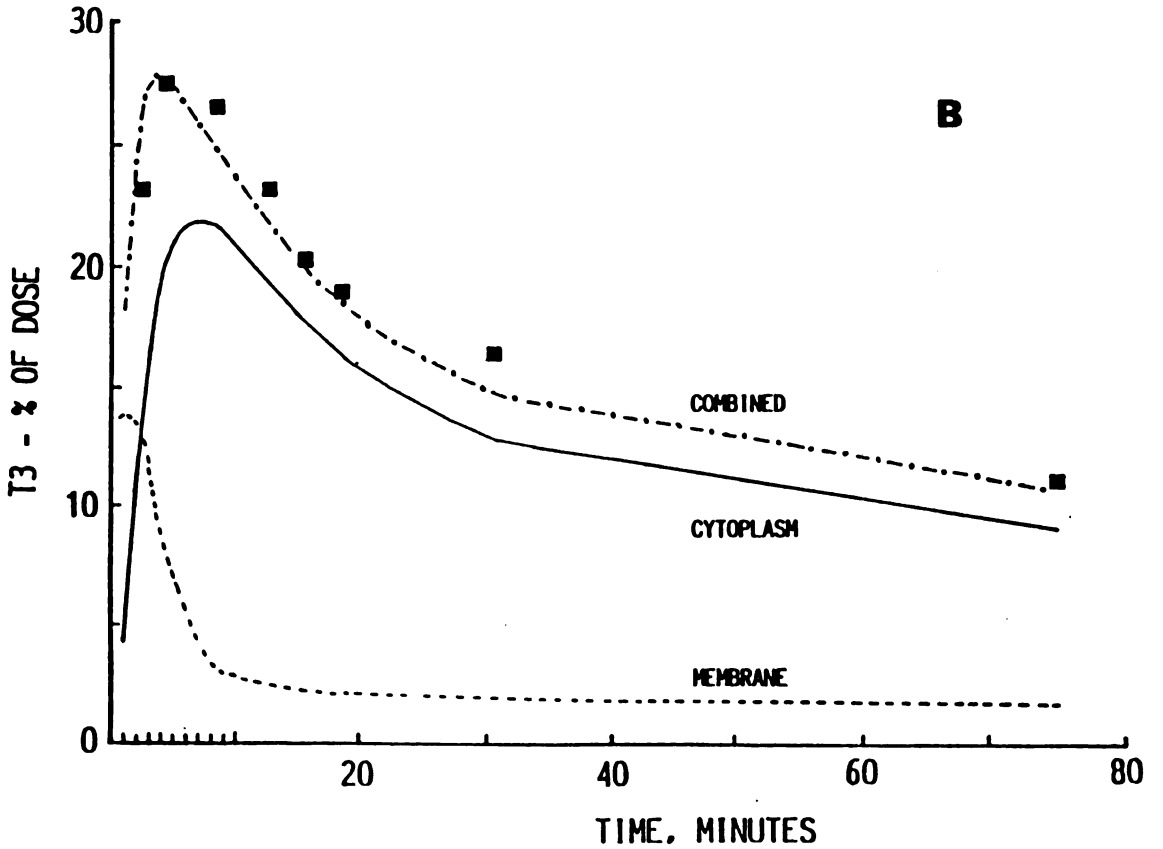
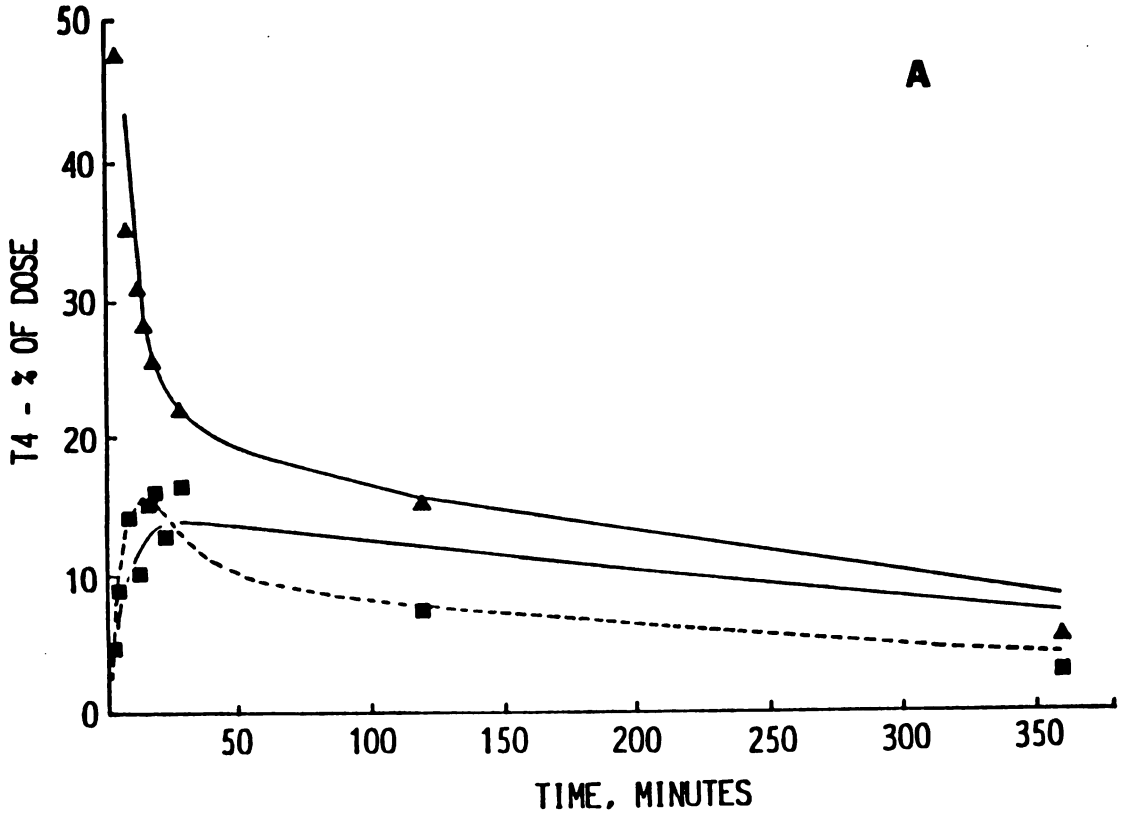

B
T3 - % OF DOSE
30
20
10
0
COMBINED
CYTOPLASM
MEMBRANE
20
40
60
80
TIME, MINUTES

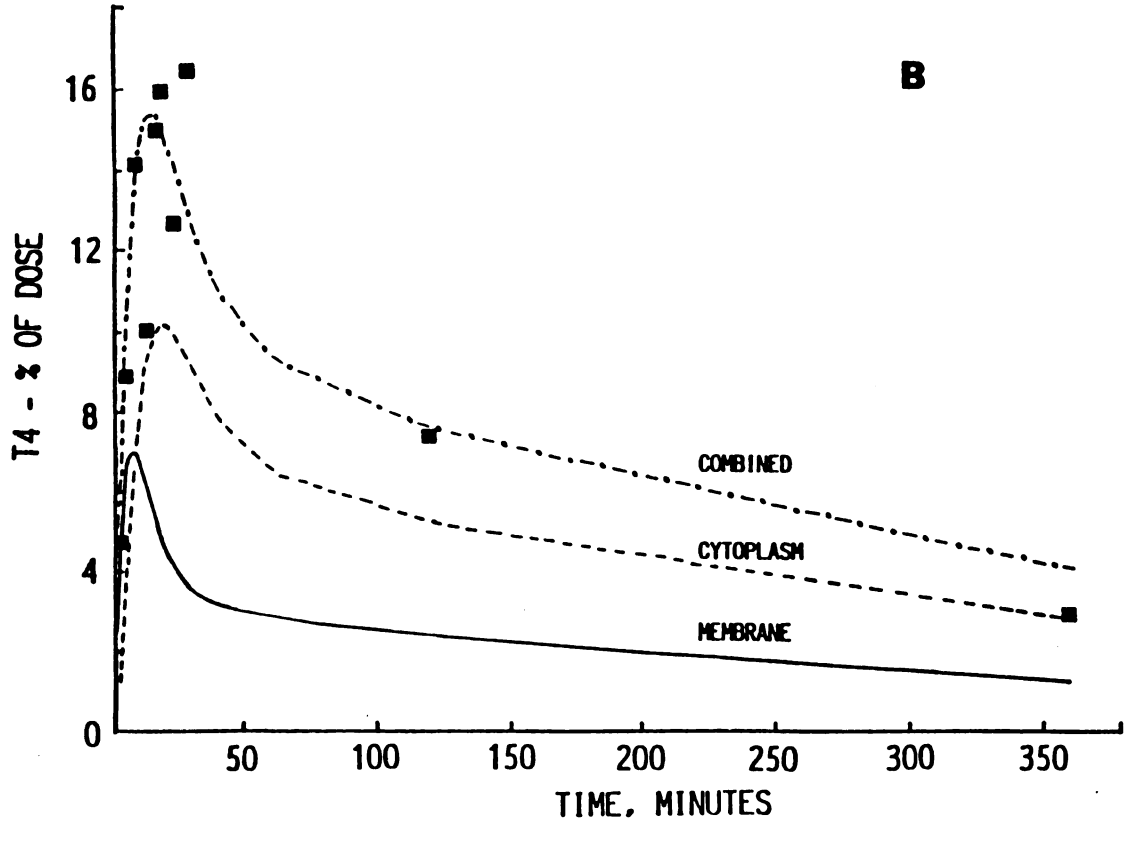
Figure 23

(a) Best fit for plasma (▲—▲) and liver (■—■) T4 tracer data in normal rats using the four (——) and five (- - - -) compartment models. Rate constants determined for total uptake, recycling, and metabolism are given in table 2.

(b) Predicted masses of T4 in the membrane, cytoplasmic, and combined compartments of the liver.

Each point represents the average of three samples.


A
T4 - % OF DOSE
50
40
30
20
10
0
50
100
150
200
250
300
350
TIME, MINUTES


B
T4 - % OF DOSE
16
12
8
4
0
COMBINED
CYTOPLASM
MEMBRANE
50
100
150
200
250
300
350
TIME, MINUTES

combined subcompartments. While it is likely that the kidney and carcass compartments would be better fit if they were further subdivided, our data for these compartments was not of sufficient quality to distinguish between additional schemes.

The transport rates for both T3 and T4 were similar in TCDD treated and control rats, as expected. The rate constant for glucuronidation of hepatic T4 was increased 4-5 fold (0.822/hr vs. 0.160/hr @ 2hrs)by TCDD, as reported in previous studies where much higher Ah-inducer doses were used (8,48). This was expected from the induction curve of cytochrome P-450c and 4-nitrophenyl glucuronosyl transferase, Ah-locus enzymes which are induced maximally at three days by a dose of 10 - 15 nmol TCDD/Kg (78).

Earlier studies did not report the relatively large amount of free thyroxine which we found in the bile of both control and treated rats (table 3). In our study, a 4-5 fold increase in T4 glucuronide (T4G) production (36 pmol/hr -> 154 pmol/hr) caused by TCDD treatment results in only a 2 fold increase in the total biliary excretion of T4 (124 pmol/hr -> 246 pmol/hr).

The transport of T4G from the liver into the bile is apparently very rapid, as the glucuronide was only detectable in the bile (figure 24). Transport and total metabolism of tracer T3 was similar in control and treated rats (table 3).

TABLE 1

RATE CONSTANTS GIVING OPTIMAL FIT FOR FIVE COMPARTMENT T3 MODEL

PROCESS	T3 RATES (X 10^3 per minute)	
	Control	TCDD-Treated
Uptake		
k_{21} (liver)	280 (252 - 302)	277 (260 - 296)
k_{31} (kidney)	98.0 (90 - 128)	101 (86 - 117)
k_{41} (carcass)	542 (500 - 703)	515 (510 - 751)
Recycling (CONSAM estimates only)		
k_{13} (kidney)	40.4	46.9
k_{14} (carcass)	30.5	31.3
k_{52}	368	434
k_{15}	770	641
Metabolism		
K_{05} (liver -> bile)	22.5 $\pm$ 6.5	22.9 $\pm$ 3.6
As approximately equal quantities of T2, T3, & glucuronide		
K_{03} (kidney -> urine)	3.47 $\pm$ 0.88	2.52 $\pm$ 0.40
K_{04} carcass residual)	0.75	0.92

TABLE 2

RATE CONSTANTS GIVING OPTIMAL FIT FOR FIVE COMPARTMENT T4 MODEL

PROCESS	T4 RATES (X 10^3 per minute)	
	Control	TCDD-Treated
Uptake		
k_{21} (liver)	21.1 (11.6 - 34.1)	32.2 (15.6 - 44.7)
k_{31} (kidney)	7.28 (2.20 - 4.10)	7.58 (1.40 - 6.50) *
k_{41} (carcass)	94.1	122.0
Recycling (CONSAM estimates only)		
k_{13} (kidney)	97.9	85.3
k_{14} (carcass)	29.1	43.03
k_{52}	247.0	206.0
k_{15}	35.8	78.7
Metabolism		
K_{05} (liver -> bile)	7.99 $\pm$ 1.70	19.23 $\pm$ 0.27
glucuronide	2.23 $\pm$ 0.45(30 %)	10.70 $\pm$ 3.3(60 %)
K_{03} (kidney -> urine)	1.11 $\pm$ 3.9	1.78 $\pm$ .61
K_{04} (carcass/residual)	2.19	3.87

* CONSAM estimates provided better fit, original estimates were based on very low counts (about 2 X background).

TABLE 3

Metabolites of T3 and T4 in Bile and Urine after 6 Hours

T3 Disposition (% of dose/hr)

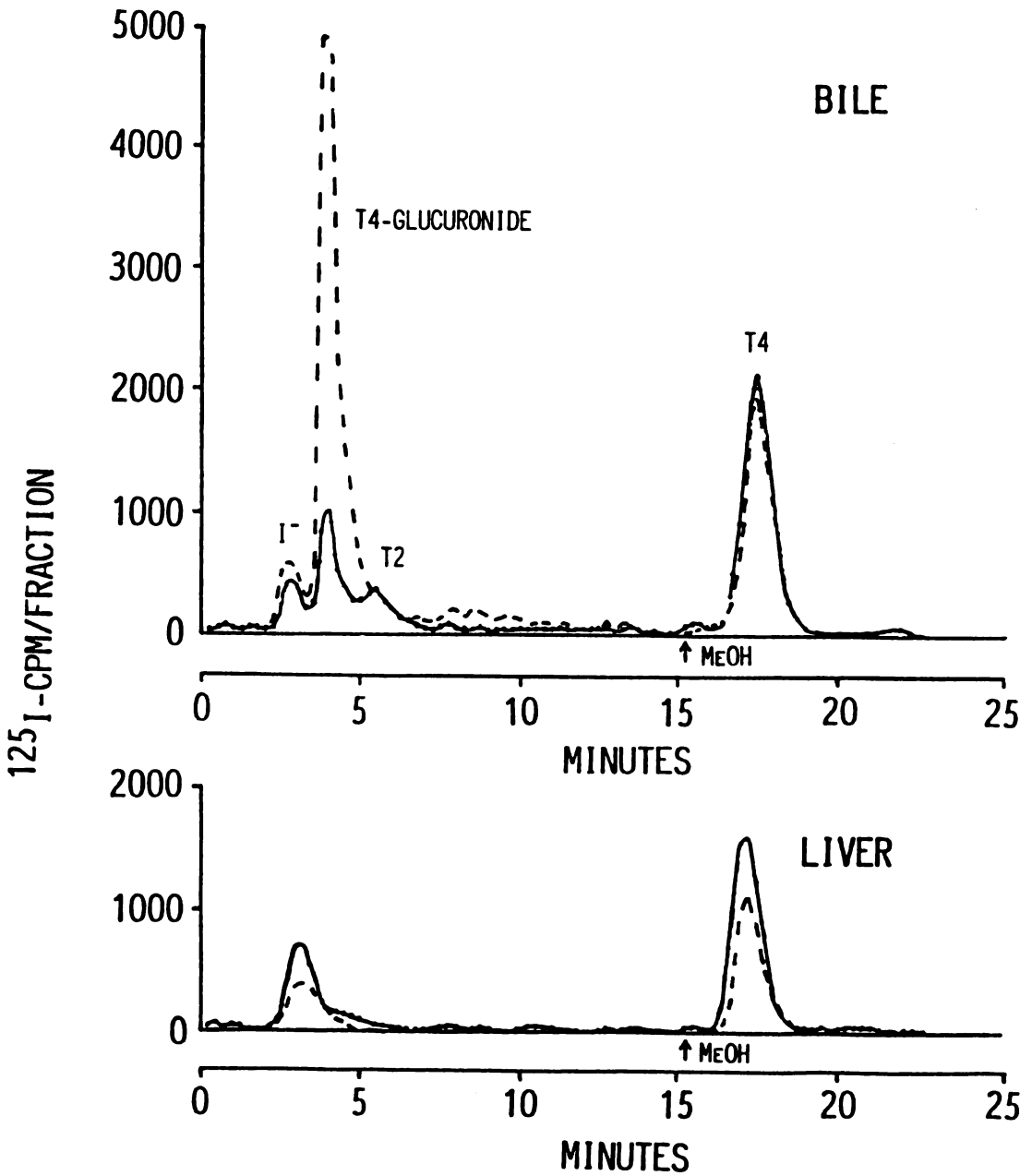
	Urine		Bile			
	I^-	T3	I^-	Glucuronide	T2	T3
Control	2.667	0.0256	0.329	0.547	0.431	0.445
± S.D.	1.636	0.0017	0.050	0.067	0.032	0.016
% Total	(89 %)	(5.4 %)	(10.9 %)	(38 %)	(30 %)	(32 %)
TCDD	5.619	0.0205	0.284	0.761	0.346	0.421
± S.D.	0.251	0.0038	0.004	0.086	0.004	0.021
% Total	(99 %)	(0.3 %)	(15.6 %)	(49 %)	(22 %)	(27 %)

T4 Disposition (% of dose/hr)

	Urine		Bile		
	I^-	T4	I^-	Glucuronide	T4
Control	1.634	0.0719	0.107	0.377	1.125
± S.D.	0.459	0.0339	0.0097	0.0098	0.011
% Total	(96 %)	(4.2 %)	(5.9 %)	(20.9 %)	(62.4 %)
TCDD	1.257	0.0909	0.127	1.092	1.125
± S.D.	0.708	0.0266	0.013	0.148	0.063
% Total	(93 %)	(6.7 %)	(5.3 %)	(45.6 %)	(41.3 %)

Figure 24

Upper figure : Chromatographic profiles of 100 ul bile samples from normal (——), and TCDD-treated (----) rats 6 hours after T4 tracer injection. Lower figure: Profile of 250 mg equivalent liver extracts obtained from the same animals as bile samples in the upper figure. For this figure fractions were collected every 15 sec. for 25 min. Mobile phase was 500:498:1, methanol:water:acetate, with 1 ml of methanol injected onto the column at fraction 64 to wash out T4. For routine separations, fractions were collected every 30 sec for 20 min, with methanol injection at fraction 32.


BILE
T4-GLUCURONIDE
T4
I⁻
T2
MeOH
LIVER
MeOH
5000
4000
3000
2000
1000
0
0
5
10
15
20
25
MINUTES
MINUTES
125I-CPM/FRACTION

A small increase in glucuronide metabolites was observed in the bile, but was less interesting than an apparent shift from a metabolite with a slightly longer retention time than T3 (triac ?) to T3 as a major product of beta-glucuronidase digestion (figure 25). Although Hearn et al. (62) have achieved separation of the thyroacetic acids from their thyronine counterparts using an acidic, unbuffered mobile phase, the difference in retention time between T3 and triac in our buffered system (pH 6) is only about 1 min. (18 vs 19 min. @ 1.5 ml/min.).

Our measurements of hepatic T3 produced by deiodination of T4 may reflect reports that type I deiodinase is a plasma membrane enzyme, rather than an enzyme contained in the endoplasmic reticulum or cytoplasmic compartment (24,75,79), for reasons described below.

Liver T3 is produced in a rapidly rising peak that lags, but falls off as quickly as does plasma T4 (figure 26). A pulse of iodide (and thus rT3, since the tracer is 3,5-labeled) appears at the same time, in agreement with reports that T3 and rT3 production rates are about equal.

If the deiodinase acted on T4 in the cytoplasmic compartment, T3 levels should follow the slowly rising and falling T4 levels in the liver. The results of simulations of this process using the integrated 10 compartment model (figure 27) are shown in figure 28. Attempts to model the pulse of T3 production by the addition of an endocytosis process to the ten (making it 11) compartment model were

Figure 25

(a) Chromatographic profile of bile samples of control rats 6 hrs after injection of T3 tracer, before (●—●), and after (O–O) incubation with beta-glucuronidase.

(b) Chromatographic profile of bile samples from TCDD-treated rats 6 hrs after injection of T3 tracer, before (●–●), and after (O–O) incubation with beta-glucuronidase.

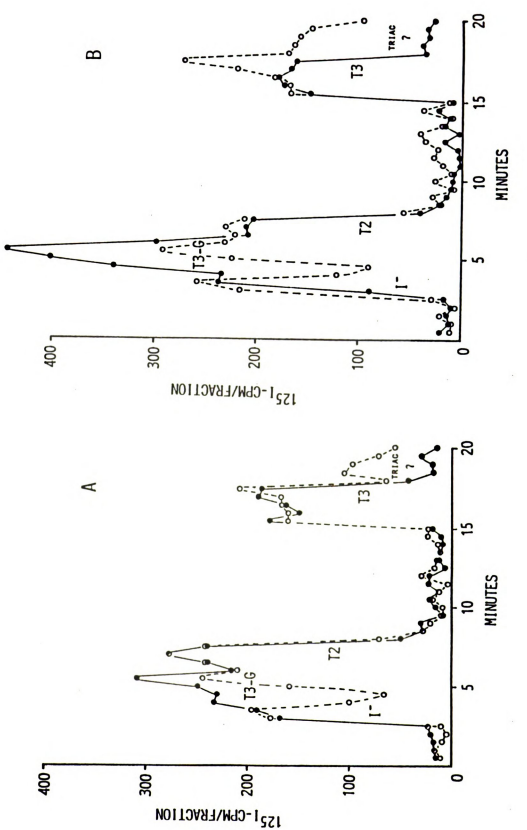

A
B
^{125}I-CPM/FRACTION
MINUTES
I⁻
T3-G
T2
T3
TRIAC
0
100
200
300
400
5
10
15
20

Figure 26

Hepatic uptake of T4, and production of T3 after injection of T4 tracer. Each symbol represents the average of samples from three rats. Controls are indicated by open symbols, TCDD-treated rats by filled symbols. Note that both I^- and T3 have maxima at 15 minutes. The peak for uptake of plasma T3 tracer occurs at 5-6 min (figure 22). Data for iodide in controls has been omitted for clarity.

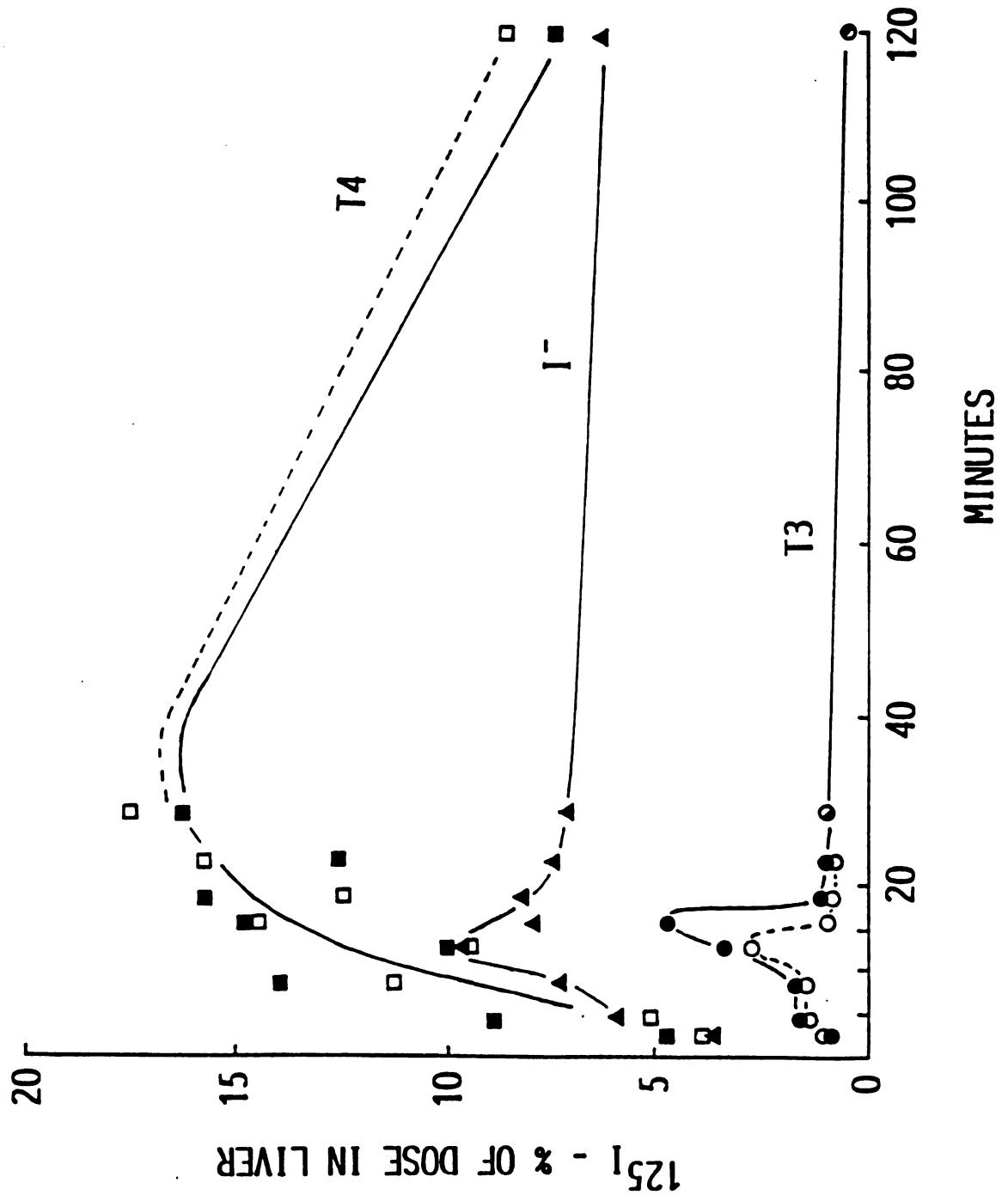

T4
I⁻
T3
MINUTES
0
20
40
60
80
100
120
5
10
15
20
125I - % OF DOSE IN LIVER

Figure 27

Integrated model for production of T3 from T4 . Compartments 1 -> 5 are associated with T4 uptake and metabolism, 6 -> 10 are assigned to T3 metabolism. Dashed lines labeled with the rate constant k_{di} represent deiodination pathways from T4 -> T3. Other dashed lines represent routes of hormone inactivation.

TEN COMPARTMENT MODEL OF T4 AND T3 METABOLISM

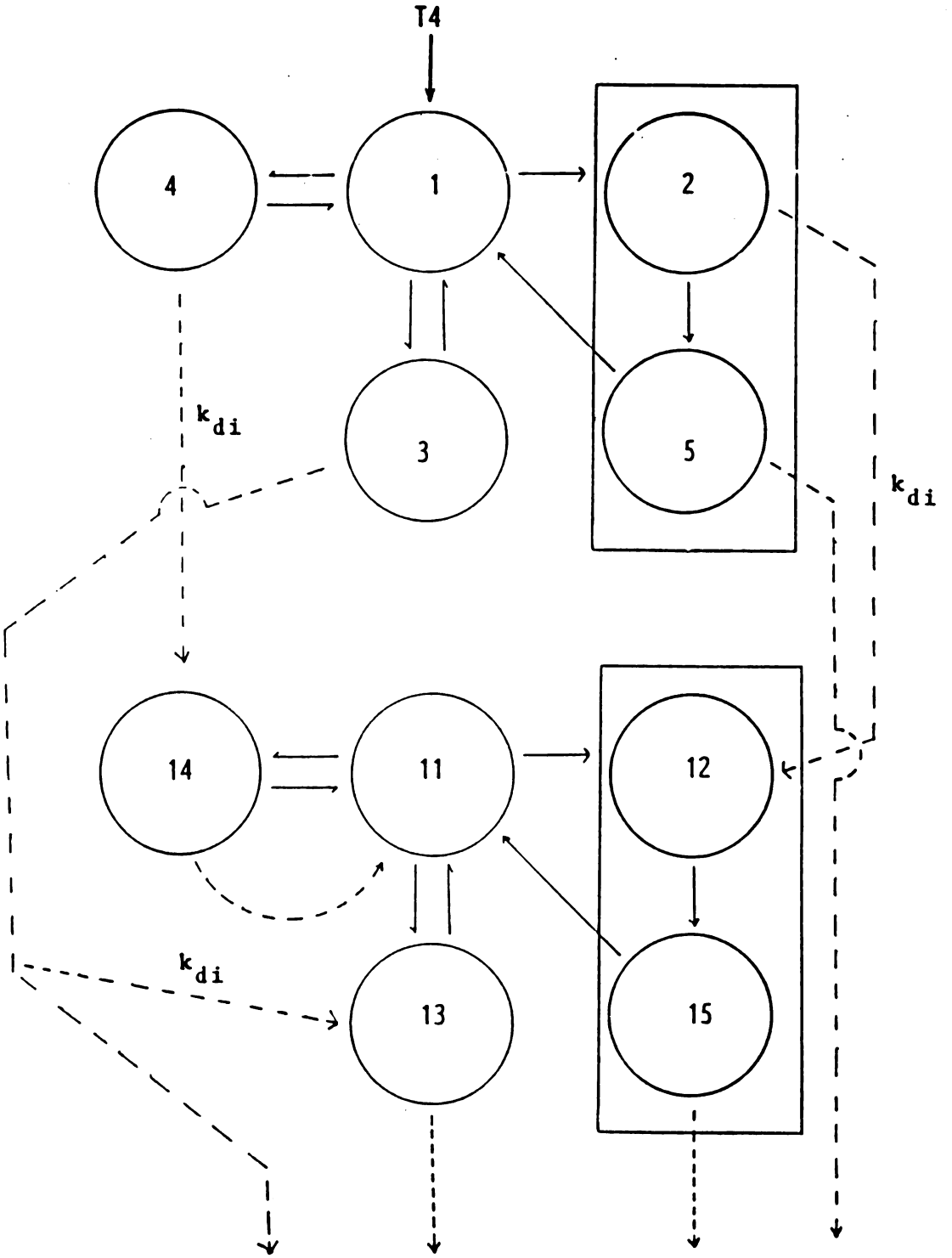
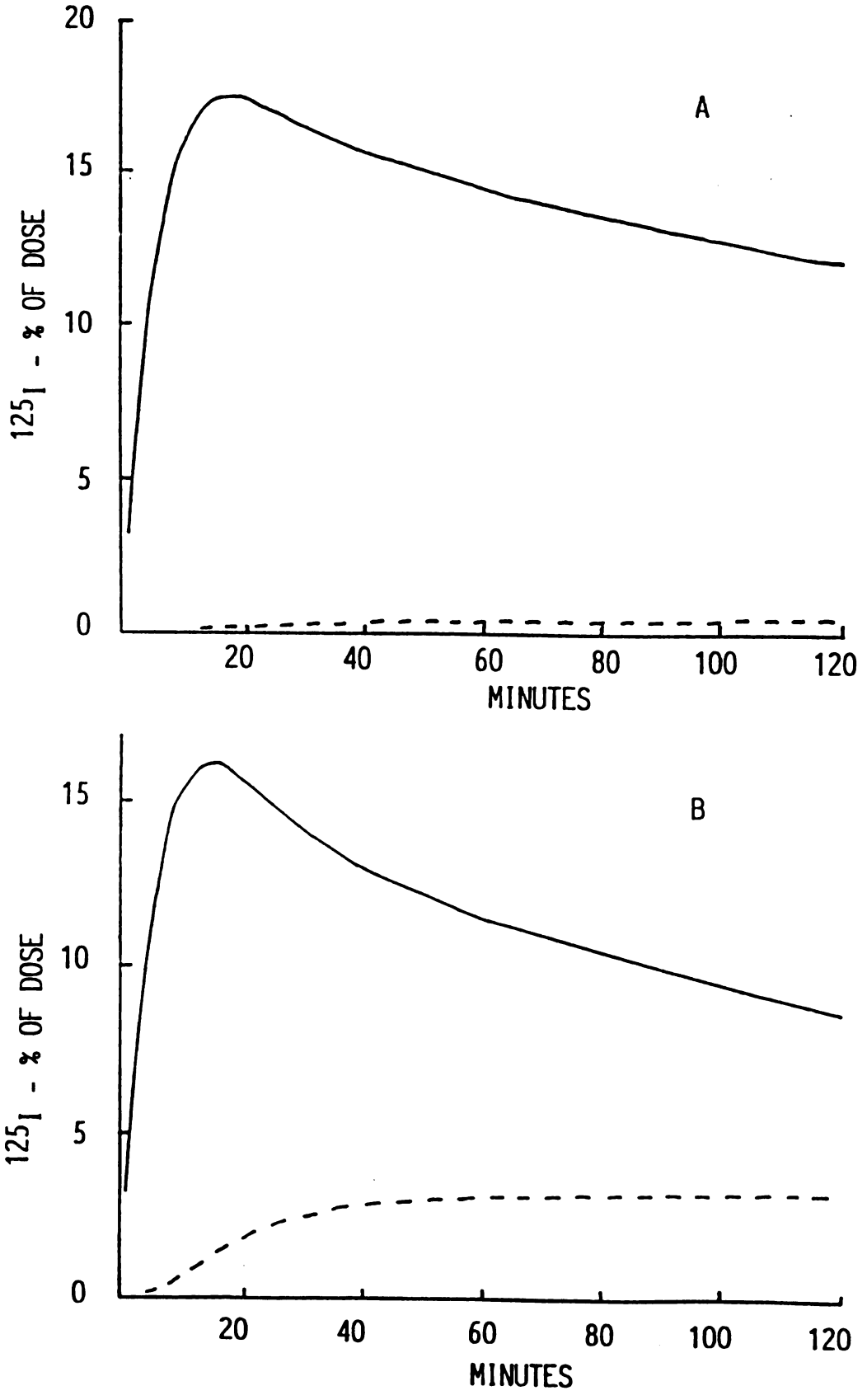


Figure 28

Predicted levels of T4 (——) and T3 (- - - -) in the liver assuming that cytosolic T4 is the deiodinase substate. Rate constants used to simulate T3 production were Vmax = 438 fmol/min-mg protein and Km = 3.6 uM (ref. 68) A first order mass rate constant was computed for the whole liver using these values (appendix A). The final rate constants for deiodination were :

A) k_{di} = 0.0027/min B) 10 X (A), k_{di} = 0.027

Note the change in the T4 curve caused by the increased deiodination rate in (B) relative to (A).


20
15
10
5
0
^{125}I - % OF DOSE
A
20
40
60
80
100
120
MINUTES
15
10
5
0
^{125}I - % OF DOSE
B
20
40
60
80
100
120
MINUTES

unsuccessful, presumably because of insufficient information about intracellular transport of T3.

Although the T3/T4 ratio was much higher in the kidney than in the liver, a pulse of T3 production like that observed in the liver was not observed (figure 29).

Very little T3 or T4 was excreted by the kidney . The only significant metabolite of these compounds observed in the urine was I^- (figure 30), in agreement with earlier studies of thyroid hormone metabolism (41).

DISCUSSION

Many biochemical, physiological, and histological changes are associated with exposure to Ah-inducers (34). Most of these changes have been observed only at doses of these compounds which produce gross pathology accompanied by anorexia. Thus, from the literature, it is difficult to distinguish between primary effects of Ah locus enzyme induction, and effects which are secondary to the anorexia, fatty liver, and other pathology which occurs at high doses. One effect which was thought to be well explained by induction of these enzymes was the drop in plasma T4 following Ah inducer treatment. Several investigators have reported increased fecal and/or bile excretion of thyroxine, accompanied by a 50-75 % drop in serum T4, in rats treated with various Ah-inducers (8,48,89). It was assumed that the change in T4 levels was a secondary result of the induction of glucuronosyl transferase .

Figure 29

Uptake of T4, production of T3 and I^- in kidneys of control (●—●), and TCDD-treated (○—○) rats following tracer injection. The arrow indicates the time at which a pulse of T3 production occurred in the liver.

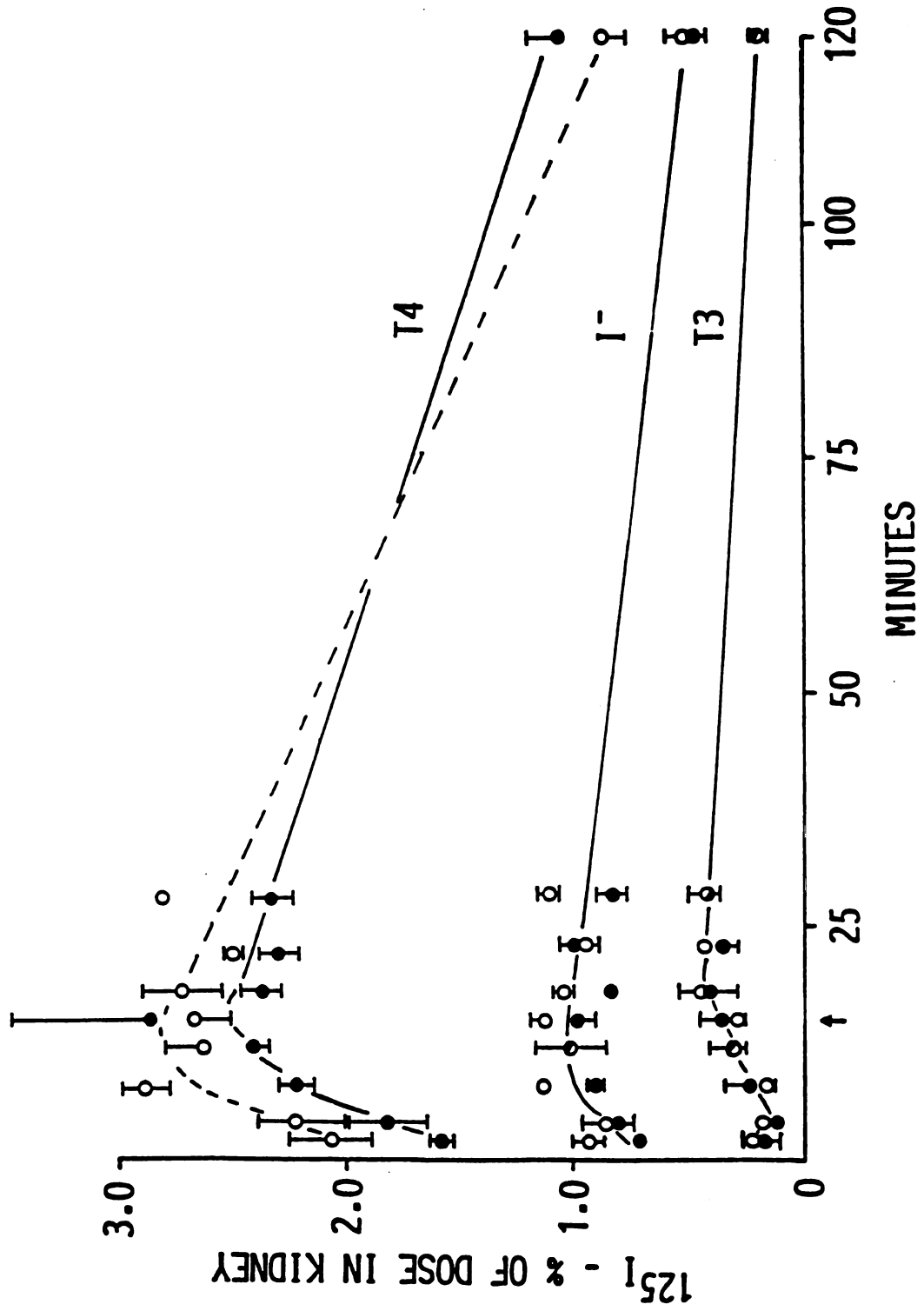
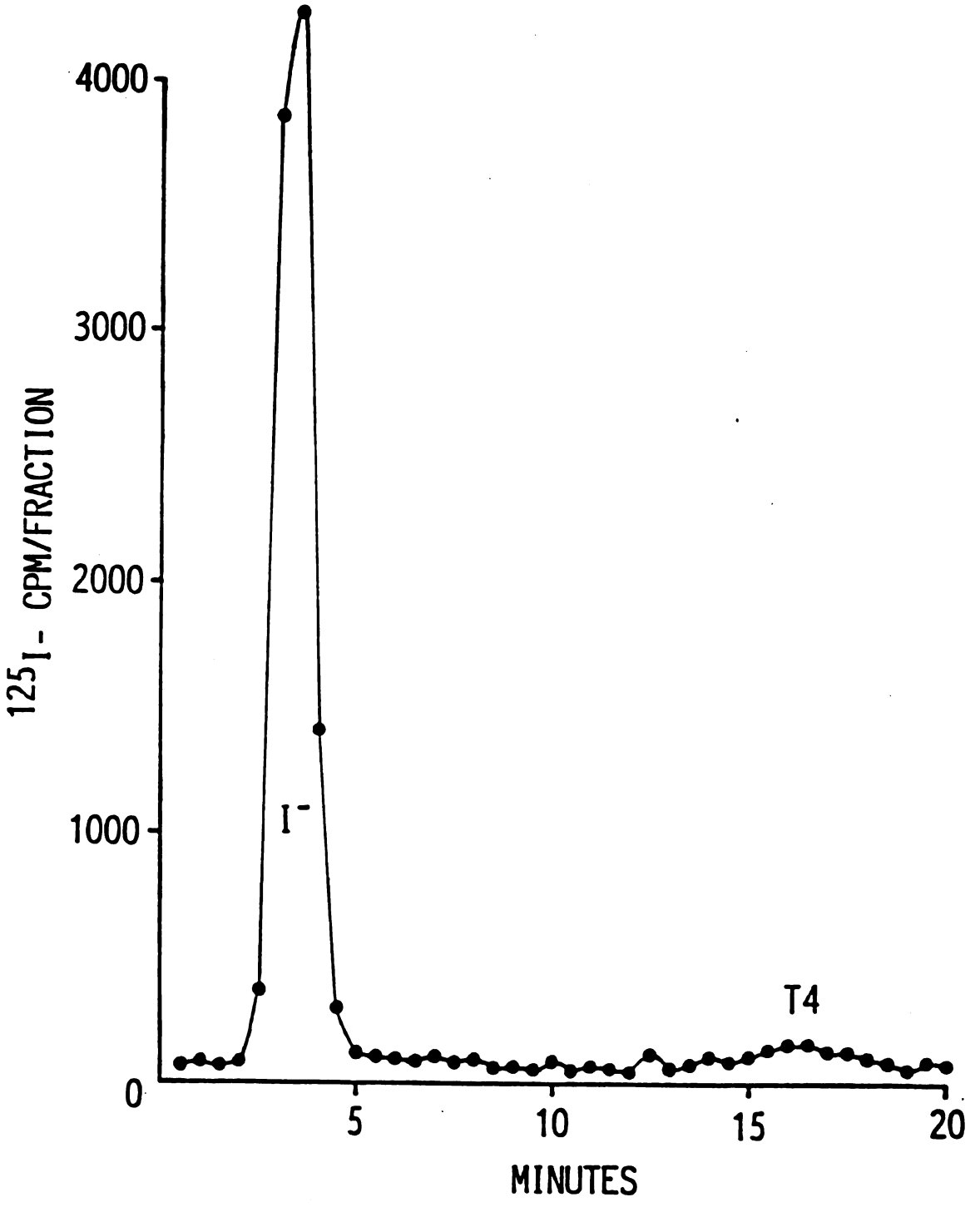

^{125}I - % OF DOSE IN KIDNEY
3.0
2.0
1.0
0
25
50
75
100
120
MINUTES
T4
I^-
T3

Figure 30

Chromatographic profile of urine from a control rat 6 hr after injection of T4 tracer. No significant difference was observed between urine from control and TCDD-treated rats. The urinary profiles following T3 injection were qualitatively similar to those for T4. Mobile phase for urine was methanol:water:acetate, 575:452:1 .


4000
3000
2000
1000
0
^{125}I- CPM/FRACTION
I⁻
T4
5
10
15
20
MINUTES

However, these studies were all performed at inducer doses which result in anorexia, fatty liver, and other gross pathology. As reported in studies from our laboratory (111) and others (78,103), induction of cytochrome p-450c and the glucuronosyl transferases is maximal at a TCDD dose of 10 nmol/Kg. Food consumption and body weight gain are normal at this dose, and no acute morbidity is observable.

Nonetheless, we have shown (111) that plasma T4 drops by 40-50 % at this dose. Liver concentrations drop by 10-20 %, and fall precipitously at higher doses (chapter 2). In the present study we have shown that glucuronidation is already as high at 10 nmol/Kg as reported by others at larger doses, suggesting that the continuing drop in T4 at higher doses is brought on by anorexia, rather than by a further increase in glucuronidation. We propose that the high rate of thyroxine disposal creates a moderate iodine deficiency, which is aggravated by the reduced iodine intake and negative protein balance (including tyrosine) associated with anorexia. A lower thyroxine production rate might also account for the failure of earlier studies to find the large amount of free T4 excreted in the bile with T4 glucuronide.

The appearance of a pulse of T3 with a magnitude proportional to the specific activity of T4 in the plasma, rather than being a constant fraction of the tracer dose, indicates that a constant amount of T4 was converted to T3 rather than an amount which was proportional to the total

flux of tracer into the liver - which was the same for control and TCDD-treated rats.

We suggest that liver deiodination is controlled by a component of the plasma to liver T4 flux which is constant, rather than by the total T4 flux or liver T4 concentration. Such a scheme would explain the ability of our animals to maintain T3 levels as T4 concentrations are dropping. The net T4 flux from plasma to liver is actually larger in TCDD treated rats than controls, as evidenced by the bile disposal rate. It has been reported that both T3 and T4 are transported by two parallel uptake systems:
1) a high affinity, low capacity system that can be inhibited by colchicine, ouabain, and cyanide, which is saturated well below physiological concentrations of T4, and (2) a low affinity, high capacity system which is not saturated at 10 X the normal physiological levels of T3 or T4 (42,56,73). The high affinity system is thought to represent receptor-mediated endocytosis of thyroid hormones (23). The lag time for appearance of the pulse of T3 which we observed in liver is similar to the time required for endocytosis of epidermal growth factor (22) and lysosomal marker proteins (76). The low affinity, high capacity system probably represents passive diffusion of thyroid hormones through the plasma membranes driven by their lipophilic character (59,98). Leonard et al. (75) suggested that the plasma membrane location of the deiodinase "...may represent a physiological mechanism to allow efficient

production of... T3, from an intrinsically inactive precursor T4, without the obligatory penetration by T4 of the intracellular space." Our results indicate that an association of the high affinity uptake system and the plasma membrane 5'-deiodinase might serve to deliver a controlled flux of T4 to the deiodinase, while excluding the much larger flux of T4 which passively diffuses into the cell.

Control of T3 production from T4 is an area of controversy. DiStefano et al. have recently found that as much as 75 % of the total mass of T3 in the rat is contained within the intestinal lumen, only 1/6 of which is conjugated (41). T4 does not accumulate in the intestine, but an interesting transformation does occur. Of the T4 entering the intestine from the bile, 30-50 % is conjugated in a normal rat. During its passage through the intestine, most of this glucuronide is hydrolysed by bacterial beta-glucronidase, and can be partially resorbed as T4 (27), rather than simply excreted, as our model and many others have assumed. T3 is even more readily absorbed, and the large T3/T4 ratio in the intestine relative to the plasma (1:2 vs. 1:50 in plasma) suggests that deiodination of T4 to T3 could be occuring in the lumen, although deiodination in the intestine has yet to be shown. Even if the large amount of T3 in the intestine is simply the result of the high rate of disposal of T3, the existance and recycling of this large, previously ignored pool of T3 dramatically

differs from accepted models for thyroid hormone regulation. Our results suggest an association between two processes whose properties, independently, could not explain the behavior of the integrated control system in vivo. While our results are not conclusive, the association we suggest between uptake and deiodination better explains the relationship between T4 concentrations and T3 production observed in animals treated with Ah-inducers, than does the model assuming independence of uptake and deiodination, and may provide a rationale for the the plasma membrane location of the 5'-deiodinase.

SUMMARY

SUMMARY

TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin) is the most toxic of a class of polycyclic aromatic hydrocarbons which cause the induction of enzymes associated with the Ah locus. This set of enzymes includes cytochrome P-450c, P-450d, at least one glucuronosyl transferase, glutathione-S-transferase, and other enzymes which have not been as well characterized.

Some of the effects of Ah inducers on metabolism appear to be explainable as secondary effects of the induction of the Ah locus enzymes mentioned above. Although these enzymes were discovered by virtue of their catalytic activity towards carcinogenic compounds such as benzo[a]-pyrene and 3-methylcholanthrene, they are known to hydroxylate and or conjugate fatty acids, steroids, and thyroxine (T4).

Since (T4) conjugation and excretion was known to be increased on treatment with Ah-inducers, we attempted to determine whether either plasma or liver T3 levels dropped with T4. Our initial experiments demonstrated that T3 levels did not change with T4 levels.

Since studies of T3 production and metabolism *in vitro* (liver and kidney homogenates or microsome suspensions) indicated that deiodination of T4 to produce T3 was linearly dependent on T4 concentration, we decided to perform experiments *in vivo* to determine whether transport

mechanisms destroyed by tissue homogenization could explain the discrepancy between in vitro and in vivo results.

In pharmacokinetic studies, T3 was produced from T4 in the liver in a pulse which lagged T4 uptake by 4 - 5 min. The relative size of this pulse in treated vs control rats was similar to the ratio of specific activities for T4 between the respective groups. In light of reports on the passive and active uptake of T3 and T4, this data suggested that deiodination of T4 is regulated by a saturable uptake system which runs in parallel with a nonsaturable, diffusive uptake of T4, driven by its hydrophobicity.

Our results suggest an association between two processes whose properties, independently, could not explain the behavior of the integrated control system in vivo. While our results are not conclusive, the association we suggest between uptake and deiodination better explains the relationship between T4 concentrations and T3 production observed in animals treated with Ah-inducers, than does the model assuming independence of uptake and deiodination, and may provide a rationale for the the plasma membrane location of the 5'-deiodinase.

While attempting to determine the thyroid status of Ah-inducer treated rats, we discovered that liver malic enzyme (normally regulated by insulin, glucagon, glucocorticoids, and thyroid hormones) was increased by Ah-inducers in a thyroid hormone dependent manner. The induction of malic enzyme by glucocorticoids is thyroid hormone dependent, ie.

thyroid hormones must be present for induction to occur. Certain symptoms of Ah-inducer toxicity, such as thymic involution, hyperlipidemia, and fatty liver development can also be produced by glucocorticoid administration, and might be explained if Ah-inducers had corticomimetic properties in addition to their interactions with the Ah receptor. For these reasons, we performed experiments to determine whether TCDD could act as a ligand for the glucocorticoid receptor. These experiments demonstrated no affinity of TCDD for the glucocorticoid receptor, but left open the possibility that the Ah receptor may be able to regulate some of the same genes as does the glucocorticoid receptor.

APPENDIX A

ESTIMATION OF T4 ACTIVE UPTAKE AND DEIODINATION RATES FOR T3 PRODUCTION MODELS.

ESTIMATION OF T4 ACTIVE UPTAKE AND DEIODINATION RATES FOR T3 PRODUCTION MODELS.

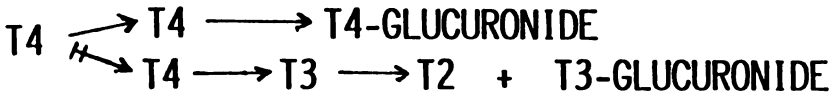
Studies of T3 production from T4 have generally have been performed in homogenous mixtures of 5'-deiodinase, T4 , and glutathione or a synthetic thiol supplied in vitro.

Michealis-Menten rate constants have been determined using whole liver homogenates , aqueous suspensions of liver or kidney microsomes, or cytosol-microsome mixtures. The maximal velocities (Vmax) determined in these preparations are generally very low : 0.2-2.0 pmol/min. The half-saturation concentrations (Km) have been very high(1-5 uM), relative to the physiological concentrations of T4. Because of the high Km values, the observed deiodination rates are essentially first-order with respect to T4 concentration.

Since specific steps in the metabolism of thyroid hormones occur in specific tissues and/or discrete subcompartments of these tissues, the precise relationship between steady-state T4 and T3 concentrations in vivo cannot be predicted from in vitro deiodination rates alone.

Crabtree and Newsholme (29) have developed nomenclature and a theoretical treatment for the control of metabolism which describe the fluxes of substrates and metabolites in multi-compartment/multienzyme systems, and have shown that these fluxes, rather than concentrations of metabolites, are the controlling variables in complex metabolic systems. Using

their notation, the metabolism of T4 can be described as follows :



In the above scheme, each arrow represents the flux of metabolites from one compartment to another or its conversion into a new metabolite. The symbol ⊢⟶ indicates a process which is flux limited, ie. in terms of Michealis-Menten kinetics, the enzyme (or in this case, transporter) is saturated. Thus beyond a certain concentration of T4 (about 5 nM using the data from Doctor, reference 42) the flux from T4 in the plasma to the membrane and to T3 is constant. Since both deiodination and glucuronidation/-catabolism are dependent on the fluxes of T4 and T3 provided by the parallel passive and active transport systems, analysis of in vivo production data requires the determination of these transport rates before an integrated model of the process can be developed. We determined total liver uptake rates (diffusive + active) from our tracer data, but did not measure saturable uptake rates. We therefore used literature data to estimate the ratio of active to passive T4 uptake, and compared these values with the observed T3 production as described below.

Determination of the Uptake of T4
by the High Affinity System

According to Doctor et al.(42) the uptake of T4 by cultured parenchymal cells can be described as the sum of two processes represented with Michealis-Menten kinetic constants as

$$v = \frac{Vmax_1[T4]}{Km_1 + [T4]} + \frac{Vmax_2[T4]}{Km_2 + [T4]}$$

where the subscripts denote the high and low affinity systems, respectively. For the low affinity system, Km = 500 nM, and Vmax = 1.9 nmol/.1488 gm-min. Since the concentration of T4 in the plasma of a normal rat is about 50 nM, the transport rate would be

$$\frac{v}{Vmax} = \frac{50 \times 10^{-9}}{500 \times 10^{-9} + 50 \times 10^{-9}} = 0.0909$$

which indicates that the velocity is well below saturation with substrate, and will behave as a first-order process. However, this number cannot be used directly as a rate constant to predict behavior of this system in vivo. Given a liver weight of 12.5 gm/300 gm rat, Vmax = 159 nmol/liver-min. Substituting this value to determine the uptake velocity gives

v = 0.0909*Vmax or v = 14.45 nmol/liver-min.

The total plasma pool (8 ml) contains only 416 pmol of T4 !

High Affinity System

Similar calculations can be performed using rate constants for the high affinity system :

$$Km = 900 \text{ pM} \qquad Vmax = 3.2 \text{ pmol/0.1488 gm}$$

$$\frac{v}{Vmax} = \frac{50 \times 10^{-9}}{0.9 \times 10^{-9} + 50 \times 10^{-9}} = 0.982$$

Note that the velocity has almost reached the maximal velocity (v/Vmax = 1.00 when the enzyme is saturated). Again using a liver weight of 12.5 gm/300 gm rat, the forward velocity is v = 264 pmol/liver-min. As before, this number is too high, but the ratio of the low and high affinity systems can be used with in vivo total rates to estimate fractional rates.

In rats treated with 10 nmol TCDD/Kg-BW, the concentration of T4 in the plasma is about 32 nM. When this value is substituted into the velocity equation for high affinity uptake, very little change in rate is observed :

$$\frac{v}{Vmax} = \frac{32 \times 10^{-9}}{0.9 \times 10^{-9} + 32 \times 10^{-9}} = 0.9726$$

To get a 30 % drop in T4 uptake by this system, the plasma T4 concentration must drop to about 2 nM. The passive uptake of T4 by the low affinity system meanwhile, is dropping linearly with T4 concentrations. These values were determined by Doctor et al. in an incubation medium containing 1 % albumin. In later work (72), it was shown

that albumin concentration was critical to the transport of T3 by its high affinity uptake system. Increasing the albumin concentration raised the Vmax 5-fold. Increased albumin raised the rate for low affinity (passive) uptake only about 2-fold. Increases in temperature had a similar effect - the high affinity system uptake increased 3-fold between 25 and 37°C, while passive uptake was increased 1.24-fold. Assuming that the T4 high affinity system responds to these parameters in the same way as the T3 system, the ratio between the two under physiological conditions can be estimated :

Low Affinity System @ 37° + 20 mg albumin/ml :

$$\frac{14.45 \text{ nmol}}{\text{min}} \times 2 \times 1.24 = \frac{35.8 \text{ nmol}}{\text{min}}$$

High Affinity System @ 37° + 20 mg albumin/ml :

$$\frac{264 \text{ pmol}}{\text{min}} \times 5 \times 3 = \frac{3.962 \text{ nmol}}{\text{min}}$$

which implies that high affinity uptake of T4 is about 11 % of the total uptake in vivo.

Since the concentrations of T4 in both normal and treated animals are at steady-state during the experiments (as opposed to the concentrations of ^{125}I-T4), the uptake velocities are constant with respect to the T4 pools. In the high affinity transport process, uptake of T4 is a constant percentage of the total mass of tracer in the plasma pool. Since the amount of tracer is declining during the experiment, uptake of tracer is the product of % high

affinity uptake and the % of Dose remaining in the plasma pool. The specific activity of tracer in TCDD treated animals is higher than in normal rats (since total T4 is lower), hence the amount of tracer taken up when a constant mass of T4 is transported will be greater for TCDD treated animals than controls if the system is unaffected by TCDD. This effect does not occur with the passive system since it is operating well below saturation, and all uptake is described by the equation

$$\frac{dX_j}{dt} = k_{ji}X_i$$

X_i = mass of ^{125}I-T4 in plasma compartment

X_j = mass of ^{125}I-T4 in tissue compartment

Specific activity calculations indicate that the high affinity uptake of tracer (but not total T4) would be 1.62 X controls, using known plasma T4 concentrations :

Normal 52 nM X 8 ml plasma = 416 pmol/pool

TCDD 32 nM X 8 ml plasma = 256 pmol/pool

328 fmol tracer @ 4400 Ci/mmol injected

implies

Specific Activity (SA) normal = 0.07878 % of total T4

Specific Activity (SA) TCDD = 0.12796 % of total T4

or SA TCDD = 1.624 X SA normal

Although the total ^{125}I-T4 uptake was the same in treated and control rats, as expected from the above analysis, with passive uptake predominating, the pulse of tracer T3 which appeared in the livers of TCDD-treated rats (figure 26) was

1.5 - 2.0 X larger than that for controls, as would be expected if the same mass of T4 was deiodinated, but with the higher specific activity in treated animals outlined above.

The ratio of active/passive uptake estimated above was used with the total uptake rate determined for the five compartment sub-model for tracer T4, in an attempt to simulate the pulse of T3 production using a modification of the 10 compartment model. The high affinity uptake was modeled as an endocytosis process with the active component comprizing 11 % of the total uptake in controls, and 1.62 X 11 % = 17.82 % of the total T4 uptake in treated rats. A pulse of T4 uptake could be obtained with the same delay as was observed in the data, but T3 did not fall off rapidly after the pulse maximum as did the observed T3 levels. At this point it was clear that there was insufficient information available about the mechanisms of T3 transport in the cytoplasm and nucleus to intelligently modify the ten compartment model so that the T3 production pulse could be fitted.

Derivation of a First-Order Deiodination Rate From in vitro Data for the in vivo Model.

First-order deiodination rates for whole liver (as opposed to a Michealis-Menten rate in mass/mg-protein units) were derived in the same fashion as the transport rates derived above. The most complete set of in vitro Michealis-Menten

rate constants found in the literature were those of Kaplan (reference 69). These rate constants were obtained in studies of deiodination of T4 to T3 in liver homogenates and microsomes from rats kept in hypothyroid, euthyroid, or hyperthyroid states, respectively. Homogenization of these livers resulted in a 1:3 dilution of the original liver activity. Computation of the activity in the whole liver was determined by using this dilution factor along with the protein concentrations determined for whole homogenates (45 mg/ml), and microsomal suspensions (5.7 mg/ml when diluted to the starting homogenate volume). Correcting these values for dilution gives

45 mg protein/ml X 4 = 180 mg protein/gm liver
(total homogenate)
5.7 mg/ml X 4 = 22.8 mg protein/gm liver (as microsomes)

For euthyroid rats, the Vmax and Km determined in the microsomal mixture were

Vmax = 438 fmol T3/mg-min Km = 3.6 uM

Using this Vmax value, the estimated microsomal protein concentration in the liver, and the liver mass for a 300 gm rat (12.5 gm/liver), a maximal velocity for the whole liver was computed :

Vmax = (438 fmol T3/mg-min)(22.8 mg/gm)(12.5 gm/liver)
= 124 pmol T3/min-liver

The expected velocity at the physiologic T4 concentration (18 nM in liver - chapter 3) is then computed from the

Michealis-Menten expression

$$v = \frac{d[T3]}{dt} = \frac{Vmax[T4]}{Km + [T4]} = \frac{(124\ pmol/min)[18\ nM]}{3600\ nM + [18\ nM]}$$

$$= 617\ fmol/min\text{-}liver$$

Since [T4] is at steady state in a living animal, this velocity does not change. Changes in tracer activity are not indicative of changes in reaction velocity as the tracer concentration changes.

For this reason a first-order mass rate constant can be computed from the above whole liver reaction velocity, since the mass of T4 in the liver is known :

Liver T4 mass = 18 nM X .0125 liter (12.5 gm liver)

= 225 pmol (control)

The mass balance equation for the deiodination reaction is

$$v = \frac{dX_2}{dt} = k_{deiodination} \cdot X_1$$

where X_1 = mass of T4 in liver.

X_2 = mass of T3 in liver

Therefore the mass rate constant, $k_{deiodination}$ is

$$k_{deiodination} = v/X_1 = \frac{617\ fmol/min\text{-}liver}{225\ pmol}$$

$$= 2.74 \times 10^{-3}/min\text{-}liver$$

This value was used in the ten compartment model of thyroid hormone metabolism to simulate T3 production, configured so that cytosolic T4 was the 5'-deiodinase substrate

(figure 28). Rate constants for uptake, recycling, and metabolism were determined by fitting tracer data to the 5-compartment sub-models for T3 and T4, as described in chapter 4.

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