ABSTRACT

STUDIES ON THE RELATIONSHIPS OF TCDD TOXICITY AND VITAMIN A

By

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The effect of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and similarly acting compounds on the mobilization and transport of vitamin A, the depletion of hepatic retinoids and the accumulation of retinoids in the kidneys of Sprague-Dawley rats were investigated in several different experiments. In addition, the effect of the thyroid hormone T3 on the above parameters was also investigated. TCDD was determined to be a non-competitive inhibitor of hepatic retinyl palmitate hydrolase, (RPH). Inhibition of RPH in vivo, by treatment with either nonadecafluorodecanoic acid or 3,4,3',4'-tetrachloro-biphenyl resulted in lowered serum retinol levels. Inhibition of RPH was determined not to be of toxicological significance in the case of TCDD treatment. The retinoid material which accumulated in the kidney of TCDD-treated rats was determined to be retinyl esters. The activity of renal acyl CoA retinyl:acyl transferase (ACARAT) was elevated, and correlated with the levels of retinyl esters. A similar accumulation and enzyme induction was observed in rats fed a vitamin A deficient diet. TCDD treatment increased the rate of hepatic retinoid degradation caused

by microsomal retinol oxidase, and retinoyl UDP-glucuronosyl transferase. Oxidation of retinal to retinoic acid and microsomal oxidation of retinoic acid were unaffected by treatment with TCDD. Inclusion of T3 in the diet of rats treated with TCDD enhanced the toxic response, and also increased the depletion of hepatic retinoids and accumulation of retinyl esters in the kidneys of treated rats.

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ACARAT.....Acyl CoA:Retinol Acyl Transferase
AHH.....Aryl Hydrocarbon Hydroxylase

BNF.....Beta-Napthoflavone

C.....Celsius
CRABP.....Cellular Retinoic Acid Binding Protein
CRBP.....Cellular Retinol Binding Protein
CoA.....Coenzyme A

DT.....Dithiothreitol
Dal.....Daltons
DPPD.....N,N'-diphenyl-p-phenylene-diamine

EDTA.....Ethylene Diamine Tetraacetate
em.....Emission
ex.....Excitation

Fig. .....Figure

g.....Gram

HCB.....Hexachlorobiphenyl
HPLC.....High Pressure Liquid Chromatography
hr.....Hour
i.p. .....Intraperitoneal

Kd.....Coefficient of Dissociation
K_I.....Coefficient of Inhibition
Kg.....Kilogram
Km.....Michalis-Menton Coefficient

LPC.....Lipoprotein Complex
LD_{50}.....Lethal Dose, (50%)

M.....Molar
MC.....3-Methylcholanthrene
Me-NDFDA.....Methyl Nonadecafluorodecanoate
min.....Minute
mg.....Milligram
ml.....Millilitre
mM.....Millimolar
mm.....Millimeter
MOPS.....3-(N-morpholino)-propane-sulfonic acid

NDFDA.....Nonadecafluorodecanoic Acid
nmol.....Nanomole
nm.....Nanometer
PAH.....Polyhalogenated Aromatic Hydrocarbon
PCB.....Polychlorinated Biphenyl
pg.....Picogram
PGE.....Phosphate-Glycerol-EDTA
PNP.....(Para) Nitrophenol

r.....Correlation Coefficient
RBP.....Retinol Binding Protein
RPH.....Retinyl Palmitate Hydrolase

SD.....Standard Deviation
S.E. .....Standard Error (of mean value)

T3.....Triiodothyronine
T4.....Thyroxine
TCAB.....3,4,3',4'-Tetrachloroazobenzene
TCB.....3,4,3',4'-Tetrachlorbiphenyl
TCN.....2,3,6,7-Tetrachloronaphthalene
TCDD.....2,3,7,8-Tetrachlorodibenzo-p-dioxin
TCDF.....2,3,7,8-Tetrachlorodibenzofuran
TTR.....Transthyretin

UDP.....Uridine 5' Diphosphate
UDPGT.....Uridine Diphosphate Glucuronosyl Transferase
ug.....Microgram
ul.....Microliter
uM.....Micromolar

v.....Volume
VA.....Vitamin A
Vmax.....Maximum Velocity

INTRODUCTION

TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin, dioxin) is often described as the most toxic man-made compound yet identified. This chemical has been implicated as the toxic species in a number of serious incidents of environmental contamination. TCDD also represents the archetypical species of an ubiquitous group of environmental contaminants known as the polyhalogenated aromatic hydrocarbons (PAH's).

Despite the fact that TCDD and the PAH's have been recognized as the most significant environmental contaminants yet identified in the biosphere, there has been little progress made in the determination of the mechanism by which these chemicals are toxic. The toxic symptoms caused by these compounds have been well described and characterized, and the ability of many of the structurally diverse PAH's to cause essentially the same toxic response in treated animals has also been clearly demonstrated.

Much of the research reported to date has focused on the ability of TCDD and the PAH's to bind to a specific cytosolic receptor, resulting in activation of a particular enzyme locus. Structure-activity relationships suggest that the ability for a compound to bind to the receptor

correlates with its ability to cause TCDD-type toxicity. However, despite intensive investigation, no clear linkage between increased activity of any of the induced enzymes, and a plausible mechanism for toxicity has been suggested.

The research presented in this thesis has grown out of the observation that the characteristic signs of TCDD-type toxicity are very similar to those expressed by animals suffering from the advanced stages of a vitamin A deficiency. Further, it has been demonstrated that TCDD and similarly-acting compounds cause a depletion of vitamin A levels in treated animals, thereby supporting the hypothesis that some aspects of TCDD-type toxicity are caused by a vitamin A deficiency. The mechanism by which this depletion of vitamin A may be caused by TCDD has become the central focus of this research.

This topic, while seemingly straightforward, has nevertheless proven difficult to evaluate in terms of vitamin A metabolism, and the effects of TCDD on that metabolism. Indeed, one bit of common ground that exists between research on TCDD and on vitamin A is that, despite years of in-depth study, there has been no clear mechanism of action elucidated for either chemical, with the sole exception of the role of retinol/retinal in the visual cycle. Therefore, research on the mechanism by which TCDD affects vitamin A metabolism becomes, to an extent, an investigation of vitamin A metabolism.

In attempting to further define the problem, it became

readily apparent that the effects of TCDD and similarly acting compounds on hepatic vitamin A loss could be thought of in terms of only a few possibilities. Specifically, these compounds could be (a) affecting the uptake of dietary vitamin A, (b) increasing the rate of normal mobilization of vitamin A from the liver, (c) increasing the rate of utilization of vitamin A by target tissues, (d) enhancing the rate of normal degradative pathways of retinoids in the liver, or (e) enhancing or initiating a new, as yet unidentified pathway of degradation of retinoids in the liver. Naturally, the possibility also exists that some combination of any or all of these effects could occur.

In Chapter 1, experiments describing the effect of TCDD and TCDD-like toxins on the mobilization of vitamin A from the liver are described. The research presented in this chapter demonstrates the effect of TCDD and similarly acting compounds on serum retinol levels and the activity of hepatic retinyl palmitate hydrolase. In addition, the effect of TCDD on the ternary protein complex responsible for the transport of retinol in serum is examined.

One biochemical consequence of both TCDD toxicity and a deficiency of vitamin A is an accumulation of retinoid material in the kidney. In Chapter 2, research is described suggesting that the mechanism of this phenomenon in both cases may be the elevation of the activity of an acyl-CoA transferase enzyme. Further, the dose-response and time course for both TCDD- and vitamin A deficiency-induced

renal retinoid accumulation is presented.

The results from experiments on the effects of TCDD treatment of rats on the hepatic storage, oxidative degradation, and conjugation of retinoids are presented in chapter 3. Specifically, the effects of TCDD on the storage of retinyl esters, the degradation of retinol, (oxidation to retinoic acid), and microsomal oxidation of retinol and retinoic acid are presented in this chapter. Further, effects of on the conjugation of retinoic acid or retinol with glucuronic acid are presented and discussed.

TCDD and similar compounds have been shown to affect the thyroid hormone status of treated animals, and an inverse correlation between thyroid hormone levels and vitamin A storage levels has been reported. In Chapter 4, experiments on the effects of dietary T3 on both the TCDD-induced hepatic depletion of retinyl esters and the accumulation of retinoids in the kidney are presented.

Each chapter is written in a format similar to that of many scientific journals, and contains its own Abstract, Introduction, Materials and Methods, Discussion, and List of References. Since this subject involves questions of basic vitamin A metabolism, as well as TCDD toxicity, a literature review of each of these topics, containing its own List of References, precedes Chapter 1. A summary of the research is presented as Chapter 5, as well as a discussion of possible directions for future research.

LITERATURE REVIEW

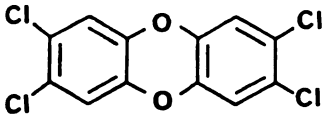
TCDD and PAH's; Structure and Toxicological Significance

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is often described as the most toxic man-made compound yet identified. A more significant fact however, is that the toxic response caused by this compound is also characteristic of the toxicity caused by the "Polyhalogenated Aromatic Hydrocarbons" (PAH's; Poland and Knutson, 1982). This group of compounds, containing TCDD, is comprised of many structurally diverse chemicals, including such species as the polyhalogenated-dibenzo-p-dioxins, -dibenzofurans, -biphenyls, -naphthalenes, and -azo-, -azoxy-, and -hydrazo-benzenes (Figure 1; Goldstein, 1980).

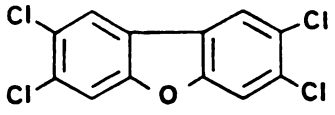
Nearly all the PAH's are man-made, having been produced either intentionally for use in a variety of applications, (i.e., coolant fluids, fire retardants, chemical feedstocks) or as contaminants formed during the synthesis of other chemical species, (i.e., the formation of trace amounts of polychlorinated-dibenzofurans and -dioxins during the synthesis of trichlorophenol (Brinkman and De Kok, 1980; Rappe and Busler, 1980)).

The PAH's have become increasingly recognized as significant and widespread environmental contaminants, primarily because of two factors. First, specific PAH's have been implicated as the causative agent in several incidents of localized contamination (i.e., Sevaso, Italy; Missouri, U.S.A.; Yusho, Japan) resulting in acute and/or

FIGURE 1. REPRESENTATIVE POLYHALOGENATED AROMATIC HYDROCARBONS



2,3,7,8-TETRACHLORODIBENZO-P-DIOXIN (TCDD)



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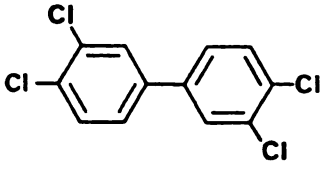
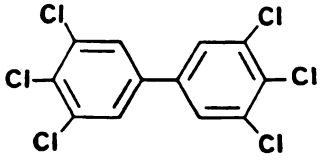
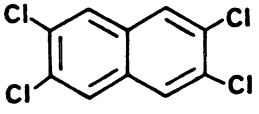


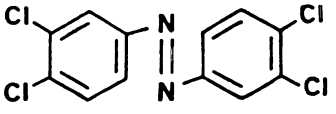
3,4,3',4'-TETRACHLOROBIPHENYL (TCB)



3,4,5,3',4',5'-HEXACHLOROBIPHENYL (HCB)



2,3,6,7-TETRACHLORONAPHTHALENE (TCN)



3,4,3',4'-TETRACHLOROAZOBENZENE (TCAB)

chronic symptoms of toxicity among a significant population (Kuratsune, 1980; Reggiani, 1980). Secondly, there have been significant advances made in the analytical technology and methods used for the identification and quantification of this type of compound at trace levels, and as components of complex mixtures (Rappe and Busler, 1980). Because of the increased analytical capability, many PAH's have now been identified both as ubiquitous environmental contaminants, and also as fairly routine contaminants of many human populations (Landrigan, 1980).

Despite both the increased attention, and intense research interest, the mechanism by which these compounds elicit their characteristic toxic response has eluded clear identification.

Much of the research on the mechanism of PAH action has focused on the well-defined ability of these compounds to bind to a specific intracellular receptor (Dencker, 1985). This receptor shows a high affinity for TCDD (Kd = 0.27 x 10^{-9}M) that is relatively constant in a number of species (Poland, 1984). No endogenous ligand has yet been identified for this receptor, and it has therefore become commonly referred to as the "TCDD Receptor."

A model for TCDD toxicity proposed by Poland, et al., (1979) suggests that the consequence of TCDD binding to the receptor is the translocation of the receptor-TCDD complex into the nucleus, binding to the chromatin material, and resulting in the activation of at least one structural gene.

Presumably then, the products of this gene activation cause the altered physiology referred to as TCDD-type toxicity. Significantly, this model for toxicity provides a mechanism whereby a group of structurally distinct compounds can cause essentially the same toxic response, and suggests that the basis for differences in toxicity would be the affinity the receptor has for a particular ligand.

Poland's model also suggests that since TCDD is the most potent member of the PAH's, it should be the best ligand for the receptor. Therefore, the closer a molecule is to the overall size, shape, and electrostatic configuration of TCDD, the better ligand for the receptor, and the more potent toxin it should be. This is supported by structure-activity data suggesting that the toxic PAH's are laterally halogenated, and either exist in a coplanar configuration, or are not energetically restricted from approaching coplanarity, thereby mimicking the laterally chlorinated, coplanar, TCDD. Hence, 3,4,5,3',4',5'-hexachlorobiphenyl (HCB) is a potent TCDD-type toxin, while the more sterically hindered 2,4,5,2',4',5'-HCB isomer is not (Nagayama, et al., 1983; Safe et al., 1981).

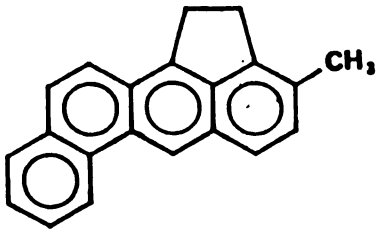
The model is supported by experimental evidence showing the translocation of the TCDD-receptor complex to the nucleus both in vivo (Okey et al., 1979) and in cultured cells (Okey et al., 1980). Further, it has been shown that TCDD-type toxicity results in the activation of at least one genomic locus leading to the synthesis of a group of

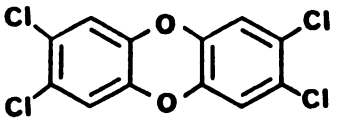
enzymes, including aryl hydrocarbon hydroxylase (AHH; Poland and Glover, 1973), several UDP-glucuronsyl transferases (Owens, 1977), DT diaphorase (Beatty and Neal, 1976), ornithine decarboxylase (Nebert, et al., 1980), and aldehyde dehydrogenase (Dietrich et al., (1978).

The locus responsible for the production of the cytosolic receptor is referred to as the "Ah locus." TCDD sensitivity has been shown to be associated with both the Ah locus, and the ability to induce AHH activity (Poland and Knutson, 1982). Further, the ability of TCDD to cause such signs as thymic atrophy, teratogenicity and mortality in treated animals, also appear to be associated with the presence of the Ah locus (Poland and Glover, 1980; Denker, 1985).

The ability for the cytosolic receptor to bind several dibenzo-p-dioxin congeners correlates with AHH induction (Poland, et al., 1976). Also, there is a strong correlation between the toxicity of specific PAH's and their ability to induce AHH activity (Goldstein, 1980).

The primary enzymatic activity linked to the locus controlled by the Ah receptor is the cytochrome P-450-dependent AHH. 3-Methylcholanthrene (MC) has been suggested as a prototypical compound for the AHH induction (Nebert, et al., 1972):





3-Methylcholanthrene

2,3,7,8-Tetrachlorodibenzo-p-dioxin

TCDD is a significantly more potent inducer of AHH activity than is MC, although experiments based on simultaneous administration of the two compounds suggest that they act by exactly the same mechanism (Poland and Glover, 1974).

While Polands's model provides logical explanations for many of the phenomena associated with TCDD-type toxicity, there are several problems with the proposal. It has been noted for example, that the dose of TCDD required to give maximal induction of the AHH enzyme activity is substantially lower than that required to cause many of the other aspects of toxicity (Golstein and Hardwick, 1984). This suggests that the products of gene locus activation by the Ah receptor-TCDD complex are not the enzymes or proteins leading directly to the toxic response. Also, the relatively slight change in receptor concentration between species does not at all correlate with the large magnitude in difference of the LD_{50}. Specifically, while the oral TCDD-LD_{50} for the (Hartley) guinea pig and (Syrian Golden) hamster are ~1.5 ug/kg (Schwetz et al., 1973) and ~1200

ug/kg (Olson, et al., 1980), respectively, there was no significant difference found between the levels of TCDD specifically bound in the liver cytosol from the two species. Perhaps the most serious problem is that one of the more sensitive animals to TCDD toxicity is the guinea pig, yet TCDD does not appear to induce AHH activity in this animal (Goldstein and Hardwick, 1984). Finally, many of the features of TCDD toxicity, including the wasting syndrome and lethality can be caused by a perfluorinated alkanoic acid which shows no affinity for the Ah receptor, and does not induce AHH activity (Olson et al., 1982).

These objections are addressed, with the exception of the last, by the suggestion that TCDD induces a pleiotropic response of which AHH-type induction is only one identified consequence, and that the toxic signs are consequences of protein synthesis not necessarily linked with the activity of AHH or coordinately induced enzymes (Goldstein and Hardwick, 1984).

Further, it is suggested that the presence of a functional receptor may not necessarily evoke the pleiotropic response. Clearly, changes in structure that affect either the affinity of the receptor for the ligand, or of the receptor-ligand complex for the nuclear binding site would be expected to have a pronounced effect on the expression of the pleiotropic response.

Effects of TCDD Toxicity

TCDD-type toxicity results in a plethora of pathological and biochemical changes in treated animals which may, in the case of sufficient dose, be lethal. There are pronounced differences in the toxic lesions observed between species in the response to TCDD toxicity, i.e., pericardial edema in chickens, chloracne and acneform eruption in primates, and "X-disease" (a skin hardening) in cattle. However, there remains a group of symptoms that appear to be common to all affected animals, which has become generally referred to as the "wasting syndrome." The consequence of this condition is a period of pronounced reduced weight gain, or in the case of near-lethal or lethal dose, weight loss, continuing until the death of the animal. Weight loss prior to mortality may be as much as 50% of the body weight at the time of dose (Poland and Knutson, 1982).

Results of experiments with animals pair-fed to dioxin treated animals suggest that the weight loss caused by the PAH's is more than simply a function of reduced food intake (Harris, et al. 1973; Allen et al., 1977; Gasiewicz, et al., 1980; Ball and Chhabra, 1981). However, more recent work suggests that the early experiments were incorrectly interpreted, and that the reduced food intake is an adequate explanation for the TCDD-induced weight loss (Seefeld and Peterson, 1984; Seefeld et al., 1984; Kelling et al., 1985). In either case, it is clear that the PAH-induced hypophagia places the animal under severe nutritional stress.

Peterson et al., have suggested (1984) that TCDD acts as an anorectic agent (Stunkard, 1982) and thereby causes treated animals to alter their basic body weight "set point." The set point may be thought of as the body weight the animal tends to maintain through a balancing of energy intake and expenditure (Seefeld, et al., 1984). Therefore, in the case of lethally treated animals, the caloric intake appropriate to the new set point is nutritionally insufficient to keep the animal alive. An objection to this proposal is that replacing the caloric deficit resulting from hypophagia does not protect the animals from all the the toxic effects of TCDD. Gasiewicz et al. (1980) demonstrated that rats treated with TCDD and given total parenteral nutrition still died as a consequence of treatment (Gasiewicz, et al., 1980). It should be noted however, that the cause of death has been suggested to be a consequence of the effects of overnutrition (Peterson et al., 1984).

In terms of identifiable pathologic changes caused in organs, or organ systems, TCDD toxicity characteristicly results in hepatic enlargement, lymphoid involution and immunosuppression, and morphological alteration of epithelial tissues in all affected species (Poland and Knutson, 1982).

The hepatic enlargement caused by TCDD appears to be a function primarily of smooth endoplasmic reticulum proliferation, resulting in both hyperplasia and

hypertrophy (Fowler, et al. 1973; Hinton, et al., 1978). The hepatomegaly occurs even at TCDD doses well below lethal, and is seen in all affected species (McConnell, 1980). The endoplasmic reticulum proliferation is correlated with cytochrome P-450 induction. This phenomenon has not been clearly linked to a toxic mechanism, although Stohs et al. (1984) have proposed that a consequence of P-450 induction may be elevated rates of lipid peroxidative damage. While 13 this area is being actively researched, and data suggest that, to some degree, TCDD does induce elevated levels of hepatic lipid peroxidation, the physiological significance of the phenomenon remains to be established. Some degree of hepatotoxicity may also be expressed, although not necessarily in all affected species (Thunberg, 1983). In chickens, rabbits, and to a small degree, certain mouse strains, TCDD causes a pronounced hepatic necrosis of lethal severity. Such a lesion is not observed in rats, guinea pigs, cattle, or non-human primates (McConnell, 1984).

The PAH-induced lymphoid involution as observed in the thymus was first described by Buu-Hoi, et al. (1972), and has been characterized primarily as a loss or depletion of cortical lymphocytes, with some lymphocytic necrosis depending on species (Vos, et al., 1973, Gupta et al., 1973, McConnell, et al., 1978). This sign of toxicity is observed in all affected species. There is also an associated depression of immune function, which is particulary notable in young animals (Vos, et al., 1980; McConnell, 1980).

TCDD-induced lymphoid involution segregates with the Ah locus, and is suggested to be mediated by the Ah receptor (Poland and Glover, 1980). Recent work by Greenlee et al., (1985) suggests that in addition to the effects described above, TCDD exerts a direct effect on thymic epithelium, and a consequence of this effect may be an altered maturation of thymus-dependent T-lymphocyte precursors. Other lymphoid tissue may also be reduced in size in treated animals, but the most pronounced changes are observed in the thymus (McConnell and Moore, 1979).

Many of the effects of TCDD intoxication are reflected by changes in epithelially-derived tissues, and are generally either hyperplastic/metaplastic or hypoplastic, and have been extensively reviewed (Poland and Knutson, 1982; McConnell et al., 1978; McConnell, 1980).

The most prominent lesion of TCDD-type toxicity observable in man is chloracne (Taylor, 1974), which may be associated with hyperkeratosis of the dermis, a function of transformation of sebaceous epithelium to squamous tissue (Gunlife and Cotterill, 1975). This condition was first linked to TCDD in 1957 (Kimmig and Schultz, 1957), and similar hyperkeratotic acneform eruptions have been observed in rabbits (Jones and Krizek, 1962), and monkeys (Allen, et al., 1977; (Poland and Knutson, 1982).

Vitamin A: Structure and Physiological Significance

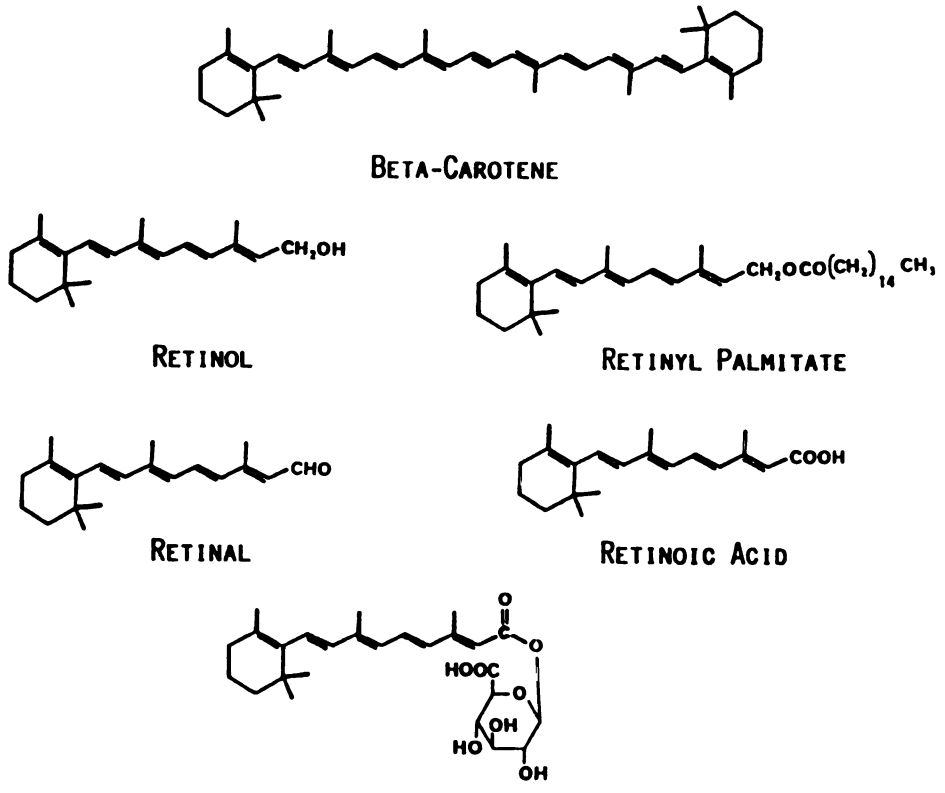
"Vitamin A" is a catch-all term used to designate the particular nutrient activity exhibited by a group of structurally related compounds: the "retinoids." Vitamin A was first isolated and described as "fat soluble factor A" by McCollum and Davis (1915) and has been recognized as a discrete and essential nutrient since that time.

Various forms of vitamin A have long been incorporated in folk remedies and records exist describing the use of preparations rich in the vitamin for the treatment of specific disease states in cultures as early as the Egyptian (Wolf, 1980). The history of the use and recognition of vitamin A, as well as the early biochemical research, has been extensively reviewed by Moore (1957).

The free alcohol form of the vitamin, retinol (Figure 2) is able to support all vitamin A dependent processes in the body, and is the basic retinoid to which the activity of other vitamin A active species are compared. Vitamin A activity may be demonstrated, in whole or in part, by a number of different compounds, all of which may be thought of as derivatives or oxidation products of retinol. As researchers discovered or designed other retinoid species with the ability to exhibit aspects of vitamin A activity, confusion over both structure and nomenclature led to the adoption of a standard nomenclature for the retinoids, selected examples of which are shown in Figure 2.

Despite early discovery and extensive research, the

FIGURE 2. STRUCTURE AND NOMENCLATURE OF SELECTED RETINOIDS (ALL-TRANS ISOMERS SHOWN)



BETA-CAROTENE

RETINOL

RETINYL PALMITATE

RETINAL

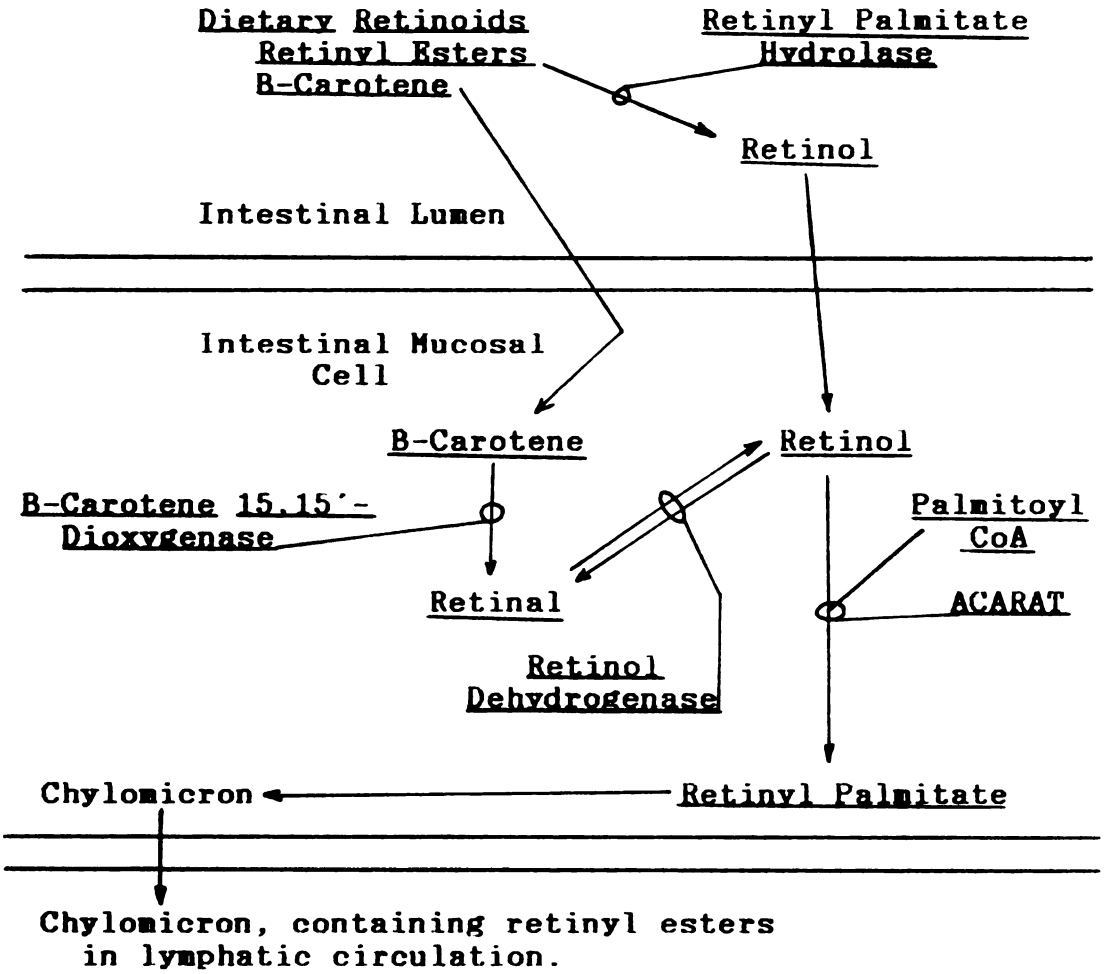
RETINOIC ACID

RETINOYL-BETA-GLUCURONIDE

exact biochemical roles for the various vitamin A species have not yet been elucidated, with the notable exception of the function of retinol/retinal in the visual cycle, as detailed by Wald, (1960). It is clear however, that the vitamin is required to maintain growth, and more specifically, that it functions in the processes of epithelial cell differentiation, and facilitates the maintenance of epithelial structures (Zile and Cullum, 1983). One other major physiological function involving vitamin A is reproduction, where a sufficient quantity of vitamin A is essential for the maintenance of both spermatogenesis and oogenesis, as well as for structural growth of both the placenta and embryo (Thompson, et al., 1964). Retinoic acid and other C-15 carboxyl-retinoid species are able to maintain the epithelial growth and differentiation functions dependent on vitamin A, while only retinol or retinyl esters appear to be able to support the reproductive function (Zile and Cullum, 1983).

Interestingly, there is a well defined toxicity associated with excessive intake of vitamin A. However, because uptake of the vitamin is at least partially modulated by the nutritional status of the individual, this is an extremely rare phenomenon (Underwood, 1984). Further, the toxicity seems to be a function of the membrane-disrupting effects of excessive retinyl esters, rather than the consequence of excessive retinol-dependent metabolic processes (Smith and Goodman, 1976).

Figure 3. Intestinal Hydrolysis, Absorption and Esterification of Dietary Retinoids



Intestinal Uptake and Transport to Liver of Vitamin A

Vitamin A is not synthesized by animals, rather, it is gained directly or indirectly from plants, in the form of carotenes, (primarily beta). In the intestine, beta-carotene is absorbed into the intestinal mucosa and cleaved by the action of a 15-15' dioxygenase, yielding two molecules of retinal (Figure 3; Goodman et al., 1967). Retinal thus formed is reduced by the action of a non-specific aldehyde oxidase to retinol (Fidge and Goodman, 1968).

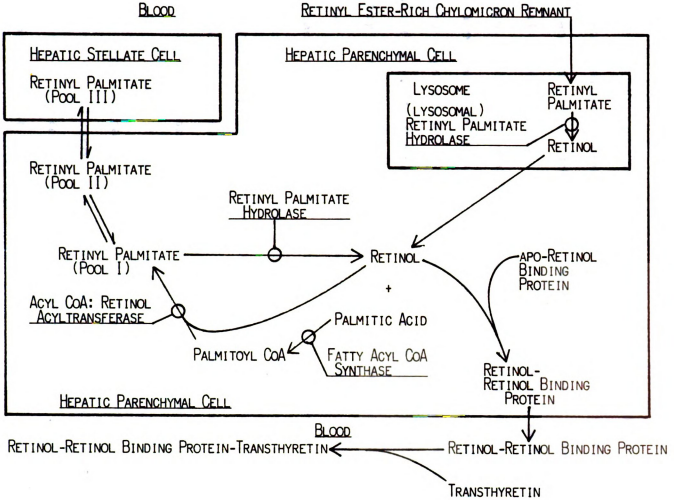
Another major source of retinoid material for carnivorous or omnivorous animals is pre-formed retinyl esters. These compounds are broken down in the intestine into retinol and free fatty acids by hydrolases secreted by the intestinal mucosal cells. Retinol thus formed is absorbed by the intestinal mucosal cells (Goodman and Blaner, 1984). Retinyl esters are not absorbed to any significant extent from normal physiological doses of vitamin A (Mahadevan et al., 1963).

The process of hydrolysis/esterification is thought to proceed several times before the retinol is eventually absorbed (Lawrence et al., 1966) and both hydrolytic and ester synthase activities have been identified in the intestinal lumen (Mahadevan et al., 1961).

Hepatic Uptake, Storage and Mobilization of Vitamin A

Retinol in the intestinal mucosal cells is esterified with fatty acid residues, (primarily palmitate, or stearate with small amounts of oleate and linoleate by the action of

FIGURE 4. HEPATIC UPTAKE, STORAGE AND MOBILIZATION OF RETINOL



acyl CoA:retinol acyl transferase (ACARAT; Huang and Goodman, 1965; Goodman, 1966). The retinyl esters thus formed are incorporated into chylomicrons, released into the lymphatic circulation and rapidly broken down in the blood to yield chylomicron remnants (Redgrave, 1970). The depleted chylomicron remnant, enriched in retinyl esters (Hazzard and Bierman, 1976), is absorbed by the hepatic parenchymal cells via a process of receptor-mediated endocytosis (Sherrill and Dietschy, 1978). The endocytic vesicles are eventually fused with lysosomes and the retinyl esters are hydrolysed by a lysosomal retinyl palmitate hydrolase (Figure 4; Goodman and Blaner, 1984). Retinol thus formed may be subject to esterification and storage, oxidation, metabolism, complexation with cellular binding proteins and participation in cellular vitamin A-mediated events or complexation with the plasma-transport protein and mobilization into plasma (Goodman and Blaner, 1984).

Much of the retinol released from the lysosome is re-esterified by the same ACARAT enzyme as is found in the intestinal mucosa. This enzyme utilizes fatty acyl CoA's and retinol as substrates and exhibits the greatest affinity for palmitoyl-, stearoyl- and oleoyl-CoA (Ross, 1982). Retinyl esters thus formed are stored in the liver in three "pools", the first of which, and the most readily available for mobilization, is that associated with a lipoprotein complex in the hepatic parenchymal cell (described below; Heller, 1979, Sklan, 1982). A second pool of retinyl esters

exists within the hepatic parenchymal cell lipid droplets, and is thought to be less readily available for mobilization. The hepatocyte pools however, correspond to only a small fraction of the total quantity of retinyl esters that may be stored in the liver (Goodman and Blaner, 1984). By far, the greatest quantity of retinyl esters are stored as lipid droplets in the stellate cells lining the perisinusoidal rays. In the well-fed state, with respect to vitamin A, a marked fluorescence of these cells can be observed. While this pool of retinyl esters is the largest store of vitamin A in the body, it is not readily available to the body as retinol; rather, the esters must be transferred by an as yet unidentified mechanism to the parenchymal cells. Mechanisms for the control of uptake and mobilzation of retinyl esters from the stellate cells have not been identified.

Mobilization and Transport of Retinol

The process of delivery of retinol to target tissues may be thought of as beginning with the hydrolysis of retinyl esters by retinyl palmitate hydrolase (RPH). RPH is located in association with a lipoprotein complex (LPC) that is cytoplasmic, in a "loose" association with the endoplasmic reticulum (Heller, 1979, Chen and Heller, 1979). Several other enzymic activities are also found in the LPC, i.e., triolein, cholesteryl oleate and phosphatidylcholine hydrolases (Sklan, et al., 1982). Retinyl esters appear to be associated with the LPC, as does cellular retinol binding

protein (CRBP). Retinol produced by the action of the hydrolase is presumed to bind to either CRBP or the serum transport binding protein, retinol binding protein (RBP).

RBP is normally present in excess in the hepatic parenchymal cells. Therefore the limiting factor in the mobilization of retinol to plasma appears to be the availabilility of retinol, and hence, the hydrolysis of retinyl esters (Smith et al., 1973) The mechanism(s?) for the control of either the activity of RPH or the availability of its substrate retinyl esters is as yet, unelucidated.

The retinol-RBP complex in serum rapidly binds to the multifunctional globular protein transthyretin (TTR). TTR in plasma is a tetramer composed of ~14,000 dal subunits, which also serves to transport the thyroid hormone thyroxine. Several studies have shown that while there is no interdependence of the binding of either thyroxine or holo-RBP to TTR (van Jaarsveld, et al., 1973; Raz and Goodman, 1969), the binding of holo-RBP to TTR does significantly stabilize the retinol-RBP interaction (Peterson, 1971: Goodman and Raz, 1972).

The retinol-RBP-TTR ternary complex is the form in which vitamin A is carried and delivered to target tissues. The mechanism of transfer of retinol from the ternary complex to the target tissues has not been completely elucidated, and although there is some evidence that the process is receptor mediated (Rask and Peterson, 1976), it

appears that neither the TTR nor RBP molecules are internalized along with the retinol molecule. A consequence of the transfer process seems to be a proteolytic modification of the RBP molecule such that it is unable to bind retinol, and also exhibits a markedly diminished affinity for the TTR tetramer. This "free" RBP (as opposed to apo-RBP, which is not released from the liver) is thought to be rapidly cleared from serum and catabolized by the kidney.

In the target tissue, the fate and role of retinol remain unclear. The presense of specific binding proteins for both retinol and retinoic acid (cellular retinol binding protein, CRBP; cellular retinoic acid binding protein, CRABP) suggests that the activities or delivery of the retinoids to particular sites within the cell may be mediated by the retinoid-protein complexes. It is not at all inconceivable that there exists several metabolic roles, some of which are mediated by the binding proteins.

Degradation and Loss of Vitamin A.

Vitamin A may be lost from the body by several routes. The process of loss of vitamin A equivalents however, appears to be initiated in all cases by an oxidative process. Oxidation may produce functionally active species, such as retinoic acid, or inactive species, such as 4-oxoretinoic acid.

Retinol and presumably, retinaldehyde and retinoic acid may be oxidized at the four position of the B-ionone ring by

microsomal oxidases. While several of the 4-hydroxy and 4-oxo compounds have been shown to demonstrate some vitamin A activity, it is generally thought that oxidation at this position removes the molecule from the pool of active vitamin A compounds, and may serve to "target" the molecule for elimination from the body, perhaps by a process of further oxidation.

Neither retinol nor retinaldehyde seem to be effectively conjugated to glucuronic acid. However, retinoic acid serves as an effective substrate, and it has been suggested that the retinoyl-B-glucuronide is the major metabolite of vitamin A appearing in the bile (Frolik, 1984).

Other degradative products may be formed by the decarboxylation of retinoic acid, a process that was demonstrated *in vivo* by Roberts and DeLuca (1967), who reported the expiration of 35% of a dose of $^{14}C(15)$ retinoic acid as CO_2. Recent data demonstrating the process as one of physiological significance are provided by the work of Skare, *et al.*, (1982), who have demonstrated the presence of several retinoid metabolites with shortened side chains in urine.

Clinical Signs and Lesions of a Vitamin A Deficiency

A depression in the rate of weight gain in growing animals has been noted as one of the earliest clinical signs of the onset of a vitamin A deficiency. Additional signs, such as abnormal epithelial development, depressed immune

function and decreased reproductive competence develop as the deficiency persists (Underwood, 1984).

1. Growth

In weanling rats given a diet complete except for vitamin A, growth generally ceases in 4-6 weeks in most strains (Coward et al., 1969). The cessation of growth appears following the loss of hepatic retinoids, as suggested by the work of Lamb et al., (1974), who reported an essentially complete depletion between one and two weeks prior to the weight plateau stage.

Further suggestion that the effects on growth rate may not reflect the actual onset of deficiency, is provided by the depression in efficiency of protein utilization which was noted prior to the actual onset of the weight plateau (Hayes, 1971), as was hypophagia (Anzano et al. 1979). Mechanistically, Zile et al., (1979; 1981) have suggested that the failure to grow is a consequence of inefficient utilization of nutrients needed to maintain cellular proliferation.

2. Epithelial Differentiation

The appearance of a keratinizing metaplasia of mucosal epithelial tissues has been noted in association with vitamin A deficiency states since the first reports on vitamin A deficiency by Wolbach and Howe (1925) and Wolbach (1937). The lesion is not expressed uniformly in all epithelial tissues (Olson, 1972). Mucosal epithelium that

does not respond to deficiency by keratinization may still be affected by deficiency, i.e., a decline in intestinal mucosal goblet cells (De Luca et al., 1969). Normally keratinizing tissues, such as skin, become hyperkeratinized (Matoltsy, 1976). Epithelial tissues that contain both goblet and keratinizing cells lose goblet cells and undergo squamous metaplasia. This phenomenon has been observed in cornea and conjunctiva (Sommer and Green, 1982) and respiratory, urinary and genital tracts (Wolbach, 1937; Wilson and Warkany, 1947). In germ-free rats, a similar lesion is noted as the deficiency develops, suggesting that the tissue changes are a consequence of deficiency, rather than a secondary effect of infection (Beaver, 1961; Rodgers et al., 1969).

3. Immune Function

Vitamin A was recognized as having significant anti-infectious properties in the early work of Green and Mellanby (1928), and seems to be required for both the humoral and cell-mediated immune responses (Jurin and Tannock, 1972). Vitamin A deficiency has such pronounced effects on the immune system that the most common identifiable cause of death in non-germ free rats is infection. Further, as the deficiency develops there is a pronounced lymphoid involution, most notable in the thymus tissue, a function of loss of cortical lymphocytes.

4. Reproduction

The reproductive system has a well defined requirement for retinol to maintain both spermatogenisis in males, (Palludan, 1966; Aluwalia and Bieri, 1971), and to support full gestation, embryonic development and delivery in females (Thompson et al., 1964; Takahashi et al., 1975).

As early as the 1930's, Mason (1935) demonstrated that vitamin A deficiency in rats causes a pronounced atrophy of the testes, such that the germinal epithelia degenerate and sperm development does not progress past the spermatid stage. Histologically, even at the early stages of deficiency, the germinal epithelia contain primarily spermatogonia, with some spermatocytes, but no spermatids are observed.

In 1957, Moore reported that the ovaries of vitamin A-deficient rats are distinctly smaller than those from control animals and acutely deficient female rats fail to conceive when mated with normal males. However, mildly deficient female rats do conceive, but fetal resorption at about day 14 or severe malformation of the fetus is usually observed.

Vitamin A deficient female rats have a consistently cornified vaginal epithelia, which is normally expressed for only about 30 hours of the rat estrous cycle. The vaginal cornification, a result of a normally hormonally-controlled keratinizing epithelium becoming constantly active and unresponsive to hormonal control, appears to precede other manifestations of the deficiency (Mason and Ellison, 1934).

TCDD and Vitamin A Status

Effects of TCDD on Vitamin A Storage

The sensitivity of hepatic vitamin A levels to exposure to certain xenobiotics was first noted in 1939 by Goerner and Goerner with dibenz(a,h)anthrocene and 2-amino-6-azotoluene in rats. The ability of the PAH's to deplete vitamin A stores was reported by Olafson (1947) and Marsh et al., (1956), who noted the lowered serum vitamin A levels in cattle exposed to polychlorinated naphthalenes. McConnell et al., (1979) observed significantly depressed hepatic retinyl ester stores in monkeys accidently contaminated by PCB's. In rodents, depletion of hepatic stores of vitamin A and depressed serum levels have been observed with both poly-chlorinated and -brominated biphenyls, (Villeneuve, et al., 1971; Innami, et al., 1974; Akoso, et al., 1982; Brouwer and van den Berg, 1983; Darjono, et al., 1983). TCDD has also been shown to cause a rapid and dose-dependant depletion of hepatic retinyl ester stores in rats (Thunberg, et al., 1979; 1980).

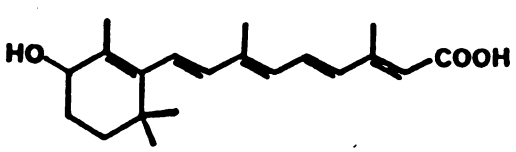
Four main theories have been proposed to explain the TCDD-induced loss of hepatic vitamin A stores; a) increased rates of lipid peroxidative degradation of retinoids (Kato, et al., 1978; Ikegami, et al., 1980;); b) increased microsomal oxidative degradation of vitamin A by non-lipid peroxidative mechanisms (Hausworth and Brizuela, 1976; Innami, et al., 1976; Saito, et al., 1982); c) increased formation of retinol-glucuronide by induction of UDPGT

activity (Kato et al., 1978; Thunberg, 1983); and d) disruption of the ternary complex responsible for the transport of retinol, with the subsequent clearance of free retinol by the kidney (Brouwer and van den Berg, 1986).

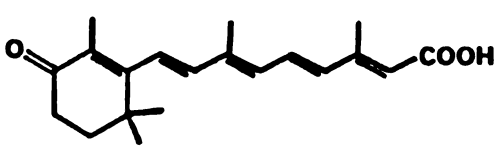
The basis of the suggestion that increased levels of lipid peroxidation could be the cause of hepatic vitamin A loss is twofold. First, there is the marked proliferation of cytochrome P-450 isozymes capable of catalyzing the single-electron reduction of oxygen (O_2) yielding superoxide (O_2^-) , suggested to be the precursor of such reactive oxygen species as the hydroxyl radical (OH•; Thomas and Aust, 1986). Secondly, a role of vitamin A as a lipophilic free radical scavenger, analogous to that proposed for vitamin E (McCay, 1985) has been suggested by Draper (1980). At least one aspect of vitamin A metabolism, the oxidative decarboxylation of retinoic acid has been shown to proceed via a free radical mechanism in vitro, although the reaction does not seem to be of great significance in vivo (Roberts and DeLuca, 1969). These authors showed that the potent free radical scavenger N,N'-diphenyl-p-phenylene-diamine (DPPD) was able to protect against free-radical mediated decarboxylation in the former instance, but not the latter. However, the ethanol-caused depletion of vitamin A does appear to be affected to at least some degree by this mechanism, in that a similar experiment performed in ethanol-treated rats showed a significant protective effect of DPPD (Ryle, et al., 1986). Attractive as this theory

may be in explaining the TCDD-caused depletion of hepatic retinoids, Saito et al., (1982) clearly demonstrated the lack of correlation between the level of lipid peroxidation in PCB-treated rats and the depletion of hepatic retinoids, as well as the lack of effect of dietary antioxidants (such as DPPD) in protecting against such depletion.

Many of the proposed reactions of vitamin A degradation are postulated to occur in the endoplasmic reticulum (Goodman and Blaner, 1984), and accordingly, Hausworth and Brizula (1976) and Kato (1978) have suggested that induction of microsomal enzymes might influence the rate of vitamin A degradation. A cytochrome P-450 dependent oxidation of retinoic acid has been demonstrated in both hamsters (Roberts et al., 1979) and rats (Sato and Leiber, 1982). Most significant to the hypothesis, Leo et al., (1984) have demonstrated that microsomal retinoic acid degradation can be induced in rats fed excessive amounts of retinoic acid. These microsomal oxidation reactions are proposed to result in the hydroxylation and further oxidation to the keto-acid (Roberts et al., 1980):



4-hydroxy-retinoic acid



4-keto-retinoic acid

It has been suggested that molecules thus modified are more readily conjugated with either glutathione or UDP-glucuronic acid, and eliminated from the body. This hypothesis is indirectly supported by data showing the endoplasmic proliferation and induction of several microsomal hydroxylases, (i.e., AHH), caused by TCDD. While this hypothesis has not been directly evaluated, recent work by Zile and Cullum (1985), has shown an increased excretion of polar retinoid metabolites as a result of a dose of 3,4,5,3',4',5'-HBB to rats.

The UDP-glucuronosyl transferases (UDPGT's) are a family of related enzymes catalyzing the formation of esters, amides or ethers from molecules containing a nucleophilic center and UDP-glucuronic acid (Neal, 1980). Several isozymes of this group are induced in treated animals as a function of the pleiotropic response to PAH toxicity (discussed above). It has been proposed that the increased rate of glucuronide formation plays a role in both the vitamin A (Thunberg, 1983) and thyroxine (T4) depletion (Bastomsky and Murthy, 1976). However, no increased UDPGT activity towards retinol has yet been demonstrated (Thunberg, 1983).

The theory that PAH's disrupt the serum transport of retinol has been suggested and researched by Brouwer and van den Berg (1986). They suggest that one consequence of PAH toxicosis is a disruption of the retinol-RBP complex as a function of the binding of the PAH, or a PAH metabolite to

transthyretin. The free retinol-RBP complex would then be filtered by the kidney, where presumably, both retinol and the RBP molecule would be catabolized.

Similarities of TCDD Toxicity and Vitamin A Deficiency

The similarities between the various manifestations of a vitamin A deficiency and PAH toxicity were noted as descriptions of the clinical and pathological characteristics of PAH toxicosis became available (Kimbrough, 1974). Most notably, both conditions produce extensive epithelial lesions, characterized by the squamous metaplasia of normally columnar epitheliam. Further, there is a characteristic transformation of epithelial tissues to a keratinizing type (Thunberg, 1983). Another significant similarity is the pronounced involution of lymphoid tissue, and the resultant depression of immune function. Lastly, the marked weight loss, a function of hypophagia and perhaps anorexia, produces the so called "wasting syndrome" in both cases. Indeed, it is extremely difficult from an empirical viewpoint to differentiate a terminal vitamin A deficient animal from one given a lethal dose of TCDD. However, because of a lack of understanding of vitamin A mechanisms, and the limitations of analytical methodology, these observations could not be carried beyond the descriptive level. More importantly, the question of whether animals treated with TCDD express symptoms of a vitamin A deficiency because TCDD actually causes such a deficiency, or merely

causes the symptoms by an alternate mechanism, have not yet been answered.

BIBLIOGRAPHY

Ahluwalia, B., and Bieri, J.G. (1971). Local stimulatory effect of vitamin A on spermatogenesis in the rat. J. Nutr. 101:141-151.

Akoso, B.T., Sleight, S.D., Aust, S.D., and Stowe, H.D. (1982). Pathologic effects of purified polybrominated biphenyl congeners in rats. J. Am. Coll. Toxicol. 1:1-21.

Allen, J.R., Bersotti, D.A., Van Miller, J.P., Abrahamson, L.J., Lalich, J.J. (1977). Morphological changes in monkeys consuming a diet containing low levels of 2,3,7,8-tetrachlorodibenzo-p-dioxin. Food Cosmet. Toxicol 15:401-410.

Anzano, M.A., Lamb, A.J., and Olson, J.A. (1979). Growth, appetite, sequence of pathological signs, and survival following the induction of rapid synchronous vitamin A deficiency in the rat. J. Nutr. 109:1419-1431.

Ball, L.M., and Chhabra, R.S. (1981). Intestinal absorption of nutrients in rats treated with 2,3,7,-tetrachlorodibenzo-p-dioxin (TCDD). J. Toxicol. Environ. Health. 8:629-638.

Bastomsky, C.H., and Murthy, P.V.N. (1976). Enhanced <u>in vitro</u> hepatic glucuronidation of thyroxine in rats following cutaneous application or ingestion of polychlorinated biphenyls. Can. J. Physiol. Pharmacol. 54:23-26.

Beatty, P., and Neal, R.A. (1976). Induction of DT-diaphorase by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Biochem. Biophys. Res. Commun. 68:197-204.

Beaver, D.L. (1961). Vitamin A deficiency in the germ free rat. Am. J. Pathol. 38:335-357.

Brinkman, U.A.T., and De Kok, A. (1980). Production, properties and usage. In: Halogenated biphenyls, terphenyls, naphthalenes, dibenzodioxins and related products. (Kimbrough, R.D. ed.) Elsevier/North-Holland Biomedical Press, Amsterdam; pp 1-40.

Brouwer, A., and van den Berg, K.J. (1983). Early decrease in retinoid levels in mice after exposure to low doses of polychlorinated biphenyls. Chemosphere 12:555-558.

Brouwer, A. and van den Berg, K.J. (1986). Binding of a metabolite of 3,4,3',4'-tetrachlorobiphenyl to transthyretin reduces serum vitamin A transport by inhibiting the formation of the protein complex carrying both retinol and thyroxine. Toxicol. Appl. Pharmacol. 85:301-312.

Buu-Hoi, N.P., Chanh, P.H., Sesque, G., Asum-Gelade, M.C., and Saint-Ruf, G. (1972). Organs as targets of dioxin (2,3,7,8-tetrachlorodibenzo-p-dioxin) intoxication. Naturwissenschaften 59:174-178.

Chen, C.C., and Heller, J. (1979). Retinyl esterase activity of purified rat liver retinyl ester lipoprotein complex. Arch. Biochem. Biophys. 198:572-579.

Coward, W.A., Howell, J.McC., Thompson, J.N., and Pitt, G.A.J. (1969). The retinol requirements of rats for spermatogenesis and vision. Br. J. Nutr. 23:619-626.

Darjono, Sleight, S.D., Stowe, H.D., and Aust, S.D. (1983). Vitamin A status, polybrominated biphenyl (PBB) toxicosis, and common bile duct hyperplasia in rats. Toxicol. Appl. Pharmacol. 71:184-193.

DeLuca, L.M., Little, E.P., and Wolf, G. (1969). Vitamin A and protein synthesis by rat intestinal mucosa. J. Biol. Chem. 244(3):701-708.

Dencker, L. (1985). The role of receptors in 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) toxicity. Arch. Toxicol., Suppl. 8:43-60.

Dietrich, R.A., Bludeau, P., Roger, M., and Schmuck, J. (1978). Induction of aldehyde dehydrogenases. Biochem. Pharmacol. 27:2343-2347.

Draper, H.H. (1980). Nutrient interrelationships. In: Vitamin E: a comprehensive treatise. (Machlin, L.J. ed.) Marcel Dekker, Inc. New York. pp: 272-288.

Firestone, D. (1984). Chlorinated aromatic compounds and related dioxins and furans: production, uses and environmental exposure. In: Biological mechanisms of dioxin action. (Poland, A. and Kimbrough, R.D. eds.) Banbury Report #18. Cold Spring Harbor Laboratory pp: 3-16.

Fowler, B.A., Lucier, G.W., Brown, H.W., and McDaniel, O.S. (1973). Ultrastructural changes in rat liver cells following a single oral dose of TCDD. Environ. Health Perspect. 5:141-148.

Frolik, C.A. (1984). Metabolism of Retinoids. In: The Retinoids. (Sporn, M.B., Roberts, A.B. and Goodman, D.S. eds.) Academic Press, Inc. Orlando. Vol II; pp: 177-208.

Gasiewicz, T.A., Holscher, M.A., and Neal, R.A. (1980). The effect of total parenteral nutrition on the toxicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin in the Rat. Toxicol. Appl. Pharmacol. 54:469-488.

Gasiewicz, T.A., and Rucci, G., (1984). Cytosolic receptor for 2,3,7,8-tetrachlorodibenzo-p-dioxin. Evidence for a homologous nature among various mammalian species. Mol. Pharmacol. 26:90-98.

Goerner, A., and Goerner, M.M. (1939). The influence of a carcinogenic compound on the hepatic storage of vitamins. J. Nutr. 18:441-446.

Goldstein, J.A. (1980). Structure-activity relationships for the biochemical effects and the relationship to toxicity. In: Halogenated biphenyls, terphenyls, naphthalenes, dibenzodioxins and related products. (Kimbrough, R.D. ed.) Elsevier/North-Holland Biomedical Press, Amsterdam; pp 151-190.

Goldstein, J.A., and Hardwich, J. (1984). Regulation of a multigene family of P-450 isozymes by 2,3,7,8-tetrachlorodibenzo-p-dioxin and related compounds. In: Biological mechanisms of dioxin action. (Poland, A. and Kimbrough, R.D., eds.) Banbury Report #18; Cold Spring Harbor Laboratory. pp: 119-133.

Goodman, D.S., Blomstrand, R., Werner, B., Huang, H.S., and Shiratori, T. (1966). The intestinal absorbtion and metabolism of vitamin A and B-carotene in man. J. Clin. Invest. 45:1615-1623.

Goodman, D.S., Huang, H.S., Kanai, M., and Shiratori, T. (1967). The enzymatic conversion of all-trans B-carotene into retinal. J. Biol. Chem. 242:3543-3554.

Goodman, D.S., and Raz, A. (1972). Extraction and recombination studies of the interaction of retinol with human plasma retinol-binding protein. J. Lip. Res. 13:338-347.

Goodman, D.S., and Blaner, W.S. (1984). Biosynthesis, Absorption and Hepatic metabolism of Retinol. In: The Retinoids. (Sporn, M.B., Roberts, A.B. and Goodman, D.S. eds.) Academic Press, Inc. Orlando. Vol II;pp 1-39.

Green, H.N., and Mellanby, E. (1928). Vitamin A as an anti-infectious agent. Br. Med. J. 2:691-696.

Greenlee, W.F., Dold, K.M., Irons, R.D., and Osborne, R. (1985). Evidence for direct action of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on thymic epithelium. Toxicol. Appl. Pharmacol. 79:112-120.

Gunlife, W.S., and Cotterill, J.A. (1975). Occupational acne. Major Problems in Dermatology. 6:28-31.

Gupta, B.N., Vos, J.G., Moore, J.A., Zinkl, J.G., and Bullock, B.C., (1973). Pathologic effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin in laboratory animals. Environ. Health Persp. 5:125-140.

Harris. M.W., Moore, J.A., Vos, J.G., and Gupta, B.N. (1973). General biological effects of TCDD in laboratory animals. Environ. Health Perspect. 5:101-109.

Hausworth, J.W., and Brizuela, B.S. (1976). The differential effects of chemical carcinogens on vitamin A status and on microsomal drug metabolism in normal and vitamin A deficient rats. Cancer Res. 36:1941-1946.

Hayes, K.C. (1971). On the pathophysiology of vitamin A deficiency. Nutr. Rev. 29:3-6.

Hazzard, W.R., and Bierman, E.L. (1976). Delayed clearance of chylomicron remnants following vitamin-A-containing oral fat loads in broad-B disease (Type III hyperlipoproteinemia). Metab. Clin. Exp. 25:777-801.

Heller, J. (1979). Characterization of rat liver cytosol retinyl ester lipoprotein complex. Arch. Biochem. Biophys. 198:562-571.

Hinton, D.E., Glaumann, H., and Trump, B.F. (1978). Studies on the cellular toxicity of polychlorinated biphenyls (PCB's). I. Effects of PCBs on microsomal enzymes and on synthesis and turnover of microsomal and cytoplasmic lipids of rat liver - a morphological and biochemical study. Virchows Arch. B. 27:279-306.

Huang, H.S., and Goodman, D.S. (1965). Vitamin A and carotenoids. I. Intestinal absorption and metabolism of C-labeled vitamin A alcohol and B-carotene in the rat. J. Biol. Chem. 240:2839-2844.

Ikegami, S., Nakamura, A., Ono, S., Nagayama, S., and Innami, S. (1980). Effect of combination of PCB and BHC on vitamin A storage and lipid peroxide formation in liver of rats. J. Jpn. Soc. Food Nutr. 33:327-333.

Innami, S., Nakamura, A., and Nagayama, S. (1974). Polychlorinated biphenyl toxicity and nutrition. II. PCB toxicity and vitamin A. J. Nutr. Sci. Vitaminol. 20:363-370.

Innami, S., Nakamura, A., Miyazaki, M., Nagayama, S., and Nishide, E. (1976). Further studies on the reduction of vitamin A content in the livers of rats given polychlorinated biphenyls. J. Nutr. Sci. Vitaminol. 22:409-418.

Jones, E.L., and Krizek, H.A. (1962). A technic for testing acnegenic potency in rabbits applied to the potent acnegen 2,3,7,8-tetrachlorodibenzo-p-dioxin. J. Invest. Dermatol. 39:511-517.

Jurin, M., and Tannock, I.F., (1972). Influence of vitamin A on immunological response. Immunology 23:282-287.

Kato, N., Kato, M., Kimura, T., and Yoshida, A. (1978). Effect of dietary addition of PCB, DDT or BHT and dietary protein on vitamin A and cholesterol metabolism. Nutr. Rep. Int. 18:437-445.

Kelling, C.K., Christian, B.J., Inhorn, S.L. and Peterson, R.E. (1985). Hypophagia-induced weight loss in mice, rats and guinea pigs treated with 2,3,7,8-tetrachlorodibenzo-p-dioxin. Fundam. Appl. Toxicol. 5:700-712.

Kimbrough, R.D. (1974). The toxicity of polychlorinated polycyclic compounds and related compounds. Crit. Rev. Toxicol. 2:445-489.

Kimmig, J., and Schultz K.H. (1957). Occupational chloracne caused by aromatic cyclic ethers. Dermatologica 115:540-546.

Kuratsune, M. (1980). Yusho. In: Halogenated biphenyls, terphenyls, naphthalenes, dibenzodioxins and related products. (Kimbrough, R.D. ed.) Elsevier/North-Holland Biomedical Press, Amsterdam; pp: 287-302.

Lamb, A.J., Apiwatanaporn, P., and Olson, J.A. (1974). Induction of rapid synchronous vitamin A deficiency in the rat. J. Nutr. 104:1140-1148.

Landrigan, P.J. (1980). General population exposure to environmental concentrations of halogenated biphenyls. In: Halogenated biphenyls, terphenyls, naphthalenes, dibenzodioxins and related products. (Kimbrough, R.D. ed.) Elsevier/North-Holland Biomedical Press, Amsterdam; pp: 267-286.

Lawrence, C.W., Crain, F.D., Lotspeich, F.J., and Krause, R.F. (1966). Absorption, transport, and storage of retinyl-15-^{14}C palmitate-9,10 ^{3}H in the rat. J. Lip. Res. 7:226-229.

Leo, M.A., Iida, S., and Lieber, C.S. (1984). Retinoic acid metabolism by a system reconstituted with cytochrome P-450. Arch. Biochem. Biophys. 234:305-312.

Mahadevan, S., Murthy, S.K., Krishnamurthy, S., and Ganguly, J. (1961). Studies on vitamin A esterase. 4. The hydrolysis and synthesis of vitamin A esters by rat intestinal mucosae. Biochem J. 79:416-424.

Mahadevan S., Seshardri Sastry, P., and Ganguly, J. (1963). Studies on metabolism of vitamin A. 3. The mode of absorption of vitamin A esters in the living rat. Biochem J. 88:531-534.

Marsh, C.L., Olson, C., and Blore, I.C. (1956). Observations on collagen, vitamin A and ascorbic acid in bovine hyperkeratosis. Am. J. Vet. Res. 17:410-414.

Mason, K.E. (1935). Foetal death, prolonged gestation and difficult parturition in the rats as a result of vitamin A deficiency. Am. J. Anat. 57:303-315.

Mason, K.E., and Ellison, E.T. (1934). The demonstration of the oestrus in the vitamin A deficient rat by supravital study of the vaginal smear. J. Nutr. 10(1):1-11.

Matoltsy, A.G. (1976). Keratinization. J. Invest. Dermatol. 67:20-25.

McCay, P.B. (1985). Vitamin E: Interactions with free radicals and ascorbate. Ann. Rev. Nutr. 5:323-340.

McCollum, E.B., and Davis, M. (1915). The nature of the dietary deficiencies of rice. J. Biol. Chem. 23:181-246.

McConnell, E.E., Hass, J.R., Altaman, N., and Moore, J.A. (1979). A spontaneous outbreak of polychlorinated biphenyl (PCB) toxicity in Rhesus monkeys (Macaca mulatta): Toxicopathology. Lab. Animal Sci. 320:131-137.

McConnell, E.E., (1980). Toxicity, carcinogenesis, reproduction, teratogenesis and mutagenesis. In: Halogenated Biphenyls, terphenyls, naphthalenes, dibenzodioxins and related products. (Kimbrough, R.D. ed.) Elsevier/North-Holland Biomedical Press, Amsterdam; pp: 109-150.

McConnell, E.E., Moore, J.A., Haseman, J.K., and Harris, M.W., (1978). The comparative toxicity of chlorinated dibenzo-p-dioxins in mice and guinea pigs. Toxicol. Appl. Pharacol. 44:335-356.

McConnell, E.E. (1984). Clinicopathologic concepts of dibenzo-p-dioxin intoxication. In: Biological mechanisms of dioxin action. (Poland, A and Kimbrough, R.D., eds.) Banbury Report #18; Cold Spring Harbor Laboratory. pp: 27-38.

Moore, T, (1957). "Vitamin A". Elsevier Publishing Co., New York

Nagayama, J., Kurok, H., Masuda, Y., and Kuratsune, M. (1983). A comparative study of polychlorinated dibenzofurans, polychlorinated biphenyls and 2,3,7,8-tetrachlorodibenzo-p-dioxin on aryl hydrocarbon hydroxylase inducing potency in rats. Arch. Toxicol. 53:177-184.

Neal, R.A. (1980). Metabolism of toxic substances. In: "Casaret and Duoll's Toxicology: the basic science of poisons." (Duoll, J., Klassen, C.D., and Amdur, M.O. eds.) MacMillan Pub. Co. Inc. New York pp: 56-69.

Nebert, D.W., Goujon, F.M., and Gielen, J.E. (1972). Aryl hydrocarbon hydroxylase induction by polycyclic hydrocarbons: simple autosomal dominant trait in the mouse. Nat. New Biol. 236:107-110.

Nebert, D.W., Eisen, H.J., Negishi, M., Land, M.A., and Hjelmeland, L.M. (1981). Genetic mechanisms controlling the induction of polysubstrate monooxygenase (P-450) activities. Ann. Rev. Pharmacol. Toxicol. 21:431-462.

Okey, A.B., Bondy, G.P., Mason, M.E., Kahl, G.F., Eisen, H.J., Guenthner, T.M., and Nebert, D.W. (1979). Regulatory gene product of the Ah locus: Characterization of the cytosolic inducer-receptor complex and evidence for its nuclear translocation. J. Biol. Chem. 254:11636-11648.

Okey, A.B., Bondy, G.P., Mason, M.E., Nebert, D.W., Forster-Gibson, C.J., Muncan, J., and Dufresne, M.J. (1980). Temperature-dependant cytosol-to-nucleus translocation of the Ah receptor for 2,3,7,8-tetrachlorodibenzo-p-dioxin in continuous cell culture lines. J. Biol. Chem. 255:11415-11422.

Olafson, P. (1947). Hyperkeratosis (X-disease) of cattle. Cornell Vet. 37:279-291.

Olson, C.T., Anderson, M.E., George, M.E., VanRafelghem, M.J. and Bach, A.M. (1982). The toxicology of perfluorodecanoic acid in rodents. In: Proc. Thirteenth Ann. Conf. on Environ. Toxicol. University of Calif. Irvine: Overlook Branch, Dayton, Ohio. 20:287-303.

Olson, J.A. (1972). The biological role of vitamin A in maintaining epithelial tissues. Isr. J. Med. Sci. 8:1170-1178.

Olson, J.R., Holscher, M.A., and Neal, R.A., (1980). Toxicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin in the golden syrian hamster. Toxicol. Appl. Pharmacol. 55:67-78.

Owens, I.S., (1977). Genetic regulation of UDP-glucuronosyltransferase induction by polycyclic aromatic compounds in mice. J. Biol. Chem. 252:2827-2833.

Palludan, B. (1966). Direct effect of vitamin A on boar testis. Nature (London) 211:639-640.

Peterson, P.A. (1971). Studies on the interaction between prealbumin, retinol-binding protein and vitamin A. J. Biol. Chem. 246:44-49.

Peterson, R.E., Seefeld, M.D., Christian,. B.J., Potter, C.L., Kelling, C.K., and Keesey, R.E. (1984). The wasting syndrome in 2,3,7,8-tetrachlorodibenzo-p-dioxin toxicity; Basic features and their interpretation. In: Biological mechanisms of dioxin Action. Banbury Report #18; Cold Spring Harbor Laboratory pp: 291-308.

Poland, A. (1984). Reflections on the mechanism of action of halogenated aromatic hydrocarbons. In: Biological mechanisms of dioxin action. Banbury Report #18; Cold Spring Harbor Laboratory pp: 109-117.

Poland, A., Greenlee, W.F., and Kende, A.S. (1979). Studies on the mechanism of action of the chlorinated dibenzo-p-dioxins and related compounds. Ann. N.Y. Acad. Sci. 30:214-230.

Poland, A., and Knutson, J.C. (1982). 2,3,7,8-Tetrachlorodibenzo-p- dioxin and related halogenated aromatic hydrocarbons: examination of the mechanism of toxicity. Ann. Rev. Pharmacol. Toxicol. 22:517-554.

Poland, A., and Glover, E. (1973). Chlorinated dibenzo-p-dioxins: potent inducers of aminolevulinic acid synthetase and aryl hydrocarbon hydroxylase. II. A study of the structure-activity relationship. Mol. Pharmacol. 9:736-747.

Poland, A., and Glover, E. (1974). Comparison of 2,3,7,8-tetrachlorodibenzo-p-dioxin, a potent inducer of aryl hydrocarbon hydroxylase with 3-methylcholanthrene. Mol. Pharmacol. 10:349-359.

Poland, A., Glover, E., and Kende, A.S. (1976). Stereospecific, high affinity binding of 2,3,7,8-tetrachlorodibenzo-p-dioxin by hepatic cytosol. J. Biol. Chem. 251: 4936-4946.

Poland, A., and Glover, E. (1980). 2,3,7,8-tetrachlorodibenzo-p-dioxin: Segregation of toxicity with the Ah locus. Mol. Pharmacol. 17:86-94.

Rappe, C., and Buser, H.R., (1980). Chemical properties and analytical methods. In: Halogenated biphenyls, terphenyls, naphthalenes, dibenzodioxins and related products. (Kimbrough, R.D. ed.) Elsevier/North-Holland Biomedical Press, Amsterdam; pp: 41-76.

Raz, A., and Goodman, D.S. (1969). The interaction of thyroxine with human plasma prealbumin and with the prealbumin-retinol binding protein complex. J. Biol. Chem. 244:3230-3237.

Redgrave, T.G. (1970). Formation of cholesteryl ester-rich particulate lipid during metabolism of chylomicrons. J. Clin. Invest. 49:465-471.

Regianni, G. (1980). Localized contamination with TCDD - Seveso, Missouri and other areas. In: Halogenated biphenyls, terphenyls, naphthalenes, dibenzodioxins and related products. (Kimbrough, R.D. ed.) Elsevier/North-Holland Biomedical Press, Amsterdam; pp: 303-371.

Roberts, A.B., and Deluca, H.F. (1967). Pathways of retinol and retinoic acid metabolism in the rat. Biochem. J. 102:600-605.

Roberts, A.B., and DeLuca, H.F. (1969). The effect of DPPD on the decarboxylation of retinoic acid in vitro and in vivo. Arch. Biochem. Biophys. 129:290-295.

Roberts, A.B., Lamb, L.X., and Sporn, M.B. (1980). Metabolism of all-trans-retinoic acid in hamster liver microsomes: Oxidation of 4-hydroxy to 4-keto retinoic acid. Arch. Biochem. Biophys. 199:374-383.

Roberts, A.B., Nicholas, M.D., Newton, D.L., and Sporn, M.B. (1979). In Vitro metabolism of retinoic acid in hamster intestine and liver. J. Biol. Chem. 254:6296-6302.

Rodgers, W.E.Jr., Bieri, J.G., and McDaniel, E.G. (1969). Vitamin A deficiency in the germ-free state In:"The Fat Soluble Vitamins" (DeLuca, H.F., and Suttie, J.W. eds.) Univ. of Wisconsin Press, Madison pp:241-255.

Ross, A.C. (1982). Retinol esterification by rat liver microsomes. Evidence for a fatty acyl coenzyme:retinol acyltransferase. J. Biol. Chem. 257:2453-2459.

Ruf, G. (1972). Organs as targets of dioxin (2,3,7,8-tetrachlorodibenzo-p-dioxin) intoxication. Naturwissen-chaften 59: 174-175.

Ryle, P.R., Hodges, A., and Thomson, A.D. (1986). Inhibition of ethanol-induced hepatic vitamin A depletion by administration of N,N-Diphenyl-p-phenylene-diamine (DPPD). Life Sciences 38:696-702.

Safe, S., Parkinson, A., Robertson, L., Cockerline, R., and Safe, L., (1981). PCB's as microsomal enzyme inducers: structure-activity rules. In: Toxicology of halogenated hydrocarbons. (Khan, M.A.Q. and Stanton, R.H. eds) Pergamon Press, New York pp: 106-132.

Sato, M., and Leiber, C.S. (1982). Increased metabolism of retinoic acid after chronic ethanol consumption in rat liver microsomes. Arch. Biochem. Biophys. 213:557-564

Saito, M., Ikegami, S., Ito, Y., and Innami, S. (1982). Influence of dietary antioxidants on polychlorinated biphenyls (PCB)-induced hepatic lipid peroxide formation and vitamin A reduction in rats. J. Nutr. Sci. Vitaminol. 28:455-466.

Schwetz, B.A., Norris, J.M., Sparschu, G.L., Rowe, V.K., Gehring, P.J., Emerson, J.L. and Gerbig, C.G. (1973). Toxicology of chlorinated dibenzo-p-dioxins. Environ. Health Perspect. 5:87-99.

Seefeld, M.D., and Peterson, R.E. (1983). The starvation-like syndrome and 2,3,7,8-tetrachlorodibenzo-p-dioxin: New ideas on the mode of action at the whole animal level. In: Proc. Thirteenth Ann. Conf. on Environ. Toxicol. University of Calif. Irvine, Overlook branch, Dayton, Ohio. 20:237-249.

Seefeld, M.D., Keesey, R.E, and Peterson, (1984). Body weight regulation in rats treated with 2,3,7,8-tetrachlorodibenzo-p-dioxin. Toxicol. Appl. Pharmacol. 76:526-536.

Seefeld, M.D., and Peterson, R.E. (1984). Digestible energy and efficiency of feed utilization in rats treated with 2,3,7,8-tetrachlorodibenzo-p-dioxin. Toxicol. Appl. Pharmacol. 74:214-222.

Sherrill, B.C., and Dietschy, J.M. (1978). Characterization of the sinusoidal transport process responsible for uptake of chylomicrons by the liver. J. Biol. Chem. 253:1859-1867.

Skare, K.L., Schnoes, H.K., and DeLuca, H.F. (1982). Biliary metabolites of all-trans retinoic acid in the rat: Isolation and identification of a novel polar metabolite. Biochemistry 21:3308-3317.

Sklan, D., Blaner, W.W., Adachi, N., Smith, J.E., and Goodman, D.S. (1982). Association of cellular retinol-binding protein and several lipid hydroxylase activities with a vitamin A-containing high-molecular-weight lipid-protein aggregate from rat liver. Arch. Biochem. Biophys. 214:35-44.

Smith, F.R., Goodman, D.S., Zaklama, M.S., Galor, M.K., El Maraghy, S., and Patwardhan, V.N. (1973). Serum vitamin A, retinol-binding protein and prealbumin concentration in protein-calorie malnutrition. 1. A functional defect in hepatic retinol release. Am. J. Clin. Nutr. 26:973-981.

Smith, F.R., and Goodman, D.S. (1976). Vitamin A transport in human vitamin A toxicity. N. Engl. J. Med. 294:805-808.

Sommer, A., and Green, W.R. (1982). Goblet cell response to vitamin A treatment for corneal xeropthalmia. Am. J. Opthamol. 94:213-215.

Stunkard, A.J., (1982). Anorectic agents lower a body weight set point. Life Sci. 30:2043-2055.

Takahashi, Y.I., and Underwood, B.A. (1974). Effect of long and medium chain length lipids upon aqueous solubility of a-tocopherol. Lipids 9:855-859.

Taylor, J.S. (1974). Chloracne-A continuing problem. Cutis 13:585-591.

Thomas, C.E., and Aust, S.D. (1986). Free radicals and environmental toxins. Ann. Emer. Med. 15:1075-1083.

Thompson, J.N., Howell, J. McC., and Pitt, G.A.J. (1963). Vitamin A and reproduction in rats. Proc. Roy. Soc. London. 159:510-535.

Thunberg, T., Ahlborg, U.G., and Johnsson, H. (1979). Vitamin A status in the rat after a single oral dose of 2,3,7,8-tetrachlorodibenzo-p-dioxin. Arch. Toxicol. 42:265-274.

Thunberg, T., Ahlborg, U.G., Hakansson, H., Krantz, C., and Monier, M. (1980). Effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin on the hepatic storage of retinol in rats with different dietary supplies of vitamin A (retinol). Arch. Toxicol. 45:273-285.

Thunberg, T, Ahlborg, U.G., and Wahlstrom, B. (1984). Comparison between the effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin and six other compounds on the vitamin A storage, the UDP-glucuronosyltransferase and the aryl hydrocarbon hydroxylase activity in the rat liver. Arch. Toxicol. 55:16-19.

Underwood, B.A. (1984). Vitamin A in Animal and Human Nutrition. In: "The Retinoids" (Sporn, M.B., Roberts, A.B. and Goodman, D.S., eds.) Academic Press, Inc. Orlando. pp 282-392.

Van Jaarsveld, P.P., Edelhoch, H., Goodman, D.S., and Robbins, J. (1973). The interaction of human plasma retinol-binding protein with prealbumin. J. Biol. Chem. 248:4698-4705.

Villeneuve, D.C., Clark, M.L., and Clegg, D.J. (1971). Effects of PCB administration on microsomal enzyme activity in pregnant rabbits. Bull. Environ. Contamin. Toxicol. 2:120-128.

Vos, J.G., Moore, J.A., and Zinkl, J.G. (1973). Effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin on the immune system of laboratory animals. Environ. Health Persp. 5:149-162.

Vos, J.G., Faith, R.E., and Luster, M.I. (1980). Immune Alterations. In: Halogenated biphenyls, terphenyls, naphthalenes, dibenzo-dioxins and related products. (Kimbrough, R.D. ed.) Elsevier/North-Holland Biomedical Press, Amsterdam; pp 109-150.

Wald, G., (1960). The visual function of vitamin A. In: Vitamins and Hormones. New York, Academic Press, 18:417-430.

Wilson, J.G. and Warkany, J. (1947). Epithelial keratinization as evidence of fetal vitamin A deficiency. Soc. Exp. Biol. Med. Proc. 64:419-422.

Wolbach, S.B., and Howe, D.R. (1925). Tissue changes following deprivation of fat-soluble A vitamin. J. Exp. Med. 42:753-778.

Wolbach, S.B. (1937). The pathologic changes resulting from vitamin A deficiency. J. Am. Med. Assoc. 108:7-13.

Wolf, G. (1980). Vitamin A. In: "Human Nutrition" (Alfin-Slater, R.B., and Kritchevsky, D., eds.), Plenum, New York pp: 97-203.

Wolf, G. (1984). Multiple Functions of Vitamin A. Physiological Reviews 64(3):873-937.

Zile, M.H., Bunge, E.C., and DeLuca, H.F. (1979). On the basis of vitamin A-stimulated Growth. J. Nutr. 109:1787-1796.

Zile, M.H., Bunge, E.C., and DeLuca, H.F. (1981). DNA labeling of rat epithelial tissues in vitamin A deficiency. J. Nutr. 111:777-788.

Zile, M.H., and Cullum, M.E. (1983). The function of vitamin A: current concepts. Proc. Soc. Exp. Biol. & Med. 172:139-155.

Zile, M.H., and Collum, M.E. (1985). Acute polybrominated biphenyl toxicosis alters vitamin A homeostasis and enhances degradation of vitamin A. Toxicol. Appl. Pharmacol. 81:177-181.

CHAPTER 1

Effects of TCDD, Nonadecafluorodecanoic Acid and Three PCB Congeners on Hepatic Retinyl Palmitate Hydrolase Activity, Plasma Retinol Levels and Transport.

ABSTRACT

The effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), three PCB congeners (3,4,3′,4′-tetrachlorobiphenyl [TCB], 3,4,5,3′,4′,5′-hexachlorobiphenyl [HCB], and 2,4,5,2′,4′,5′-HCB), and nonadecafluorodecanoic acid (NDFDA) on plasma retinol levels, hepatic retinyl palmitate hydrolase activity (RPH) and the stability of the retinol-retinol binding protein-transthyretin (retinol-RBP-TTR) complex were determined in Sprague-Dawley rats in three separate experiments.

The effects of nonadecafluorodecanoic acid (NDFDA) on serum retinol levels and hepatic retinyl palmitate hydrolase (RPH) activity were investigated in male Sprague-Dawley rats given a single i.p. dose of 0, 50, or 100 mg/kg NDFDA and sacrificed at 2, 8 or 11 days. Treated animals had depressed serum retinol levels, pronounced lymphoid involution and a depressed rate of weight gain in proportion to dose. Hepatic RPH activities were depressed in rats treated with NDFDA at all time points and were positively correlated with serum retinol levels. Hepatic retinol levels were also depressed by day 11. Extraction of hepatic homogenates with acetone removed NDFDA and increased RPH activities 2 and 3 fold for the low and high dose groups, respectively. Analysis of partially purified RPH showed both NDFDA and TCDD to be non-competitive inhibitors; K_I = 450 and 750 uM, respectively. We conclude that NDFDA causes a decrease in the mobilization of vitamin A from the liver

by non-competitive inhibition of RPH, and that levels of TCDD present in the liver following even a toxic dose would be insufficient to cause significant inhibition of hepatic RPH.

A single i.p. dose of 1, 5, or 15 mg/kg 3,4,3',4'-tetrachlorobiphenyl (TCB) caused a dose-dependent depression of plasma retinol levels 24 hours after treatment of female Sprague-Dawley rats. The loss of plasma retinol appeared to be a function of depressed levels of the retinol-RBP-TTR complex. No free retinol-RBP was observed in plasma from treated animals. Hepatic retinyl palmitate hydrolase (RPH) activity was also depressed and was highly and positively correlated to the plasma retinol levels. TCB was determined to be a non-competitive inhibitor of partially purified RPH with a K_I of 91 uM. Incubation of TCB with liver microsomes and NADPH decreased the inhibition of RPH. Doses of either 2,4,5,2',4',5'-hexachlorobiphenyl (HCB) or 3,4,5,3',4',5'-HCB (equimolar to the 15 mg/kg TCB dose) failed to cause a similar depression of plasma retinol in treated female rats. We conclude that, in contrast to other PCB congeners, TCB causes a depression of plasma retinol by inhibition of hepatic RPH.

The effects of TCDD on plasma retinol levels were determined in male Sprague-Dawley rats treated with 0, 0.1, 1.0, 10, 30, 100, or 300 nmol/kg body weight (oral), and killed on days 3, 7, or 12 after treatment. In contrast to both PCB and NDFDA treatment, plasma retinol levels were

maximally elevated to ~2x control values in the 100 nmol/kg group 12 days following dose. Changes in plasma retinol levels were highly and positively correlated with changes in retinoid absorbance of the retinol-retinol binding protein-transthyretin peak.

INTRODUCTION

The polyhalogenated aromatic hydrocarbons (PAH's) typified by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), cause a pronounced toxic response in treated animals that resembles a vitamin A deficiency in many respects (Thunberg, 1983a). The ability of the PAH's to cause a depletion of hepatic retinoid levels has been reported in many different species using polychlorinated biphenyls (Villeneuve et al., 1971; Innami et al., 1976,), polybrominated biphenyls (Cecil et al., 1973; Akoso et al., 1982) and TCDD (Thunberg, 1983a).

The effect of these compounds on serum or plasma retinol levels does not appear to be as uniform as hepatic retinoid depletion. Thunberg (1979) has demonstrated that TCDD causes a short-lived elevation of serum retinol, followed by a decline to levels lower than those of control rats. Brouwer and van den Berg (1984, 1985) have reported that at least one PCB congener (3,4,3',4'-tetrachlorobiphenyl; TCB) caused a rapid and pronounced depression of plasma retinol in both mice and rats. McConnell et al., (1979) have reported that PCB contaminated monkeys showed no statistically different changes of plasma retinol levels when compared to uncontaminated animals.

Nonadecafluorodecanoic acid (NDFDA) is structurally different from TCDD and other PAH's, yet has been shown to cause symptoms similar to those of both TCDD-type toxicity and a vitamin A deficiency (Olson et al., 1982). Preliminary

experiments in our laboratory showed that a single i.p. dose of NDFDA rapidly caused a depression in levels of serum retinol not observed in pair-fed control animals (Bank et al., 1986).

In a recent paper, Brouwer and van den Berg (1986) have proposed a mechanism by which TCB and other PAH's may cause a depression of plasma retinol levels following treatment. It was suggested that a metabolite of TCB, which has a high affinity for transthyretin (TTR), binds to and de-stabilizes the ternary complex consisting of retinol, retinol-binding protein (RBP) and transthyretin. This ternary complex is responsible for the transport of retinol in plasma (Goodman, 1984). Presumably, TCB-caused dissociation of the ternary-complex allows the retinol-RBP binary complex to be filtered and subsequently degraded by the kidney. The authors have suggested this model as an explanation for the depletion of hepatic retinoids caused by the PAH's.

An alternative explanation for the observed depression of plasma retinol levels following a TCB or an NDFDA dose would be the inhibition of the enzyme responsible for the release of retinol from stored retinyl esters within the hepatic parenchymal cells, retinyl palmitate hydrolase (RPH; Goodman and Blaner, 1984). Inhibition of this enzyme could limit the amount of retinol available for complexation with apo-RBP, normally present in excess in the liver (Goodman, 1984).

We have therefore hypothesized that the primary mechanism by which TCB and NDFDA rapidly cause a depression in plasma retinol levels in treated rats is the inhibition of hepatic RPH. Further, we have hypothesized that TCDD, contrary to the suggestion of Brouwer and van den Berg, will not cause a depression of retinol levels, nor inhibit RPH, nor cause a dissociation of the ternary complex. We also hypothesize that TCB, unlike other PCB's but similar to NDFDA, causes a depression of serum retinol levels by inhibition of hepatic RPH.

We have examined these hypotheses in three separate experiments. In experiment 1, we have determined the effect of NDFDA on rat serum retinol and hepatic RPH in vivo, and both NDFDA and TCDD on hepatic RPH activity in vitro. In experiment 2, we evaluated similar parameters in rats following treatment with TCB. In experiment 3, we evaluated the dose-response characteristics of the effect of TCDD on plasma retinol, and the composition of the ternary complex.

MATERIALS AND METHODS

Animals; treatment and tissue preparation

Experiment 1 (NDFDA):

Male Sprague-Dawley rats, 120-140 g, were procured from Charles River Co. (Portage, MI.) and acclimated for 1 week on hardwood bedding, with access to a standard diet (Rodent-Blox(R); Wayne Feeds, Chicago, IL.) and tap water *ad libitum*. The animals were given a single i.p. dose of NDFDA at 0, 50, or 100 mg/kg in peanut oil (10 ml/kg). Three animals per dose were killed at 2, 8 and 11 days post treatment by CO_2 anesthesia and exsanguination via open chest cardiac puncture. Serum was prepared by allowing the blood to clot for 1 hr at 4^{o} C, followed by centrifugation at 2000 x g for 10 min. Serum was aspirated and stored at -20 ^{o}C. Liver and thymus were excised, rinsed in cold aq. 1.15% KCl (w/v), weighed, immediately frozen on dry ice, and stored at -20^{o} C. Liver homogenates were prepared by homogenization of liver in 10 volumes of 50 mM 3-(N-morpholino)-propane-sulfonic acid, 0.02% sodium azide, pH 7.2, containing 2% (w/v) sodium cholate (MOPS - cholate), in a Potter-Elvehjem homogenizer. Acetone-dried preparations of the liver homogenates were made by mixing the homogenate with 17 volumes reagent grade acetone. The mixture was centrifuged at 2000 x g for 5 min and the supernatant decanted and discarded. The extraction and centrifugation step was repeated two additional times, and the resultant pellets were solubilized in 1.0 ml MOPS - cholate buffer by

sonication. The mixture was centrifuged at 2000 x g for 5 min and the supernatant was stored at 4° C until use.

Experiment 2 (TCB and HCB):

Female Sprague-Dawley rats, 175-195 g were procured from Charles River Co. (Portage, MI) and acclimated for 1 week on hardwood bedding with access to a standard rodent diet (Rodent-Blox(R); Wayne Feeds, Chicago, IL) and tap water ad libitum. Three rats were randomly assigned to each of four treatment groups. All rats were given a single i.p. dose of 3,4,3',4'-tetrachlorobiphenyl (Analabs, North Haven, CT) at 0, 1.0, 5.0, or 15.0 mg/kg in corn oil (10 ml/kg). In a separate experiment, rats were similarly treated with 0 or 18 mg/kg of 2,4,5,2',4',5'- or 3,4,5,3',4',5'-hexachlorobiphenyl (Analabs). All rats were fasted overnight and killed 24 hours after dosing by exsanguination via cardiac puncture following CO_2 anesthesia. Blood was drawn into 7 ml EDTA-Vacutainer tubes (Becton-Dickinson, Rutherford, NJ) and stored on ice. Plasma was prepared by centrifugation (1500 x g, 10 min at 4° C) and stored at -20° C until analysed for retinol. Liver was perfused in situ with 0.9% NaCl, excised, and stored in the same solution until homogenization. Kidneys were excised, frozen on dry ice, and stored at -20° C prior to homogenization. Livers were weighed and homogenized in 4 ml 0.9% sodium chloride per g wet weight in a Potter-Elvehjem homogenizer. Kidneys were later thawed, weighed,

and homogenized in 9 ml 0.9% NaCl per g wet weight, as per liver. Homogenates thus prepared were frozen and stored at -20^{o} C prior to analysis for retinol and RPH activity.

Experiment 3, (TCDD):

Male Sprague-Dawley rats, 140-160 g were procured from Charles River Co. (Portage, MI) and acclimated for 1 week on hardwood bedding. The rats were given a single oral dose of corn oil containing 0.0, 0.1, 1.0, 10, 30, 100, or 300 nmol TCDD/ml. Three animals per dose level were killed 3, 7, and 12 days after dose as per experiment 2, above. An additional group of three rats were dosed with corn oil alone on day 0, placed on a vitamin A-deficient diet (AIN-76, VA(-); US Biochemical Corp., Cleveland, OH) and killed on day 12. Blood was sampled and plasma prepared from all animals as per experiment 2.

Retinoid Levels

Serum (or plasma), hepatic and/or renal retinol levels were determined by a modification of the method of Dennison and Kirk (1977). Briefly, a 200 ul aliquot of plasma or tissue homogenate was mixed with 2 ml sodium chloride-saturated water and 2 ml reagent grade ethanol. Retinol was extracted into 1.0 ml of HPLC-grade hexane. Phase separation was facilitated by centrifugation (2500 x g, 2 min). The supernatant was analysed for retinol by HPLC using a fluorescence detector (Shimadzu RS-530-S, λex = 330 nm, λem = 470 nm) and a 4 x 250 mm column packed with

Lichrosorb SI-60 (Alltech, Deerfield, IL). Isocratic elution of retinol was performed using 25% hexane in chloroform (v/v). Retinol was eluted at a retention time of 5.5 min with a detection limit of 500 pg. Retinyl palmitate in 1.0 ml kidney homogenate samples was determined by the same method, with the exception that the mobile phase used was 8% chloroform in hexane (v/v). Retinol and retinyl palmitate standards (Sigma Chemical Co., St. Louis, MO) were prepared in ethanol and verified by $E^{1\%}_{324\ NM} = 1835$ and $E^{1\%}_{328\ NM} = 975$, respectively (Windhols, 1976). HPLC standards of 1.0 and 0.1 ng/ul for both compounds were prepared by dilution of the stock standard with hexane. All standards were stored at -20° C and remained stable for at least six months.

Analysis of RPH Activity

RPH activity in hepatic homogenates was determined by a modification of the method described by Prystowsky et al., (1981). A 200 ul aliquot of the hepatic homogenate described above was added to 1.78 ml MOPS-cholate buffer (50 mM 3-(N-morpholino)-propane sulphonic acid, pH 7.2, 2.0% sodium cholate, 0.02% sodium azide) and 20 ul of 10 nmol/ul retinyl palmitate (in ethanol) incubated for 20 min at 37° C in a shaking water bath. The reaction was terminated by the addition of 1 ml ethanol and 1 ml sodium chloride-saturated water. Retinol in the incubation mixture was extracted with hexane and quantitated by HPLC as described

above for plasma retinol. Samples were corrected for the amount of retinol present in unincubated samples. Protein concentration of the homogenate was determined by a bicinchoninic acid microassay (Redinbaugh and Turley, 1985). RPH activity was calculated and expressed as pmol retinol formed per min per mg protein.

Partial Purification of RPH

Retinyl palmitate hydrolase was partially purified from rat livers by a modification of the method described by Prystowsky et al. (1981). Briefly, an acetone-dried powder was prepared by homogenization of liver in acetone (0.02 g/ml) and removal of the acetone by evaporation. The acetone-dried powder was solubilized in MOPS-cholate buffer at 2 ml/g of original liver weight. Material in this solution which precipitated between 33% and 70% saturation with ammonium sulfate was collected by centrifugation, resolubilized in and dialyzed against MOPS-cholate, and used in the experiments described below.

In Vitro Inhibition of RPH by NDFDA and TCDD

The effect of NDFDA and TCDD on the kinetics of the hydrolysis of retinyl palmitate by RPH was determined using the standard RPH incubation described above, modified as follows: substrate (retinyl palmitate) was provided at 0, 2.5, 5.0, 10, 20, and 40 uM. Fifty ul of partially purified RPH solution were used for all assays, (6.9 mg/ml protein), adjusted to a final volume of 2.0 ml with MOPS-cholate

buffer. The effect of NDFDA or TCDD on the activity of RPH was studied by including each compound at a concentration of 500 uM in separate assays. All assays were performed in triplicate, the experiment repeated in the absence of NDFDA and TCDD, and the results expressed as a mean $\pm$ SD. Kinetic constants Km, Vmax, and K_I for NDFDA and TCDD were calculated from Lineweaver-Burke (double-reciprocal) plots of the data.

In Vitro Inhibition of RPH by TCB and Two HCB isomers

The ability of TCB to inhibit RPH in vitro was evaluated by examining the effect of TCB on the kinetics of the hydrolysis of retinyl palmitate by the enzyme preparation described above. Control incubations were performed in triplicate with 200 ul of partially- purified RPH (2.2 mg/ml) and substrate (retinyl palmitate) supplied at 0, 2.5, 10, 20, and 40 uM. The Km and Vmax were calculated from a Lineweaver-Burke plot of the data. The effect of TCB on RPH activity was determined by repeating the above assays in the presence of 100 uM TCB (added to the incubation buffer in 5 ul ethanol). The Km and Vmax for the inhibited RPH activity were calculated from a Lineweaver-Burke plot, as above.

Analysis of Hepatic NDFDA Levels

A 0.2 ml aliquot of the liver homogenate (described above) was transferred to a centrifuge tube containing 1 ml NaCl-saturated H_2O, and 1 ml ethanol (containing 0.1% sodium

ascorbate, w/v), and mixed thoroughly. This mixture was extracted three times with 1 ml of hexane, and the extracts were combined and evaporated to dryness at 35° C. A 2.0 ml volume of 14% boron trifluoride/methanol (Pierce Chemical Co., Rockford, IL) was added and the sample heated at 60 °C. for 90 min. The sample was allowed to cool, diluted with 2 ml NaCl-saturated H_2O, and extracted with 1.0 ml hexane. NDFDA methyl esters (Me-NDFDA) in the extract were quantitated by gas chromatography using an electron-capture detectore. The chromatographic system consisted of a 6' x 4 mm column packed with 3% OV-101 on 80/100 Gas Chrom Q at 80 °C with N_2 at 15 ml/min. Standards were prepared by a quantitative derivatization and extraction of 200 mg of NDFDA (Sigma). Standard purity was evaluated by GC-Mass Spectrometry and found to be >98% Me-NDFDA, by total ion current.

Fractional Distribution of Retinol in Plasma

Plasma proteins were separated on the basis of molecular weight on an HPLC using a Spherogel TSK G3000 SW column (Beckman, Berkeley, CA) and a mobile phase of 1.15% potassium chloride, 0.04% sodium azide. Aliquots of plasma (100 ul) from each rat per treatment group were combined and mixed with an equal volume of mobile phase. Aliquots of 20 ul of the combined diluted plasma were injected into the HPLC and column effluent was monitored for absorbance at 280 nm with a Schoeffel SF770 UV/visible detector and for

fluoresence (λex = 330 nm, λem = 470 nm) with a Shimadzu RF-530-S detector, connected in series. The peak corresponding to the retinol-RBP-TTR complex was identified by retinoid fluoresence and estimated molecular weight. The chromatographic system was standardized by the use of molecular weight marker proteins analysed on separate chromatographic runs.

Effect of Microsomal Incubation on the Ability of TCB to Inhibit RPH In Vitro

Microsomes were prepared from the livers of rats treated for 3 days at 40 (mg/kg)/day, oral, with the methyl cholanthrene (MC)-type inducer B-naphthoflavone (BNF), as previously described (Millis et al., 1985). Reaction mixtures consisted of 40 ul of resuspended microsomes (25 mg protein/ml), 1 ml PGE buffer (10 mM phosphate, pH 7.4, 20% glycerol and 0.1mM EDTA), TCB (100 uM), and/or 100 ul of an NADPH-generating system, and were incubated for 1 hr at 37^{o} C in a shaking water bath, conditions previously shown to produce metabolic degradation of TCB (Millis et al., 1985). Following this incubation, the mixture was evaluated for endogenous RPH activity by the addition of 100 ul 20% sodium cholate and 20 ul 10 nmol/ul retinyl palmitate and incubation in the dark at 37^{o} C for 20 min in a shaking water bath. The reaction was terminated, retinol extracted, and the amount of retinyl palmitate hydrolysis determined as described above for RPH activity analyses.

Statistical Analysis

Statistical analyses were performed using Student's t-test with the Bonferroni correction for multiple comparisons (Godfrey, 1985).

RESULTS

Experiment 1 (NDFDA)

Body and Organ Weights

A rapid and pronounced dose-related effect was observed on the body weight of the NDFDA-treated animals, with the high dose group showing a progressive decline in weight (Figure 1). However, while the low dose group initially lost weight, they seemed to recover to a near normal growth rate from day 5 through day 11. An increase in the liver wt./body wt. ratio and lymphoid involution, as measured by a decline in the thymus wt./body wt. ratio, were observed in treated animals, as shown in Table 1.

Serum and Hepatic Retinol

A significant effect of NDFDA treatment on serum retinol levels was observed (Figure 2). By two days post-dose, the average serum retinol concentration in the high-dose treated group was decreased to only 18% that of the control animals. Serum retinol concentration in the low dose treated group was similarly depressed to 39% of the control level. While the low-dose treated animals showed some recovery by day 11 (to 78% of control values), the high dose group was unable to significantly increase serum retinol levels. Hepatic retinol levels did not correlate with serum values, and were only significantly depressed relative to control animal values at day 11, as shown in Figure 3.

Figure 1. Body-weight changes in control and NDFDA-treated rats. Values are means for groups of n = 3 rats treated with 0 (—○—), 50 (—△—), or 100 (—□—) mg/kg.

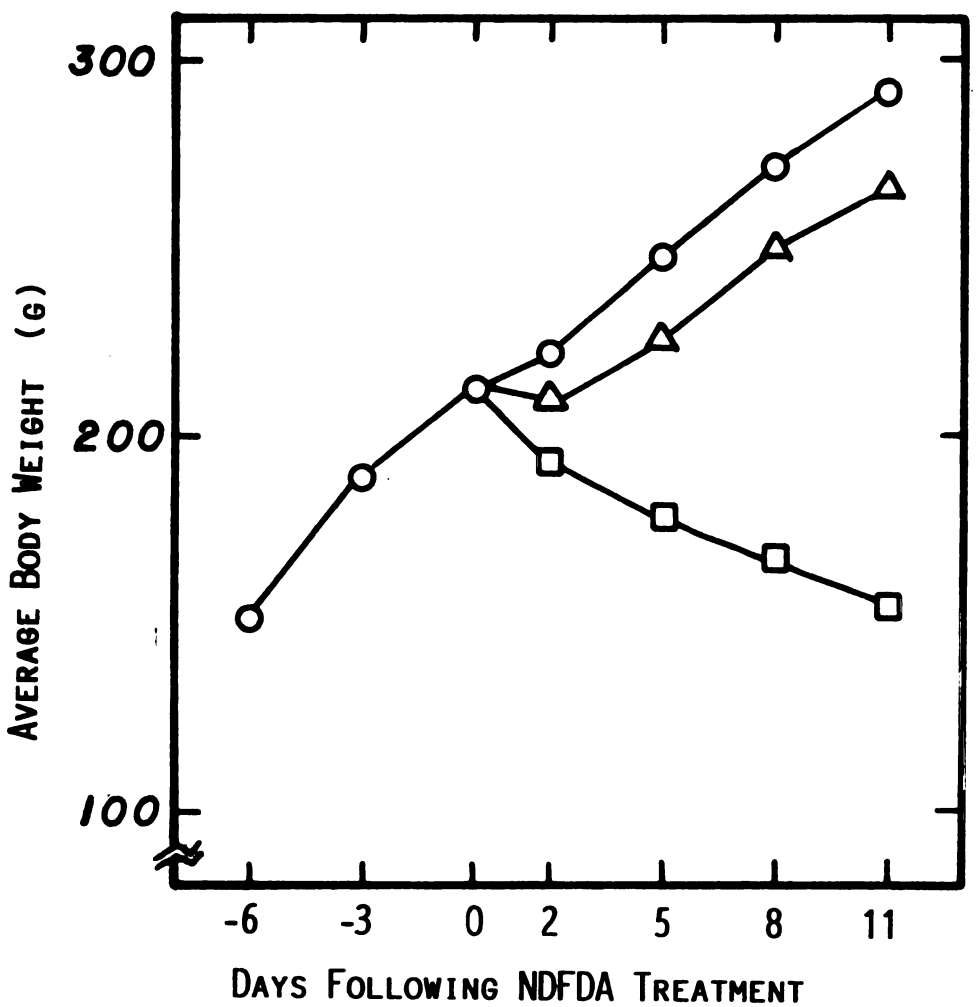

300
200
100
AVERAGE BODY WEIGHT (G)
-6
-3
0
2
5
8
11
DAYS FOLLOWING NDFDA TREATMENT

Table 1. The Effect of a Single i.p. Dose of NDFDA on Hepatic and Thymic Body Weight Ratios in Male Sprague-Dawley Rats ($x10^3$).

	Days Following Treatment			
Group	0	2	8	11
Control:				
Liver wt./Body wt.	52±3	52±2	55±5	46±2
Thymus wt./Body wt.	3.7±0.5	2.8±0.2	2.5±0.1	2.2±0.3
50 mg/kg:				
Liver wt./Body wt.	----	59±2	71±4[a]	67±3[a]
Thymus wt./Body wt.	----	3.3±0.3	2.4±0.1	2.2±0.2
100 mg/kg:				
Liver wt./Body wt.	----	54±1	60±7	69±7[a]
Thymus wt./Body wt.	----	2.8±0.2	1.2±0.7[a]	0.9±0.5[a]

[a]: Significantly different from control values; $p < 0.05$.

Figure 2. Serum retinol levels in control and NDFDA-treated rats. Values are means ± S.E. for groups of n = 3 rats treated with 0 (—○—), 50 (—△—), or 100 (—□—) mg/kg. Significantly different from control value: a = p <0.025, b = p <0.010, c = p <0.005.

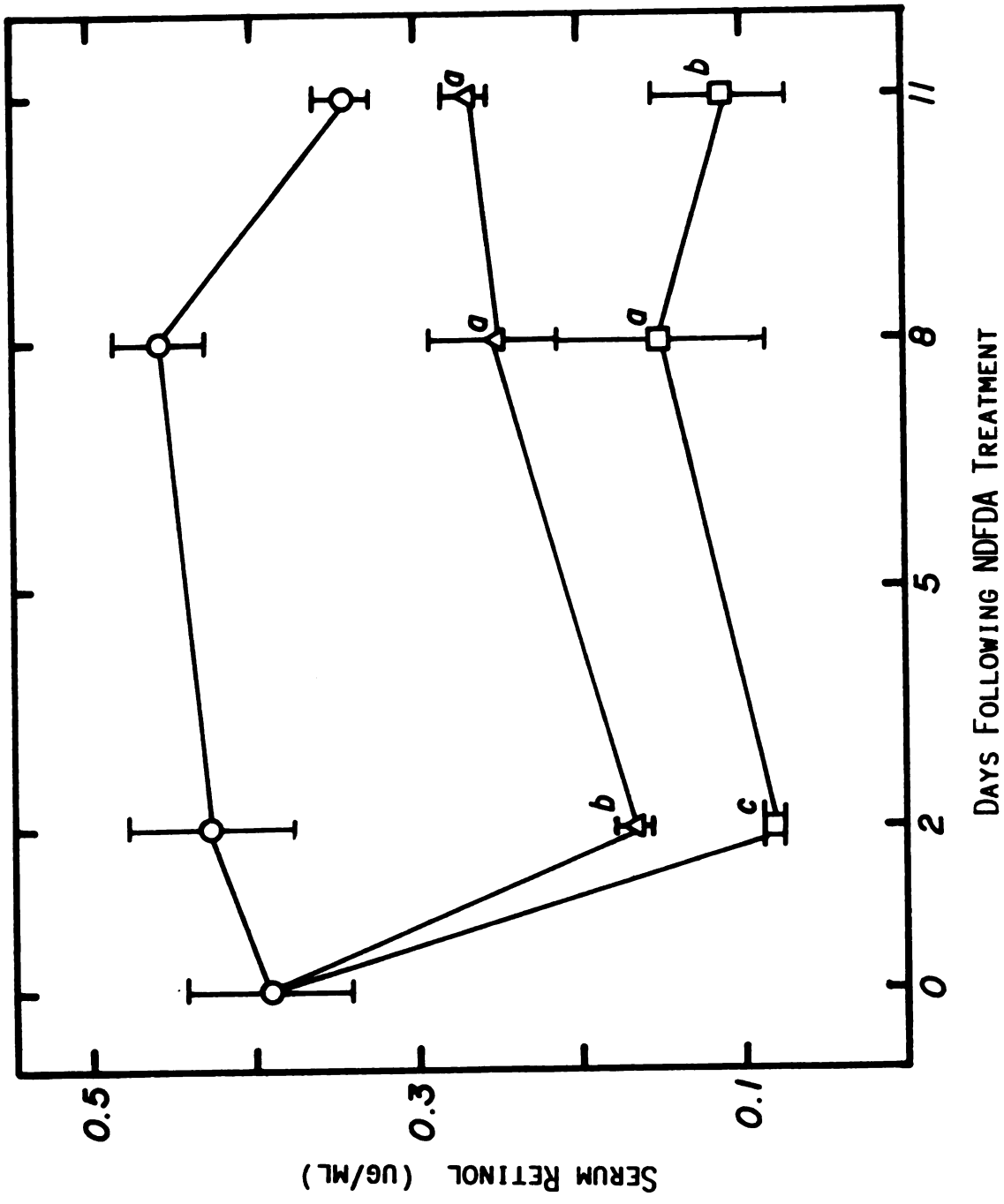
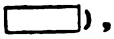
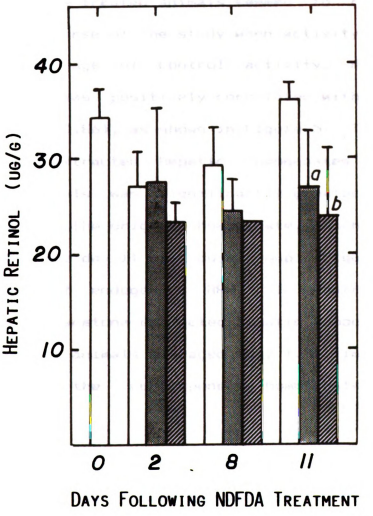

SERUM RETINOL (UG/ML)
0.5
0.3
0.1
0
2
5
8
11
DAYS FOLLOWING NDFDA TREATMENT
a
a
a
b
b
c

Figure 3. Hepatic retinol levels in control and NDFDA-treated rats. Values are means + S.E. for groups of n = 3 rats treated with 0 (), 50 (), or 100 () mg/kg. Significantly different from control value: a = $p < 0.1$, b = $p < 0.05$.


40
30
20
10
HEPATIC RETINOL (UG/G)
a
b
0
2
8
11
DAYS FOLLOWING NDFDA TREATMENT

Hepatic RPH Activity

A pronounced depression in RPH activity in the hepatic homogenates from both NDFDA-treated groups was noted at day 2 and activities remained low with respect to control values throughout the study (Figure 4). As with other parameters, the low dose treated animals seemed to recover somewhat during the course of the study when activity was considered as a percentage of control activity. RPH activity in homogenates was positively correlated with serum retinol levels ($r = 0.86$), as shown in Figure 5. The RPH activity in acetone-extracted hepatic homogenates prepared from treated animals was significantly greater than the RPH activity in the original homogenate, reaching a ratio of 4.5:1 in the day 11 high dose group (Figure 6), showing inhibition of endogenous RPH. In contrast, the RPH activity in acetone-extracted hepatic homogenates prepared from control animals averaged only 1.35 times that of the activity of the corresponding homogenate for all time points.

In Vitro Inhibition of RPH by NDFDA and TCDD

In vitro analysis of the ability of NDFDA and TCDD to inhibit the activity of partially purified RPH at a concentration of 500 uM showed that each compound was able to cause significant inhibition of the enzyme (Figure 7). Analysis of the data by a double reciprocal (Lineweaver-Burke) plot suggests that both compounds act as non-

Figure 4. Hepatic retinyl palmitate hydrolase activity of control and NDFDA-treated rats. Values are means ± S.E. for groups of n = 3 rats treated with 0 (—○—), 50 (—△—), or 100 (—□—) mg/kg. Significantly different from control value: a = p <0.025, b = p <0.010, c = p <0.005.

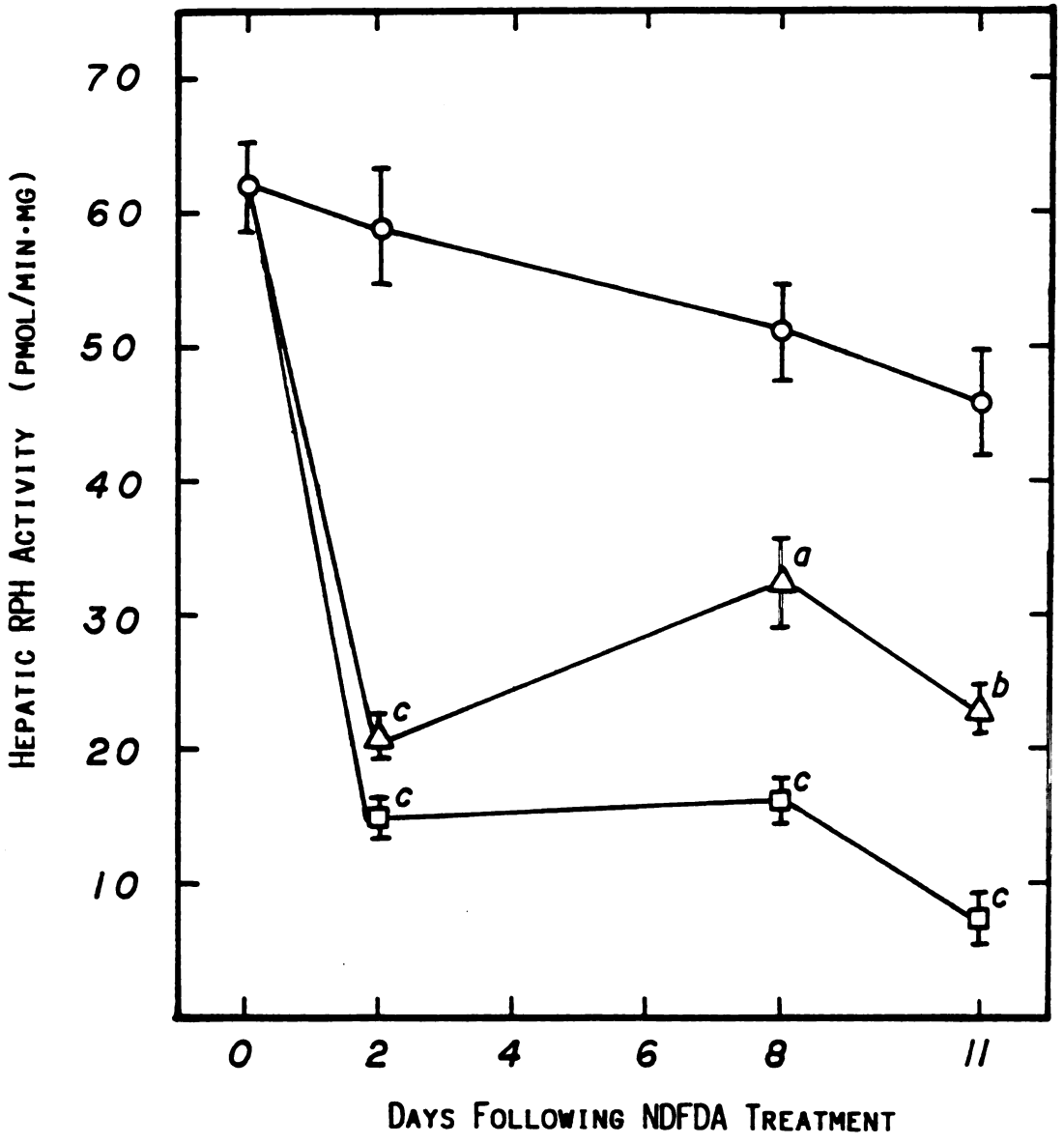

HEPATIC RPH ACTIVITY (PMOL/MIN·MG)
70
60
50
40
30
20
10
0
2
4
6
8
11
DAYS FOLLOWING NDFDA TREATMENT
a
b
c
c
c
c

Figure 5. Correlation of serum retinol levels with hepatic RPH activity in control and NDFDA-treated rats. Values are means $\pm$ S.E. for groups of n = 3.

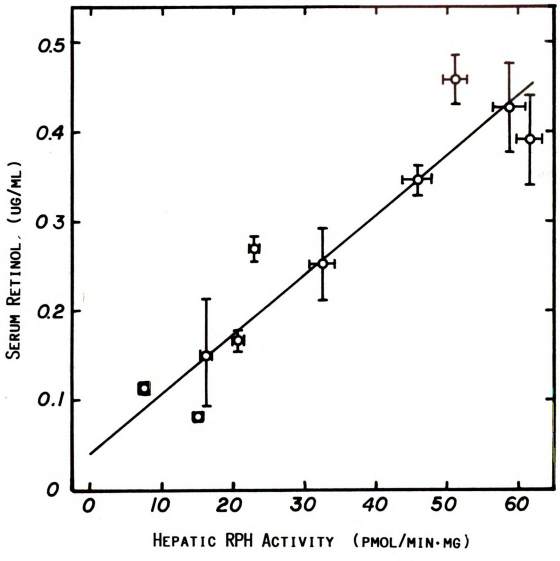

0.5
0.4
0.3
0.2
0.1
0
SERUM RETINOL (UG/ML)
0
10
20
30
40
50
60
HEPATIC RPH ACTIVITY (PMOL/MIN·MG)

Figure 6. Increase in RPH activity in acetone-extracted hepatic homogenates from control and NDFDA-treated rats. Values are means + S.E. of the ratio of the acetone-extracted homogenate to the original homogenate activity for groups of n = 3, treated with 0, (), 50 (), or 100 () mg/kg. Significantly different from control value: a = $p < 0.10$, b = $p < 0.05$, c = $p < 0.01$.

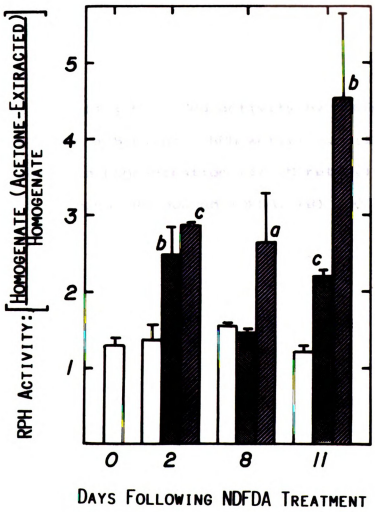
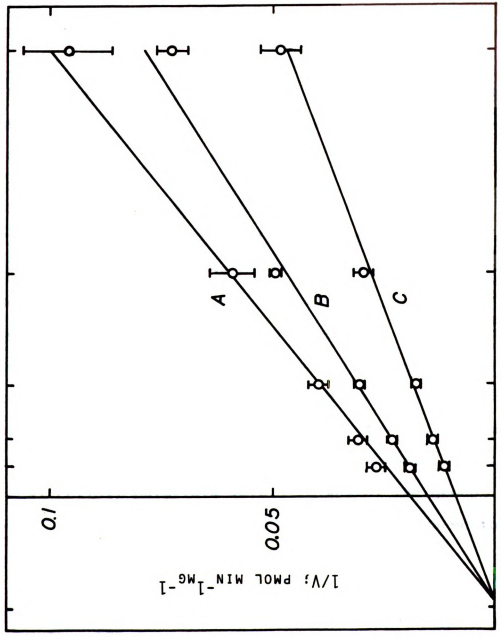

RPH Activity: [Homogenate (Acetone-Extracted) / Homogenate]
5
4
3
2
1
b
c
b
a
c
b
0
2
8
11
Days Following NDFDA Treatment

Figure 7. Inhibition of RPH activity by NDFDA and TCDD. Values are means ± S.E. of 1/RPH activity, (1/pmol/min-mg) versus 1/substrate concentration (1/[uM retinyl palmitate]). Incubation contining (A) 500 uM NDFDA, (B) 500 uM TCDD, (C) control.


0.1
0.05
1/V; pMOL MIN^{-1}MG^{-1}
-0.1
0
0.5
0.1
0.2
0.4
A
B
C
1/S; 1/[RETINYL PALMITATE], uM

competitive inhibitors, with a K_I = 450 uM for NDFDA and 750 uM for TCDD.

Hepatic NDFDA Levels

Hepatic NDFDA concentration was inversely correlated with serum retinol levels (Day 2, r = -0.66; Day 8, r = -0.93; Day 11, r = -0.92) and liver RPH activity (Day 2, r = -0.64; Day 8, r = -0.58; Day 11, r = -0.85). NDFDA levels in the treated animals were found to decline throughout the study, as shown in Table 2.

Experiment 2 (TCB and 2 HCB isomers):

Plasma, Hepatic, and Renal Retinoid Levels

A single i.p. dose of TCB caused, at the two highest dose rates, at 24 hours, a depression in plasma retinol levels that was dose-dependent and significantly different from control rat levels, as shown in Figure 8. Neither the 2,4,5,2',4',5',- nor 3,4,5,3',4',5'-HCB congeners caused a similar depression of plasma retinol levels. No significant differences were noted in hepatic or renal retinol, or renal retinyl palmitate levels in any of the groups treated with 3,4,3',4'-tetrachlorobiphenyl when compared to control group values (data not shown).

Hepatic RPH Activity

The TCB dose caused a significant and dose-dependent depression of hepatic RPH activity of all treated groups when compared to control group animals, as shown in Figure 9. Further, the hepatic RPH activity was positively

Table 2. Hepatic NDFDA Levels[a] Following a Single i.p. Dose in Male Sprague-Dawley Rats.

Treatment Group	Days Following Treatment			
	0	2	8	11
Control	ND[b]	ND[b]	ND[b]	ND[b]
NDFDA, 50 Mg/Kg	-	120±20	110±40	69±9
NDFDA, 100 Mg/Kg	-	270±50	120±40	130±30

[a]: Mean PPM NDFDA for groups of n = 3, ± SD.

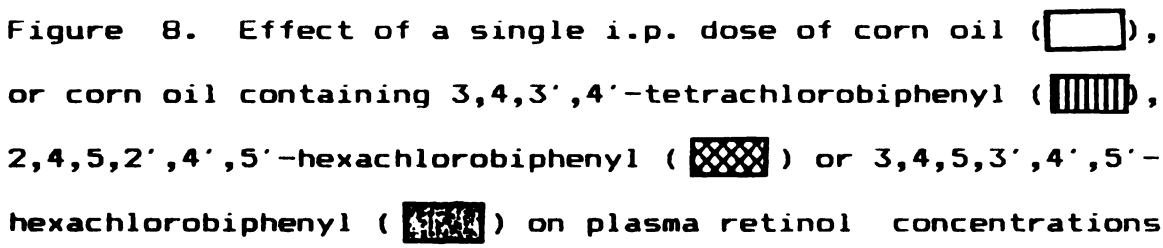


Figure 8. Effect of a single i.p. dose of corn oil ( ), or corn oil containing 3,4,3′,4′-tetrachlorobiphenyl ( ), 2,4,5,2′,4′,5′-hexachlorobiphenyl ( ) or 3,4,5,3′,4′,5′-hexachlorobiphenyl ( ) on plasma retinol concentrations in female Sprague-Dawley rats 24 hours post-treatment. Each bar represents the mean ± SD of three rats and asterisks denote significant difference from controls ($p < 0.05$). Numbers in parentheses at the top of each bar represent the percentage of control value.

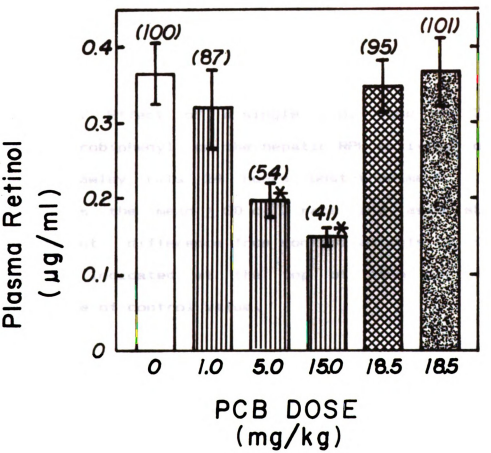
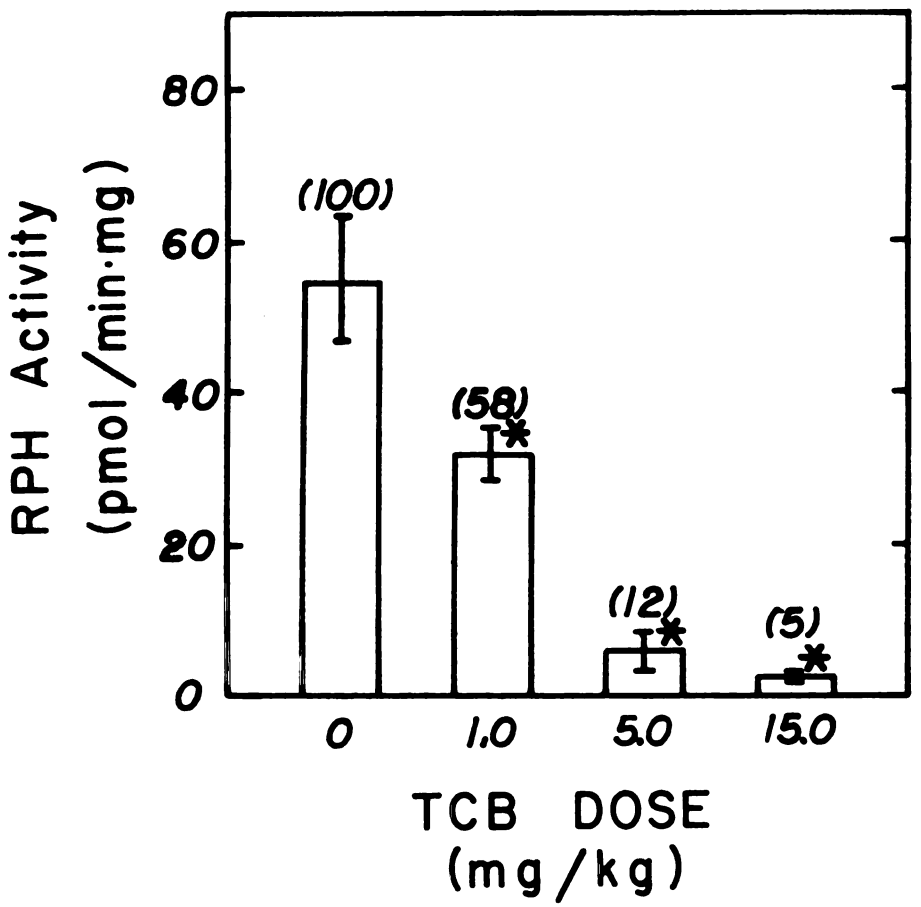

(100)
(87)
(54)
(41)
(95)
(101)
0.4
0.3
0.2
0.1
0
0
1.0
5.0
15.0
18.5
18.5
Plasma Retinol
(µg/ml)
PCB DOSE
(mg/kg)

Figure 9. Effect of a single i.p. dose of 3,4,3',4'-tetrachlorobiphenyl on the hepatic RPH activity of female Sprague-Dawley rats 24 hours post-treatment. Each bar represents the mean $\pm$ SD of 3 rats and asterisks denote significant difference from control animals ($p < 0.05$). Numbers indicated at the top of each bar represent percentage of control value.


RPH Activity
(pmol/min·mg)
80
60
40
20
0
(100)
(58)
(12)
(5)
*
*
*
0
1.0
5.0
15.0
TCB DOSE
(mg/kg)

correlated (r = 0.90) with plasma retinol, as shown in Figure 10. This relationship could be considered as a linear (plasma retinol = 0.164 + 3.69 x 10^{-3} (RPH Activity); SD of fit = 3.56 x 10^{-2}) or a second degree polynomial function (plasma retinol = 0.133 + 8.81 x 10^{-3} (RPH Activity) + 9.08 x 10^{-5} (RPH Activity); SD of fit = 7.47 x 10^{-3}).

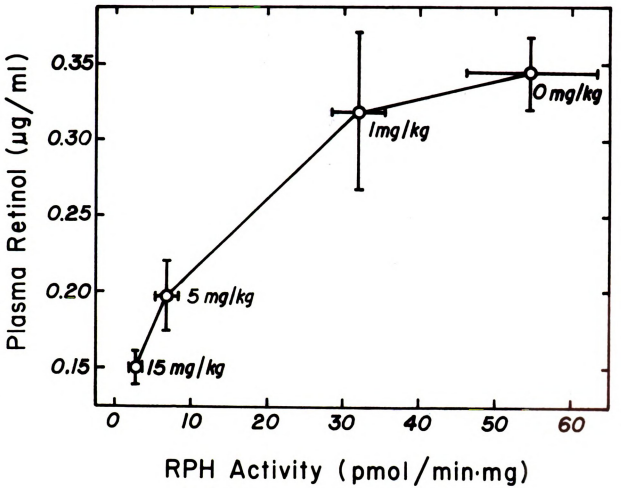
In Vitro Inhibition of RPH by TCB

TCB significantly inhibited the activity of retinyl palmitate hydrolase when included in an RPH assay incubation at 100 uM, as shown in Figure 11. The Km for retinol (8.6 uM), determined by analysis of a double-reciprocal plot of the substrate-velocity data, appeared to be unaffected by TCB. However, the apparent Vmax (17 pmol/min-mg) was significantly lower than that for control incubations (41 pmol/min-mg), indicating that the inhibition was non-competitive with a K_I of 91 uM.

Fractional Distribution of Retinol Among Plasma Proteins

The analysis of combined plasma samples from the different treatment groups by HPLC yielded a fluorescent peak corresponding to the ternary complex of retinol-RBP-TTR. It appeared that the difference in retinol levels between the control and treated groups could be accounted for entirely by changes in the retinol-RBP-TTR peak (Figure 12). No peaks corresponding to free retinol-RBP were observed.

Figure 10. Relation of plasma retinol levels and hepatic RPH activity in female Sprague-Dawley rats 24 hours following a single i.p. dose of 3,4,3′,4′-tetrachlorobiphenyl. Each point represents the mean ± SD of 3 rats for each parameter.


Plasma Retinol (μg/ml)
0.35
0.30
0.25
0.20
0.15
0
10
20
30
40
50
60
RPH Activity (pmol/min·mg)
0 mg/kg
1mg/kg
5 mg/kg
15 mg/kg

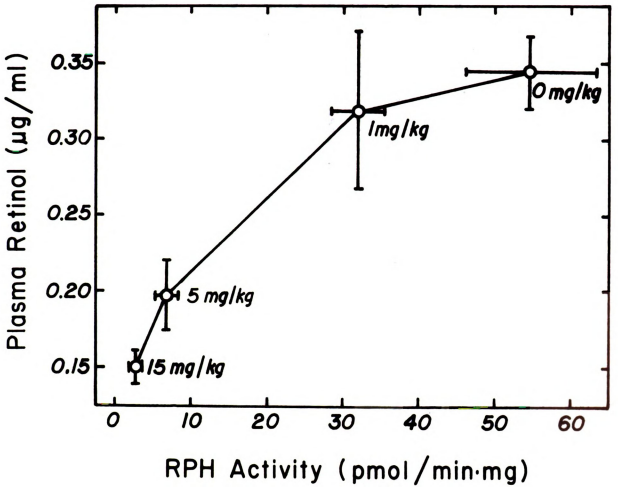

Plasma Retinol (µg/ml)
0.35
0.30
0.25
0.20
0.15
0
10
20
30
40
50
60
RPH Activity (pmol/min·mg)
0 mg/kg
1mg/kg
5 mg/kg
15 mg/kg

Figure 11. Inhibition of RPH activity by 3,4,3',4'-tetrachlorobiphenyl. Values are means ± SD of 1/RPH activity (1/pmol $min^{-1}mg^{-1}$) versus 1/retinyl palmitate concentration (1/[retinyl palmitate], uM) for three replicate RPH assays, as described in "Methods." Insert values are the corresponding means ± SD of RPH activity (pmol/min mg) versus retinyl palmitate concentration (uM retinyl palmitate) for three replicate assays.

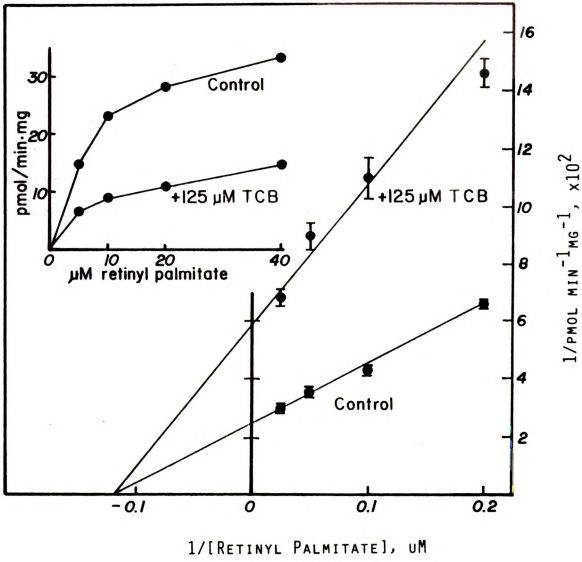
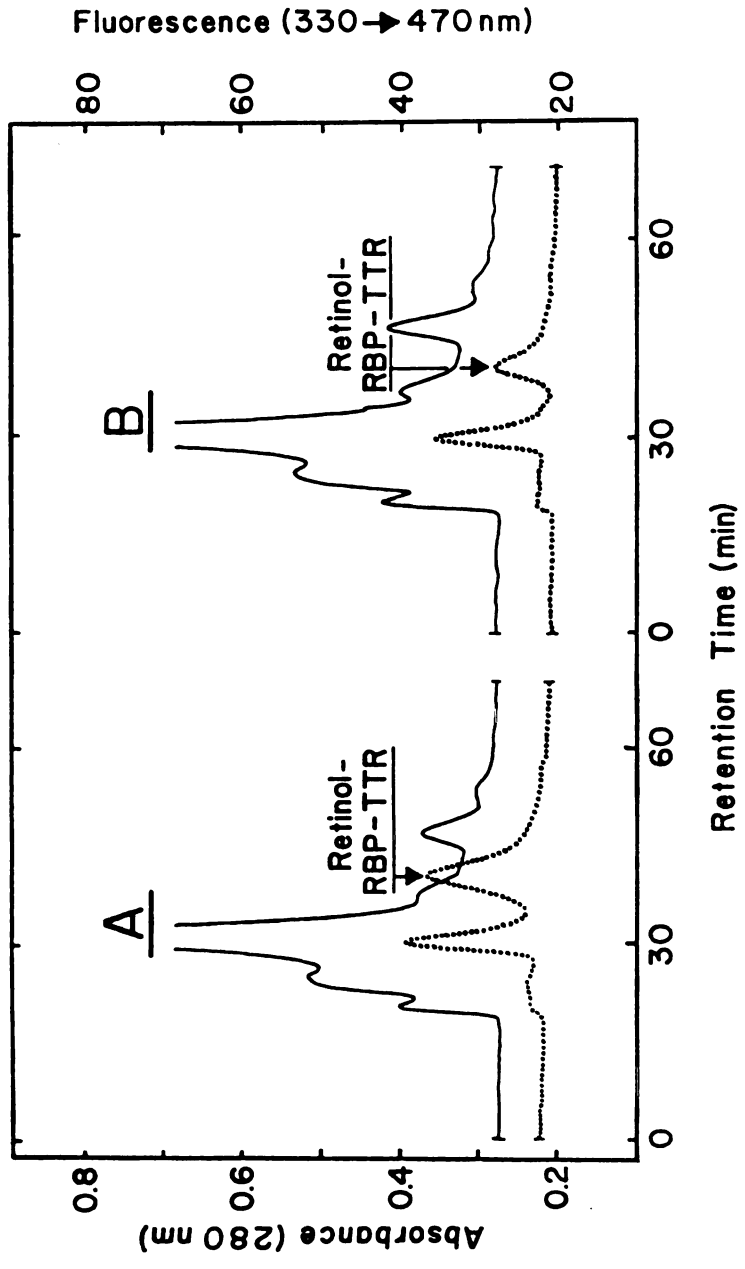

pmol/min.mg
30
20
10
Control
+125 μM TCB
0
10
20
40
μM retinyl palmitate
+125 μM TCB
Control
16
14
12
10
8
6
4
2
-0.1
0
0.1
0.2
1/[Retinyl Palmitate], uM
1/pmol min-1mg-1, x10^2

Figure 12. HPLC analysis of combined plasma samples from rats treated with 0 (A) or 15 mg/kg (B) 3,4,3',4'-tetrachlorobiphenyl. Samples were 10 ul aliquots of combined plasma diluted 1:1 with mobile phase, as described in "Methods." Column effluent was monitored for absorbance at 280 nm (———) and fluorescence at 330 nm excitation, 470 nm emission (·····).


Fluorescence (330→470nm)
80
60
40
20
A
B
Retinol-
RBP-TTR
Retinol-
RBP-TTR
0.8
0.6
0.4
0.2
Absorbance (280 nm)
0
30
60
0
30
60
Retention Time (min)

Effect of Incubation of TCB with Microsomes on the Inhibition of RPH in vitro

Inhibition of endogenous microsomal RPH activity by TCB was significantly reduced by an initial incubation of the microsome-TCB mixture with an NADPH-generating system, as shown in Table 3.

Experiment 3, (TCDD)

Plasma Retinol Levels

A single dose of TCDD in male Sprague-Dawley rats caused a dose-dependent elevation of plasma retinol levels through the 100 nmol/kg dose level 12 days after treatment, as shown in Figure 13. Animals treated with 300 nmol/kg did not have an elevation of the plasma retinol level, and had actually a lower average level than did controls, although the difference was not statistically significant.

Distribution of Retinol Among Plasma Proteins.

The retinoid fluorescence of the ternary complex of combined plasma samples from both control and treated groups were strongly and positively correlated ($r = .978$; Figure 14). There was no evidence of retinoid fluorescence in the region of the HPLC chromatogram corresponding to the retinol-RBP complex.

Table 3. The Effect of Incubation with Microsomes and NADPH on the Ability of 3,4,3',4'-Tetrachlorobiphenyl to Subsequently Inhibit Endogenous Retinyl Palmitate Hydrolase Activity[a].

Incubation Conditions	Retinyl Palmitate Hydrolase Activity: pmol min-mg (x + SD)	Retinyl Palmitate Hydrolase Activity: % Control Activity
+ NADPH	37 ± 1	100
+ TCB	8.2 ± 0.7[b]	22
+ NADPH, TCB	33 ± 2	88

[a]: Microsomes were incubated for 60 min at 37° C with an NADPH generating system, TCB (100 uM) or both. RPH activity was measured in a subsequent assay following the addition of 100 ul sodium cholate (20%), as per "Methods."

[b]: Significantly different ($p < 0.05$) from either the + NADPH, or + NADPH, TCB samples.

Figure 13. The effect of a single i.p. dose of TCDD, or a vitamin A-deficient (VA(-)) diet on the plasma retinol levels of male Sprague-Dawley rats 12 days following either TCDD dose (—○—) or start of the VA(-) diet (—●—). Values are means, ± SD.

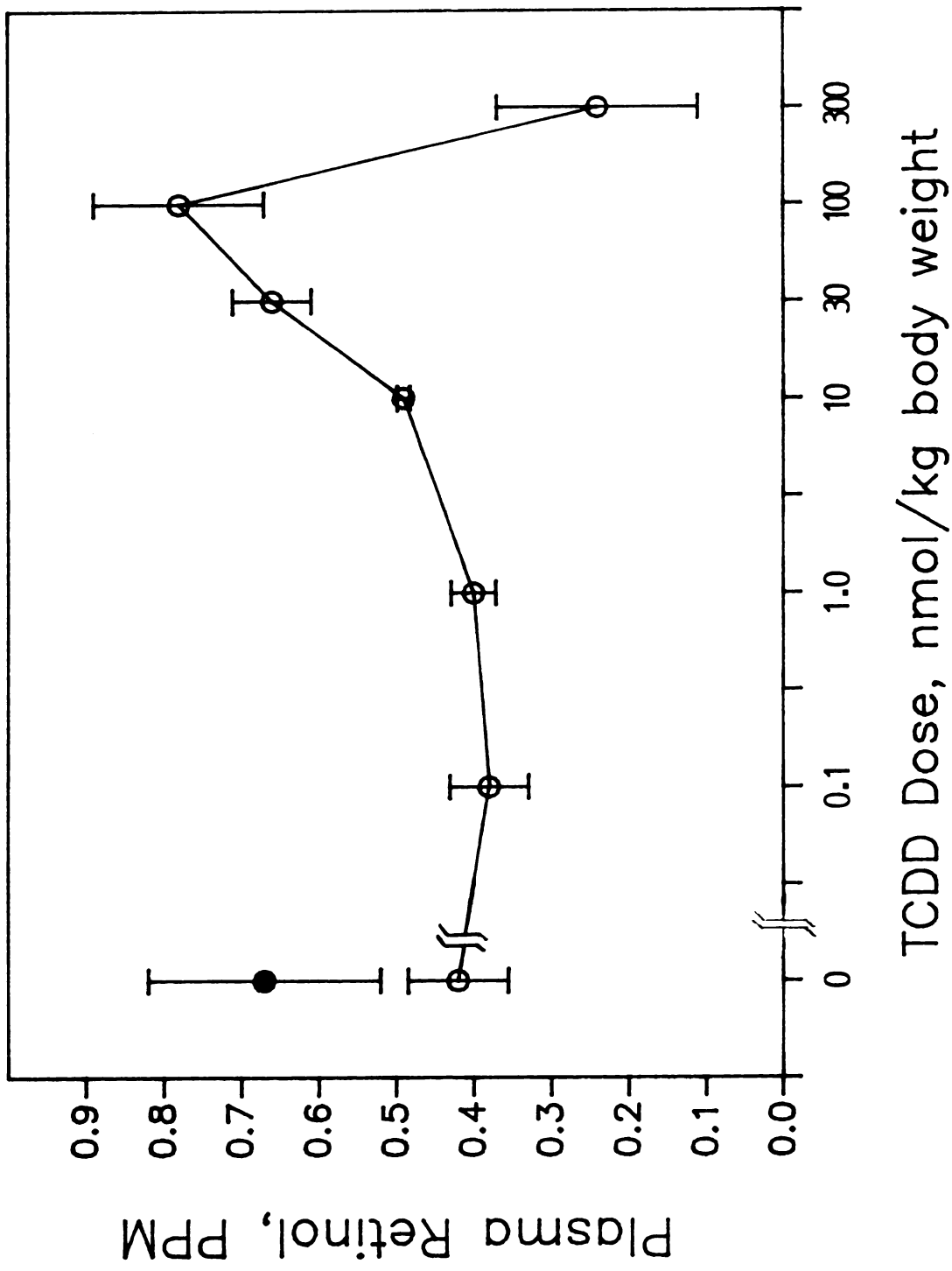
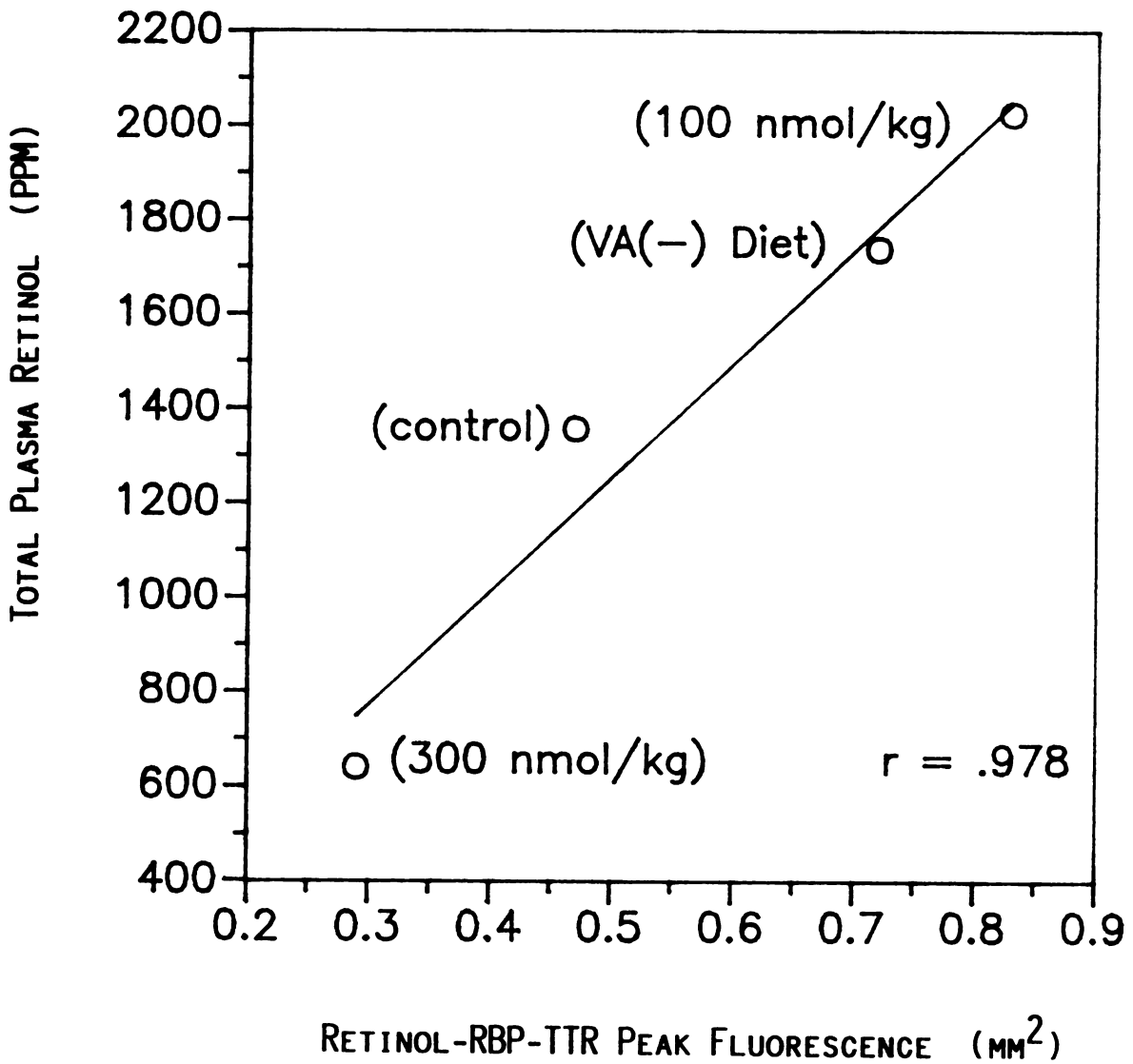

Plasma Retinol, PPM
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
0
0.1
1.0
10
30
100
300
TCDD Dose, nmol/kg body weight

Figure 14. Relation between total plasma retinol levels and retinoid fluorescence of the retinol-RBP-TTR complex of rats 12 days following treatment with a single i.p. dose of TCDD, or placed on a vitamin A deficient diet for 12 days. Values are means of groups of 3 rats (total plasma retinol) or total retinoid fluorescence from HPLC separation plasma retinol pooled from all rats in a particular group.


2200
2000
1800
1600
1400
1200
1000
800
600
400
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
(100 nmol/kg)
(VA(−) Diet)
(control)
(300 nmol/kg)
r = .978
TOTAL PLASMA RETINOL (PPM)
RETINOL-RBP-TTR PEAK FLUORESCENCE (MM2)

DISCUSSION

Vitamin A uptake, storage, and mobilization is a highly regulated process, such that plasma retinol in normally nourished and healthy animals is maintained within well-defined limits (Underwood, 1984). Even in the case of a developing vitamin A deficiency, serum retinol levels tend to be maintained until hepatic retinyl ester stores are nearly exhausted (Underwood et al., 1979). Therefore, the rapid and pronounced drop in plasma retinol levels in both rats and mice given a dose of TCB (Brouwer and van den Berg, 1985,1986) and in rats treated with NDFDA (Bank et al., 1985) suggests that these chemicals may provide an interesting means of investigating aspects of vitamin A mobilization, transport, and delivery. Further, clarification of the differences between the effects of NDFDA and TCB on plasma retinol and those caused by TCDD might provide significant insights into the mechanism by which TCDD is able to cause symptoms of a vitamin A deficiency in treated animals.

The rapid depression in serum or plasma retinol levels observed following administration of either NDFDA or TCB to rats suggested that the compounds most likely caused this effect by one of three mechanisms: (a) causing a rapid depletion of hepatic retinyl ester stores, (b) affecting the formation or secretion of the retinol-retinol-binding protein complex by the liver, or (c) causing a lesion in the process of hepatic retinyl ester hydrolysis.

Because the onset of severely depressed serum retinol levels occurred so rapidly following administration of either NDFDA or TCB, it seemed highly unlikely that depletion of hepatic retinyl ester stores could be a contributory mechanism. The amount of stored retinyl esters in the liver is generally well in excess of metabolic needs, even in young and growing animals, and continues to increase with age. This reflects the fact that dietary vitamin A availability is routinely in excess of daily requirements, resulting in the relatively large quantity of stored retinyl esters (Underwood, 1984). The process of causing signs of a vitamin A deficiency in rats by even complete elimination of dietary availability of retinoids may take many weeks (Wolf, 1980). In contrast to the rapid effect caused by NDFDA or TCB treatment, depressed serum retinol levels in the case of a severe vitamin A deficiency are seen only in the final stages when the hepatic reserves of retinyl esters are nearly exhausted (Underwood, 1984). Treatment of animals with TCDD, or similarly acting compounds, seems to cause an initial moderate rise of serum retinol, followed by an eventual decline in those levels, as hepatic retinyl ester stores are depleted (Kimbrough, 1974; Brouwer and van den Berg, 1984; Neal, et al., 1979)

Vitamin A is mobilized from the liver as retinol bound to a specific retinol-binding protein (RBP) following the hydrolysis of stored retinyl esters by retinyl palmitate hydrolase (RPH; Goodman and Blaner, 1984). The control

mechanisms for this process have not yet been clearly elucidated. It had been previously reported that apo-RBP is normally present in excess in the liver (Goodman, 1984); therefore, because of the extremely short period between the NDFDA or TCB dose and the onset of lowered serum retinol levels, it seemed unlikely that an interference with RBP synthesis could produce the results described above. If either compound did interfere with the synthesis or levels of apo-RBP, the binding of retinol to apo-RBP, or the secretion of the R-RBP complex, an increase in hepatic retinol levels in proportion to the dose might be expected. However, while hepatic retinol levels were not significantly depressed by the NDFDA dose until Day 11, there was no evidence for an accumulation of retinol in the liver. Similarly, there was no accumulation of retinol in the livers of TCB-treated rats 24 hours following treatment, despite the pronounced depression of serum retinol levels.

Prystowsky et al. (1981) found that hepatic RPH activity of control animals varied widely, and did not correlate with serum retinol values, suggesting that RPH activity may not be a limiting or controlling factor in the mobilization of retinol from the liver. However, Napoli and Beck, (1984) demonstrated that RPH was subject to non-competitive inhibition by vitamin E and phylloquinone. This suggested that inhibition of the enzyme could result in the depression of serum retinol levels by limiting the availability of retinol for complexation with apo-RBP.

Our hypothesis, that the mechanism by which NDFDA and TCB cause lowered serum or plasma retinol levels is the inhibition of hepatic RPH, is supported by several observations. The strong, positive correlation of plasma retinol levels with hepatic RPH activity in control, NDFDA- and TCB-treated animals suggests that the enzyme activity and the depressed plasma levels are linked in a cause-effect relationship. Also, the dose-dependent depression of hepatic RPH activity observed in the treated animals supports the idea that TCB or NDFDA is directly responsible for that depression, as does the kinetic data showing that both compounds cause non-competitive inhibition of the enzyme *in vitro*.

It is significant to note that the inhibition of RPH *in vitro* was accomplished by concentrations of TCB and NDFDA that corresponded roughly to the concentrations one would expect to observe in livers of treated animals, and were seen in the case of NDFDA (recognizing that considerations of molar concentrations may not necessarily apply when membrane or highly hydrophobic enzymes such as RPH are being considered). Further, the concentration of TCDD that was able to cause significant inhibition of the enzyme (500 uM) is much higher than levels expected to occur in the livers of TCDD-treated animals. This suggests that even though TCDD is able to inhibit RPH in a manner similar to that of NDFDA, the much lower LD_{50} of TCDD (50 ug/kg; Neal *et al.*, 1979; Thunberg, 1983a) compared to that of NDFDA (41 mg/kg;

Olson et al., 1982) precludes this mechanism from contributing to the toxicity of TCDD.

Further support for the hypothesis that NDFDA and TCB inhibit RPH is provided by the fact that removal of the inhibiting species, by extraction with solvent in the case of NDFDA, or incubation of microsomes under conditions consistent with metabolic degradation in the case of TCB, was shown to ameliorate the subsequent inhibition of RPH in both cases. With respect to TCB, these data suggest that it is the parent compound, rather than a metabolite, that is primarily responsible for the inhibition of RPH. This last point may partially explain the recovery of plasma retinol towards normal levels observed in rats given a single dose of TCB.

Another explanation for the decrease in plasma retinol has been recently published by Brouwer and van den Berg (1986). They have suggested an interference by either TCB or one of its metabolites in the binding of holo-RBP to transthyretin. Such a destabilization of the ternary complex would presumably result in the removal of free plasma RBP-retinol by the kidney and its degradation or storage. The authors have extended this theory to be a general model for PCB- and TCDD-type toxicity.

A logical consequence of this theory would be the accumulation of retinoids in the kidney, since this organ effectively scavenges any free retinol-RBP complex, and is thought to catabolize RBP (Goodman, 1984). Curiously, both

in vitamin A deficiency (Moore and Sharman, 1950) and TCDD toxicity (Thunberg, 1983b), there is a marked tendency of the kidney to store retinoids in the form of retinyl esters. We did not observe any increase in the concentration of retinoids in the kidneys of TCB-treated rats, but the degradation of these species could be so rapid, or the time course of this experiment too short, to allow our observation of such an elevation. The effect of TCDD on renal retinoid accumulation is the subject of Chapter 2. An additional consequence of the above hypothesis would be the increase in the plasma concentration of free retinol-RBP, as a result of ternary complex dissociation. However, upon HPLC analysis of plasma from control and TCB-treated rats, we did not observe any protein peak that would correspond to retinol-RBP. However, it should be noted that renal clearance of the RBP-retinol could be rapid enough to preclude observation of the free retinol-RBP. Further, the changes in plasma retinol levels observed in control, VA(-), and TCDD-treated rats were positively correlated with the amount of retinol associated with the RBP-TTR complex. This suggests that the entire range of changes in plasma retinol levels in the 100 experiment are strictly a function of changes in the amount of the ternary complex, and do not reflect any contribution from the proposed partial dissociation of the retinol-RBP complex from TTR.

TCB is one of the toxic PCB congeners, and like TCDD,

has been shown to cause the depletion of hepatic retinyl esters and symptoms of a vitamin A deficiency in treated animals. Similar symptoms are caused by NDFDA. The depressed plasma retinol levels caused by the inhibition of RPH may then hasten the onset or exacerbate the magnitude of the vitamin A deficiency apparently caused by TCB, NDFDA, and similarly-acting compounds.

It is clear, however, that the depressed plasma retinol levels caused by TCB and NDFDA should not be thought of as being characteristic of TCDD-type toxicity for several reasons. The plasma retinol level depression following a dose of TCB is observed both in strains of mice that are considered sensitive (C57/BL6) and insensitive (DBA/2) to TCDD-type toxicity, while the hepatic retinoid depletion occurs only in the "sensitive" strains (Brouwer and van den Berg, 1984, 1985). Also, TCDD itself does not cause this phenomenon, instead it causes a temporary rise in plasma retinol levels (Thunberg et al., 1979). Conversely, while several compounds which do not cause symptoms of dioxin-type toxicity (e.g., retinoic acid (Keilson et al., 1979), endosulfan (Sriram and Misra, 1983) and cadmium (Sugawara and Sugawara, 1978)) depress plasma retinol levels. NDFDA very effectively causes a depression of plasma retinol levels along with acute toxic effects similar to those observed in TCDD-type toxicity.

In summary, we have attempted to elucidate the mechanism for one of the toxic effects of the PCB congener

3,4,3',4'-tetrachlorobiphenyl and the perfluorodecanoic acid, NDFDA. Our findings suggest that TCB is an effective inhibitor of RPH activity both *in vitro* and *in vivo*, and in so doing causes a depression of plasma retinol levels. This also points out a significant difference between the toxic response of rats to TCB and other PCB congeners. Additionally, the data demonstrate the consequences of the inhibition of hepatic RPH on the levels of the retinol-RBP-TTR ternary complex in plasma.

BIBLIOGRAPHY

Akoso, B.T., Sleight, S.D., Aust, S.D., and Stowe, H.D. (1982). Pathologic effects of purified polybrominated biphenyl congeners in rats. J. Am. Coll. Toxicol. 1:1-21.

Bank, P.M., Powers, R.H. and Aust. S.D. (1986). The effect of perfluorodecanoic acid (PFDA) on vitamin A homeostasis in rats. Toxicologist. 6:315 (abs.# 1266).

Brouwer, A., and van den Berg., K.J. (1984). Early and differential decrease in natural retinoid levels in C57BL/Rij and DBA/2 mice by 3,4,3'4'-tetrachlorobiphenyl. Toxicol. Appl. Pharmacol. 73:204-209.

Brouwer, A., and van den Berg, K.J. (1985). Time and dose responses of the reduction in retinoid concentrations in C57BL/Rij and DBA/2 mice induced by 3,4,3',4'-tetrachlorobiphenyl. Toxicol. Appl. Pharmacol. 78:180-189.

Brouwer, A., and van den Berg, K.J. (1986). Binding of a metabolite of 3,4,3',4'-tetrachlorobiphenyl to transthyretin reduces serum vitamin A transport by inhibiting the formation of the protein complex carrying both retinol and thyroxine. Toxicol. Appl. Pharmacol. 85:301-312.

Cecil, H.C., Harris, S.J., Bitman, J., and Fries, G.F. (1973). Polybrominated biphenyl-induced decrease in liver vitamin A in Japanese quail and rats. Bull. Environ. Contam. Toxicol. 3:179-185.

Dennison, D.B. and Kirk, J.R. (1977). Quantitative analysis of vitamin A in cereal products by high pressure liquid chromatography. J. Food Sci. 42(5):1376-1379.

Godfrey, N.K. (1985). Comparing the means of several groups. New Eng. J. Med. 313:1450-1456.

Goodman, D.S., and Blaner, W.S. (1984). Biosynthesis, Absorption, and Hepatic Metabolism of Retinol. In: The Retinoids (Sporn, M.B., Roberts, A.B., and Goodman, D.S. eds.) Academic Press, Orlando, Florida. Vol 2; pp: 1-39

Goodman, D.S. (1984) Plasma Retinol-Binding Protein. In: The Retinoids (Sporn, M.B., Roberts, A.B., and Goodman, D.S., eds.) Academic Press, Orlando, Florida. Vol 2; pp: 41-88.

Innami, S., Nakamura, A., Miyazaki, M., Nagayama , S. and Nishide, E. (1976). Further studies on the reduction of vitamin A content in the livers of rats given polychlorinated biphenyls. J. Nutr. Sci. Vitaminol. 22:409-418.

Keilson, B., Underwood, B.A., and Loerch, J.D. (1979). Effects of retinoic acid on the mobilization of vitamin A from the liver in rats. J. Nutr. 109:787-795.

Kimbrough, R.D. (1974). The toxicity of polychlorinated polycyclic compounds and related compounds. Crit. Rev. Toxicol. 2:445-489.

McConnell, E.E., Hass, J.R., Altaman, N., and Moore, J.A. (1979). A spontaneous outbreak of polycholorinated biphenyl (PCB) toxicity in Rhesus monkeys (Macaca mullatta). Toxicopathol. Lab. Animal Sci. 320:131-137.

Millis, C.D., Mills, R.A., Sleight, S.D., and Aust, S.D. (1985). Toxicity of 3,4,5,3',4',5'-hexabrominated biphenyl and 3,4,3',4'-tetrabrominated biphenyl. Toxicol. Appl. Pharmacol. 78:88-95.

Moore, T., and Sharman, I.M. (1950). Vitamin A in the kidneys of male and female rats. Biochem J. 47:xlii-xliv.

Napoli, J.L. and Beck, C.D. (1984). Tocopherol and phylloquinone as non-competative inhibitors of retinyl ester hydrolysis. Biochem J. 223:267-270.

Neal, R.A., Beatty, P.W., and Gasiewicz, T.A. (1979). Studies on the mechanisms of toxicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). In: "Health effects of halogenated aromatic hydrocarbons" (Nicholson W.J., and Moore, J.A., eds.) Ann. N.Y. Acad. Sci. 20:204-213.

Olson, C.T., Anderson, M.E., George, M.E., VanRafelghem, M.J. and Back, A.M. (1982) The toxicology of perfluorodecanoic acid in rats. Proceedings: 13th conference on environmental toxicology. University of California, Irvine: Overlook Branch, Dayton, Ohio. #20:287-303.

Prystowsky, J.H., Smith, J.E., and Goodman, D.S. (1981). Retinyl palmitate hydrolase activity in normal rat liver. J. Biol. Chem. 256:4498-4503.

Redinbaugh, M.G., and Turley, R.B. (1985). Adaptation of the bicinchoninic acid protein assay for use with microtitre plates and sucrose gradient fractions. Analyt. Biochem. 153:267-271.

Sriram, K., and Misra, U.K. (1983). Effect of endosulfan on liver and plasma vitamin A levels in rats. Nutr. Rep. Int. 28(4):731-734.

Sugawara, C., and Sugawara, N. (1978). The effect of cadmium on vitamin A metabolism. Toxicol. Appl. Pharmacol. 46:19-27.

Thunberg, T., Ahlborg, U., and Johnsson, H. (1979). Vitamin A (retinol) status in the rat after a single oral dose of 2,3,7,8-tetrachlorodibenzo-p-dioxin. Arch. Toxicol. 42:265-274.

Thunberg, T. (1983a). Studies on the effect of 2,3,7,8-tetrachlorodibenzo-p-dioxin on vitamin A: A new aspect concerning the mechanism of toxicity. PhD Thesis, Dept. of Toxicology. Karolinska Institute, Stockholm, Sweden.

Thunberg, T. (1983b). The effect of TCDD and vitamin A in normal and in vitamin A deficient rats. Chemosphere 12:577-580.

Underwood, B.A., Loerch, J.D., and Lewis, K.C. (1979). Effects of vitamin A deficiency, retinoic acid and protein quality on serially obtained plasma and liver levels of vitamin A in rats. J. Nutr. 109:796-806.

Underwood, B.A., (1984). Vitamin A in animal and human nutrition. In: The Retinoids (Sporn, M.B., Roberts, A.B., and Goodman, D.S., eds.) Academic Press, Orlando, Florida. Vol 1; pp: 282-392.

Villeneuve, D.C., Clark, M.L., and Clegg, D.J. (1971). Effects of PCB administration on microsomal enzyme activity in pregnant rabbits. Bull. Environ. Contamin. Toxicol. 2:120-128.

Wolf, G. (1980). Vitamin A. In:"Human Nutrition" (Alfin-Slater, R.B., and Kritchevsky, D., eds). Plenum, New York. pp: 97-203.

Windhols, M. (Ed.) (1976) Merck Index. Merck & Co., Inc. Rahway, N.J. 9:1297.

CHAPTER 2

The Effects of TCDD and Vitamin A Deficiency on Renal Retinoid Accumulation

ABSTRACT

The effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and vitamin A deficiency on renal retinoid accumulation in male Sprague-Dawley rats was examined in two separate experiments. Rats given a single oral dose of TCDD showed a pronounced, dose-dependant elevation of renal retinoid levels within twelve days following treatment. Retinol levels in the maximally affected dose group (100 nmol TCDD/kg body wt.) were elevated to levels ~2x those of control rats. Retinyl palmitate concentrations of the same group were similarly elevated, but to levels ~8x those of control group animals. Renal retinyl palmitate hydrolase (RPH) activity was unaffected by TCDD treatment, but renal acyl CoA:retinol acyl transferase (ACARAT) activity was elevated in a dose dependent fashion, with ACARAT activity in the 100 nmol/kg TCDD treatment group elevated to levels ~8x those of control-group animals. Renal ACARAT activity and retinyl palmitate concentration were highly and positively correlated (r = 0.93). Vitamin A depletion, produced by 26 days of feeding a diet completely lacking vitamin A to male Sprague-Dawley rats (VA(-)), also caused renal retinoid accumulation. Retinol levels in the VA(-) rats were slightly, but significantly elevated over those of rats given a normal diet. Retinyl palmitate levels were also elevated, to ~8x the levels of control rats fed a normal diet. RPH activity was unaffected in the VA(-) group, but ACARAT activity was elevated to ~6.5x those of

control animals. We conclude that a consequence of TCDD toxicity is an accumulation of retinyl palmitate in the kidney of treated animals, by a mechanism that is identical to that of the accumulation of retinyl palmitate observed in mildly vitamin A deficient rats.

INTRODUCTION

TCDD and similarly acting toxins cause a characteristic toxicity in treated animals which has been referred to as the "wasting syndrome." Features of this toxic response include hypophagia, anorexia, lymphoid involution and immune suppression, hyperkeratosis of epithelial tissues and hormonal abnormalities (Poland and Knutson, 1982; McConnell, 1980; Peterson et al., 1984). The similarity between many aspects of a vitamin A deficiency state and TCDD-type toxicity was suggested by Kimbrough, (1974) and has been reviewed by Thunberg (1983a). Further, the ability of these toxins to cause a depletion in hepatic retinoid stores has been demonstrated in many species (Villeneuve et al., 1971; Innami et al., 1974, 1976; Akoso et al., 1982 Brouwer and van den Berg, 1983; Thunberg, et al., 1979).

One aspect of a developing vitamin A deficiency is the pronounced increase in the concentration of retinoids in the kidney (Moore and Sharman, 1950; Morita and Nakano, 1982). Many authors have suggested that the kidney may play a major role in vitamin A homeostasis, and further, that in the case of a vitamin A deficiency, becomes "the major storage organ" for the vitamin (Moore, 1957; Wolf, 1980, 1984). Because the analytical methods employed in many of the analyses of retinoid material in the kidney did not discriminate between retinyl esters and retinol, the nature of the accumulation of retinoid material in the vitamin A deficiency state has not been characterized, nor were suggestions proposed as to

the mechanism by which this accumulation occurred.

In addition to causing toxic signs that resemble a vitamin A deficiency, TCDD has been shown to cause accumulation of retinoid material in the kidney of treated rats (Thunberg, 1983b; Hakansson 1985). We have hypothesized that the accumulation of retinoids in the kidney is the response of the animal to the onset of a vitamin A deficiency, caused in this case as a consequence of the TCDD dose. We therefore expected that the mechanism of renal retinoid accumulation would be similar between TCDD-treated animals, and those suffering from the early stages of a vitamin A deficiency. Experimentally, we have examined the dose response characteristics of the accumulation of retinoids in the kidney of rats treated with TCDD, and evaluated the activities of the renal enzymes proposed to operate in the storage and/or mobilization of retinoids. Further, we have then compared the activities of these enzymes with those from rats in the early stages of a deficiency induced by dietary vitamin A restriction.

MATERIALS AND METHODS

Animals; treatment and tissue preparation

Experiment 1 (TCDD):

Male Sprague-Dawley rats, 140-160 g were procured from Charles River Co. (Portage, MI) and acclimated for 1 week on hardwood bedding. The rats were given a single oral dose of corn oil containing 0.0, 0.1, 1.0, 10, 30, 100, or 300 nmol TCDD/ml. Three animals per dose level were killed 3, 7, and 12 days after dosing as per Chapter 1, "Methods." An additional group of three rats were dosed with corn oil alone on day 0, fed a vitamin A free diet (AIN-76, VA(-); US Biochemical Corp. Cleveland, OH) and killed on day 12. Blood was sampled and plasma prepared from all animals as per Chapter 1, "Methods." Kidneys were carefully excised and stored in 1.15% KCl/H_2O on ice until homogenization. Kidneys were weighed and homogenized in 9 ml 1.15% KCl/H_2O per g wet weight in a Potter-Elvehjem homogenizer. Homogenates thus prepared were frozen and stored at -20^{o} C prior to analysis for retinol, retinyl palmitate, and acyl CoA:retinol acyl transferase (ACARAT).

Experiment 2, (Vitamin A Deficient Diet):

Male Sprague-Dawley rats, 50-60 g were procured from Charles River Co. (Portage, MI) and acclimated for 1 week on hardwood bedding, with access to feed (Rodent-Blox, Wayne Feeds, Chicago, Il), and water *ad libitum*. The animals were divided into two groups, and "control" animals were fed a complete diet, (AIN-76A, US Biochemicals, Cleveland, OH),

while "Vitamin A Depleted" rats received the equivalent diet less vitamin A. Three rats from each group were killed 26 days following the initiation of the defined diets by exsanguination (cardiac puncture) following deep CO_2 anesthesia. Blood was collected and stored as per Chapter 1. Kidneys were excised, homogenized and homogenates stored as per Chapter 1, "Methods."

Retinoid Levels

Renal retinol and retinyl palmitate levels were determined by hexane extraction of homogenate, and HPLC quantitation as described in Chapter 1, "Methods."

Analysis of ACARAT Activity

The activity of ACARAT in renal homogenates (described above) was determined by a modification of the method described by Ball et al. (1985). Briefly, samples containing 100 uM palmitoyl Co A (Sigma Chemical Co., St. Louis, MO), 100 uM retinol (Sigma), 10 mM dithiothreitol, 18 uM Bovine Serum Albumin, and a 200 ul of the renal homogenate were incubated in a final volume of 2.0 ml 200 mM phosphate buffer, pH 7.4 at 37^{o} C. in a shaking water bath. The reaction was terminated by the addition of 2.0 ml ethanol and 2.0 ml NaCl-saturated water. Retinyl palmitate in the incubation mixture was extracted with 1.0 ml of hexane and quantitated by HPLC as described in Chapter 1, "Methods." Samples were corrected for the amount of retinyl palmitate present in unincubated samples. Protein

concentration of the homogenate was determined by a bicinchoninic acid microassay (Redinbaugh and Turley, 1985).

Renal RPH Activity:

RPH activity in renal homogenates was determined by as described in Chapter 1, for hepatic homogenates.

Statistical Analysis

Statistical analysis was performed using Student's t-test with the Bonferroni correction for multiple comparisons (Godfrey, 1985). All references to statistical significance were at the $p < 0.05$ level.

RESULTS

Renal Retinol and Retinyl Palmitate

The effect of the single TCDD dose on the level of retinol in the kidney of treated rats from experiment 1 is shown in Table 1. We observed a slight, significant elevation of retinol levels in rats 12 days following treatment with 10, 30 or 100 nmol/kg TCDD. An elevation of similar magnitude was noted in the kidneys of rats placed on a vitamin A deficient diet for 26 days, (experiment 2), as shown in Table 2.

The single dose of TCDD caused a pronounced increase in the concentration of renal retinyl palmitate, as shown in Figure 1. The accumulation of retinyl palmitate was only apparent in rats treated with doses greater than 1.0 nmol/kg, and reached a maximum of ~8x control levels in the 100 nmol/kg dose group. At 12 days following the initiation of a vitamin A deficient diet, there was no statistically significant difference in the amount of retinyl palmitate in the kidney, when compared to control animals (Figure 1). However, in experiment 2, the rats given a vitamin A deficient diet for 26 days showed a significant elevation of renal retinyl palmitate levels, again to ~8x control values (Table 2).

Table 1. The Effect of a Single Dose of TCDD or Feeding a Vitamin A Deficient (VA(-)) Diet on Renal Retinol Levels and Retinyl Palmitate Hydrolase Activity Levels in Male Sprague-Dawley Rats, 12 Days Following Dose, or Start of VA(-) Diet.[a]

TCDD Dose (nmol/kg)	0	0, VA(-) Diet	1.0	10	30	100	300
Renal Retinol; PPM, ± SD	0.69 ±0.07	0.8 ±0.2	0.7 ±0.2	1.1[b] ±0.2	1.4[b] ±0.4	1.5[b] ±0.3	0.8 ±0.3
Renal RPH; pmol/min/mg, ± SD	5.3 ±0.8	4.3 ±0.7	6 ±1	7 ±1	6.9 ±0.5	5 ±1	5.1 ±0.7

[a]: Values are means ± SD for groups of 3 rats.

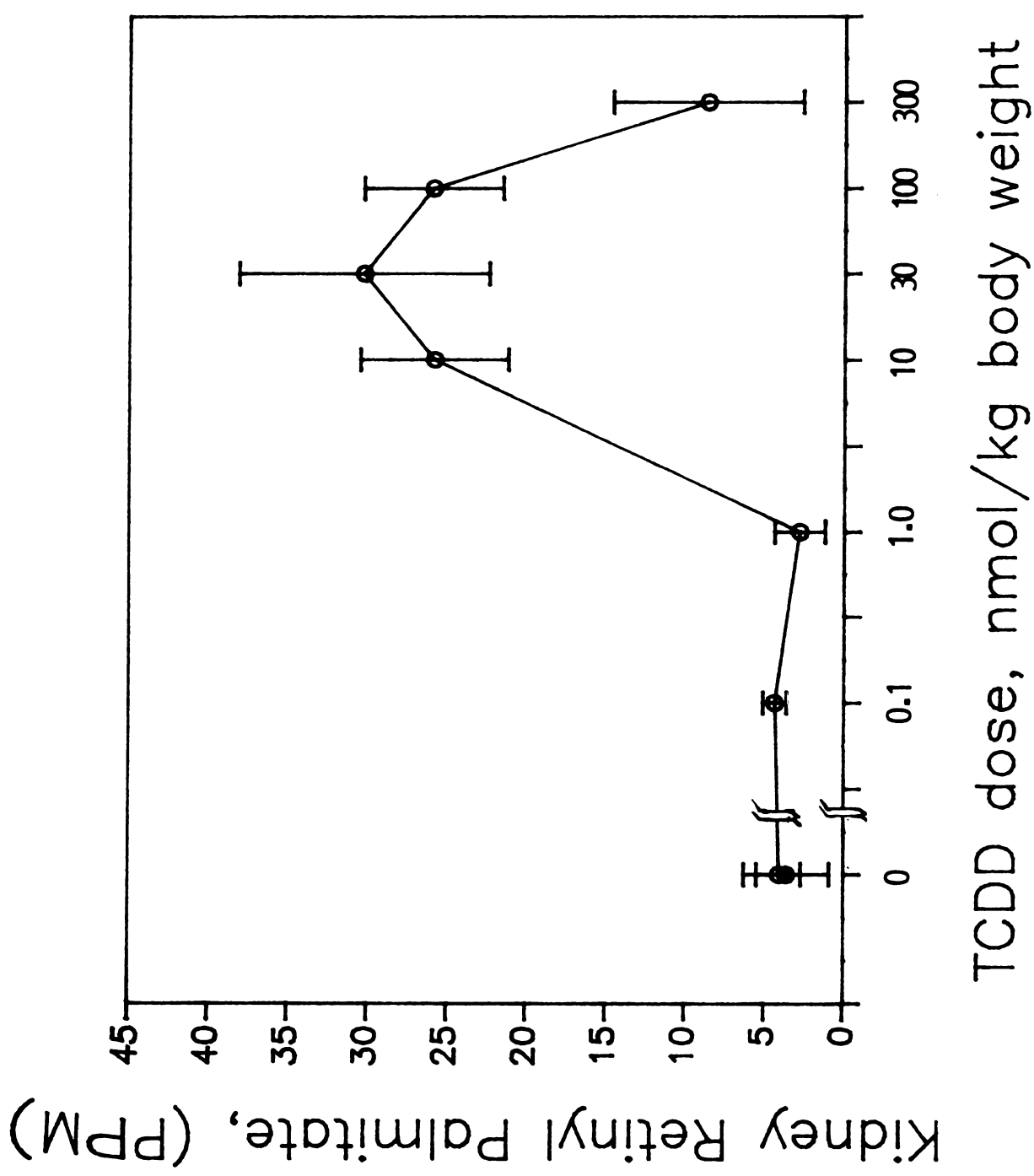
[b]: Significantly different from control group, $p < 0.05$.

Table 2. The Effect of Feeding a Control or Vitamin A Deficient Diet for 26 days to Male Sprague-Dawley Rats on Plasma Retinol, Liver Retinyl Palmitate, Renal Retinol and Retinyl Palmitate and Acyl CoA:Retinol Acyl Transferase.

Dietary Vitamin A Content	Plasma Retinol (PPM±SD)	Liver Retinyl Palmitate (PPM±SD)	Kidney			
			Retinol (PPM±SD)	Retinyl Palmitate (PPM±SD)	ACARAT (pmol/min/mg) (±SD)	RPH (pmol/min/mg) (±SD)
VA (-)	0.41[a] ±0.06	18[a] ±8	1.25[a] ±0.05	23[a] ±4	5[a] ±3	5 ±4
VA (+)	0.52 ±0.03	45 ±9	1.1 ±0.2	2.8 ±0.3	0.8 ±0.4	6 2

[a]: Statistically different from VA (+) diet group, $p < 0.05$.

Figure 1. Effect of a single oral dose of TCDD or feeding a vitamin A-deficient diet (VA(-)) on kidney retinyl palmitate levels in male Sprague-Dawley rats 12 days following treatment or start of VA(-) diet. Values are means $\pm$ SD for groups of n = 3. (—○—): TCDD treated, (—●—): VA(-) diet.


Kidney Retinyl Palmitate, (PPM)
45
40
35
30
25
20
15
10
5
0
0
0.1
1.0
10
30
100
300
TCDD dose, nmol/kg body weight

Renal RPH Activity

No effect of the single dose of TCDD on renal RPH activity was observed (Table 1). Neither was there an effect caused by a 26 days of feeding a diet deficient in vitamin A, as shown in Table 2.

Renal ACARAT Activity

Renal ACARAT Activity in the TCDD treated animals was elevated with increasing dose, as shown in Figure 2. Further, this enzyme activity was strongly and positively correlated with kidney retinyl palmitate, $r = .93$, as shown in Fig. 3. The renal ACARAT activity was also elevated in in rats fed a vitamin A deficient diet for 26 days, when compared to animals fed a control diet, as shown in Table 2.

Figure 2. Effect of a single oral dose of TCDD or feeding a vitamin A-deficient (VA(-)) diet on kidney ACARAT activity in male Sprague-Dawley rats, 12 days following either treatment or start of VA(-) diet. Values are means ± SD for groups of n = 3. (—○—): TCDD treated, (—●—): VA(-) diet.

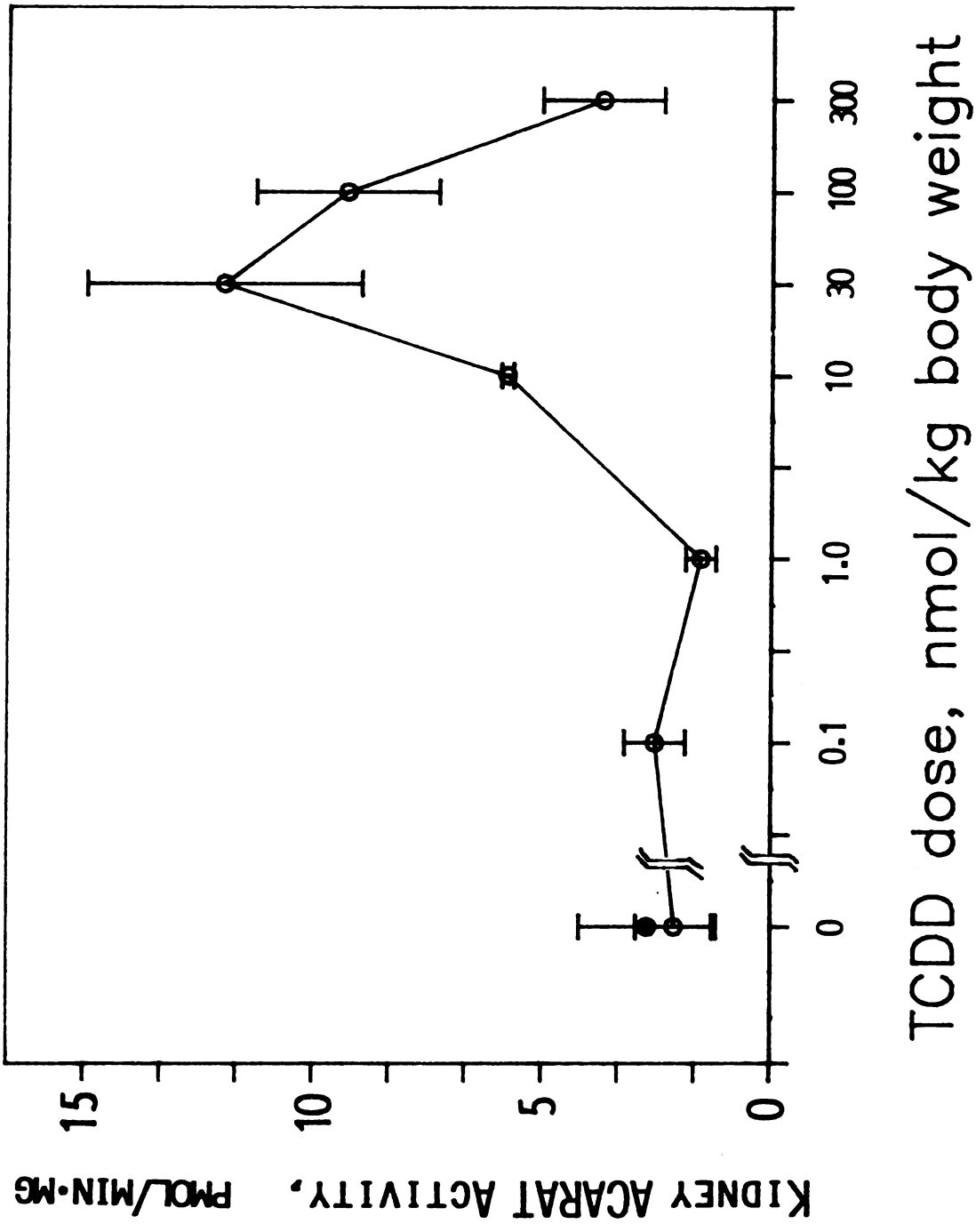
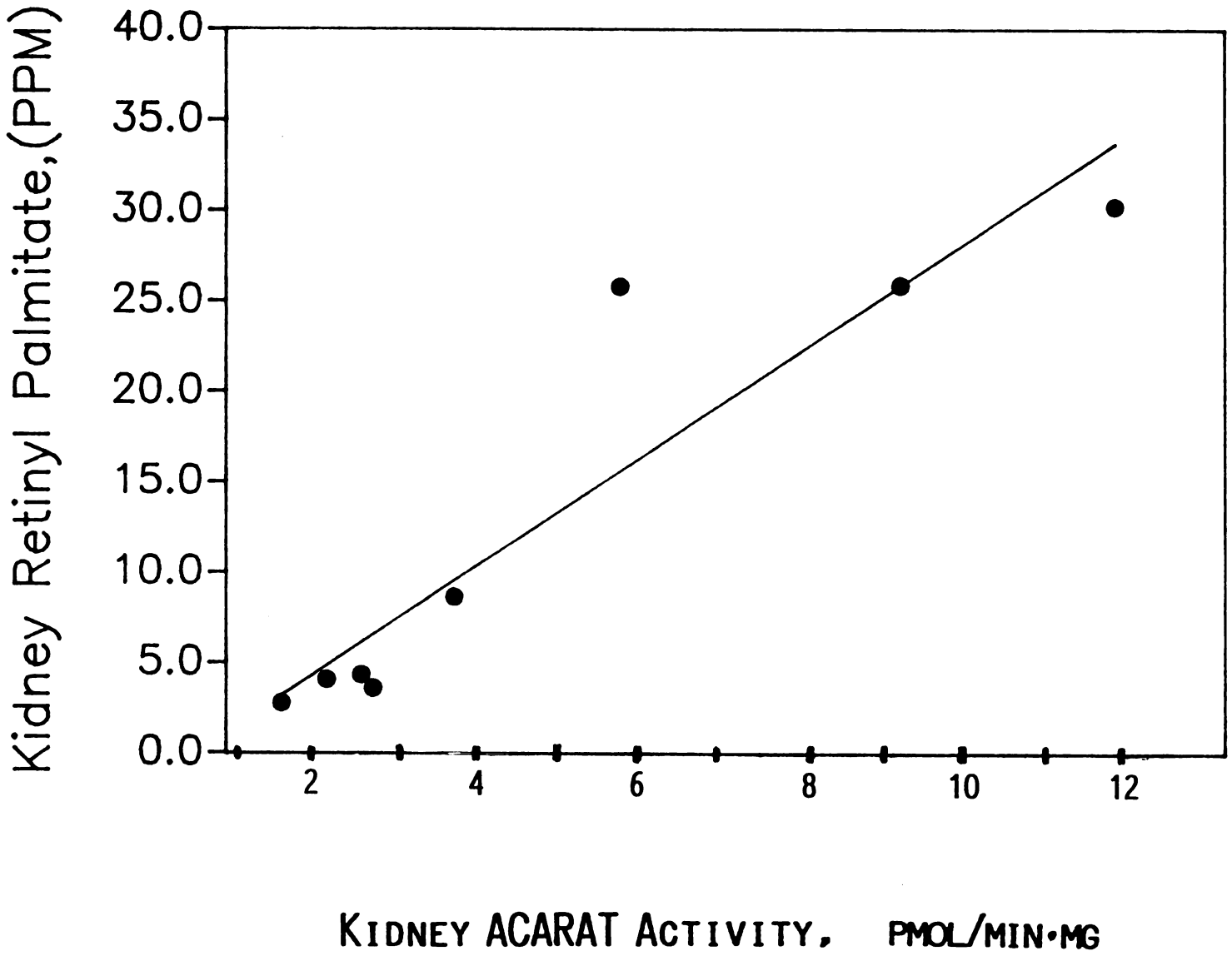

KIDNEY ACARAT ACTIVITY, PMOL/MIN•MG
0
5
10
15
0
0.1
1.0
10
30
100
300
TCDD dose, nmol/kg body weight

Figure 3. Correlation of kidney retinyl palmitate concentration with kidney ACARAT activity in male Sprague-Dawley rats 12 days following a single oral dose of TCDD or fed a vitamin A deficient diet for 12 days. Values are the means of groups of n = 3.


Kidney Retinyl Palmitate,(PPM)
40.0
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
2
4
6
8
10
12
KIDNEY ACARAT ACTIVITY, PMOL/MIN•MG

DISCUSSION

It has been clearly established in the literature, and demonstrated again in the experiment described herein, that a toxic dose of TCDD results in elevated levels of retinoids in the kidneys of treated animals (Thunberg, 1983b; Hakensson, 1985), as does the onset of a vitamin A deficiency (Morita and Nakano, 1982; Moore, 1957). Further, we have shown that the retinoid accumulation in the kidney caused by TCDD appears to be primarily an increase in the amount of retinyl esters, a consequence of elevated ACARAT activity. Similar accumulation and elevation of ACARAT were noted in animals fed a vitamin A-deficient diet.

Several authors have suggested that the renal accumulation of retinoids in the case of vitamin A deficiency may be of some physiologic significance, even to the point of postulating that in the case of severe deficiency, the kidney becomes "the major storage organ for vitamin A" (Moore, 1957; Wolf, 1980). This explanation for the phenomenon however, would require that a major change occurs in the way the body both stores and mobilizes vitamin A. Also, such a hypothesis requires that the kidney have the means of synthesizing apo-RPB, and other cellular mechanisms involved in the mobilization of retinol-RBP. While significant quantities of apo-RBP can be demonstrated to be present in the liver, the RBP in the kidney is reported to be distinct from apo-RBP, and probably arises from the absorption of filtered RBP. RBP from this source,

having delivered a molecule of retinol to a target tissue, is suspected to be structurally altered from apo-RBP, and is no longer able to bind retinol (Goodman, 1984).

A more reasonable hypothesis may be that the role of the kidney during times of restricted vitamin A availability is simply to minimize loss of the vitamin, and to recycle either retinol or retinyl esters back into circulation. While not deliberately designed to evaluate this hypothesis, this experiment has provided some interesting data as to the role the kidney may play in moderating the effects of a developing vitamin A deficiency state.

In the case of both TCDD toxicity, and the vitamin A deficiency induced by elimination of dietary retinoids, the concentration of retinyl esters in the kidney were increased to a much greater degree than the increase in the amount of retinol, presumably as a consequence of elevated ACARAT activity. These data suggest that mechanistically, the kidney is accumulating retinol, and subsequently esterifying it to form retinyl esters. We suspect that these retinyl esters are released back into the circulation to return to the liver for subsequent re-mobilization as retinol-RBP. The significance of the similarity between the mechanism of accumulation of retinoids in the kidney of both TCDD-treated and vitamin A deficient animals may be that the data clearly show that TCDD causes biochemical symptoms of a vitamin A deficiency. Hence, a treated animal is responding, at least partially, to a vitamin A deficiency

state. Considering the similarities between vitamin A deficiency symptoms and TCDD toxicity, this suggests that some of these symptoms are a consequence of a vitamin A deficiency, rather than a toxic lesion caused directly by TCDD.

BIBLIOGRAPHY

Akoso, B.T., Sleight, S.D., Aust, S.D., and Stowe, H.D. (1982). Pathologic effects of purified polybrominated biphenyl congeners in rats. J. Am. Coll. Toxicol. 1:1-21.

Ball, M.D., Furr, H.C., and Olson, J.A. (1985). Enhancement of acyl CoA:retinol acyltransferase in rat liver and mammary tumor tissue by retinyl acetate and its competitive inhibition by N-(4-hydroxyphenyl) retinamide. Biochem. Biophys Res. Comm. 128:7-11.

Brouwer, A., and van den Berg, K.J. (1983). Early decrease in retinoid levels in mice after exposure to low doses of polychlorinated biphenyls. Chemosphere 12:555-558.

Godfrey, N.K. (1985). Comparing the means of several groups. New Eng. J. Med. 313:1450-1456.

Hakansson, H., and Ahlborg, U.G. (1985). The effect of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on the uptake, distribution and excretion of a single oral dose of 11,12-^{3}H-retinylacetate and on the vitamin A status in the rat. J. Nutr. 115:759-771.

Innami, S., Nakamura, A., and Nagayama, A. (1974). Polychlorinated biphenyl toxicity and nutrition. II. PCB Toxicity and vitamin A. J. Nutr. Sci. Vitaminol. 20:363-370.

Innami, S., Nakamura, A., Miyazaki, M., Nagayama, S., and Nishide, E. (1976). Further studies on the reduction of vitamin A content in the livers of rats given polychlorinated biphenyls. J. Nutr. Sci. Vitaminol. 22:409-418.

Kimbrough, R.D. (1974). The toxicity of polychlorinated polycyclic compounds and related compounds. Crit. Rev. Toxicol. 2:445-489.

McConnell, E.E., (1980). Toxicity, carcinogenesis, reproduction, teratogenesis and mutagenesis. In: Halogenated Biphenyls, terphenyls, naphthalenes, dibenzodioxins and related products. (Kimbrough, R.D. ed.) Elsevier/North-Holland Biomedical Press, Amsterdam; pp: 109-150.

Moore, T. (1957). "Vitamin A." Elsevier Publishing Co. New York.

Moore, T., and Sharman, I.M. (1950). Vitamin A in the kidneys of male and female rats. Biochem. J. 47:xliii-xliv.

Morita, A., and Nakano, K. (1982). Change in vitamin A contents in tissues of rats fed on a vitamin A-free diet. J. Nutr. Sci. Vitaminol. 28:343-350.

Peterson, R.E., Seefeld, M.D., Christian,. B.J., Potter, C.L., Kelling, C.K., and Keesey, R.E. (1984). The wasting syndrome in 2,3,7,8-tetrachlorodibenzo-p-dioxin toxicity; basic features and their interpretation. In: Biological mechanisms of dioxin action. Banbury Report #18; Cold Spring Harbor Laboratory pp: 291-308.

Poland, A., and Knutson, J.C. (1982). 2,3,7,8-Tetrachlorodibenzo-p-dioxin and related halogenated aromatic hydrocarbons: Examination of the mechanism of toxicity. Ann. Rev. Pharmacol. Toxicol. 22:517-554.

Redinbaugh, M.G., and Turley, R.B. (1985). Adaptation of the bicinchoninic acid protein assay for use with microtitre plates and sucrose gradient fractions. Analyt. Biochem. 153:267-271.

Thunberg, T., (1983a). Studies on the effect of 2,3,7,8-tetrachlorodibenzo-p-dioxin on vitamin A: A new aspect concerning the mechanism of toxicity. PhD Dissertation, Carolinska Institute, Stockholm.

Thunberg, T., (1983b). The effect of TCDD on vitamin A in normal and vitamin A-deficient rats. Chemosphere 12:577-580.

Thunberg, T., Ahlborg, U.G., and Johnsson, H. (1979). Vitamin A status in the rat after a single oral dose of 2,3,7,8-tetrachlorodibenzo-p-dioxin. Arch. Toxicol. 42:265-274.

Villeneuve, D.C., Clark, M.L., and Clegg, D.J. (1971). Effects of PCB administration on microsomal enzyme activity in pregnant rabbits. Bull. Environ. Contam. Toxicol. 2:120-128.

Wolf, G. (1980). Vitamin A. In: "Human Nutrition" (R.B. Alfin-Slater and D. Kritchevsky, eds.), Plenum, New York pp: 97-203.

Wolf, G.(1984). Multiple Functions of Vitamin A. Physiological Reviews 64(3):873-937.

CHAPTER 3

Effects of TCDD and Vitamin A Deficiency on Hepatic Retinoid Storage and Degradation

ABSTRACT

The effects of a single oral dose of TCDD of 0, 0.1, 1.0, 10, 30, 100 or 300 nmol/kg or feeding a diet deficient in vitamin A (VA(-)) to male Sprague-Dawley rats 12 days following either TCDD treatment or start of VA(-) diet on body weight, food intake, organ weight, hepatic retinyl palmitate concentration, retinal oxidase activity, microsomal UDPGT activity and microsomal NADPH-dependent retinoid degradation was examined. TCDD treated animals exhibited a dose-dependent depression in rate of weight gain, and hypophagia. Further indications of TCDD-type toxicity noted were hepatic enlargement and thymic involution. No effect on the rate of retinal oxidase activity was observed, but the rate of microsomal NADPH-dependent retinol degradation was significantly elevated in TCDD treated rats, and depressed in rats given a VA(-) diet. Both PNP and retinoic acid UDPGT activities were elevated in TCDD treated rats. No activity of retinol-UDPGT was observed. We conclude that animals treated with TCDD have elevated enzyme activities that would expected to exacerbate the depletion of hepatic retinoids.

INTRODUCTION

The ability of TCDD and similar compounds to cause a toxic response that resembles in many respects a deficiency of vitamin A has been demonstated in many animal species (Thunberg, 1983). One aspect of this toxic response includes the depletion of hepatic retinoids (Thunberg et al., 1979; Brouwer and van den Berg, 1983; Innami, et al., 1976). However, a mechanism by which hepatic retinoids could be rapidly lost as a consequence of TCDD toxicity has not yet been demonstrated.

The degradation and loss of retinoids from the body does not appear to be simply a function of the oxidation of retinol, to retinal and retinoic acid, since these two oxidation products, are able to support all (retinal) or some (retinoic acid) vitamin A-dependent functions (Underwood, 1984). Even the glucuronosyl retinoic acid conjugate has been shown to have significant retinoid activity (presumably as retinoic acid arising from the hydrolysis of the retinoyl-glucuronide bond; Nath and Olson, 1967). However, because retinoic acid cannot be reduced to either retinal or retinol in animals, it is clear that oxidation and/or conjugation processes provide the major mechanism for the degradation of retinoids in the normal animal (Frolik, 1984).

The reversible oxidation of retinol to retinal occurs by an NAD-linked cytosolic oxidase (Bliss, 1951; Zachman and Olson, 1961). The further oxidation to retinoic acid has

been reported to be carried out by a distinct NAD-linked cytosolic oxidase (Mahadevan et al., 1962).

Both retinol and retinoic acid may be subject to other reactions leading to inactive retinoid species. A microsomally-catalysed oxidation of the B-ionene ring of retinol has been demonstrated, presumably yielding either 4-hydroxy or 4-keto retinol. Further oxidation may produce a number of other species such as the corresponding 4-hydroxy or 4-keto retinoic acid. Retinoic acid may also be subject to several degradation pathways. Like retinol, retinoic acid may be oxidized at the 4 position of the B-ionone ring yielding the 4-hydroxy or 4-keto species, presumably via the same microsomally catalysed P-450-dependent system responsible for retinol hydroxylation. The decarboxylation of retinoic acid has been demonstrated both in vivo and in vitro, and there is considerable evidence suggesting that decarboxylation may provide a significant pathway for the loss of retinoid equivalents. However, the formation of retinoyl-B-glucuronide conjugates in the liver has been clearly demonstrated in several species, and has been shown to constitute the major fraction of biliary excreted retinoids observed following administration of many different retinoids. Lippel and Olson (1968) have suggested the formation of the analogous retinol-glucuronic acid conjugate and demonstrated its formation in the bile of rats given a pharmacologic dose of retinol. However, the extent to which this compound represents a degradation pathway for

retinol has not been established.

Several experiments have demonstrated that the fate of a dose of radiolabeled retinoid is the formation of a large number of radiolabeled products in both urine and feces. Because of the significant fraction of retinol-b-glucuronide formed, it is thought that many of the degradation products identified in the in vivo radiolabeled experiments mentioned above arise from the action of intestinal bacteria on the retinoyl-glucuronide conjugate. Clearly, hydrolysis of the retinoyl-b-glucuronide, and subsequent reabsorption of the molecule would provide the basis for a significant enterohepatic circulation of retinoic acid (Frolik, 1984).

With respect to the toxicity of TCDD-type toxins, there is evidence to suggest that this type of toxicity results in an increased rate of excretion of oxidized retinoid compounds in the feces of treated rats (Cullum and Zile, 1985; Hakansson and Ahlborg, 1985).

We have hypothesized that TCDD treatment causes an increase in the rate of enzyme-catalyzed oxidation of retinoids. We have examined this hypothesis in a single experiment wherein groups of male Sprague-Dawley rats were given single oral doses of TCDD ranging from 0.1 to 300 nmol/kg. We examined the effects of these TCDD dose levels on the rate of microsomal P-450 dependent oxidation of both retinol and retinoic acid, the rate of formation of both retinol- and retinoyl-B-glucuronides, and the rate of oxidation of retinal to retinoic acid. In addition, the

toxic effects of the various TCDD doses on rate of weight gain, food intake, and organ/body weight ratios were determined. Further, the effects of feeding rats a diet deficient in vitamin A on the same enzymes, signs of TCDD toxicity and vitamin A effects were similarly evaluated.

MATERIALS AND METHODS

Animals; treatment and tissue preparation

Male Sprague-Dawley rats, 140-160 g were procured from Charles River Co. (Portage, MI) and acclimated for 1 week on hardwood bedding. The rats were given a single oral dose of corn oil containing 0.0, 0.1, 1.0, 10, 30, 100, or 300 nmol TCDD/ml. Feed intake and body weights were determined daily. Three animals per dose level were killed 3, 7, and 12 days after dose as per Chapter 1, "Methods." An additional group of three rats were dosed with corn oil alone on day 0, fed a vitamin A free diet (AIN-76, VA(-); US Biochemical Corp. Cleveland, OH) and killed on day 12. Blood was sampled and plasma prepared from all animals as per Chapter 1, "Methods." The thymus was carefully excised, trimmed and frozen on dry ice. Liver and kidney were carefully excised and stored in 1.15% KCl/H_2 on ice until homogenization. Livers and kidneys were weighed and homogenized in 9 ml 1.15% KCl/H_2O per g wet weight in a Potter-Elvehjem homogenizer. Homogenates thus prepared were frozen and stored at -20^{o} C prior to analysis. Hepatic microsomes were prepared by centriguation of an aliquot of homogenate at 12,000 x g for 20', re-centrifugation of the supernatant at 105,000 x g for 90' and suspension of the pellet formed in 20 mM phosphate buffer pH = 7.4, containing 20% glycerol and 0.1 mM EDTA, to yield a final concentration of ~10 mg/ml protein, as measured by a bicinchoninic acid microassay (Redinbaugh and Turley, 1985). Microsomes thus

prepared were stored at -20° C until use. The supernatant from the 105,000 x g centrifugation was reserved as "cytosol" and stored at -20° C until use.

Retinoid Levels

Hepatic retinol and retinyl palmitate levels were determined by hexane extraction of homogenate, and HPLC quantitation as described in Chapter 1, "Methods."

Retinal Oxidase Activity

Retinal oxidase activity of hepatic cytosol was determined by a modification of the method described by Mahadevan et al., (1962). Briefly, a 0.2 ml aliquot of the cytosolic fraction was incubated for 15 min at 37° C with 0.8 ml buffer (50 mM MOPS (3-(N-morpholino)-propane sulfonic acid), 0.02% sodium azide, pH = 7.4), and 100 nmol retinal (Sigma Chem. Co.) delivered in 10 ul acetone. The reaction was terminated by the addition of 1.0 ml ethanol (containing 0.1% sodium ascorbate) and 2.0 ml sodium chloride-saturated ammonium acetate (0.5M, pH = 4.6). Retinoic acid formed was extracted into 0.5 ml ethyl acetate (containing 50 mg/ml BHT), and quantitated by injection on an HPLC with a reverse phase column (0.4 x 25 cm, u-Bondopack, 10 micron). Retinoic acid was eluted using a mobile phase of 90:10:0.125 acetonitrile:H_2O:acetic acid, yielding a retention time of ~4 min. Retinoic acid in incubation samples was quantitated by external standards of retinoic acid (Sigma Chem. Co.) dissolved in ethyl acetate (containing 50 mg/ml BHT).

Microsomal Retinol and Retinoic Acid Oxidation

Microsomal retinoid oxidase activity was determined by incubation of the particular retinoid (500 ng; in 5 ul ethanol) with ~20 ug of microsomal protein, 100 uM Desferol, and 100 ul of an NADPH generating system (isocitrate dehydrogenase) in a final volume of 1.0 ml with 50 mM Tris buffer, pH 7.4, for 30' at 37^{o} C. The reaction was terminated with 1 ml 0.1% sodium ascorbate/ethanol and 1 ml sodium chloride-saturated water (retinol) or 0.5 M sodium acetate, pH 4.6 (retinoic acid). Retinoids present following incubation were extracted into 1.0 ml hexane (retinol) or ethyl acetate containing 50 mg/ml BHT (retinoic acid). Retinol in hexane extracts was quantitated by HPLC as described in Chapter 1, "Methods." Retinoic acid in ethyl acetate extracts was quantitated by injection on an HPLC equipped with a photometric detector (340 nm) and a reverse-phase column (4 x 250 mm) with 90:10:0.125 acetonitrile:water:acetic acid as the mobile phase. Microsomal NADPH dependant retinoid degradation was calculated by correction of retinoid degradation in complete incubations less NADPH.

Retinol and Retinoyl, and p-Nitrophenol UDP Glucuronosyl Transferase Activity

P-Nitrophenol UDP glucuronosyl transferase activity was assayed by a modification of the method described by Bock et al, 1983). Briefly, 100 - 200 ug microsomal protein was

incubated in Tris-HCl (50 mM, pH 7.4) containing 20 ul 0.25% Triton X-100, 50 ul $MgCl_2$ (50mM) and 50 ul p-nitrophenol (5 mM), in a final volume of 0.5 ml. Following a 1 min pre-incubation the reaction was initiated with 50 ul UDPGA (30 mM). Disappearance of PNP was determined by monitoring absorbance at 405 nm.

Retinol and retinoyl UDPGT activities were determined by similar assays, however, incubations were for 30 min at 37^{o} C, and the glucuronides were extracted as described for retinoic acid, except that three 1 ml extracts of ethyl acetate were combined, and adjusted to a final volume of 5.0 ml prior to HPLC analysis. Retinoyl-glucuronide was quantitated using E = 43,510 A.U./mol cm as per Miller and DeLuca, (1985).

Statistical Analysis

Data were analysed using Student's t-test with the Bonferroni correction for multiple comparisons (Godfrey, 1985). All references to statistical significance were at the $p < 0.05$ level.

Body weight, food intake and organ weights

The single oral dose of TCDD caused a dose-dependent loss of weight or depressed rate of weight gain following treatment. The greatest effect was observed with the 100 and 300 nmol/kg doses, as shown in Figures 1 and 2. Food intake (Figure 3) was similarly affected and at 12 days after dose was highly and positively correlated with the body weight ($r = .988$). No significant differences were observed in the body weights or rate of weight gain of the animals fed a vitamin A-deficient diet.

Significant hepatic enlargement was observed in groups treated with 1.0 nmol TCDD/kg and higher doses, with the exception of the group of rats treated with 300 nmol TCDD/kg in which hepatic necrosis was noted when the animals were killed. Animals fed a vitamin-A deficient diet for 12 days also had significant hepatic enlargement (Table 1).

No trend in renal weight was noted, and there was significant thymic involution observed in rats treated with 30 nmol TCDD/kg or greater. An enlargement of the thymus gland in rats fed a vitamin A-deficient diet for 12 days was also observed.

Hepatic Retinyl Palmitate

The single dose of TCDD caused, 12 days following treatment, a significant depression in the levels of hepatic retinyl palmitate in all treatment groups. Feeding a vitamin A deficient diet for the 12 day period resulted in a

Figure 1. The effect of a single oral dose of TCDD on the body weights of male Sprague Dawley rats. Values are means of groups of 4 (0 nmol/kg) or 3 (30, 100 and 300 nmol/kg) rats each.

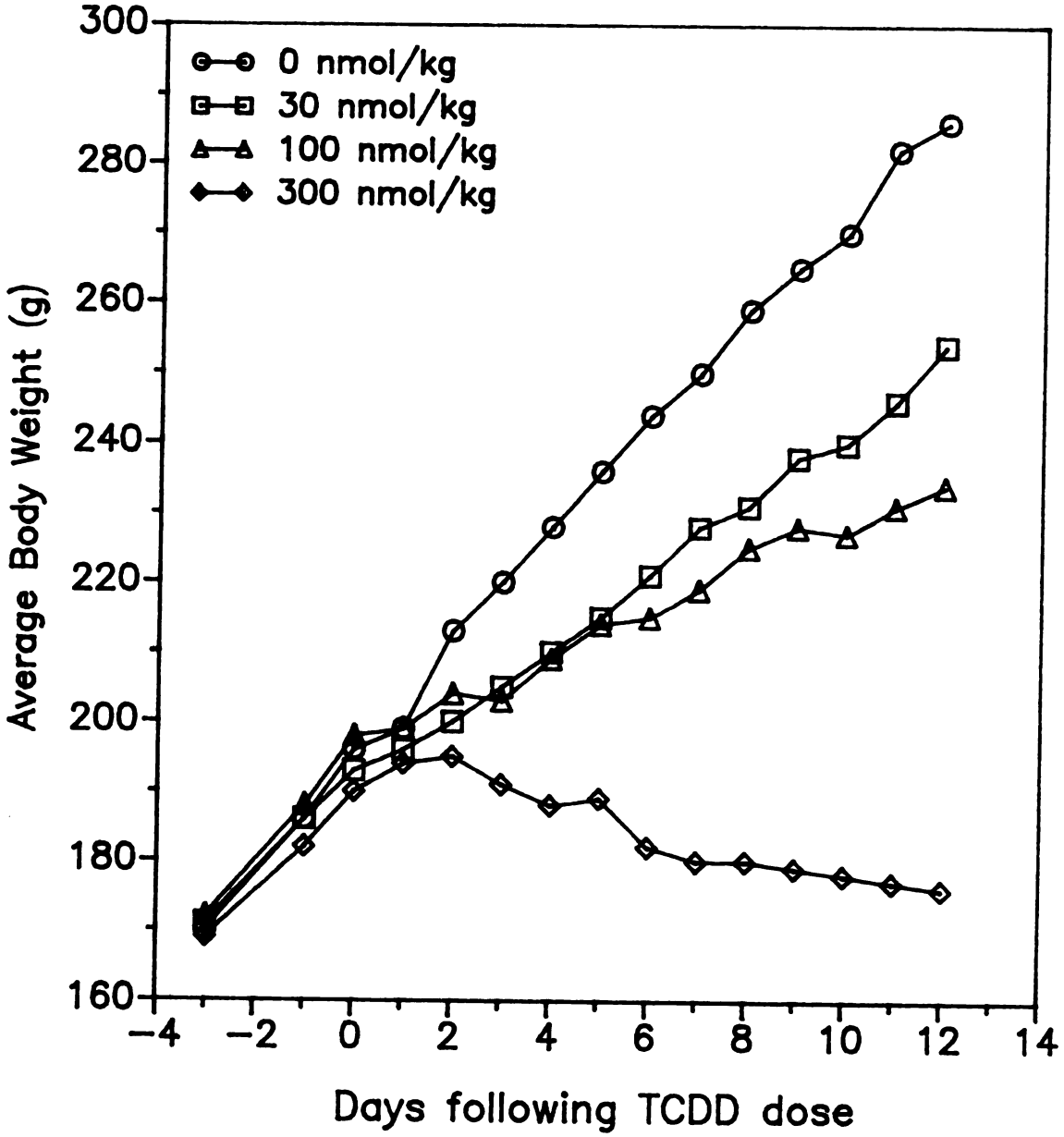

0 nmol/kg
30 nmol/kg
100 nmol/kg
300 nmol/kg
Average Body Weight (g)
300
280
260
240
220
200
180
160
−4
−2
0
2
4
6
8
10
12
14
Days following TCDD dose

Figure 2. Effect of a single oral dose of TCDD or feeding a diet deficient in vitamin A (VA(-)) on the average body weight of male Sprague-Dawley rats, 12 days following TCDD dose or start of VA(-) diet. Values are means, ±SD for groups of n = 3 rats (all TCDD dose groups) or n = 4 rats (control and VA(-) diet groups). (—○—): Control and TCDD treatment groups, (—●—): VA(-) diet group.

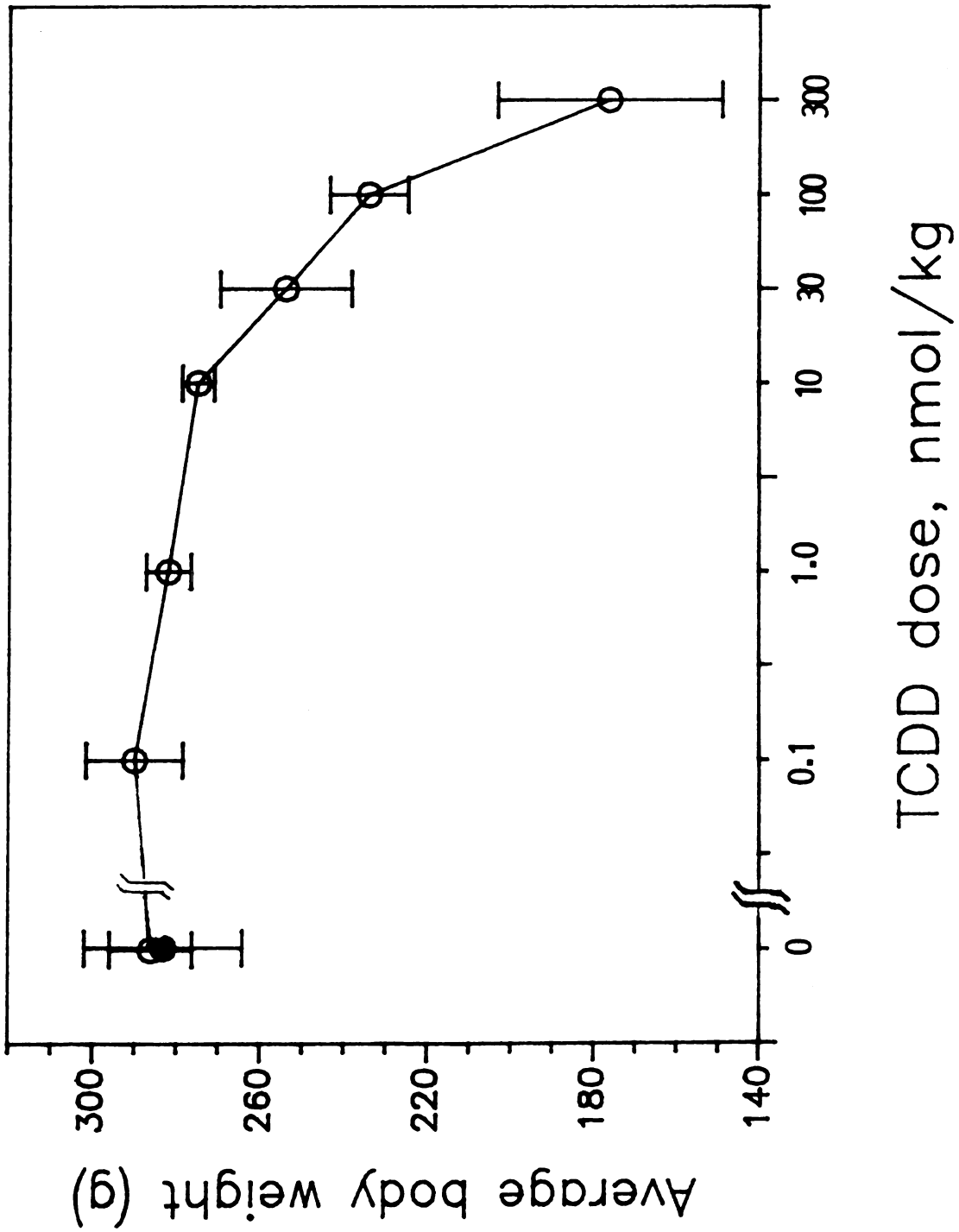

Average body weight (g)
300
260
220
180
140
0
0.1
1.0
10
30
100
300
TCDD dose, nmol/kg

Figure 3. Effect of a single oral dose of TCDD on the cumulative food intake of male Sprague Dawley rats for a 12 day period following dose. Values are cumulative group consumption for the test period, expressed on a per rat basis.

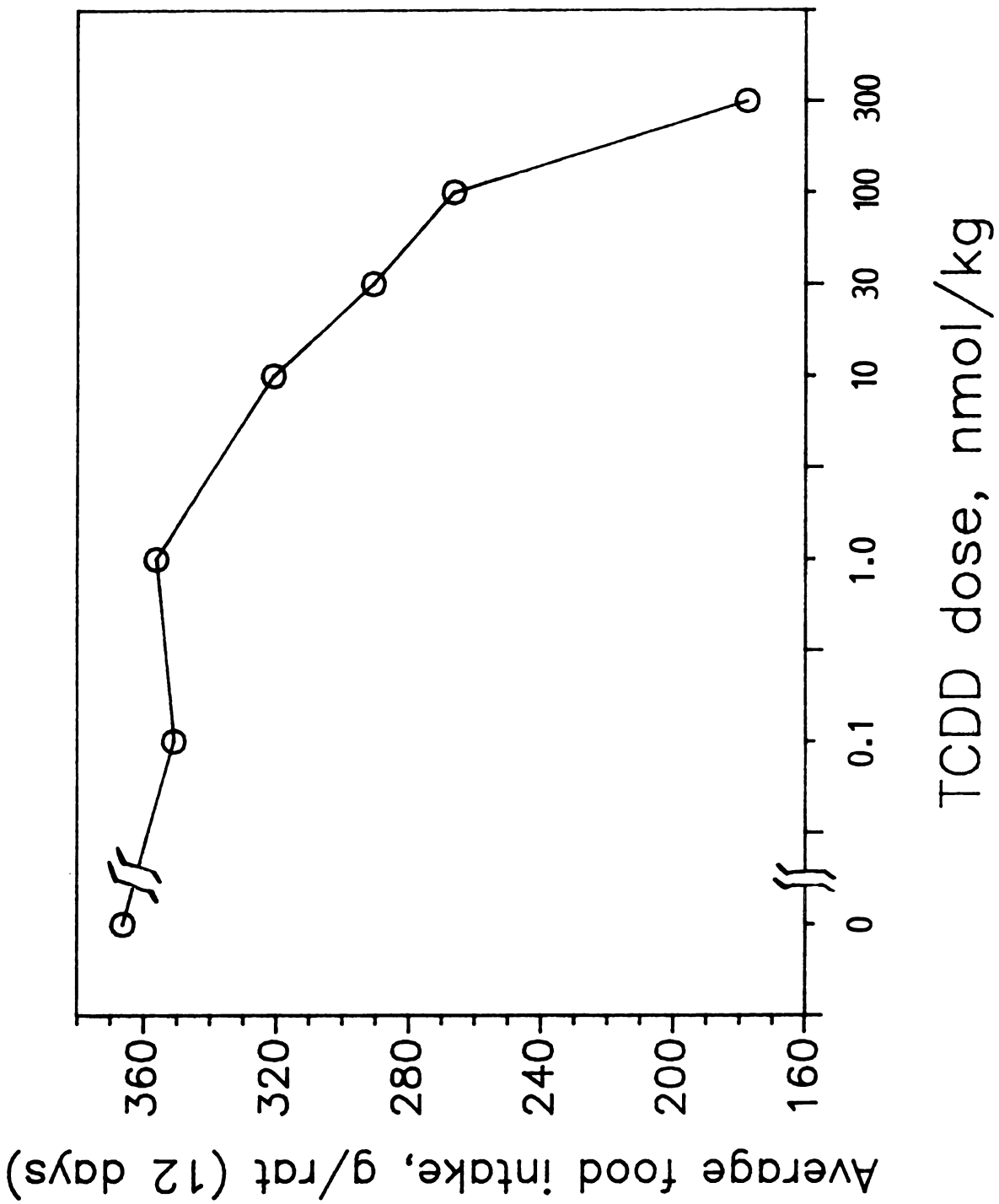

Average food intake, g/rat (12 days)
360
320
280
240
200
160
0
0.1
1.0
10
30
100
300
TCDD dose, nmol/kg

Table 1. The Effect of a Single Dose of TCDD or Feeding a Vitamin A-Deficient (VA(-)) Diet on Organ/Body Weight Ratios in Male Sprague-Dawley Rats, 12 Days Following Either TCDD Treatment or Start of the VA(-) Diet.[a]

Dose Group	Liver Weight / Body Weight ($\times 10^3$)	Kidney Weight / Body Weight ($\times 10^4$)	Thymus Weight / Body Weight ($\times 10^4$)
Control	54 ±2	94 ±2	19 ±1
VA(-) Diet	63[b] ±4	92 ±4	26[b] ±3
0.1 nmol/kg TCDD	58 ±2	98 ±4	24 ±5
1.0 nmol/kg TCDD	59.7[b] ±0.2	103[b] ±2	23 ±2
10 nmol/kg TCDD	66[b] ±3	97 ±3	19 ±2
30 nmol/kg TCDD	79[b] ±7	94 ±5	9[b] ±1
100 nmol/kg TCDD	83[b] ±6	97.0 ±0.4	8[b] ±2
300 nmol/kg TCDD	49 ±8	99 ±8	3[b] ±1

[a]: Results are mean ± SD, 12 days following TCDD treatment or start of VA(-) diet.

[b]: Significantly different from control rats, $p < 0.05$.

significant depression of the levels of hepatic retinyl palmitate, roughly equal to the depression observed in the 30 and 10 nmol TCDD/kg treatment groups, as shown in Figure 4.

Microsomal Retinol and Retinoic Acid NADPH-dependent Degradation

Hepatic microsomes from TCDD-treated rats, (10 and 100 nmol/kg) had a slightly enhanced rate of NADPH-dependent microsomal degradation, as shown in Figure 5. Microsomes from rats fed a vitamin A-deficient diet were less active in degrading retinol. We were unable to observe significant microsomal NADPH-dependent retinoic acid degradation.

PNP, Retinol, and Retinoic Acid UDP Glucuronosyl Transferase Activity

The microsomal PNP UDPGT activity was markedly elevated, in a dose-dependent manner in rats treated with TCDD (Figure 6). The ED_{50} was ~8 nmol/kg. Retinoic acid UDPGT was similarly elevated, however, the ED_{50} was ~50 nmol/kg (Figure 7) We did not observe any detectable retinol UDPGT activity.

Figure 4. Effect of a single oral dose of TCDD, or feeding a vitamin A-deficient diet (VA(-)) to male Sprague-Dawley rats on hepatic retinyl palmitate levels 12 days following dose or start of VA(-) diet. Values are means, ± SD for groups of n = 3 (all TCDD treatment groups) or n = 4 (control and VA(-) diet groups). (—○—): Control and TCDD treatment groups, (—●—): VA(-) diet group.

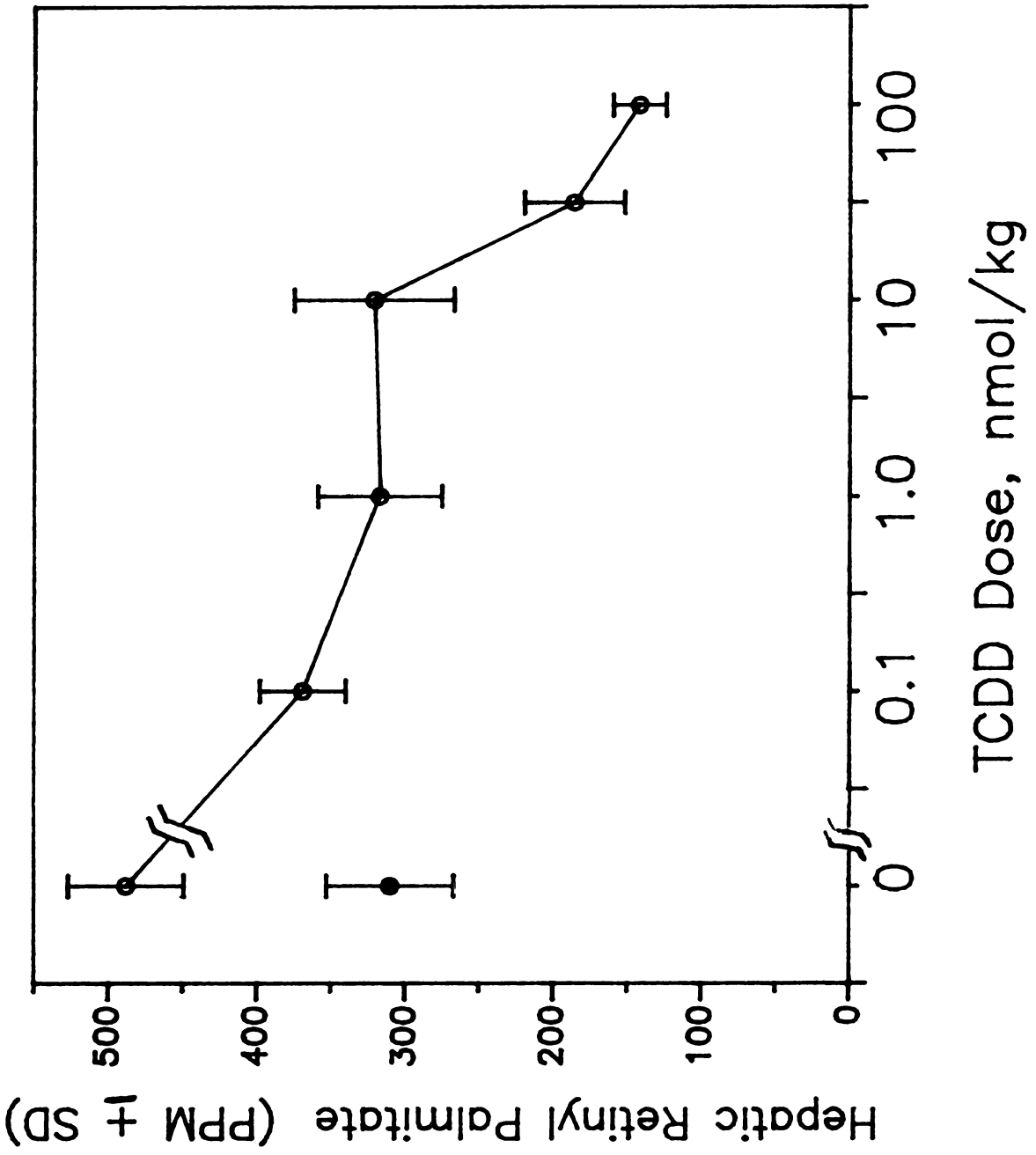

Hepatic Retinyl Palmitate (PPM ± SD)
500
400
300
200
100
0
0
0.1
1.0
10
100
TCDD Dose, nmol/kg

Figure 5. The effect of a single oral dose of TCDD or feeding a vitamin A-deficient diet (VA(-)) to male Sprague Dawley rats on hepatic microsomal retinol degredation 12 days following either TCDD dose or start of VA(-) diet. Values are means of groups of n = 3 (all TCDD treatment groups) or n = 4 (control and VA(-) diet groups)

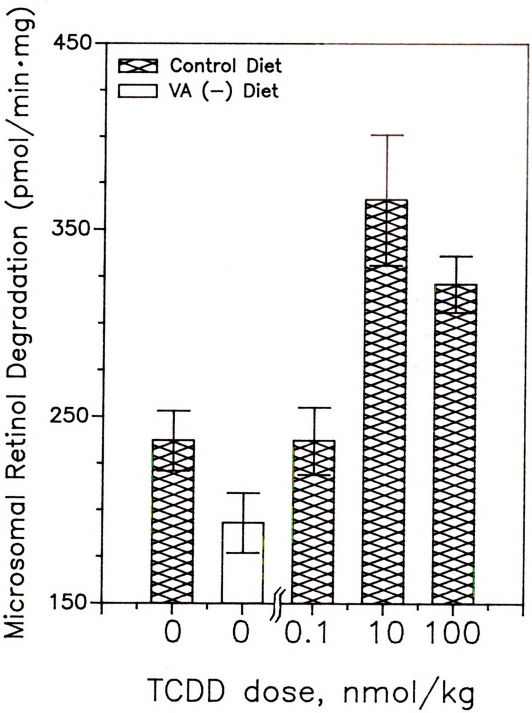

Microsomal Retinol Degradation (pmol/min•mg)
450
350
250
150
Control Diet
VA (−) Diet
0
0
0.1
10
100
TCDD dose, nmol/kg

Figure 6. The effect of a single oral dose of TCDD or feeding a vitamin A-deficient diet (VA(-)) to male Sprague Dawley rats on hepatic microsomal p-nitrophenol UDP-glucuronosyl transferase activity 12 days following either TCDD dose or start of VA(-) diet. Values are means of groups of n = 3 (all TCDD treatment groups) or n = 4 (control and VA(-) diet groups).

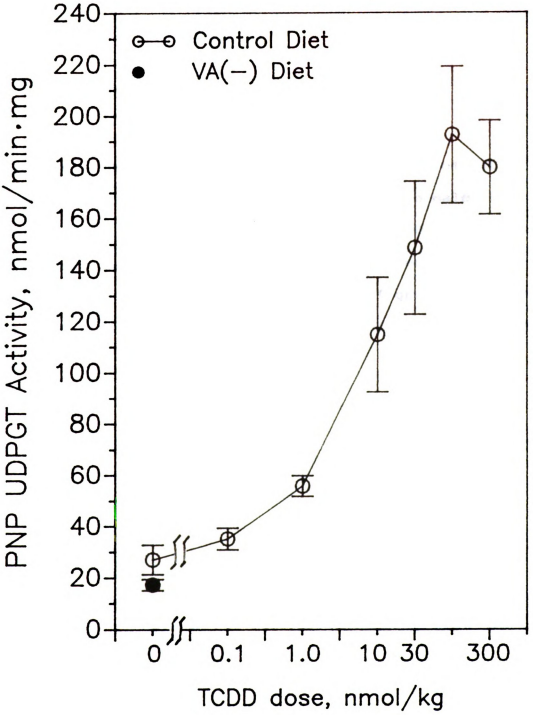
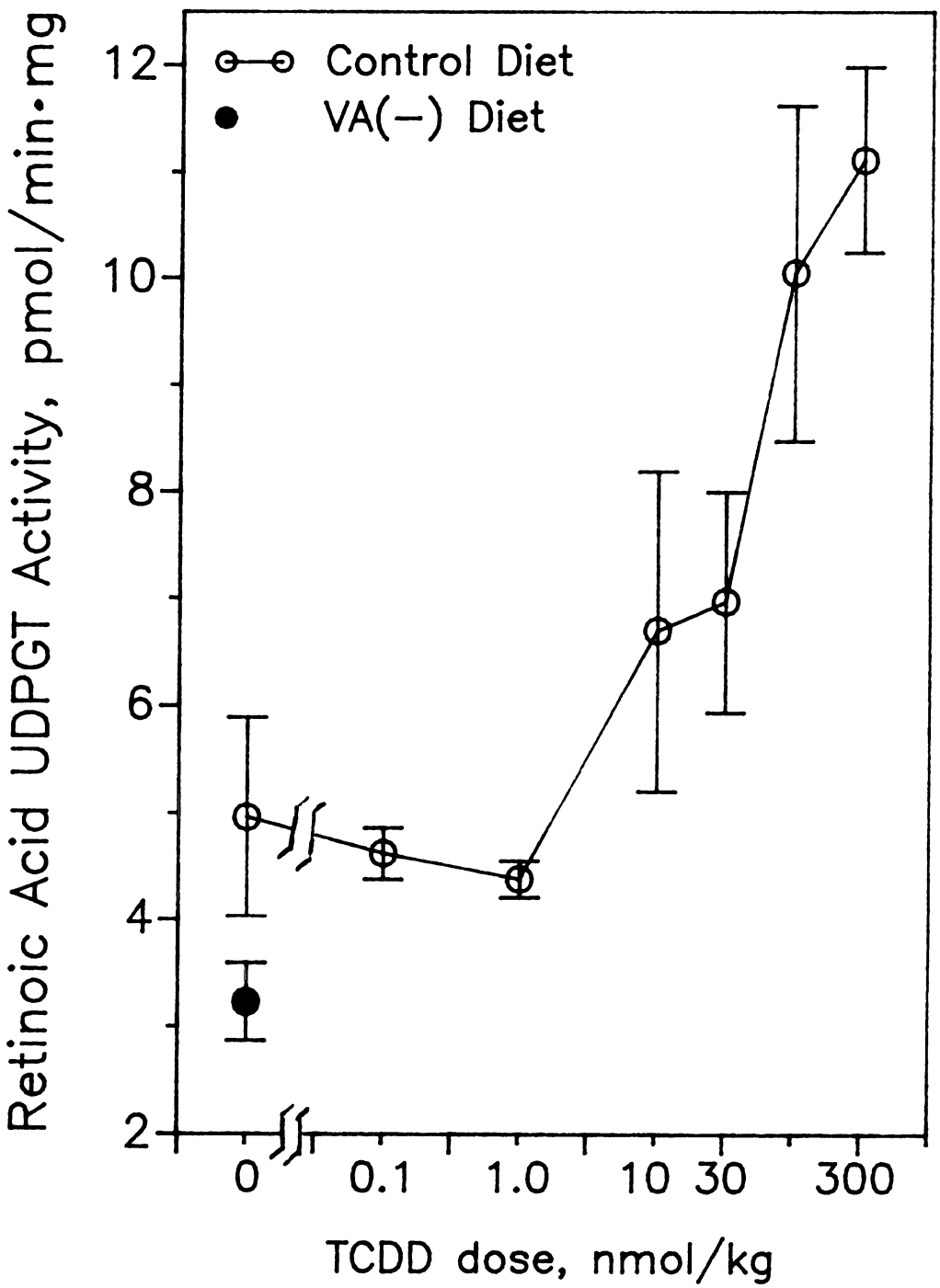

Control Diet
VA(−) Diet
PNP UDPGT Activity, nmol/min·mg
240
220
200
180
160
140
120
100
80
60
40
20
0
0
0.1
1.0
10
30
300
TCDD dose, nmol/kg

Figure 7. The effect of a single oral dose of TCDD or feeding a vitamin A-deficient diet (VA(-)) to male Sprague Dawley rats on hepatic microsomal retinoyl UDP-glucuronosyl transferase activity 12 days following either TCDD dose or start of VA(-) diet. Values are means of groups of n = 3 (all TCDD treatment groups) or n = 4 (control and VA(-) diet groups).


Control Diet
VA(−) Diet
Retinoic Acid UDPGT Activity, pmol/min•mg
12
10
8
6
4
2
0
0.1
1.0
10
30
300
TCDD dose, nmol/kg

DISCUSSION

The effect of a single oral dose of TCDD on both the overt signs of toxicity, as well as the storage of retinoids, in treated rats was quite pronounced, but in agreement with previously published data. Particularly interesting was the correlation between weight of the animals 12 days following TCDD treatment and their cumulative food intake for the period, supporting the hypothesis of Peterson et al., (Seefeld, et al., 1984a, 1984b) that hypophagia is the primary cause of the TCDD-induced weight loss. The results of this experiment however, also provide mechanistic evidence for at least some of the pathways by which vitamin A may be so rapidly lost from TCDD treated animals.

The microsomal oxidation of retinoic acid (presumably) to either the 4-hydroxy or 4-keto compound has received significant attention in the literature. This may be because of the identification of these compounds as metabolic products of physiologic doses of either retinol or retinoic acid in a number of species. The analogous microsomal oxidation of retinol has not been equally investigated. However, our research indicates that microsomes from TCDD-treated rats show a significant increase in the rate of microsomal NADPH-dependent retinol oxidation, as compared to control animals. It is important to note that the same reaction proceeds at a significantly slower rate in microsomes from rats in the early stages of a

diet-induced vitamin A deficiency. Indeed, the microsomes from rats treated with 30 nmol/kg TCDD degraded retinol at a rate over twice that of the vitamin A deficient control rats, despite the fact that both groups had lost approximately the same amount of retinyl palmitate from the liver. It seems reasonable to conclude then, that the normal reaction to a developing vitamin A deficiency is not being demonstrated in the TCDD treated animals.

Our findings with respect to the elevated activity of the microsomal PNP-UDPGT were in accord with previous reports in the literature. Indeed several authors have suggested that elevated UDPGT activities may form part of the pleiotropic response to induction enzymes controlled by the Ah receptor. Several authors have also suggested that induction of UDPGT activity towards retinol could account, in some degree, for the loss of hepatic retinoids that characterizes TCDD-type toxicity. While this type of condensation reaction is carried out by certain UDPGT isozymes, it has been demonstrated <u>in vivo</u> only following large doses of retinol (Lippel and Olsen, 1968.) We had hypothesized that if indeed UDPGT activity played a role in retinoid depletion, then it would do so by catalyzing the formation of the retinoyl-glucuronide. This compound, the first retinoic acid metabolite to be indentified (Dunagin <u>et al</u>., 1965) has been demonstrated as a significant retinoid metabolite in numerous studies. Our results tend to support this hypothesis, as we have seen a significant

elevation of the retinoyl-UDPGT activity in the microsomes from TCDD-treated rats. It again is important to note that the response of the rats to a vitamin A deficiency is to decrease the rate at which degradative reactions may proceed. In this case, the activity of the retinoyl-UDPGT was significantly depressed in rats fed a vitamin A deficient diet for 12 days as compared to controls.

BIBLIOGRAPHY

Bliss, A.F. (1951). The equilibrium between vitamin A alchohol and aldehyde in the presence of alchohol dehydrogenase. Arch. Biochem. Biophys. 31:197-204.

Bock, K.W., Burchell, B., Dutton, G.J., Hanninen, O., Mulder, G., Owens, I.S., Siest, G., and Tephly, T.R. (1983). UDP-glucuronosyltransferase activities: Guidelines for consistent interim terminology and assay conditions. Biochem. Pharm. 32:953-955.

Brouwer, A., and van den Berg, K.J. (1983). Early decrease in retinoid levels in mice after exposure to low doses of polychlorinated biphenyls. Chemosphere 12:555-558.

Cullum, M.E., and Zile, M.H. (1985). Acute PBB toxicosis alters vitamin A homeostasis and enhances degradation of vitamin A. Toxicol. Appl. Pharmacol. 81:177-181.

Frolik, C.A. (1984). Metabolism of retinoids. In:"The Retinoids" (Sporn M.B., Roberts, A.B., and Goodman, D.S. eds.) Academic Press, Inc. Orlando. Vol 2, pp:177-208.

Godfrey, N.K. (1985). Comparing the means of several groups. New Engl. J. Med. 313:145-1456.

Hakansson, H., and Ahlborg, U.G. (1985). The effect of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on the uptake, distribution and excretion of a single oral dose of 11,12-^{3}H-retinylacetate and on the vitamin A status in the rat. J. Nutr. 115:759-771.

Innami, S., Nakamura, A., Miyazaki, M., Nagayama,S., and Nishide, E. (1976). Further studies on the reduction of vitamin A content in the livers of rats given polychlorinated biphenyls. J. Nutr. Sci. Vitaminol. 22:409-418.

Lippel, K., and Olson, J.A. (1968). Biosynthesis of B-glucuronides of retinol and of retinoic acid *in vivo* and *in vitro*. J. Lip. Res. 9:168-175.

Mahadevan, S., Murthy, S.K., and Ganguly, J. (1962). Enzymic oxidation of vitamin A aldehyde to vitamin A acid by rat liver. Biochem. J. 85:326-331.

Miller, D.A., and DeLuca, H.F. (1985). Biosynthesis of retinoyl-B-glucuronide, a biologically active metabolite of all-trans-retinoic acid. Arch. Biochem. Biophys. 244:179-186.

Nath J., and Olson, J.A. (1967). Natural occurrence and biological activity of vitamin A derivatives in rat bile. J. Nutr. 93:461-469.

Seefeld, M.D., Corbett, S.W., Keesey, R.E., and Peterson, R.E. (1984). Characterization of the wasting syndrome in rats treated with 2,3,7,8-tetrachlorodibenzo-p-dioxin. Toxicol. Appl. Pharmacol. 73:311-322.

Seefeld, M.D., Keesey, R.E., and Peterson, R.E. (1984). Body weight regulation in rats treated with 2,3,7,8-tetrachlorodibenzo-p-dioxin. Toxicol. Appl. Pharmocol. 76:526-536.

Thunberg, T., Ahlborg, U., and Johnsson, H. (1979). Vitamin A (Retinol) status in the rat after a single oral dose of 2,3,7.8-tetrachlorodibenzo-p-dioxin. Arch. Toxicol. 42:265-274.

Thunberg, T. (1983). Studies on the effect of 2,3,7,8-tetrachlorodibenzo-p-dioxin on vitamin A: A new aspect concerning the mechanism of toxicity. PhD Thesis, Dept. of Toxicology. Carolinska Institute, Stockholm.

Underwood, B.A. (1984). Vitamin A in human and animal nutrition. In:"The Retinoids". (Sporn, M.B, Roberts, A.B., and Goodman, D.S., eds). Academic Press, Inc. Orlando. Vol 1, pp:281-392.

Zachman, R.D., and Olson, J.A. (1961). A comparison of Retinene reductase and alcohol dehydrogenase of rat liver. J. Biol. Chem. 236:2306-2313.

CHAPTER 4

Exacerbation of TCDD-Induced Toxicity and Hepatic Retinoid Depletion in Rats by Triiodothyronine.

ABSTRACT

The effects of triiodothyronine (T3) on both TCDD toxicity, as measured by rate of weight gain and organ hypertrophy, and hepatic retinoid depletion was determined in a single experiment in male Sprague-Dawley rats. Animals were given either a control or T3-containing (500 ug/kg) diet, and both diet groups were further treated with either 0 or 25 ug/kg TCDD and killed 12 days following treatment. Inclusion of T3 in the diet caused a slight, transitory depression in the rate of weight gain, and a pronounced exacerbation of the depression of rate of weight gain caused by the TCDD treatment. Dietary T3 had no effect on the TCDD-caused hepatic hypertrophy, but caused, unlike TCDD, a moderate hypertrophy of kidney. TCDD treatment also caused a depletion of hepatic retinoids, which was moderately, but significantly exacerbated by dietary T3. TCDD also caused a pronounced accumulation of retinoids in the kidney, which was markedly exacerbated by dietary T3. Therefore, an apparent increase in the toxicity of TCDD caused by inclusion of T3 in the diet, also caused an exacerbation of the vitamin A depletion characteristic of the toxic response to TCDD. We conclude that this finding is in accord with the depletion of hepatic retinoids and onset of vitamin A depletion as being a significant aspect of TCDD-type toxicity.

INTRODUCTION

TCDD, and related compounds, along with certain polycyclic aromatic hydrocarbons, polyhalogenated-dibenzofuran and -biphenyl congeners, all cause a characteristic toxic response in treated animals commonly referred to as the "wasting syndrome." Manifestations of toxicity include a delayed time to death, pronounced weight loss, hypophagia, cachexia, epithelial lesions, lymphoid involution, and hepatic hypertrophy (McConnell, 1980; Poland and Knutson, 1982; Peterson et al., 1984). Neither the biochemical mechanism resulting in the wasting syndrome or other toxic mechanism(s) of TCDD and similar compounds have yet been identified.

The pronounced similarity between the signs of the xenobiotic-induced wasting syndrome and those of a severe vitamin A deficiency was noted by Kimbrough (1974) and reviewed by Thunberg (1983a). The ability of TCDD and similarly-acting compounds to cause both a rapid depletion of hepatic vitamin A stores, as well as other signs of vitamin A deficiency (e.g., depressed serum retinol levels, thymic involution, failure of growth, and the accumulation of kidney retinoids), has been reported in several species (Innami et al., 1975, 1976; Brouwer and van den Berg, 1983, 1984; Spear et al., 1986). TCDD toxicity causes a short-lived rise in serum retinol levels followed by a decline to below normal levels, presumably as hepatic stores are exhausted (Thunberg et al., 1979; Thunberg, 1983a).

Similar to that observed in a vitamin A deficiency (Morita and Nakano, 1982; Moore and Sharman 1950; Wolf, 1980) TCDD causes an accumulation of retinoid material in the kidney (Thunberg, 1983b).

It has been reported that diets containing elevated vitamin A contents ameliorate, but do not eliminate, the toxic response to TCDD-type compounds (Innami et al., 1975; Thunberg et al., 1980). Therefore, it seems reasonable that some aspects of TCDD-type toxicity may be a function of the onset of an actual vitamin A deficiency caused by exposure to TCDD-type toxins.

Vitamin A is an essential nutrient, although with the exception of its role in the visual cycle its exact metabolic function(s) remains unelucidated (Wolf, 1984). It is clear, however, that an adequate supply of the vitamin is required to maintain growth, reproductive capacity, and immunological competence (Zile and Cullum, 1983). Vitamin A is not synthesized by animals, but is available in the diet in several readily utilizable forms (Underwood, 1984). The vitamin is stored in the liver as mixed retinyl esters and is hydrolyzed to the free alcohol retinol, prior to delivery to target tissues by a specific transport protein (retinol binding protein, RBP; Goodman, 1984, Goodman and Blaner, 1984). The control mechanisms for mobilization of the vitamin from the liver and of metabolism in target tissues (including the liver) remain largely unknown. Loss of the vitamin from the body seems to occur primarily when

the compound is oxidized to retinoic acid or retinoyl derivatives (Wolf, 1980; Frolik, 1984).

In addition to the effects on vitamin A levels, TCDD has also been shown to affect the thyroid hormone status of treated animals. Overtly, TCDD treatment seems to cause hyperthyroidism, as the animals demonstrate both weight loss and elevated metabolic rate (Potter et al., 1986; Rozman, 1984). Further, thyroidectomy seems to confer a significant degree of protection against TCDD toxicity (Rozman, 1984). Curiously though, TCDD treatment also causes a rapid and pronounced depression of serum thyroxine (T4) levels, normally considered symptomatic of a hypothyroid state (Bastomsky, 1977; Gupta et al., 1983).

There is evidence for some degree of interaction between levels of thyroid hormones and vitamin A. Underwood (1984) noted the inverse correlation between hepatic vitamin A reserves and thyroid state in humans. Early nutritional studies suggest that during the induction of acute hyperthyroidism there is also an onset of vitamin A deficiency (Sure and Buchanan, 1937). Bhat and Cama (1977) have suggested that the hyperthyroid state causes signs of a vitamin A deficiency by enhancing plasma clearance rates of retinol, while the rate of both apo-RBP synthesis and retinol-RBP secretion remains unchanged. This hypothesis is particularly interesting with respect to TCDD toxicity. As noted above, TCDD-treated animals express symptoms reminiscent not only of vitamin A deficiency, but of

hyperthyroidism also. Further, it has been reported that TCDD in rats increases the fractional clearance rate of retinol in plasma (Bank et al., 1987).

Preliminary experiments in our laboratory suggested that inclusion of triiodothyronine (T3) in the diet of TCDD-treated rats exacerbated the toxic response as measured by growth rate. Therefore, we hypothesized that to the extent that vitamin A depletion plays a role in the expression of TCDD toxicity, its depletion from the liver and accumulation in the kidney should be similarly exacerbated by the inclusion of T3 in the diet of treated animals. Experimentally, we wished to compare the magnitude of the retinol and retinyl ester depletion of the liver, as well as the magnitude of the retinoid accumulation in the kidney caused by a single dose of TCDD in normal and T3-fed rats. While expressly designed to demonstrate the exacerbation of TCDD-induced hepatic retinoid depletion caused by thyroid hormones, this experiment would also demonstrate the effects of dietary "hyperthyroidism" on vitamin A depletion from the liver, and the accumulation in the kidney, thereby enhancing our knowledge about the interaction of natural hormones and vitamin A metabolism.

MATERIALS AND METHODS

Animals, Diets, and Treatment

Male Sprague-Dawley rats were obtained from Charles River Laboratories (Portage, MI) and acclimated for 2 weeks on standard laboratory rat diet (Rodent-Blox (R); Wayne Feeds, Chicago, IL). Diet containing T3 (500 ug/kg) was prepared by grinding the standard diet in a Wiley mill and mixing with 10 ml of a T3 solution at 50 ug/ml in ethanol per kg of diet in a dough blender until all solvent had evaporated, and a smoothly flowing powder was obtained. Control diet was prepared in the same manner, with the exception that ethanol alone was added. One day prior to TCDD treatment, the rats were randomly assigned to 4 separate groups, and housed 3/cage in polystyrene rodent cages with food and water ad libitum.

On day 0, rats in groups not treated with TCDD ("C" and "T3" groups) received a 1.0 ml/100 g oral dose of corn oil and those in the TCDD-treated ("CT" and "T3T") groups received a similar dose containing 2.5 ug/ml TCDD. All treatment groups received their appropriate food and water ad libitum. Body weights of individual rats were determined daily. Three untreated rats were killed on day 0 by decapitation following CO_2 anesthesia and 3 rats from each group were killed on days 3, 7, and 12. Trunk blood was collected in 10 ml EDTA-Vacutainer tubes (Becton-Dickenson and Co., Rutherford, N.J.) and stored on ice. Plasma was prepared by centrifugation (1500 x g for 10 min at 4^{o} C),

frozen on dry ice, and stored at -70^{o} C until analysis for retinol. Liver was perfused *in situ* with 0.90% NaCl, excised, and stored on ice in 0.90% NaCl prior to homogenization. Liver homogenate was prepared by homogenization of an aliquot of liver at 3 ml/g in "HEDG" buffer (0.025 M HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), 1.5 mM EDTA, 1 mM DTT (dithiothreitol), 10% glycerol pH 7.4) in a Potter-Elvehjem homogenizer. Aliquots of liver homogenate were frozen on dry ice and stored at -70^{o}C until analysis for retinol and retinyl palmitate. Kidneys were excised and stored as per liver until freezing. Kidney homogenates were prepared from frozen tissues by homogenization of a single kidney at 10.0 ml/g in 1.15% KCl in a Potter-Elvehjem homogenizer. Aliquots of homogenate were used immediately for analysis of retinol and retinyl palmitate.

Retinol and Retinyl Palmitate

Plasma, hepatic, and renal levels of retinol and retinyl palmitate were determined by modifications to the method of Dennison and Kirk (1977). Aliquots of liver (0.2 ml), kidney (1.0 ml), or testes (2.0 ml) homogenates or plasma (0.2 ml) were mixed thoroughly with 2 ml NaCl-saturated water and 2 ml ethanol. Retinoids were extracted by the addition of 1.0 ml (liver and kidney samples) or 0.5 ml (plasma samples) of UV-grade hexane followed by thorough mixing. The samples were centrifuged (2500 x g for 2 min) to facilitate phase separation and the supernatants were

assayed for retinol and/or retinyl palmitate by HPLC using a fluorescence detector (Shimadzu RS-530-S, ex = 330 nm, em = 470 nm) and a 4 x 250mm column packed with Lichrosorb SI-60 (Alltech, Deerfield, IL.) Isocratic elution of retinol was performed using 25% hexane/75% $CHCl_3$. Retinyl palmitate was similarly eluted with 8% $CHCl_3$/92% hexane. Retinol was eluted by this system at a retention time of 5.5 min, retinyl palmitate at 4 min. Detection limit for each species was 500 pg. Retinol and retinyl palmitate (Sigma Chemical Co., St. Louis, MO) standards were prepared in ethanol and verified by $E_{324}(1\%) = 1835$ and $E_{326}(1\%) = 975$ A.U., respectively (Windhols, 1976). HPLC standards of 1.0 and 0.1 ng/ul for both retinoids were prepared by dilution of the stock standard with hexane. All standards were stored at $-20^{o}C$ and remained stable for at least six months.

Statistical Analysis

Data were analysed using Student's t-test with the Bonferroni correction for multiple comparisons (Godfrey, 1985). All references to statistical significance were at the $p < 0.05$ level.

RESULTS

Body Weights

The group of rats fed a normal diet (C group) and sacrificed on day 12 had gained 38.9% of their day -1 weight, as shown in Figure 1. Inclusion of T3 in the diet (T3 group) caused a short, transient depression in the rate of weight gain when compared to the C group. However, the growth rate was equal to that of the C group by day 3 and the T3 group rats averaged a 32.5% gain of their day -1 weights by day 12 (Figure 1). Typical of the response to TCDD treatment, rats fed a normal diet and treated with TCDD (CT group) failed to gain weight normally when compared to control rats, reaching only a 16.1% increase over their day -1 weights by day 12 (Figure 1). The combination of TCDD treatment and dietary T3 (T3T group) had the greatest effect on the ability of the rats to gain weight, with the day 12 T3T rats averaging an increase of only 0.27% of their day -1 weight (Figure 1).

Organ/Body Weight Ratios

Treatment of rats with TCDD caused an increase in the hepatic weight/body weight ratio, regardless of whether T3 was included in the diet (Table 1). Curiously, kidney enlargement was observed in both T3-fed groups and was unaffected by TCDD treatment. The testes weight/body weight ratio difference observed, appears to reflect the changes in the body mass of the rat, since testes weight remained essentially unaffected by any of the treatment regimens.

Figure 1. Effect of TCDD and or dietary T3 on body weight of male Sprague-Dawley rats. Each point represents the mean ± SD of groups of 3 rats: control diet (—○—); control diet + TCDD (—△—); T3 diet (—●—); T3 diet + TCDD (—▲—). [a]: Significantly different from control rats fed standard diet, $p < 0.05$.

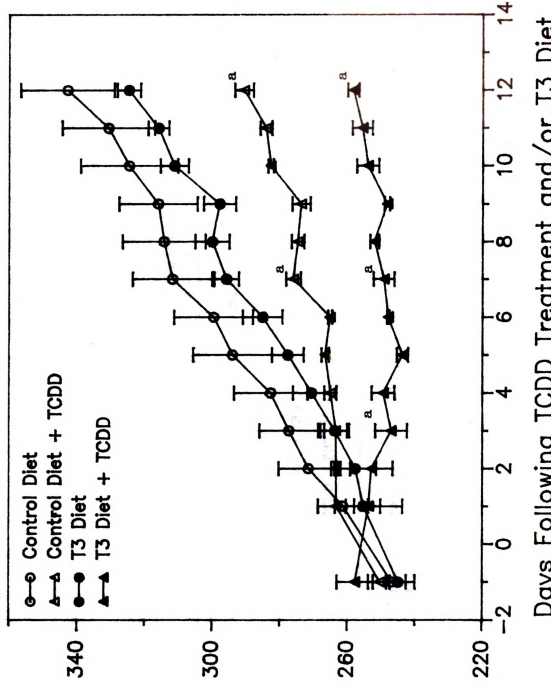

Control Diet
Control Diet + TCDD
T3 Diet
T3 Diet + TCDD
Average Body Weight (g)
340
300
260
220
−2
0
2
4
6
8
10
12
14
Days Following TCDD Treatment and/or T3 Diet
a

Table 1. The Effect of a single oral dose of TCDD and/or Dietary T3 on Organ/Body Weight Ratios in Male Sprague-Dawley Rats.[a]

Treatment		Liver Weight / Body Weight ($x10^3$)	Kidney Weight / Body Weight ($x10^4$)
Diet	Group		
Control	Control (C)	54±3	81±4
	TCDD (CT)[b]	70±2[d]	86±2
T3[c]	Control (T3)	51±3	107±5[d]
	TCDD (T3T)[b]	76±3[d]	104±7[d]

[a]: Values are means ± SD from groups of 3 rats, 12 days following either treatment with TCDD, start of T3 diet or both.

[b]: 25 ug/kg, oral.

[c]: Beginning on day 0, rats received powdered diet containing T3 (500 ug/kg of diet) ad libitum.

[d]: Significantly different from Control group (C) value, $p < 0.05$.

Tissue Retinol

Treatment of rats with either TCDD (CT group) or dietary T3 (T3 group) resulted in a significant elevation of plasma retinol when compared to control rats (C group) at 12 days post-treatment. The combination of TCDD and dietary T3 (T3T group) produced a slightly greater elevation of plasma retinol values than was observed with either treatment alone (Table 2). Plasma retinol for both TCDD-treated groups (CT and T3T) was significantly different from the control group (C) at day 7, but the level for the dietary T3 group (T3), although elevated, was not statistically significant (data not shown). Retinol levels in the kidneys followed a similar pattern, with the exception that the combination treatment (T3T group) resulted in a significant elevation of renal retinol levels, well above those observed in either of the other treatment groups (CT or T3), and over twice that of the untreated control group (C) (Table 2). In addition, some of the effects of TCDD and/or dietary T3 treatment were evident at 7 days post-treatment for plasma retinol levels and as early as 3 days post-treatment for renal retinol levels (data not shown). Retinol concentration in the liver was unaffected by TCDD or dietary T3 treatment, either alone or in combination.

Table 2. The Effect of TCDD and/or Dietary T3 on Tissue Retinol Concentration in Male Sprague-Dawley Rats.[a]

Treatment		Tissue Retinol, (PPM)		
Diet	Group	Plasma	Kidney	Liver
Control	Control (C)	0.59±0.06	1.5±0.1	0.8±0.2
	TCDD (CT)[b]	0.90±0.04[d]	2.2±0.2[d]	0.46±0.02
T3[c]	Control (T3)	0.91±0.03[d]	2.5±0.2[d]	0.78±0.06
	TCDD (T3T)	1.08±0.02[d,e,f]	3.7±0.2[d,e,f]	0.60±0.04

[a]: Values are means ± SD from groups of 3 rats, 12 days following either treatment with TCDD, start of T3 diet, or both.
[b]: 25 ug/kg, p.o.
[c]: Beginning on day 0, rats received powdered diet containing T3 (500 ug/kg of diet) ad libitum.
[d]: Significantly different from control (C group) mean value, $p<0.05$.
[e]: Significantly different from mean values of TCDD-treated rats fed control diet.
[f]: Significantly different from mean value of control rats fed T3 diet (T3 group).

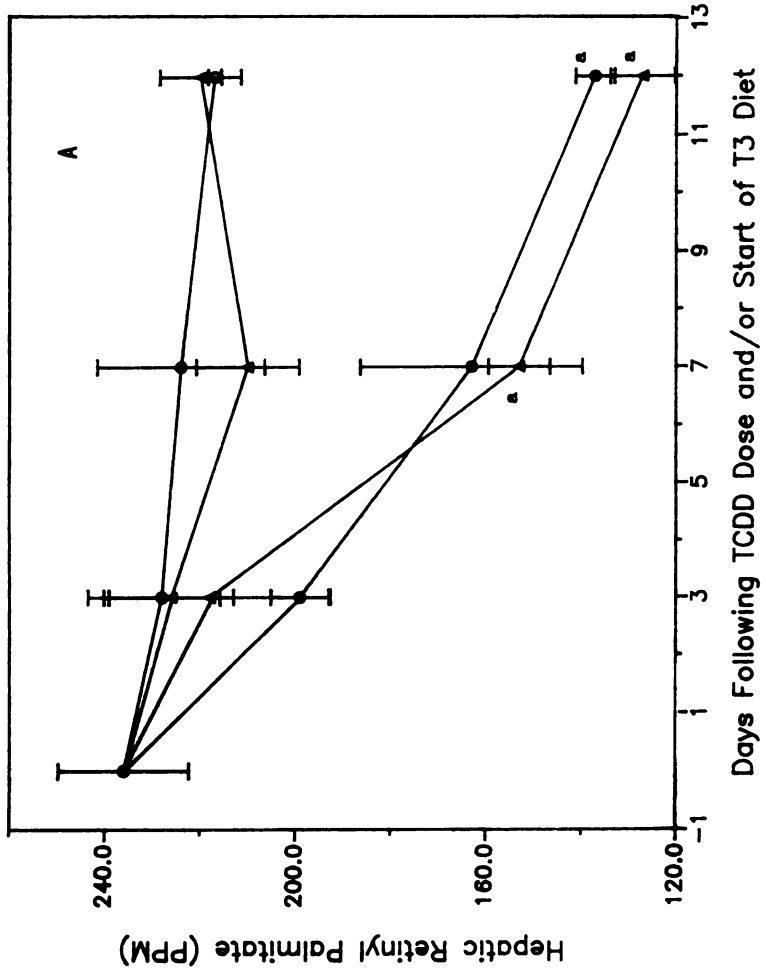
Hepatic Retinyl Palmitate

Treatment of rats with TCDD (CT group) caused a significant decrease in the concentration of hepatic retinyl palmitate to a level 63% that of the control group (C) at 12 days post-treatment (Figure 2A). Dietary T3 alone (T3 group) had no significant effect on hepatic retinyl palmitate concentration, and no additional effect over that seen with TCDD alone. However, as shown in Figure 2B, when total hepatic retinyl palmitate was considered, a different pattern emerged. Rats in both the control (C) and dietary T3 (T3) groups accumulated hepatic retinyl palmitate throughout the course of the study. The rate of accumulation for the T3 group was somewhat lower than for the control group; however, this difference was not statistically signifcant. In contrast, treatment with TCDD (CT group) caused a decline in total hepatic retinyl palmitate stores to 71% of the level in the control group (Figure 2B). Dietary T3 alone (T3 group) had no significant effect on total stores of hepatic retinyl palmitate; however, there was a small additional effect on the decline in total hepatic retinyl palmitate for the combination treatment group (T3T) to 63% of the C group level.

Renal Retinyl Palmitate

Control rats maintained renal retinyl palmitate levels in the range of 2.0-3.5 ppm throughout the study period (Figure 3). Rats treated with dietary T3 (T3 group), TCDD (CT group), or both (T3T group) showed a tendency to

Figure 2. Effect of TCDD and/or dietary T3 treatment in male Sprague-Dawley rats on the concentration (A), or the total amount of retinyl palmitate (B). Each point represents the mean ± SD of groups of 3 rats: control diet (—○—); control diet + TCDD (—●—); T3 diet (—△—); T3 diet + TCDD (—▲—). [a]: Significantly different from control rats fed standard diet, $p < 0.05$. [b]: Significantly different from TCDD-treated rats fed standard diet, $p < 0.05$.


A
Hepatic Retinyl Palmitate (PPM)
240.0
200.0
160.0
120.0
-1
1
3
5
7
9
11
13
Days Following TCDD Dose and/or Start of T3 Diet
a
a
a

Total Hepatic Retinyl Palmitate (mg)
10.0
9.0
8.0
7.0
6.0
5.0
B
a
a
a,b
−1
1
3
5
7
9
11
13
Days Following TCDD Dose and/or Start of T3 Diet

Figure 3. Effect of TCDD and/or dietary T3 on the renal retinyl palmitate concentration of male Sprague-Dawley rats. Each point represents the mean ± SD of groups of 3 rats: control diet (—○—); control diet + TCDD (—△—); T3 diet (—●—); T3 diet + TCDD (—▲—). [a]: Significantly different from control rats fed standard diet, $p < 0.05$. [b]: Significantly different from TCDD-treated rats fed standard diet, $p < 0.05$.

Kidney Retinyl Palmitate (PPM)
40.0
30.0
20.0
10.0
0.0
Control Diet
Control Diet + TCDD
T3 Diet
T3 Diet + TCDD
a, b
a
a
a
-1
1
3
5
7
9
11
13
Days following TCDD Dose and/or Start of T3 Diet

accumulate renal retinyl esters above the concentration seen in control rats. Kidneys from T3 group rats contained, at day 12, about 2.6x more retinyl esters than those from C group rats, however, this difference was not significant. At day 12, retinyl esters in the CT group had increased to a level 5.2x that of the C group. The greatest retinoid accumulation was observed in rats given a combination of dietary T3 and TCDD (T3T group), with renal retinyl palmitate levels reaching 10.2x those of control rats by day 12.

DISCUSSION

A prominent characteristic of the response of many species to TCDD is either weight loss or depressed rate of weight gain (Thunberg et al., 1983a; McConnell, 1980; Gasiewicz et al., 1980; Rozman et al., 1984; Schiller et al., 1985). We observed a depression in growth rate during the first 6 days after TCDD treatment, followed by increased growth rate during the last 6 days of the study, although the recovery was to rates still less than those of control animals. Dietary T3 alone caused only a brief period of reduced growth rate, following which the animals gained weight at a rate approximately equal to that of control rats. However, dietary T3 appeared to markedly exacerbate the TCDD-induced depression of growth rate of treated rats. These data support Rozman's (1984) suggestion that the initial phase of weight loss caused by TCDD may be at least partially thyroid hormone mediated.

Both treatment groups treated with TCDD (CT and T3T groups) had a similar degree of hepatic enlargement. We did not observe any effect of dietary T3 alone on liver weight/body weight ratio, nor did T3 exacerbate the hepatic enlargement seen in the TCDD-treated groups. This result is in accord with the suggestion that hepatic hypertrophy is primarily a consequence of endoplasmic reticulum (ER) proliferation and a direct response to TCDD, perhaps mediated by the TCDD (Ah) receptor and MC-type induction (Poland and Knutson, 1982; Dencker, 1985). Curiously, we

observed a T3-dependent enlargement of kidney, as measured by kidney weight/body weight ratio, that did not seem to be affected by TCDD.

We observed an increase in the concentration of plasma retinol following TCDD treatment similar to that reported by Thunberg (1979). The elevation of plasma retinol levels was exacerbated by treatment with dietary T3, although such treatment alone was able to cause a significant increase in plasma retinol levels. Similarly, we observed a significant increase in the renal retinol levels of TCDD-treated rats, which was also exacerbated by the inclusion of T3 in the diet. Further, dietary T3 treatment itself caused a significant elevation of renal retinol levels and the effects of the two treatments appeared to be additive. Neither TCDD, dietary T3 or the combination of the two treatments caused a significant change in the levels of hepatic retinol.

We believe that the levels of retinoids in the kidney provide a sensitive measure of the vitamin A status of the animal. Hence, the exacerbation of TCDD-caused renal retinoid accumulation caused by T3 supports the hypothesis that the depletion of vitamin A is a significant factor in the expression of TCDD-type toxicity. This argument is further supported by the fact that accumulation of retinoids in the kidney is symptomatic of a vitamin A deficiency as induced by dietary depletion. It is significant that TCDD-treated animals has not only overt

signs of vitamin A deficiency, but biochemical signs as well.

TCDD and related chemicals are able to cause a rapid depletion of hepatic retinoids (Thunberg, 1983; Brouwer and van den Berg, 1986) In this experiment, TCDD caused a depletion of hepatic retinyl palmitate that was apparent both in concentration and total amounts. Dietary T3 had no additional effect on the decrease in hepatic retinyl palmitate concentration produced by TCDD, but did cause a significant exacerbation of the decline in total hepatic retinyl palmitate. These data further support the hypothesis mentioned above, suggesting the possibility that the worsening of TCDD toxicity by T3 may be a consequence of an increase in the rate of loss of vitamin A.

BIBLOGRAPHY

Bank, P.A., Cullum, M.E., Jensen, R.K., and Zile, M.H. (1987). Chronic exposure to 3,4,5,3',4',5'-hexachlorobiphenyl alters the dynamics of vitamin A metabolism. Toxicologist 7:272 Abstract #1089.

Bastomsky, C.H. (1977). Enhanced thyroxine metabolism and high uptake goiters in rats after a single dose of 2,3,7,8-tetrachlorodibenzo-p-dioxin. Endocrinology 101:292-296.

Bhat, M.K., and Cama, H.R. (1977). Thyroidal control of hepatic release and metabolism of vitamin A. Biochim. Biophys. Acta 541:211-222.

Brouwer, A., and van den Berg, K.J. (1983). Early decrease in retinoid levels in mice after exposure to low doses of polychlorinated biphenyls. Chemosphere 12:555-558.

Brouwer, A. and van den Berg, K.J. (1984). Early and differential in natural retinoid levels in C57BL/Rij and DBA/2 mice by 3,4,3',4'-tetrachlorobiphenyl. Toxicol. Appl. Pharmacol. 73:204-209.

Brouwer, A. and van den Berg, K.J. (1986). Binding of a metabolite of 3,4,3',4'-tetrachlorobiphenyl to transthyretin reduces serum vitamin A transport by inhibiting the formation of the protein complex carrying both retinol and thyroxine. Toxicol. Appl. Pharmacol. 85:301-312.

Dencker, L. (1985). The role of receptors in 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) toxicity. Arch. Toxicol., Suppl. 8:43-60.

Gasiewicz, T.A., Holscher, M.A., and Neal, R.A. (1980). The effect of total parenteral nutrition on the toxicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin in the Rat. Toxicol. Appl. Pharmacol. 54:469-488.

Goodman, D.S. (1984). Plasma retinol-binding protein In: The Retinoids. (Sporn, M.B., Roberts, A.B. and Goodman, D.S. eds.) Academic Press, Inc. Orlando. Vol II;pp 41-88.

Goodman, D.S., and Blaner, W.S. (1984). Biosynthesis, absorption and hepatic metabolism of retinol. In: The Retinoids. (Sporn, M.B., Roberts, A.B. and Goodman, D.S. eds.) Academic Press, Inc. Orlando. Vol II;pp 1-39.

Gupta, B.N., Vos, J.G., Moore, J.A., Zinkl, J.G., and Bullock, B.C., (1973). Pathologic effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin in laboratory animals. Environ. Health Persp. 5:125-140.

Innami, S., Nakamura, A., Kato, K., and Miyazaki, M. (1975). Polychlorinated biphenyl toxicity and nutrition. IV. PCB's toxicity and vitamin A. Fukuoka Acta Med. 66:579-584.

Innami, S., Nakamura, A., Miyazaki, M., Nagayama, S., and Nishide, E. (1976). Further studies on the reduction of vitamin A content in the livers of rats given polychlorinated biphenyls. J. Nutr. Sci. Vitaminol. 22:409-418.

Kimbrough, R.D. (1974). The toxicity of polychlorinated polycyclic compounds and related compounmds. Crit. Rev. Toxicol. 2:445-489.

Moore, T., and Sharman, I.M. (1950). Vitamin A in the kidneys of male and female rats. Biochem J. 47:xliii-xliv.

Morita, A., and Nakano, K. (1982). Change in vitamin A content in tissues of rats fed on a vitamin a-free diet. J. Nutr. Sci. Vitaminol. 28:343-350.

McConnell, E.E., (1980). Toxicity, carcinogenesis, reproduction, teratogenesis and mutagenesis. In: Halogenated biphenyls, terphenyls, naphthalenes, dibenzodioxins and related products. (Kimbrough, R.D. ed.) Elsevier/North-Holland Biomedical Press, Amsterdam; pp: 109-150.

Peterson, R.E., Seefeld, M.D., Christian,. B.J., Potter, C.L., Kelling, C.K., and Keesey, R.E. (1984). The wasting syndrome in 2,3,7,8-tetrachlorodibenzo-p-dioxin toxicity; Basic features and their interpretation. In: Biological mechanisms of dioxin action. Banbury Report #18; Cold Spring Harbor Laboratory pp: 291-308.

Poland, A., and Knutson, J.C. (1982). 2,3,7,8-Tetrachlorodibenzo-p-dioxin and related halogenated aromatic hydrocarbons: Examination of the mechanism of toxicity. Ann. Rev. Pharmacol. Toxicol. 22:517-554.

Potter, C.L., Moore, R.W., Inhorn, S.I., Hagan, T.C., and Peterson, R.E. (1986). Thyroid status and thermogenesis in rats treated with 2,3,7,8-tetrachlorodibenzo-p-dioxin. Toxicol. Appl. Pharmacol. 84:45-55.

Rozman, K., Rozman, T., and Greim, H. (1984). Effect of thyroidectomy and thyroxine on 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) induced toxicity. Toxicol. Appl. Pharmacol. 72:372-376.

Spear, P.A., Moon, T.W., and Peakall, D.B. (1986). Liver retinoid concentrations in natural populations of herring gulls (Larus argentatus) contaminated by 2,3,7,8-tetrachlorodibenzo-p-dioxin and in ring doves (Streptopelia risorea) injected with a dioxin analogue. Can. J. Zool. 64:204-208.

Thunberg, T., Ahlborg, U.G., and Johnsson, H. (1979). Vitamin A status in the rat after a single oral dose of 2,3,7,8-tetrachlorodibenzo-p-dioxin. Arch. Toxicol. 42:265-274.

Thunberg, T. (1983a). Studies on the effect of 2,3,7,8-tetrachlorodibenzo-p-dioxin on vitamin A: A new aspect concerning the mechanism of toxicity. PhD Dissertation, Carolinska Institute, Stockholm.

Thunberg, T. (1983b). The effect of TCDD on vitamin A in normal and vitamin A-deficient rats. Chemosphere 12:577-580.

Thunberg, T., Ahlborg, U.G., Hakansson, H., Krantz, C., and Monier, M. (1980). Effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin on the hepatic storage of retinol in rats with different dietary supplies of vitamin A (retinol). Arch. Toxicol. 45:273-285.

Underwood, B.A. (1984). Vitamin A in animal and human nutrition. In: The Retinoids (Sporn, M.B., Roberts, A.B. and Goodman, D.S., eds.) Academic Press, Inc. Orlando. pp 282-392.

Wolf, G. (1980). Vitamin A In: Human Nutrition (R.B. Alfin-Slater and D. Kritchevsky, eds.), Plenum, New York pp: 97-203.

Wolf, G.(1984). Multiple functions of vitamin A. Physiological Reviews 64(3):873-937.

Zile, M.H., and Cullum, M.E. (1983). The function of vitamin A: Current Concepts. Proc. Soc. Exp. Biol. & Med. 172:139-155.

SUMMARY

The ability of TCDD and similarly acting compounds to cause a depletion of hepatic vitamin A reserves in treated animals, as well as certain signs consistent with those of a vitamin A deficiency state has been thoroughly described in the literature. However, relatively little research has been presented on the role this depletion may play in the development of "TCDD-type" toxicity and further, on the mechanism by which the depletion of retinoids might be caused by TCDD treatment.

It has been suggested in the literature that TCDD and similarly acting compounds affect the mobilization and transport of vitamin A. The research presented in this dissertation shows that at least one consequence of the inhibition of the hepatic enzyme responsible for the hydrolysis of retinyl esters, retinyl palmitate hydrolase (RPH), is the lowering of plasma retinol levels. This point is demonstrated as a consequence of treatment of rats with either nonadecafluorodecanoic acid (NDFDA) or 3,4,3',4'-tetrachlorobiphenyl (TCB). Both compounds are able to inhibit RPH both *in vivo* and *in vitro*. It is also shown, that while TCDD is also able to inhibit RPH *in vitro*, the K_I of this interaction is sufficiently high that significant inhibition of RPH activity would not be an expected consequence of TCDD treatment, even at an LD_{50} dose. That TCDD does not cause inhibition of RPH *in vivo* is further demonstrated by the fact that TCDD treatment causes an

increase in the levels of plasma retinol of treated rats. Other results suggest that TCDD treatment does not markedly affect the stability of the retinol-RBP-TTR ternary complex as measured by retinoid fluorescence associated with the complex. Therefore, we have concluded that TCDD does not directly affect either the mobilization or transport of vitatmin A.

The accumulation of retinoids in the kidneys has been shown to occur during the development of a vitamin A deficiency, although neither the species accumulated, nor the mechanism by which this accumulation occurs has been characterized. We have presented evidence suggesting that the retinoid accumulation in the case of dietary depriovation of vitamin A is a consequence of elevated rates of esterification of retinol by acyl CoA:retinol acyl transferase (ACARAT). Further, we have demonstrated that a dose of TCDD causes a similar accumulation of retinoids in the kidney, and increase in ACARAT activity. We have concluded that the accumulation of retinyl esters in the case of TCDD toxicity is actually a response to the depletion of retinoid reserves, analogous to the early stages of a vitamin A deficiency.

The mechanism by which TCDD toxicity results in the depletion of stored retinyl esters in the liver was investigated. Some pathways of the normal degradation of retinoid materials have been identified in the literature. We have shown that the rate of microsomal retinol oxidation is increased as a consequence of TCDD toxicity. It has been

suggested that the hydroxylated, or other oxidized retinoids are subject to more rapid oxidative degradation than the non-hydroxylated analog. We determined that the rate of oxidation of retinol to retinoic acid, via retinal, was not affected as a consequence of TCDD treatment. However, the rate of formation of retinoyl-B-glucuonide was markedly increased, and might be expected to enhance the rate of loss of retinoids from treated rats.

Lastly, an attempt was made to determine the extent to which depletion of retinoids caused as a consequence of TCDD treatment contributed to the development of the toxic response. It was shown that worsening of TCDD toxicity by inclusion of T3 in the diet caused a concomitant worsening of the depletion of vitamin A reserves.

In summary, we conclude that as a function of induction of enzymes responsible for the degradation of retinoids, TCDD-treated animals lose vitamin A at an inappropriate rate. As a consequence of this retinoid loss, the animal responds in a mannar similar to that seen shortly following removal of vitamin A from the diet, specifically, elevated plasma retinol levels, and accumulation and storage of retinyl esters in the kidney. This depletion of vitamin A may be the basis for the development of symptoms similar to those of a vitamin A deficiency state.