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Biochemical Mechanisms of Toxicity
of 2,3,7,8-Tetrachlorodibenzo-p-dioxin
in the Rat, Guinea Pig, Hamster, Rabbit, and Mouse

presented by

David William Brewster

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BIOCHEMICAL MECHANISMS OF TOXICITY OF 2,3,7,8-TETRACHLORODIBENZO-P-DIOXIN (TCDD) IN THE RAT, GUINEA PIG, HAMSTER, RABBIT, AND MOUSE

By

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ABSTRACT

BIOCHEMICAL MECHANISMS OF TOXICITY OF 2,3,7,8-TETRACHLORODIBENZO-P-DIOXIN IN THE RAT, GUINEA PIG, HAMSTER, RABBIT, AND MOUSE

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TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin) caused a gross alteration in isolated rat hepatic plasma membrane protein constituency. Changes occurred as soon as two days after administration and showed a time and dose dependent response over a 20 day observation period. Na-K ATPase, Mg ATPase, Ca ATPase, and gamma-glutamyltranspeptidase activities along with insulin, epidermal growth factor, and Concanavalin A binding were all reduced by single i.p. administration of this dioxin. Protein kinase activity was significantly increased relative to control levels. These changes were not seen in the hepatic plasma membrane of guinea pigs or hamsters, species in which TCDD produced little liver pathology. These results indicate impaired ionic transport, depressed binding of critical growth factors, and increased cellular phosphorylation in rat liver. If membrane alterations occur at vital physiological sites in other organs and tissues, specific biochemical manifestations of TCDD's toxicity may be explained.

David William Brewster

Hypertriglyceridemia in guinea pigs was found to be caused by a dose and time dependent reduction of adipose lipoprotein lipase (LPL) activity. Rabbits and hamsters showed similar effects to guinea pigs. LPL was not affected in rats, nor did hypertriglyceridemia develop. Increased serum triglycerides segregated with the Ah locus in several responsive and non-responsive mouse strains. Mink had lowered concentrations of serum triglycerides but no change in LPL. The cause for the decreased LPL activity seemed to be the result of TCDD acting at 2 different organs: the pancreas had an abnormal response to glucose administration in its insulin synthetic ability, and the adipocyte was unable to produce active LPL. Altered epidermal growth factor receptor synthesis and enhanced tyrosine kinase activity is postulated to be the underlying biochemical mechanism for TCDD's toxicity.

For Mom and Dad with love . . .

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Finally to my loving wife Janet, for your unending support, understanding, love, and countless trips to the PRC, I wish to express my deepest gratitude. Thank you Princess, and thank you for giving me a happy, healthy, and beautiful little boy. To you Jeffrey David, thank you for your love and trust and the joy you've brought into our lives.

"Whatever is true, whatever is noble, whatever is right, whatever is pure, whatever is lovely, whatever is admirable -- if anything is excellent or praiseworthy -- think about such things."

Philippians 4:8

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CHAPTER I

INTRODUCTION

An unwanted by-product in the synthesis of the herbicides 2,4- dichlorophenoxy acetic acid (2,4-D) and 2,4,5-trichloro-phenoxy acetic acid (2,4,5-T) is 2,3,7,8 tetrachlorodibenzo-p-dioxin (Figure 1). Many scientists are fascinated with TCDD because of its high toxicity to certain animal species and its wide species variation of toxicity manifested by acute oral LD_{50} determinations ranging from 0.6 ug/kg to > 3000 ug/kg (Table 1). Hepatocellular damage has been described in rats, mice, and rabbits; an edematous syndrome observed in chickens and certain mice strains; chloracne and hyperkeratosis noted in humans, rabbits, monkeys, and nude mice; hepatic porphyria witnessed in humans; and bone marrow depression seen in monkeys (Poland and Glover 1980). Recently, evidence of TCDD has been found in industrial high combustion effluent smoke stacks, and it is thought to be environmentally ubiquitous (Bumb et al. 1980).

Brief History of TCDD

Two major events are responsible for the vast amount of research concerning this compound. First, a mixture of n-butyl esters of 2,4-D and 2,4,5-T, coded as Agent Orange, and used extensively for defoliation in Vietnam between 1962

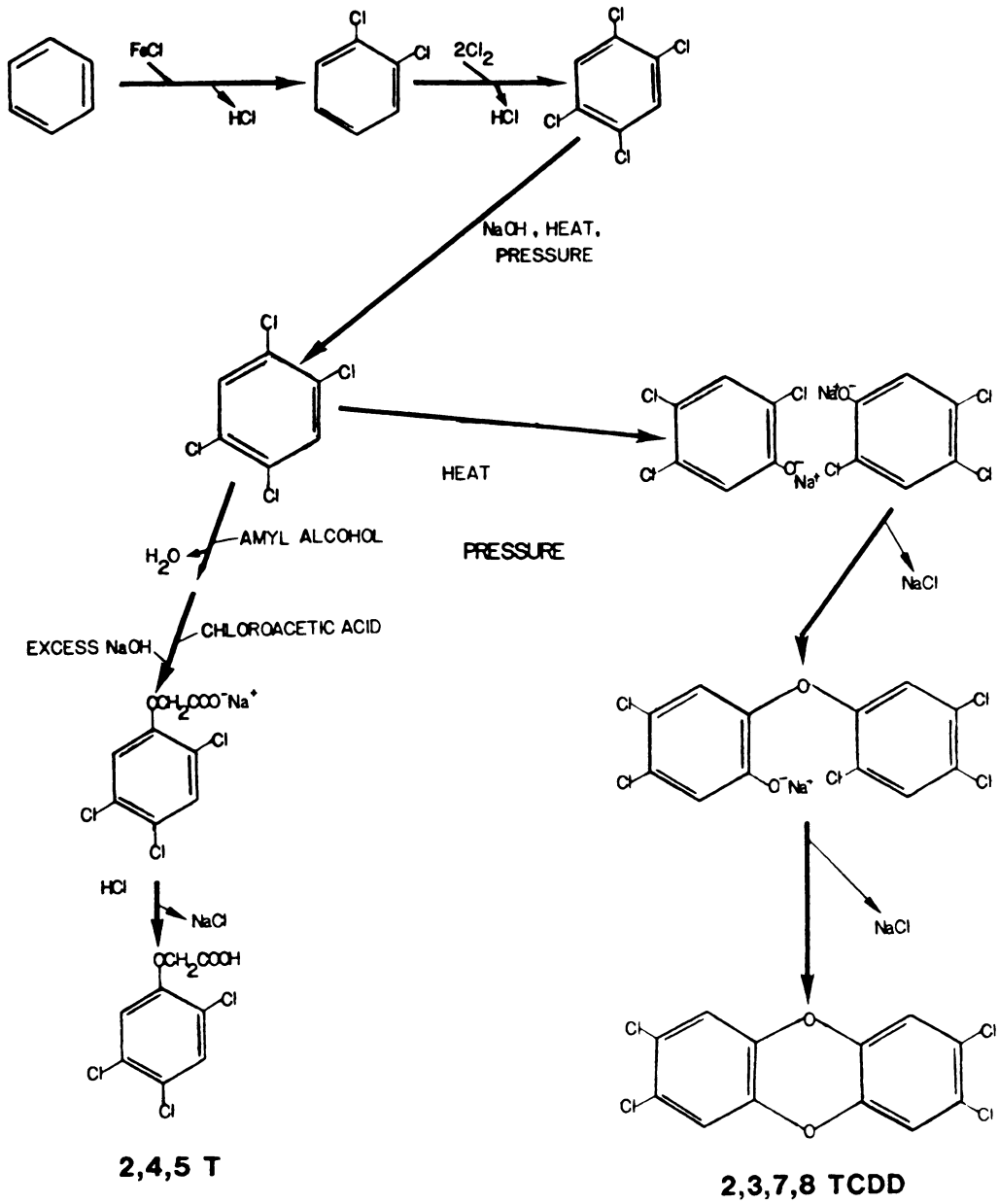


Figure 1. One possible mechanism for the spontaneous formation of 2,3,7,8 TCDD during the production of the herbicide 2,4,5 T (adapted from Esposito et al. 1980).

Table 1. 2,3,7,8 TCDD LD_{50} values for several animal species.

Species	Sex	Route of Exposure	LD_{50} (ug/Kg)	Reference
Guinea Pig	M	Oral	0.6	Schwetz et al. 1973
Guinea Pig	F	Oral	2.1	Schwetz et al. 1973
Rat	M	Oral	22	Schwetz et al. 1973
Rat	F	Oral	45	Schwetz et al. 1973
Chicken	Not Given	Oral	25-50	Greig et al. 1973
Monkey	F	Oral	70	McConnel et al. 1978a
Rabbit	Mixed	Oral	115	Schwetz et al. 1973
Mouse	M	Oral	126	Vos et al. 1974
Bullfrog	Mixed	Intraperitoneal	1000	Beatty et al. 1976
Golden Syrian Hamster	Mixed	Oral	1157	Olson et al. 1980
Golden Syrian Hamster	Mixed	Intraperitoneal	>3000	Olson et al. 1980
Human	M, F	Any	?	

and 1969 was found to contain TCDD in concentrations from an average of 0.05 ppm to a maximum of 47 ppm (Hay 1978). The toxic manifestations of this mixture on American soldiers and Vietnamese civilians are just now beginning to be examined. The second major environmental contamination episode occurred in June 1976, in Sevesso, Italy where an industrial explosion caused the release of several hundred grams of TCDD into the atmosphere. Within weeks of the explosion thousands of animals died and soil samples revealed up to 5,477 ug TCDD/m^2 (Fanelli et al. 1980).

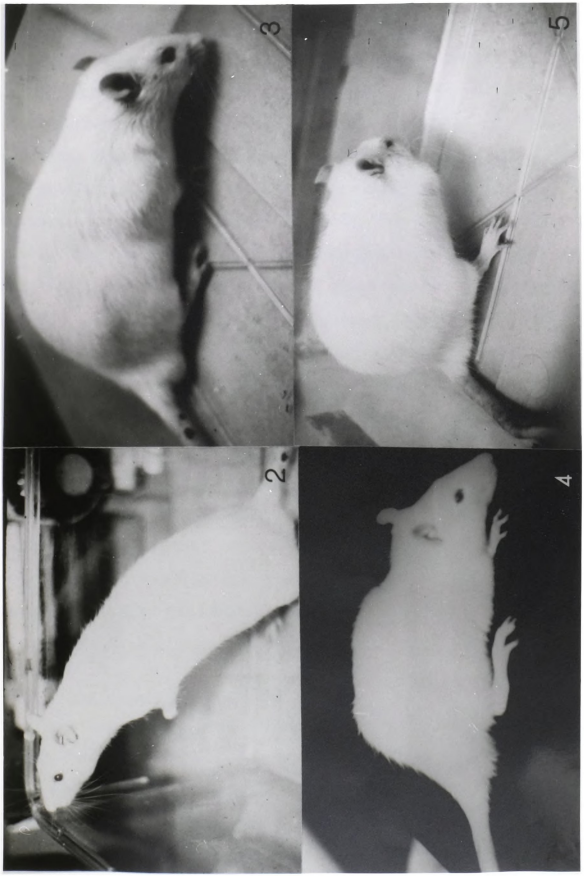
Animals treated with TCDD typically exhibit depressed behavior such as cage huddling, failure of grooming, and a general disinterest in their surroundings within 2-3 weeks post dosing (personal observation). This behavioral modification may be accompanied by a ruffled hair appearance, piloerection, and occasional subcutaneous hemorrhages seen in the tail, paws, and under the nails (Figures 2-5). Ventral hair loss and an icteric appearance of the ears, paws, tail, subcutaneous tissue, and visceral organs may follow (Gupta et al. 1973). This jaundiced appearance may result from increased levels of porphyrins since TCDD can be a powerful inducer of delta-aminolevulinic acid synthetase in some species (Poland and Glover 1973a), or from depressed uroporphyrinogen decarboxylase activity (Poland and Knutson 1982).

Figure 2. Normal appearance and curiosity of a control rat 10 days after the administration of acetone:corn oil.

Figure 3. Normal posture of a control animal with the paws under the body.

Figure 4. Typical appearance of a TCDD treated rat (10 days after administration) displaying piloerection, hair loss, and lowered carriage. Note the positioning of the fore and hind limbs.

Figure 5. Cage huddling and hunched posture of a TCDD treated animal 10 days after administration of 25 ug/kg TCDD.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Environmental Significance

The importance of TCDD as an environmental contaminant is unquestionable (see Poland and Kende 1976, Bumb et al. 1980, Holmstedt 1980, Kociba and Schwetz 1981). It is an extremely toxic compound with a wide diversity of toxic manifestations, a wide species susceptibility, and a biologically long halflife. Furthermore, Pitot et al. (1980) have shown it to be a tumor promoter. Most studies indicate the biodegradation of TCDD to be a very slow process, appearing to be mediated through microbial organisms in the soil (Esposito et al. 1980). However, Ward and Matsumura (1978) concluded the limited degradation of TCDD in aquatic systems to be favored by the presence of sediment, organic matter, and or microbial activity in the aqueous phase since the half life was longer in water without sediments than with sediments. Although biological degradation is slow, photo degradation of this chemical occurs quite readily in the presence of artificial or natural sunlight whether the compound is in solution or soil bound (Esposito et al. 1980).

Symptoms of Toxicity

The most consistent symptom of TCDD intoxication in laboratory animals is body weight loss and the consequent "wasting" syndrome (Greig et al. 1973, Harris et al. 1973, Kociba et al. 1976, Vos et al. 1973, Faith and Moore 1977, Kociba et al. 1979, Neal et al. 1979). Oral doses of 50

ug/kg to rats, resulted in an average weight loss of 38% from the time of dosing (Allen et al. 1975). They concluded that this loss was not from an anorectic effect since the dead animals had significant amounts of food in the stomach and gut. Furthermore, Gasiewicz et al. (1980) noticed this same body weight loss in their treated animals but not in the pair-fed controls. The question arises as to whether TCDD causes a decreased absorption of nutrients from the intestine or a decreased biochemical utilization of nutrients at the cellular level, since animals lose weight although food consumption seems normal and the gut apparently full. After the infusion of a "total parental nutrition" diet, containing vital minerals, vitamins, and amino acids, the body weight changes of rats administered TCDD were not found to be significantly different from controls (Gasiewicz et al. 1980). Additional evidence for malabsorption caused by TCDD stems from the work of Ball and Chhabra (1981). Doses of TCDD as low as 5 ug/kg inhibited intestinal glucose and leucine absorption.

The greatest and most consistent pathological change caused by dioxin in many species, is thymic atrophy or involution as revealed by gross necropsy (Gupta et al. 1973, Harris et al. 1973, Vos et al. 1973, Kociba et al. 1976, Neal et al. 1979, Van Logten et al. 1980, Poland and Knutson 1982). Although severely affected by TCDD, few observable pathological lesions are noticed. Involution

appears to result from increased thymocytic migration to the medulla from the cortex and consequent secretion of cortical thymocytes into the cardiovascular system (Kociba et al. 1976, Albro et al. 1978). The pyknosis noted by Buu-Hui et al. (1972) is not routinely seen except at very high doses. The pituitary gland does not cause involution since blood vessel dilatation and hemorrhages are observed in both hypophysectomized and normal rats administered 20 ug/kg TCDD (Van Logten et al. 1980). Increased serum glucocorticoid levels can result in thymic atrophy, however, adrenal hyperfunction is not responsible since adrenalectomized animals still underwent thymic involution when treated with TCDD (Neal et al. 1979). This dioxin also was not found to bind to glucocorticoid receptors which would result in a stimulation of plasma glucocorticoids. Neal et al. (1979) theorized that thymic atrophy could occur from decreased catabolism of steroid hormones, loss of feedback control of ACTH production, or hypersensitization of tissues (other than adrenals) to stimulate glucocorticoid production.

The tremendous differential species response to TCDD administration is demonstrated with liver pathology. Little hepatotoxicity is noticed in guinea pigs which are the most sensitive laboratory animal to TCDD, but in rats the liver is the organ most severely affected and exhibits the greatest number of histopathological lesions (Table 2). Briefly, the most common effects observed include

Table 2. Major pathological lesions of the rat hepatic system.

Lesion	TCDD Dose (ug/kg)	Administration Route[a]	Sex[b]	Strain[c]	Reference
Centrilobular stasis; anisokaryosis; binucleation; chromophobic vacuoles and hyaline inclusion; focal hyperplasia of Kupffer cells. Effects more severe in females.	10,000 (single)	IP	M,F	W	Buu-Hoi et al. 1972
Time course sequence--SER increase adjacent to bile duct followed by SER-RER aggregates followed by increased RER followed by decreased SER then return to normalcy.	5/25 (single)	O	M	CD	Fowler et al. 1973
Increased water content; centrilobular necrosis; dilatation of surrounding sinusoids and monocytes; Thickening of central vein at day 60 post dose; multinucleate parenchymal cells (4-30 nuclei) with no mitotic figures; pyknosis.	200 (single)	O	M,F	P	Greig et al. 1973

Table 2 (cont'd.).

Lesion	TCDD Dose (ug/kg)	Administration Route[a]	Sex[b]	Strain[c]	Reference
Necrosis; enlarged hepatocytes; disorganized cords; megalocytosis; multinucleate cells; increased mitosis of parenchymal cells; swelling; granular cytoplasm and aggregation; fatty infiltration; vacuolization.	100/50	O	M,F	CD	Gupta et al. 1973
Loss of ATPase activity along the canalicular border increasing to central vein and midzonal region; multinucleate cells.	200 (single)	O	M	P	Jones 1974
Increased hepatocyte number; increased lipid droplet; increase in SER forming concentric membrane arrays encircling mitochondria, lipid droplets and other organelles.	50 (single)	O	M	SD	Allen et al. 1975
Distorted lobules; multinucleate cells; necrosis; reticuloendothelial aggregation; increased lipid content; infiltration of some inflammatory cells in portal area.	1 (daily)	O	M,F	SD	Kociba et al. 1976

Table 2 (cont'd.).

Lesion	TCDD Dose (ug/kg)	Administration Route[a]	Sex[b]	Strain[c]	Reference
Hepatocyte swelling; necrosis; inflammatory cell infiltration in periportal and centrilobular zones; increased parenchymal reticuloendothelial cells.	10/25 (daily)	O	M	H	Croft et al. 1977
Increased microglobular lipid deposits surrounded by layers of ER; clear vacuoles surrounded by single membrane swirls similar to myeloid bodies; increased lipid, cholesterol esters, and free fatty acids.	10/50/100 (single)	O	F	F	Albro et al. 1978
Centrilobular multinucleate hepatocytes; increased lysosomes and SER; loss of centrilobular ATPase activity expanding with time to periportal area; changes in lipid content.	200 (single)	O	F	P	Greig and Osborne 1978
Hepatic degeneration; inflammatory necrotic, and proliferative changes; cytomegaly; distortion of lobular pattern and atropy of cords; cytoplasmic	0.1 (daily)	Diet	M,F	SD	Kociba et al. 1979

Table 2 (cont'd.).

Lesion	TCDD Dose (ug/kg)	Administration Route[a]	Sex[b]	Strain[c]	Reference
vacuolation; fatty change; altered tinctorial properties; hepatic necrosis and inflammation; multinucleated hepatocytes; foci or areas of hepatocellular alteration; pigment aggregates; periportal inflammation; fibrosis; hepatocellular carcinoma and hepatocellular hyperplastic nodules in the female.					
Decreased plasma membrane Na/K and Mg ATPase; decreased ouabain biliary excretion and bile flow.	10/25	O	M	H	Peterson et al. 1979
Swollen enlarged hepatocytes; vacuolization; necrosis in midzonal region; inflammation; discontinuous RER in circular structures with normal mitochondria; condensed cytoplasm; decreased glycogen stores; cystic spaces with cellular debris; hepatomegaly; cytomegaly; bile duct proliferation; binucleated cells; altered mitochondria with autophagic	100 (single)	IP	-	-	Gasiewicz et al. 1980

Table 2 (cont'd.).

Lesion	TCDD Dose (ug/kg)	Administration Route[a]	Sex[b]	Strain[c]	Reference
vacuoles; lipid droplet infiltration; most damage in centrilobular and midzonal region.					
Random single cell necrosis, swelling, and degeneration of focal areas of centrilobular zone.	20 (single)	O	F	F	Van Logten et al. 1980

[a]Administration route--O-oral, IP-intraperitoneal.
[b]Sex--M-male, F-female.
[c]Strain--W-Wistar, CD-CD, P-Porton, SD-Sprague-Dawley, H-Holtzman, F-Fischer.

hyperplasia, hypertrophy from increased smooth endoplasmic reticulum production, parenchymal necrosis, inflammatory cell infiltration, lipid accumulation, multinucleate cells, and single cell necrosis. These changes are seen most often in the centrilobular and periportal zones of the liver, and coincide with loss of some hepatic biochemical functions such as ATPase activity noted by Jones (1974) and Peterson et al. (1979b).

A survey of the literature shows the effect of TCDD on other organs of the rat to range from hemorrhages, hyperplasia, necrosis, and degeneration to significant reductions in the incidences of spontaneous lesions normally observed in chronic long term studies. Different animal species show different susceptibilities to TCDD and emphasize different manifestations of its toxic actions. The variety of lesions caused by this chemical demonstrate the difficulty in proposing a unifying theory for its biochemical mechanism of toxicity.

The inductive effects of polychlorinated dioxins on microsomal systems had been suspected as early as 1967 (see Firestone 1973), but microsomal induction as a result of TCDD administration was first shown by Greig in 1972. Subsequently, Lucier et al. (1973) demonstrated the pattern of induction by this compound to be somewhat different from that observed by the classical inducer phenobarbital. Induction by TCDD was instead similar to that seen with

3-methyl cholanthrene (3-mc) which earlier had been shown to correlate with increased aryl hydrocarbon hydroxylase (AHH) activity. AHH activity is inherited in an autosomal dominant fashion controlled by 1 or more loci designated as the Ah locus (Nebert et al. 1972). Poland and Glover (1973b) quickly realized that TCDD also induces AHH activity. Furthermore, they demonstrated that both compounds are recognized by a common cytosolic receptor in the liver which somehow stimulates the induction of AHH and other detoxifying enzymes (Poland and Glover 1976). Characterized as a heat labile protein of 2×10^5 daltons this receptor is specific for P-448 inducers since phenobarbital, pregnenolone-16 alpha carbonitrile, and steroid hormones do not compete against it. In addition, the receptor affinity for TCDD congeners is positively correlated with their induction capability as well as their biological potency. That is, those congeners having the greatest affinity for the receptor show the greatest amount of microsomal induction and the greatest amount of biological toxic manifestations. Carlstead-Duke (1979) noted the highest concentration of these cytosolic receptors to be in the thymus gland -- the organ typically exhibiting the greatest deleterious effect from TCDD. Upon binding of the polychlorinated aromatic compound to the cytosolic receptor, the receptor-substrate complex is translocated to the nucleus where a presumable interaction with DNA is

thought to occur (Greenlee and Poland 1979). The nuclear binding capability of this complex has been confirmed by Mason and Okey (1982), and the nuclear receptor substrate complex has been isolated by Poellinger et al. (1982).

Instead of examining the effect of TCDD on microsomal and other detoxification systems preliminary research will investigate alterations occurring on the plasma membrane. It is at this site where many physiological functions are performed which may be modified in such a way as to implicate toxic manifestations.

Previous reports have indicated that TCDD does influence membrane constituency. After a single oral dose of 200 ug/kg TCDD, electron microscopy revealed the formation of giant multinucleate cells which probably occurred as a result of parenchymal cell fusion (Jones and Butler 1973). Greig and Osborne (1978) further observed a loss of membrane continuity between parenchymal cells, wide intercellular spaces, and loss of ATPase activity in the centrilobular portion of the liver.

A single oral dose of 10 or 25 ug/kg TCDD inhibited biliary excretion of ouabain as soon as 2 days post treatment (Yang et al. 1977). The transport of neutral substrates across the plasma membrane is proposed to be regulated by various membrane bound ATPase's, and Peterson et al. (1979b) have demonstrated lower plasma membrane Na-K and Mg ATPase activity within 2 days of TCDD treatment.

Pair-fed control rats were not affected. TCDD is not thought to directly bind to the plasma membrane and influence enzyme activity, since only 0.01357 pmole TCDD/ug protein (equivalent to 10^{-10} to 10^{-9} M dioxin) -- accumulated in the membrane after a dose of 25 ug/kg. Plasma membrane incubated in vitro with up to 10^{-5} M dioxin showed no inhibition of activity.

Ivanovic and Weinstein (1982) noted the suppression of EGF (epidermal growth factor) binding to the cellular surface of cultured fibroblast cells after treatment with different 3-MC type microsomal inducers. They did not assay TCDD but noted the potency of the inducers paralleled their ability to alter the cellular surface and decrease binding. This potency is thought to correlate with their ability to bind with the cytosolic receptor, therefore they concluded this decreased binding phenomenon to be a result of the pleiotypic changes caused by these compounds. Pitot et al. (1980) used canalicular ATPase as a parameter to detect TCDD-promoted development of foci in the rat liver and found them to be almost devoid of this membrane bound enzyme.

Cell surface changes induced by TCDD may resemble some of those seen with precancerous cells as described by Hynes (1979). Transformed cells typically display reduced numbers of surface glycoproteins and a reduction in gap junctions. Since TCDD has been implicated as a tumor promoter by the work of Pitot et al. (1980) this dioxin may result in

decreased intercellular recognition and attachment or inhibited cell-cell communication; both are mediated by the cellular surface.

Objectives

Previous reports implicate cell membrane alterations resulting from the administration of TCDD. The objective of this research will be to elucidate some of those *in vivo* biochemical transformations. Rat liver will be used in the preliminary studies for several reasons: It is a large, easily accessible organ which displays a number of prominent pathological lesions; the size of this organ enables a high yield of relatively pure plasma membrane from a single animal; finally, many biochemical and metabolic pathways have been clearly defined in the liver. The guinea pig is the most sensitive species to this dioxin and demonstrates little liver pathology, therefore to ascertain whether these membrane alterations are related to a specific toxic manifestation associated with TCDD, other organ systems will be investigated in this species.

TCDD represents a class of chemicals (including PCB's, PBB's, and other dioxins present in the environment as pollutants) the toxicological significance of which is unclear. Previous investigations of TCDD's action have provided much information in the area of microsomal induction but no satisfactory explanation has been given for its toxic actions. The hypothesis to be tested by this

research is that manifestations of TCDD's toxicity occur as a result of organ membrane alterations. These pertubations which alter the binding capability and the enzymatic capacity of vital physiological substrates, are responsible for the toxicological implications generally associated with this dioxin. The rationale of this research is to examine toxicity by investigating TCDD's effects upon the plasma membrane. Since many vital physiological functions are dependent upon the cellular membrane, our results could yield valuable information as to the mechanism of toxicity and insight for TCDD's biochemical actions.

Significance

The importance of TCDD in environmental toxicology is unquestionable. It has a very long biological half life, is extremely toxic, seems to be ubiquitous, and represents compounds such as PCB's, PBB's, other halogenated dioxins, dibenzofurans, and polynucleated aromatics which are also present as pollutants.

This research will examine TCDD's toxicity by investigating its effects on the plasma membrane. Possibly one or more of these membrane alterations result in one of TCDD's toxic manifestations such as thymic atrophy, body weight loss, decreased immunological competence, tumorigenesis, or alterations in lipid metabolism. The significance of this project would be the elucidation of

biochemical causes of TCDD toxicity. With this research one could gain a better understanding of the mechanisms by which other hazardous environmental pollutants may cause deleterious effects. This knowledge would enable the design of effective treatments for or antidotes against these materials.

CHAPTER II

INFLUENCE OF 2,3,7,8 TETRACHLORODIBENZO-P-DIOXIN ON PROTEIN COMPOSITION OF RAT HEPATIC PLASMA MEMBRANE AND ON OVERALL BODY WEIGHT

INTRODUCTION

TCDD has been considered to be a very serious environmental pollutant (Moore 1973). The guinea pig LD_{50} is estimated to be 0.6 ug/Kg making it the most toxic small size man-made chemical known to exist (Schwetz et al. 1973), and it has been shown to be teratogenic, acengenic, and carcinogenic (Kociba et al. 1978). Toxicity is unusual because a single administration to laboratory animals produces few obvious external symptoms except body weight loss, and death usually occurs 15-30 days post-treatment depending on species.

TCDD is a potent enzyme inducer in the rat liver resulting in massive increases of microsomal protein (Poland and Glover 1973a, 1973b). Poland and Glover (1976) found a cytosolic receptor which specifically binds with TCDD and apparently leads to various biochemical changes which occur as a result of the dioxin administration. However, the induction of hepatic microsomal proteins does not adequately explain why TCDD is toxic. The animal most sensitive to TCDD (guinea pig) shows little hepatic induction while more resistant species, such as the hamster, are highly induced

(Neal 1981). There are many microsomal inducers which do not show high toxicities or cause body weight loss, i.e. phenobarbital or 3-methylcholanthrene. In view of the importance of TCDD to environmental toxicology and the lack of an explanation for its toxic action, the hepatic plasma membrane as a potential site of TCDD action has been examined.

MATERIALS AND METHODS

Male Sprague-Dawley rats (175-200 g) were obtained from Spartan Research Animals Inc. Haslett, Michigan. Food (Wayne Lab Blocks, Chicago, IL) and water were provided ad libidum and the animals were maintained on a 12 hour light, 12 hour dark cycle.

Plasma Membrane Preparation

Animals were dosed intraperitoneally with either 25 ug/kg TCDD (Dow Chemical Co. Midland, MI), dissolved in a 1:9 solution of acetone:corn oil or an equal volume of vehicle alone. At 10 days post-treatment the animals were sacrificed by decapitation, and the hepatocyte plasma membranes (PM) were isolated according to the procedure of Yunghans and Morre (1973). Examination by electron microscopy (via procedures set forth by Hooper et al. 1979) and marker enzyme assays (Na-K ATPase and ^{3}H-Concanavalin A binding) verified the presence of PM vesicles and absence of significant mitochondrial and/or microsomal contamination.

Electron microscopy was a service of the Center for Electron Optics at MSU.

Gel electrophoresis was performed with a Bio-Rad 221 Dual Slab Gel system using the method of Laemmli (1970) as modified by Hoefer Scientific (1980). Gels were subsequently dried using a Bio-Rad Gel Slab Dryer (Model 224) for densitometric scanning. The bands resulting from Coomassie blue staining were scanned for their relative intensity utilizing an ACD-18 Gelman Densitometer, and the areas under the peaks integrated for comparison between plasma membrane preparations.

Concanavalin A Binding

Concanavalin A binding was determined by mixing 25 ug membrane (in 0.25 M sucrose) with 0.21 ug ^{3}H-Concanavalin A (New England Nuclear, specific activity 25 Ci/mmole) to a total reaction volume of 0.2 ml. Each reaction tube was subsequently incubated for 10 min at 37^{o}C after which the reaction was terminated with the addition of 8 ml cold 0.1 M Tris-HCl. The mixture was then quickly passed through 0.45 u HA filter (Millipore Corp., Bedford, MA), washed with an additional aliquot of 8 ml Tris-HCl, allowed to air dry, and the remaining radioactivity assayed with liquid scintillation counting. Specific binding was determined in the presence of .01 M alpha methylmannoside (Sigma Chemical Co., St. Louis, MO). Parallel tubes were incubated for 5 to 10

min. in the presence and absence of alpha-methylmannoside before the addition of ^{3}H-Concanavalin A. Specific binding was calculated by subtracting the radioactivity remaining in the tube without alpha-methylmannoside and was on the order of 3-6% of total radioactivity added. Protein concentration was measured by the method of Lowry et al. (1951).

RESULTS

A slight difference was seen in liver weights between control and TCDD-treated animals 10 days after dosing (Table 3). Although a significant difference was noticed in the liver/body weight ratio, the difference in the yield of plasma membrane per gram of hepatic tissue between treated and control rats was indistinguishable.

The PM fractions, as examined with electron microscopy, were free from contaminating mitochondria and microsomal vesicles. Absence of microsomal contamination in these preparations was further indicated by electrophoresis and the lack of many densely stained protein bands between 52,000 and 60,000 daltons (Figure 6).

Plasma Membrane Protein Composition

Analysis of the membrane preparations via SDS-polyacrylamide gel electrophoresis clearly showed qualitative differences in the protein composition (Figure 6). Band intensity was measured with a densitometric technique and the results (Table 4) indicated the levels of

TABLE 3. Liver weight, liver/body weight ratio, liver plasma membrane yield and ^{3}H-concanavalin A binding between control rats and TCDD treated rats 10 days after dosing.

	Control	TCDD (25 ug/kg)
Liver Weight (grams)	17.00 ± 1.68 (14)[a]	15.34 ± 2.38 (16)*
Liver/Body Weight ratio (%)	5.6 ± 0.6 (14)	6.6 ± 0.5 (16)*
Plasma Membrane yield (ug)[b]	240 ± 102 (7)	226 ± 75 (9)
Total ^{3}H-Con A binding[c]	12.0 ± 4.3 (5)	9.5 ± 2.8 (5)
Specific ^{3}H-Con A binding[c]	11.0 ± 3.0 (5)	6.6 ± 2.9 (5)*

[a]Results expressed as the mean ± SD of the number of animals in parenthesis. Data analyzed using two tailed students "t" test.
*P < .05
[b]Yield of plasma membrane/gram wet weight liver tissue.
[c]Average ng bound/25 ug protein.

Figure 6. SDS-polyacrylamide slab gel electrophoresis patterns of standard protein mixture (S) and hepatic plasma membranes from control (C) or TCDD treated (T) rats. Standard protein mixture (S) contained bovine serum albumin (66k), egg albumin (45k), trypsinogen (24k) and gamma lactoglobulin (18k daltons).

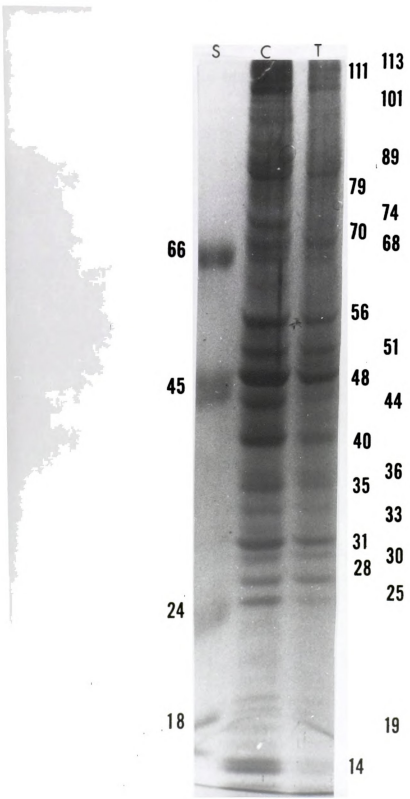
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TABLE 4. Effect of *in vivo* treatment of TCDD on the protein composition of hepatic plasma membrane. The SDS gel electropherograms such as the ones shown in Figure 4 were scanned by a densitometer and the peak area corresponding to each band was integrated and expressed as percentages of the total protein found in this molecular weight range.

Molecular Weight (X1000)	Control	TCDD-treated	% Increase (+) or decrease (-) against control
116	0.85	3.91	+360%
113	1.17	6.45	+428%
111-108	6.08	3.36	- 45%
104-101	2.96	0.99	- 67%
93-87	8.01	8.98	+ 12%
79	0.82	1.71	+108%
74	1.84	1.87	+ 2%
70	1.54	2.28	+ 48%
68	0.95	1.09	+ 15%
67-57	0.86	0.88	+ 2%
56	3.95	3.09	- 22%
51	3.60	3.14	- 13%
48	9.34	8.19	- 12%
45	3.66	2.12	- 42%
40	5.38	2.58	- 52%
36	2.83	0.61	- 78%
35	6.88	4.48	- 35%
33	4.76	2.66	- 44%
30-31	10.79	10.05	- 7%
28	2.31	2.62	+ 13%
26	3.23	7.75	+140%
25	3.42	3.28	- 4%
16-24	7.16	10.35	+ 45%
15	5.86	4.68	- 20%
14[a]	1.77	2.59	+ 46%

[a]Band 14k not pictured in Figure 6.

many proteins in the TCDD treated electrophoretograms to be severely depressed while others were intensified. The largest decreases were seen in bands at 36K, 40K, and approximately 100K daltons while significant increases were seen in bands 126K, 74K, 79K, 113K and 116K daltons. These alterations seemed to change over the 40 day observation period. As can be seen in Figure 7 significant changes were seen in the plasma membrane composition as soon as 2 days after treatment with TCDD. By day 10 the greatest number of bands were affected with an apparent recovery at day 40. This may not reflect a true reversal since the data from day 40 represent surviving animals approximating 70-80% of the total.

Con A Binding

In order to correlate these membrane changes with a specific biochemical parameter, ^{3}H-Concanavalin A binding with surface glycoproteins was measured. TCDD caused a 40% decrease in specific binding as compared to control animals (Table 3). These results are a combination from assays done both by Dr. Madhukar and myself.

Wasting

Animals treated with toxic doses of TCDD showed the typical wasting syndrome (Figure 8) described by Gasiewicz et al. (1980), Van Logten et al. (1981), and Seefeld et al. (1982). Twenty five days after administration of 25

Figure 7. SDS-polyacrylamide slab gel electrophoretogram of hepatic plasma membrane from rats 40, 20, 10, 2, or 0 (untreated control) days after TCDD treatment. Mortality at day 20 was 0% and at day 40 20-30%. Standard reference proteins (S) were bovine albumin (66k), ovalbumin (45k), trypsinogen (24k), gamma lactoglobulin (18k), and lysozyme (14k daltons).

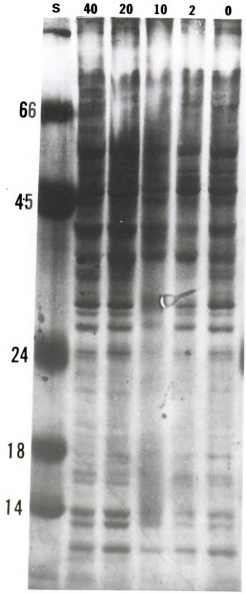
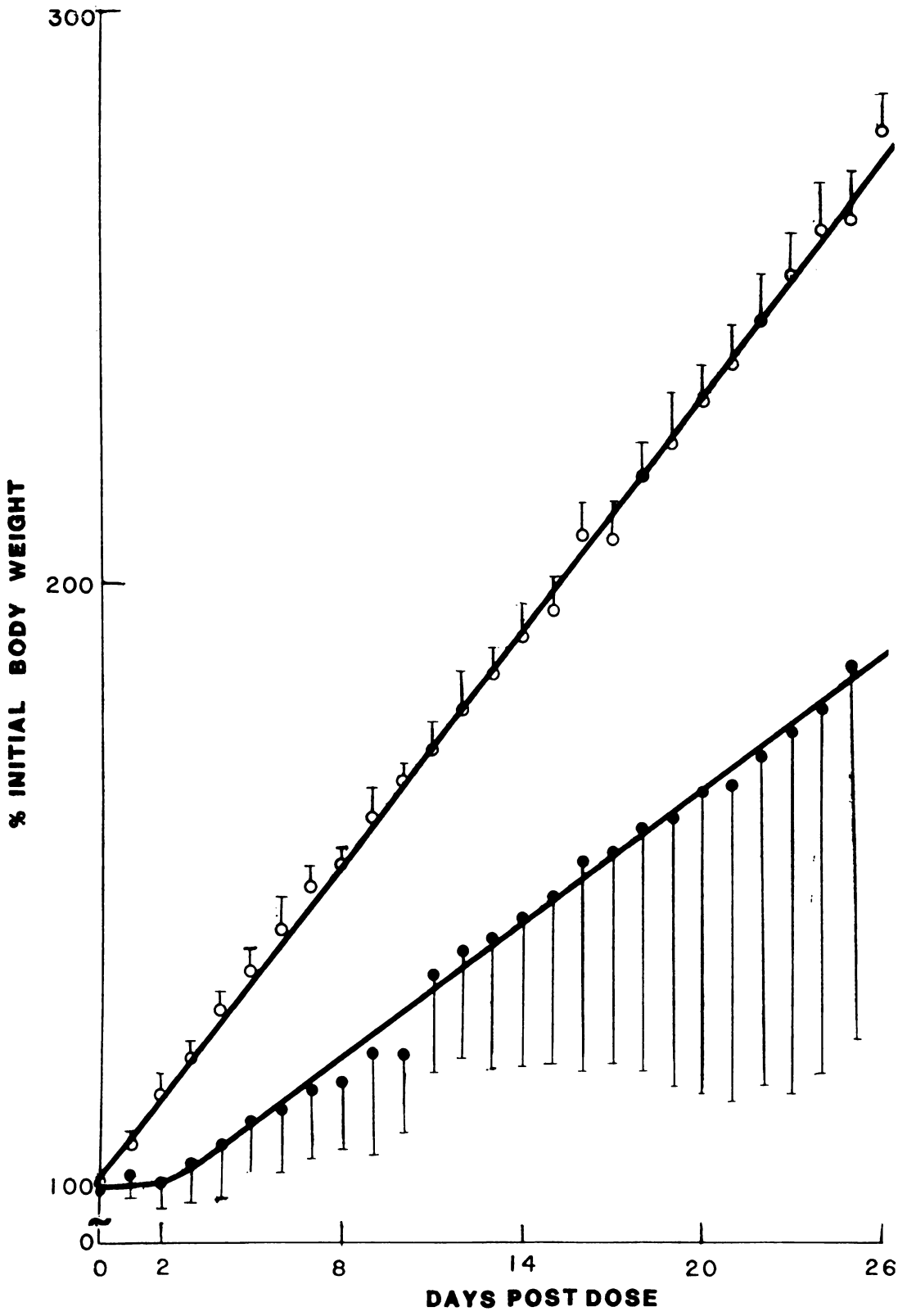

S
40
20
10
2
0
66
45
24
18
14

Figure 8. Body weight as % initial body weight over the 25 day observation period for control (o) and TCDD treated (●) rats. Each point represents the average ± SD of 5 animals. Data was tested for normality then subjected to random factorial ANOVA which indicated a significant difference between the two groups of animals at $P<.01$.


300
200
100
0
% INITIAL BODY WEIGHT
0
2
8
14
20
26
DAYS POST DOSE

ug/kg TCDD or acetone:corn oil, body weight as a % of initial body weight was 186% and 260%, respectively.

Results from subsequent experiments indicated that this loss of body weight produced by TCDD administration is variable. Some animals demonstrated a considerable loss of body weight while others neither gained nor lost weight while still others actually showed a gain in body weight. Those rats which did gain weight never did reach that attained by the control animals. This variability in response was thought to be due to inherent variability in the population. That is, some individuals were tolerant to dioxin at this dose while others were seemingly more sensitive to TCDD. The majority of TCDD treated animals neither gained nor lost weight as would be expected from a normal population. It is anticipated that a higher dose of dioxin would produce the characteristic loss of body weight in a greater proportion of animals while the reverse would occur at lower doses.

To ascertain whether the decreased weight gain was due to depressed food consumption, food and water intake and fecal output were measured over a 25 day observation period. Results indicate an initial brief drop in intake 2-3 days after dosing in both control and TCDD treated animals (Figures 9 and 10). However after that, both food and water consumption in the treated animals were significantly reduced from that of the controls, and as

Figure 9. Food consumption of 5 control (o) and 5 TCDD treated (●) rats over a 25 day observation period. Each point represents the average ± SD of the % of food or water consumed daily in relation to that provided each animal. Analysis by random factorial ANOVA after normalization by angular transformation indicated the control and TCDD populations to be significantly different in both experiments at $P<.05$.

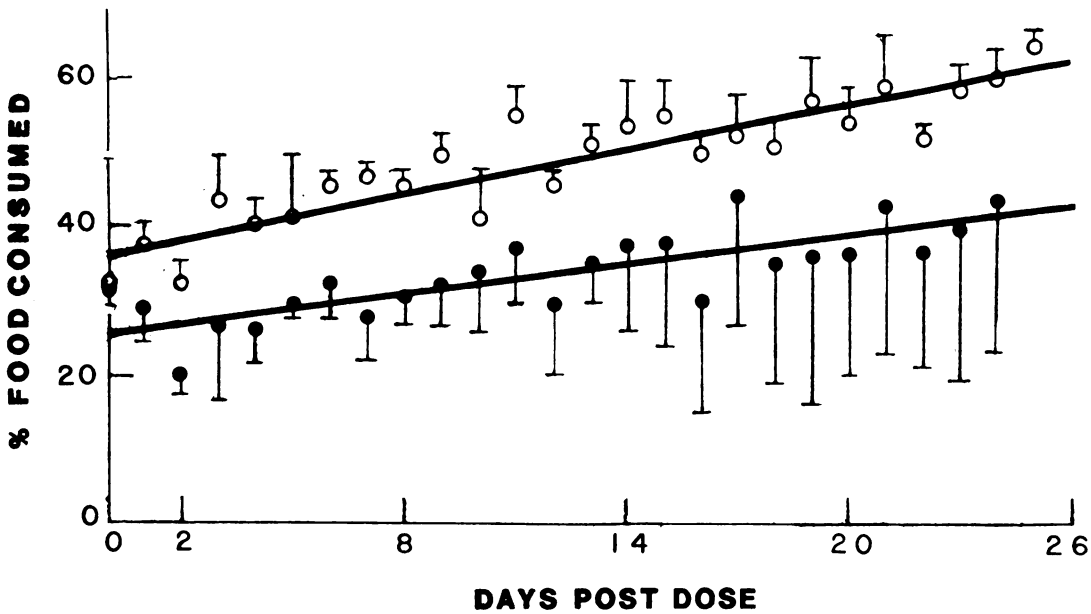
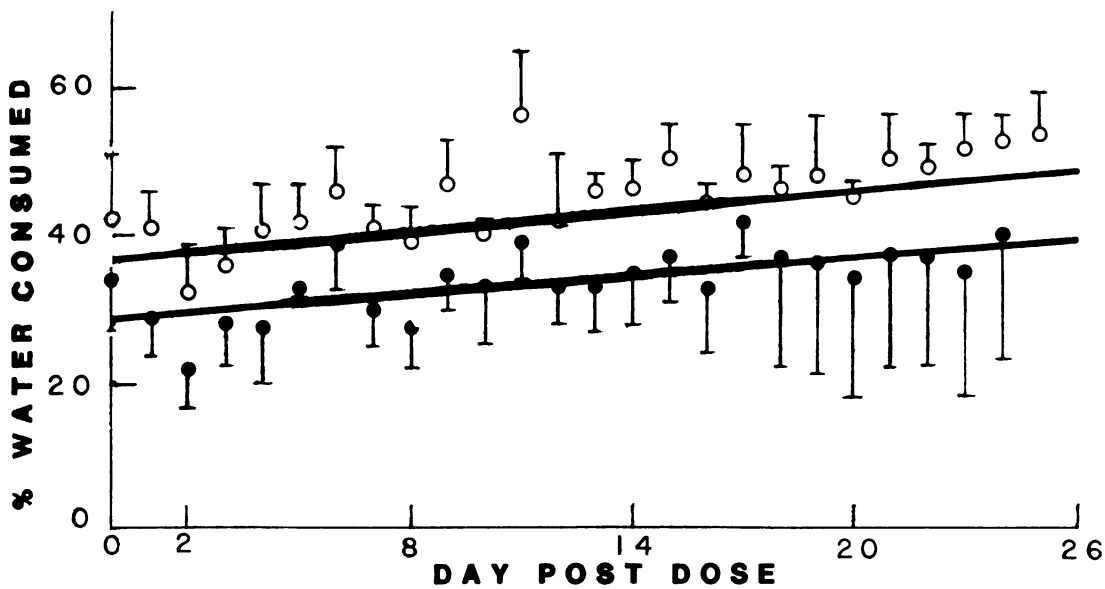

% FOOD CONSUMED
60
40
20
0
0
2
8
14
20
26
DAYS POST DOSE

Figure 10. Water consumption of 5 control (o) and 5 TCDD treated (●) rats over a 25 day observation period. Each point represents the average ± SD of the % of food or water consumed daily in relation to that provided each animal. Analysis by random factorial ANOVA after normalization by angular transformation indicated the control and TCDD populations to be significantly different in both experiments at $P<.05$.


% WATER CONSUMED
60
40
20
0
0
2
8
14
20
26
DAY POST DOSE

would be expected fecal output from the control animals was higher than that from the treated (Figure 11).

Toxicity

All animals dosed with TCDD showed noticeable hair loss beginning approximately 12 day after treatment. Some of those animals near death in the latter part of the experiment exhibited ocular hemorrhaging and lacrimation, pilo erection, minor salivation, obvious capillary dilation in the ears, and penile erection. They were considered to be abnormally aggressive and to have some difficulty in breathing. Fecal pellets were abnormally small and drier than usual and some contained a green fibrous material reminiscent of undigested food.

Observation upon dissection revealed many fatty deposits among the organs. The liver looked very granulated with enlarged cells and obvious fatty deposits. Kidneys, lungs, trachea, spleen, pancreas, heart, and thymus all appeared normal except for large fat deposits surrounding, and in some instances infiltrating, the organ. The thymus showed signs of involution and was barely detectable in some specimens. Only liver and thymus weight were significantly different from control animals (Table 5).

DISCUSSION

Although TCDD caused a 10% decrease in liver weight after 10 days, the liver to body weight ratio was actually

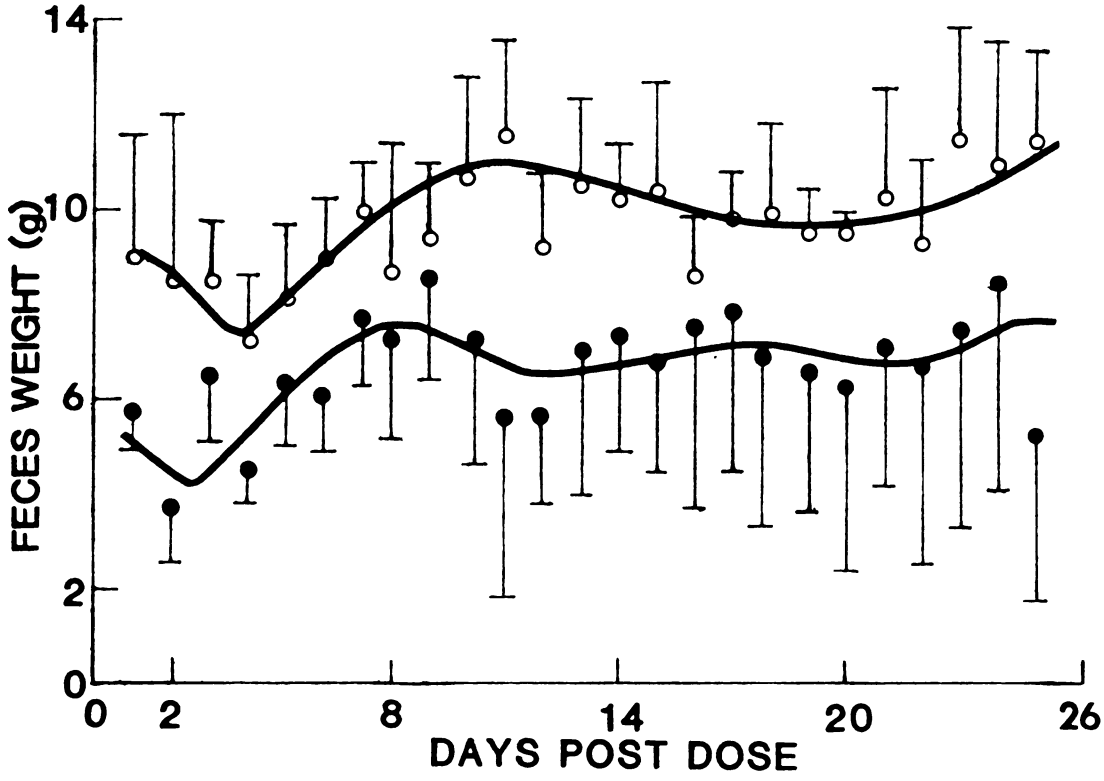


Figure 11. Fecal output (mean ± S.D., n=5) of control (o) and TCDD (●) rats as a function of time after dosing. Random factorial ANOVA indicated these populations to be significantly different at $P < .01$.

TABLE 5. Organ weights[a] of rats 25 days after dosing with either acetone:corn oil or TCDD.

Organ	Control	TCDD (25 ug/kg)
Liver	18.65 ± 3.23[a]	13.71 ± 3.14*
Kidney - Left	1.32 ± 0.26	1.52 ± 0.23
Right	1.30 ± 0.18	1.48 ± 0.09
Lungs (and trachea)	2.22 ± 0.15	2.27 ± 0.13
Heart	1.21 ± 0.12	1.29 ± 0.22
Spleen	0.93 ± 0.26	0.76 ± 0.13
Thymus	1.11 ± 0.16	0.27 ± 0.10*
Testis & epididymis - Left	3.41 ± 0.78	3.62 ± 0.70
Right	3.26 ± 0.79	3.73 ± 0.50
Urinary Bladder	1.43 ± 0.61	1.33 ± 0.48
Adrenal Gland - Left	0.04 ± 0.01	0.03 ± 0.01
Right	0.04 ± 0.01	0.03 ± 0.01
Brain	2.14 ± 0.21	2.33 ± 0.11

[a]Results expressed as mean organ weight in grams ± SD of 5 animals. Data analyzed with two tailed students "t" test.
*P <0.05

increased since there was a large difference in final body weights. Therefore in proportion to the rest of the body the liver actually became larger. Because the yield of plasma membrane did not change, this increase was probably not due to hyperplasia but rather from an increase of intracellular material. In view of the fact that TCDD is a powerful microsomal inducing agent (Poland and Glover 1973a,b) this is not surprising.

Little contamination of these preparations with other organelles was noted upon examination with electron microscopy and electrophoresis. As mentioned previously, the absence of an increase of band 54 to 56K in the treated preparation indicated minimal microsomal contamination. The 48K band is consistently found in all plasma membrane studied (Neville and Glossman 1971a,b), is probably a structural protein, and therefore was used as an internal standard. It typically made up 8-13% of the total protein in the membrane. While no effort was made to establish the identity of the specific bands that were altered, it is important to note that if these changes occur at critical sites in critical organs, symptoms of TCDD's toxicity may be explained. Therefore changes of the plasma membrane shown here may be important in understanding TCDD's toxic action.

The activity of 2 plasma membrane bound ATPases, Na-K and Mg-ATPase, in the rat liver have been previously shown to be reduced by <u>in vivo</u> treatment of TCDD (Peterson et al.

1979a, 1979b). Biliary transport of ouabain, a model neutral substrate, was also reduced. These events can be traced to the function of the plasma membrane, and it is therefore concluded that they are caused by TCDD's action *in vivo* on the plasma membrane. These pertubations are not due to a direct effect of the dioxin on the membrane since Peterson et al. (1979a) showed that TCDD added to membranes *in vitro* failed to produce the toxic effect. The protein changes may be due to membrane fluidity changes or altered synthetic and/or turnover rates of membrane components. Whether the same type of action is responsible for TCDD's toxic manifestations in other species remains to be determined. However, the importance of plasma membrane surface proteins in normal cellular functions is unquestionable, therefore it is imperative to examine these alterations in the future.

Concanavalin A is a plant lectin which binds specifically to membrane glycoproteins containing the mannose moity. The decreased Con-A binding caused by TCDD suggests altered hepatic cell surface glycoproteins in these animals. This may lead to a loss of cell-cell communication which in turn can decrease contact inhibition and result in tumor formation. Many precancerous or transformed cells are known to have reduced surface glycoproteins (Walborg et al. 1979), and TCDD has been shown to be a very good tumor promoter in the rat liver (Pitot et al. 1980).

Treated animals consumed significantly less amounts of food and water. This could account for the severe wasting syndrome experienced by these animals, however an examination of intestinal absorption along with pair-feeding needs to be evaluated. The high variability in body weight gain by the treated animals in Figure 8 and in food and water consumption (Figures 9 and 10) was due to some animals drastically cutting food intake and beginning to die at about day 14. These data confirm that of Seefeld and Peterson (1984) in that hypophagia plays a major role in the loss of body weight after TCDD treatment. These authors also measured fecal energy loss (as a percentage of feed intake) and digestible energy (percentage of feed energy absorbed by the intestine) and they found no significant differences between control and dioxin treated animals. They concluded that TCDD treated rats reduced feed intake until a new weight level abnormal for the age of the animal was obtained. Although fecal output decreased (Figure 11) there was no change in potential energy lost with the feces since feed intake was also reduced.

Symptoms of toxicity other than weight loss included: minor ocular hemorrhaging, lacrimation, pilo erection, minor salivation, and penile erection. Hair loss, a common characteristic of other polychlorinated aromatics, was also observed. The major pathological change observed grossly was the infiltration of many of the major organs with fatty

deposits. Only the liver and thymus showed significantly lower weights than control animals.

Conclusions

In conclusion, TCDD caused decreased food and water intake followed by significant loss of body weight gain and typical behavioral symptoms of starvation. Fatty infiltration of many major organ systems was noted as well as significant decreases in both liver and thymic weight. The major significance of this study is the hepatic membrane alterations. If these changes occur at critical physiological sites in the liver or other organs, some of TCDD's toxic symptoms may be explained.

CHAPTER III

ALTERATION OF RAT HEPATIC PLASMA MEMBRANE FUNCTIONS BY 2,3,7,8-TETRACHLORODIBENZO-P-DIOXIN

INTRODUCTION

Low level residues of TCDD have been detected in soil, fish, industrial and municipal fly ashes in various locations throughout the United States (Bumb et al. 1980). Because of the many diverse effects of TCDD, the search for the biochemical mechanisms of its toxic action has been difficult. Investigations to find interspecific differences among guinea pigs, rats, hamsters, etc. in the metabolism, disposition, and cytosolic receptor binding of TCDD have produced modest differences, but they probably do not explain the enormous species difference in susceptibility to this toxicant (Gasiewicz et al. 1983).

Peterson (Peterson et al. 1979a) has found that a dose-dependent reduction in plasma membrane ATPase accompanies the depression of ouabain biliary excretion in rats treated with TCDD. Although this relationship was later shown to be causally unrelated (Peterson et al. 1979b), the phenomenon that these two plasma membrane mediated functions are simultaneously reduced by dioxin treatment warrants further investigation. It was found that protein profiles of hepatic plasma membrane from TCDD-treated rats were

different from those of untreated controls (Brewster et al. 1982). Evidence is here presented that *in vivo* exposure of TCDD affects a number of physiologically important components of the rat liver plasma membrane.

MATERIALS AND METHODS

Young (125-150g) male Sprague-Dawley rats were fed Purina Laboratory Chow, *ad libitum*. TCDD was dissolved in corn oil with acetone (9:1 ratio) and used for intraperitoneal (i.p.) injection. Control rats received the same volume of corn oil-acetone. After specified time periods they were sacrificed, and the hepatocyte plasma membrane was isolated according to the method of Yunghans and Morre (1973). In an effort to compensate for the decreased food intake by the TCDD treated rats, a third group of rats underwent severe food restriction for the 10 day observation period after which liver plasma membrane was isolated.

Young (200-225g) male albino English Shorthair Guinea Pigs (Strain MDH:SR(A)) were purchased from the Michigan Department of Health. Sprague Dawley Rats and Golden Syrian Hamsters were obtained from Harlan Animals, Haslett, Michigan. Housing, plasma membrane fractionation, enzyme assays, and electrophoresis were as before.

ATPase Enzymatic Activity

ATPases were assayed by the method described by Matsumura and Clark (1980). Modifications were: 25 ug plasma membrane protein in 0.1 ml sucrose (0.25 M) added to 0.9 ml reaction buffer containing 10 ul 2,4 dinitrophenol (10 mM) was preincubated at 37°C (10 min), (gamma-^{32}P) ATP was added (final concentration 10 mM), and the mixture incubated at 37°C for an additional 10 min. The reaction was stopped with the addition of 300 ul ice cold trichloroacetic acid (10%) followed by 100 ul H_2O containing 1 mg bovine serum albumin and 1.36 mg KH_2PO_4. The solution was centrifuged (5 min, 1000 x g) and the clear supernatant decanted and mixed with 200 ug activated charcoal. The sides of the tube were rinsed with 200 ul ethanol, and after a second 5 min spin, 0.5 ml of the supernatant was mixed with aqueous scintillation cocktail for liquid scintillation counting. Buffers for determining various ATPase activities contained 30 mM imidazole (pH 7.1) with the following ion combinations: Na-K ATPase, 120 mM NaCl, 20 mM KCl, and 5 mM $MgCl_2$; Mg-ATPase, 120 mM NaCl, 5 mM $MgCl_2$; basal medium for Ca-ATPase, 120 mM NaCl, 20 mM KCl, 0.5 mM EGTA; and for Ca-ATPase, 120 mM NaCl, 20 mM KCl, 0.5 mM EGTA and 2 mM $CaCl_2$.

Protein Kinase Assay

Protein kinase activity was determined by the method of Corbin and Reiman (1974) with the following modifications:

Plasma membrane (50 ug protein) in 50 ul of 0.25 M sucrose were added to 50 ul reaction buffer containing 1 ml 50 mM potassium phosphate (pH 6.8), 1 ml 30 mg/ml histone (Sigma Chemical Co., II A-S), and 1 ml 1 mM (gamma-^{32}P)-ATP in 18 mM magnesium acetate. For the determination of c-AMP dependent protein kinase activity, 6 nmoles c-AMP were added to the reaction buffer. After incubating for 10 min at 30^{o}C, the reaction was stopped with 3 ml cold trichloroacetic acid (10%). Then 100 ul water, containing 1 mg bovine serum albumin and 1.36 mg KH_2PO_4 was added. The tubes were allowed to stand for 5 min to complete protein precipitation, spun for 5 min (1000 x g), the supernatant decanted, and the pellet redissolved with 0.5 ml NaOH (0.2N). Reprecipitation with trichloroacetic acid and a second centrifugation followed. After repeating this washing procedure once more (total 2x), the final pellet was resuspended with 0.3 ml formic acid, of which 0.1 ml was used for liquid scintillation counting.

Gamma-glutamyl Transpeptidase Assay

Gamma-glutamyl transpeptidase activity was assessed by the method of Tate and Meister (1974). Additional information on this technique is: 100 ug membrane protein (suspended in 0.25 M sucrose) in 0.1 ml tris-HCl (0.01M)-NaCl (0.15M), pH 8.0 was mixed with the assay solution and incubated 15 min (37^{o}C). After stopping the reaction with 0.1 ml glacial acetic acid, and spinning for 5 min (1000 x

g), the absorbance of p-nitroaniline was determined at 410 nm with a Varian Double Beam Spectrophotometer.

Insulin and Epidermal Growth Factor Binding

Binding of [^{125}I]-insulin and [^{125}I] epidermal growth factor (EGF) was studied, following essentially the method of O'Keefe et al. (1974). Plasma membranes (50 ug protein) were suspended in 0.2 ml of Kreb's Ringer bicarbonate buffer (pH 7.4) containing 0.1% bovine serum albumin (BSA) and incubated with or without the native ligands (insulin 2.0 ug, EGF 0.5 ug) for 10 min at 24^{o}C before the addition of 0.25 ng of the labeled ligand (insulin Sp. Act. 100 uCi/ug, EGF, Sp. Act. 150-200 uCi/ug). All of the tubes were incubated for an additional period of 20 min. At the end of the incubation, the reaction mixture was diluted with 3.0 ml of chilled Kreb's Ringer bicarbonate buffer containing 0.5% BSA and rapidly filtered through a Millipore filter (type HAWP, 0.45 u) under vacuum. The filters were washed twice with 5.0 ml aliquots of the same buffer, air dried, and counted for radioactivity. Specific binding was calculated by subtracting the amount of radioactivity bound in the presence of native ligand from that in the absence of it.

Concanavalin A Binding

Binding of [^{3}H]-Concanavalin A (Con A) was studied by the method originally used by Chandramouli et al. (1977). The following modifications were made: 25 ug of plasma

membrane protein were suspended in a 0.2 ml volume of 0.1 M tris-HCl buffer, pH 7.4 containing 0.1% BSA. Duplicate tubes were preincubated with or without alpha-methyl D-mannopyranoside (alpha-mm, 10 mM) for 10 min at 37°C before the addition of 0.25 ug of ^{3}H-Con A (Specific Activity 25-45 Ci/mmol, New England Nuclear, Boston, MA). At the end of an additional 10 min of incubation at the same temperature, 8.0 ml of cold tris-HCl (0.1 M, pH 7.4) containing 0.5% BSA was added to each tube and the contents were quickly filtered over a Millipore filter (HAWP, 0.45 u). The filter was washed with an additional 8.0 ml of the same buffer and the radioactivity was counted as described above. Specific binding was defined as the difference between the amount of radioactivity in the presence and in the absence of alpha-mm. The binding assays were performed both by B.V. Madhukar and myself. Protein concentration was determined by the method of Lowry et al. 1951.

RESULTS

Time Course of Plasma Membrane Protein Alterations

To study the biochemical characteristics of the plasma membrane, rats were treated with 25 ug/kg of TCDD (single dose) through intraperitoneal injection (i.p.), and their hepatic plasma membrane isolated after 2, 10, 20, or 40 days as before. When these plasma membrane preparations were analyzed with SDS-polyacrylamide gel electrophoresis, qualitative differences in protein composition became

apparent. In the electropherotogram shown in Figure 7 the most significant effect of in vivo administered TCDD was observed in the preparations obtained from rats sacrificed at 10 and 20 days after treatment where many of the bands between 14 and 30 K dalton were completely abolished. After 20 days it appeared as if the effect was reversed; densitometric measurement of these bands confirmed this visual conclusion. The band at 48 K, which is a structural protein comprising 8-13% of the total membrane protein, served as a good internal standard (Brewster et al. 1982) and remained constant throughout the treatment period.

Alterations of Enzymatic Activity and Receptor Binding

To study the qualitative nature of the altered plasma membrane, activites of several membrane bound enzymes and receptor proteins were measured (Tables 6 and 7). Na-K, Mg, and Ca ATPase were all significantly reduced in the treated animals. There was little change in gamma-glutamyl trans-peptidase activity. The level of protein kinase activity in the plasma membrane from treated rats was significantly higher than that of the control, indicating that TCDD treatment does not always cause a reduction in enzymatic activity. Both c-AMP stimulated and nonstimulated protein kinases from the TCDD-treated animals showed higher enzyme activity than control.

Examination of receptor binding (Table 7) revealed the EGF receptor to be most affected by TCDD. In agreement with

Table 6. Difference in enzyme activities between hepatic plasma membrane preparation from control and *in vivo* TCDD-treated rats. Results are expressed as mean ± standard deviation of the number of animals in parenthesis. Each preparation was tested twice.

Enzyme	Enzyme activities (nmoles product/mg protein/hr) Control	TCDD-treated[a]
Na-K ATPase[c]	1496 ± 142 (3)	890 ± 178 (3)[b]*
Mg ATPase[c]	1820 ± 10 (2)	1110 ± 149 (2)*
Ca ATPase[c]	608 ± 65 (4)	340 ± 140 (8)*
Gamma-Glutamyl transpeptidase[d]	887 ± 231 (4)	658 ± 35 (3)
Protein kinase[e]		
in the absence of c-AMP	36 ± 12 (4)	147 ± 67 (7)*
in the presence of c-AMP	58 ± 25 (4)	217 ± 110 (7)*

[a] TCDD at 25 ug/kg single dose intraperitoneal injection. Plasma membrane collected at 10 days post-treatment.

[b] Data were analyzed using a two tailed student's "t" test; *$P<0.05$.

[c] n moles P_i liberated/mg plasma membrane protein/hr at pH 7.1, 37°C.

[d] n moles p-nitroaniline liberated/mg plasma membrane protein/hr at pH 8.0, 37°C.

[e] n moles P_i incorporated/mg plasma membrane protein/hr at pH 8.0, 37°C.

Table 7. Effect of *in vivo* TCDD treatment on ligand binding to cell-surface membrane receptors in the rat liver. Results are expressed as mean ± SD of the number of animals in parentheses.

Ligand	Dose (ug/kg single i.p.)	Specific binding	
		Control	TCDD-treated
^{3}H-Con A[a]	25	15.2 ± 2.5 (4)	11.7 ± 0.4[c]*(3)
^{125}I-EGF[b]	25	32.0 ± 8.6 (5)	3.8 ± 0.3*(3)
^{125}I-Insulin[b]	25	8.1 ± 1.2 (7)	11.7 ± 0.9*(3)

[a] ng of ^{3}H-Con A bound/25 ug protein/10 min.
[b] pg of ^{125}I-EGF, or insulin-bound/50 ug protein/20 min.
[c] Data were analyzed using two tailed student's "t" test; *P<0.05.

previous observations (Brewster et al. 1982) Con A binding was also reduced, however, insulin binding was slightly increased at this dose and time regimen.

The time course of TCDD's effect was also studied following a single i.p. dose of 25 ug/kg (Figure 12). During the 40 day observation period, TCDD-treated rats gained consistently less body weight than did the control rats. Insulin binding was little affected at the beginning, but by 20 days after treatment it was significantly reduced ($P<0.05$). Con A and EGF binding were continuously suppressed with the maximum effect occurring 20 days after treatment.

TCDD's effects on membrane bound enzymatic activities were studied at the same time and dose regimen. Results shown in Figures 13 and 14 clearly indicate, with the exception of gamma-GT and protein kinase activity, that these enzymes followed a similar reduction pattern as the receptors: The decline becomes significant at day 10 and reaches a maximum at day 20 followed by an apparent recovery. Control values for the enzyme assayed at 2, 10, 20 and 40 days respectively are: Total ATPase 972, 1479 $\pm$ 142, 1768 and 1137 nmoles of P_i liberated/mg protein/hr; Mg-ATPase: 1211, 1820 $\pm$ 10, 1076, and 1230 nmoles of P_i/mg protein/hr, Ca-ATPase, 564 $\pm$ 119, 609 $\pm$ 65, 603 $\pm$ 105 and 638 $\pm$ 152 nmoles P_i/mg protein/hr; and gamma glutamyl transpeptidase 884 $\pm$ 116, 887 $\pm$ 231, 935 $\pm$ 223 and 666 $\pm$ 68 nmoles of p-nitroaniline produced/mg protein/hr at 37^{o}C and

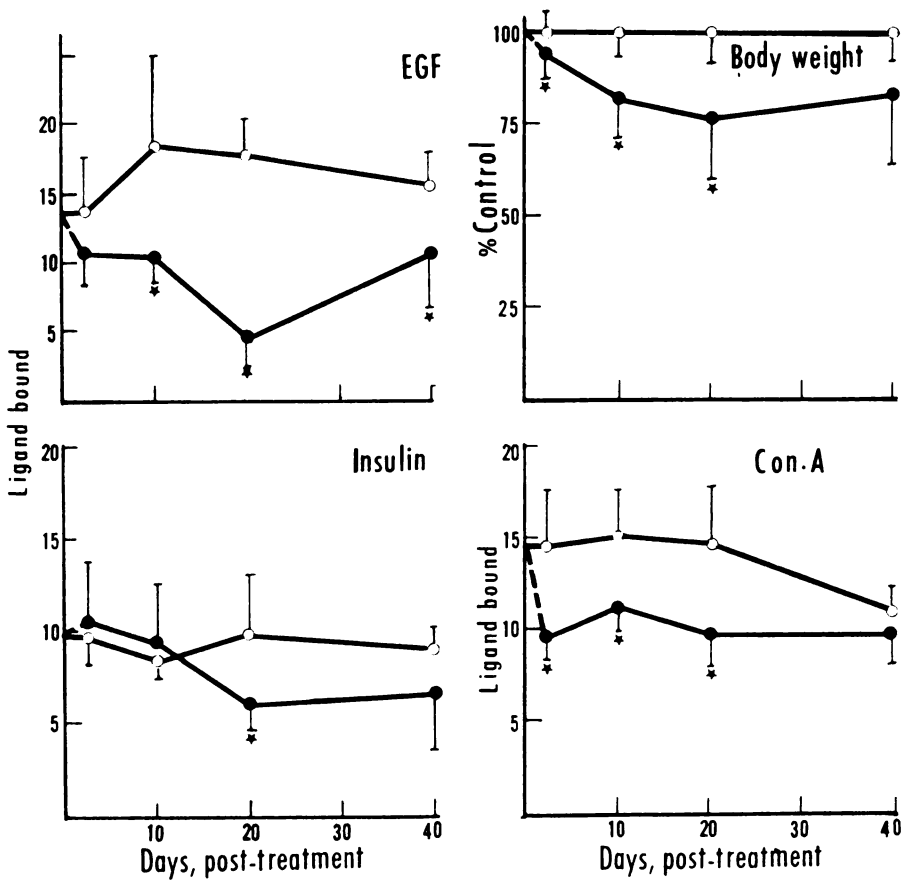


Figure 12. Time course of body weight changes and changes in specific binding of insulin, Con-A, or EGF to liver plasma membranes from TCDD-rats (●) or control rats (o). Binding is expressed as in Table 7. Changes in the body weight of the TCDD-treated rats is expressed as % of body weight of control at each time point. Each point is the mean ± S.D. of 4 to 8 animals, and the data were analyzed using random factorial ANOVA followed by Tukeys test for unconfounded comparisons. *P=0.01.

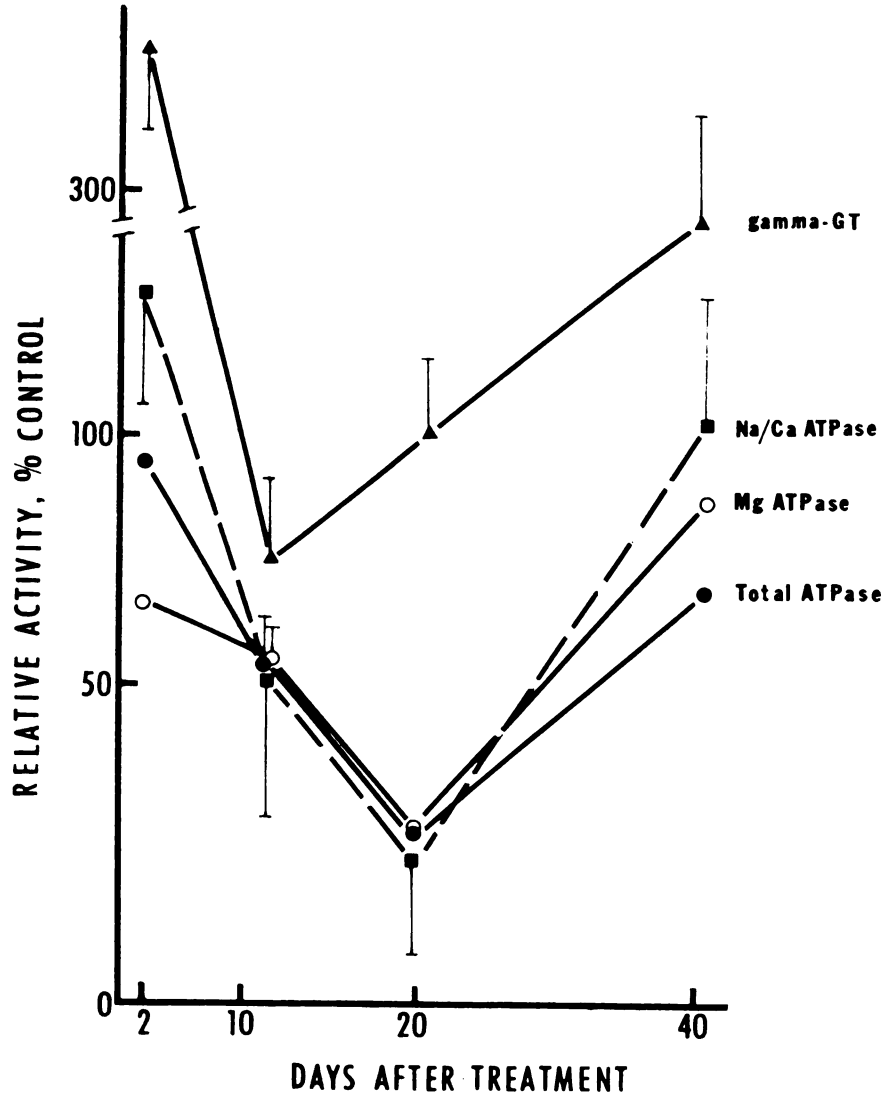


Figure 13. Time course of changes in plasma membrane associated enzyme activities in the rat liver as a result of in vivo TCDD exposure. Values are expressed as % of control activity and are represented by mean ± S.D. of at least 3 different membrane preparations.

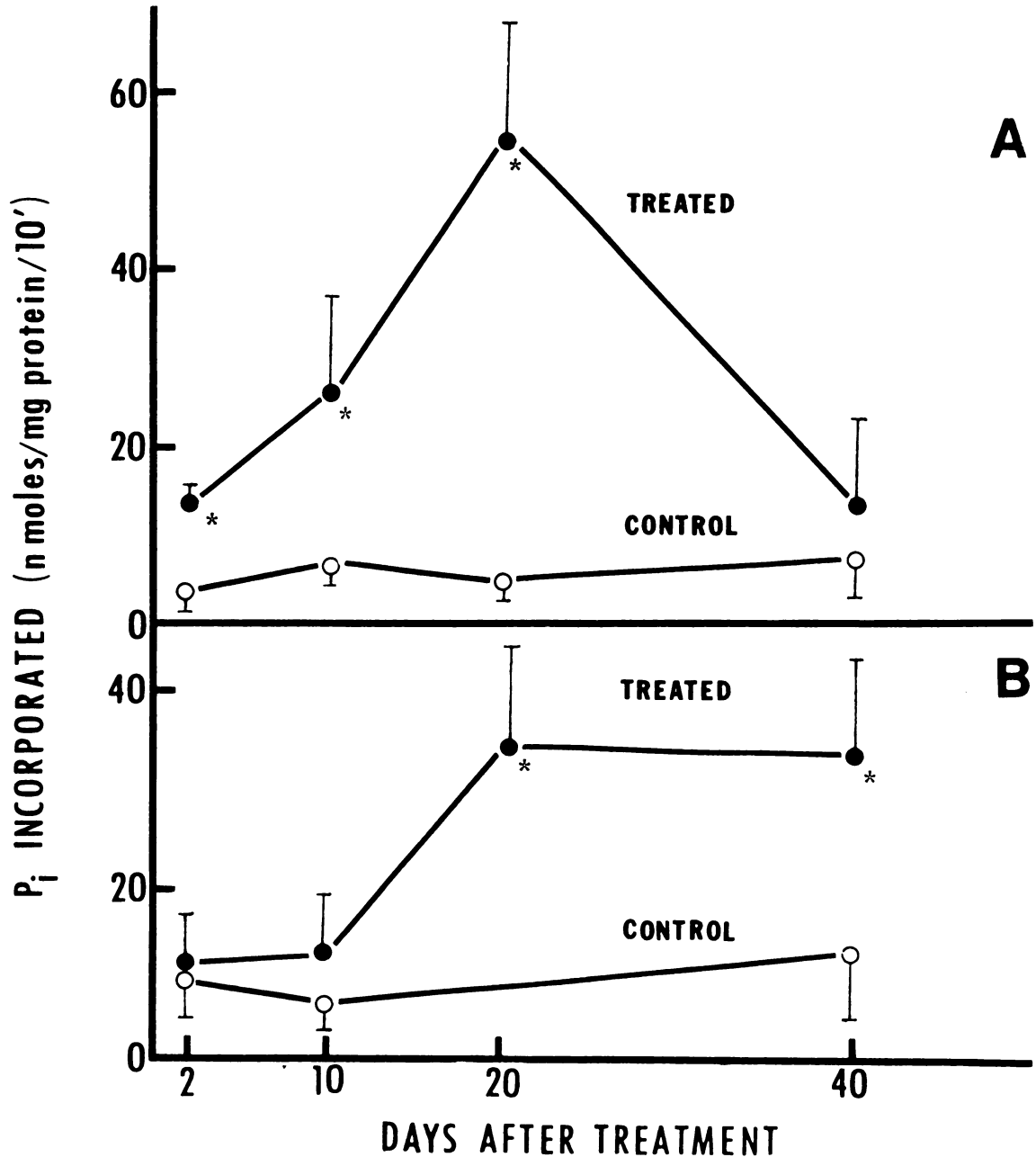


Figure 14. Time course of c-AMP independent (A) and c-AMP dependent (B) protein kinase activity for control (o) and TCDD treated (●) rats. Data were analyzed by random factorial ANOVA followed by Tukeys test for unconfounded comparisons. *P=.01.

pH 8.0. There is the possibility that this apparent recovery does not represent a true biochemical recovery, since by day 40 approximately 20 to 30% of the treated rats had died while at other times no mortality was observed.

The protein kinase activity followed a similar but reciprocal pattern as the other enzymes and receptors (Figure 14). Activity both in the presence and absence of c-AMP was significantly increased over control levels at day 10 and reached a maximum stimulation at day 20. The c-AMP independent activity returned to normal control values at day 40 but the c-AMP dependent phosphorylation remained elevated throughout the 40 day observation period.

To ascertain whether reduced food consumption and lowered body weight could affect these membrane parameters, rats were maintained on a limited diet for 10 days. Na-K ATPase activity, EGF, and insulin binding were no different from ad lib control levels (Table 8). Mg ATPase activity was reduced somewhat but not to the extent as that caused by TCDD; Ca ATPase was actually increased. It is interesting to note that the behavior seen in these animals reflected that of the TCDD treated; pilo erection, cage huddling, lack of interest, and uncommon aggressiveness were obvious.

Table 8. Activity of liver plasma membrane bound enzymes and receptors from rats maintained on limited diets or fed ad libitum for 10 days after dosing with acetone:corn oil.

Membrane Parameter	Activity	
	Control	Starved
Na-K ATPase[a]	1496 ± 142 (3)[b]	1524 ± 101 (3)
Mg ATPase[a]	1820 ± 10 (2)	1413 ± 19 (3)*
Ca ATPase[a]	608 ± 65 (4)	829 ± 42 (3)*
EGF binding[c]	32 ± 9 (5)	37 ± 1 (2)
Insulin binding[c]	8 ± 1 (7)	13 ± 12 (2)

[a]Enzyme activity is as defined in Table 6.
[b]Results expressed as mean ± SD of the number of animals in parenthesis. Data analyzed with a two tailed student's "t" test.
*P<.05.
[c]Receptor binding is as defined in Table 7.

Effect of TCDD on Enzymatic Activity in the Guinea Pig and Hamster

To ascertain whether the liver is affected by TCDD in other species, 4 enzymes were measured in the guinea pig and 3 in the hamster 10 days after treatment with either corn oil:acetone or TCDD (Table 9). Neither ATPase nor protein kinase activity in either animal was significantly affected except guinea pig Mg ATPase which tripled with dioxin treatment.

TCDD treatment to the rat decreased Na-K, Mg, and Ca ATPase in a dose dependent manner (Figure 15). In the guinea pig only Ca ATPase was dose dependent, however only at the higher doses was activity below control levels (Figure 16). The Na-K and Mg activity showed no dose dependence in this species. The hamster was unique in that increasing doses of TCDD increased activity of all 3 enzymes (Figure 17).

DISCUSSION

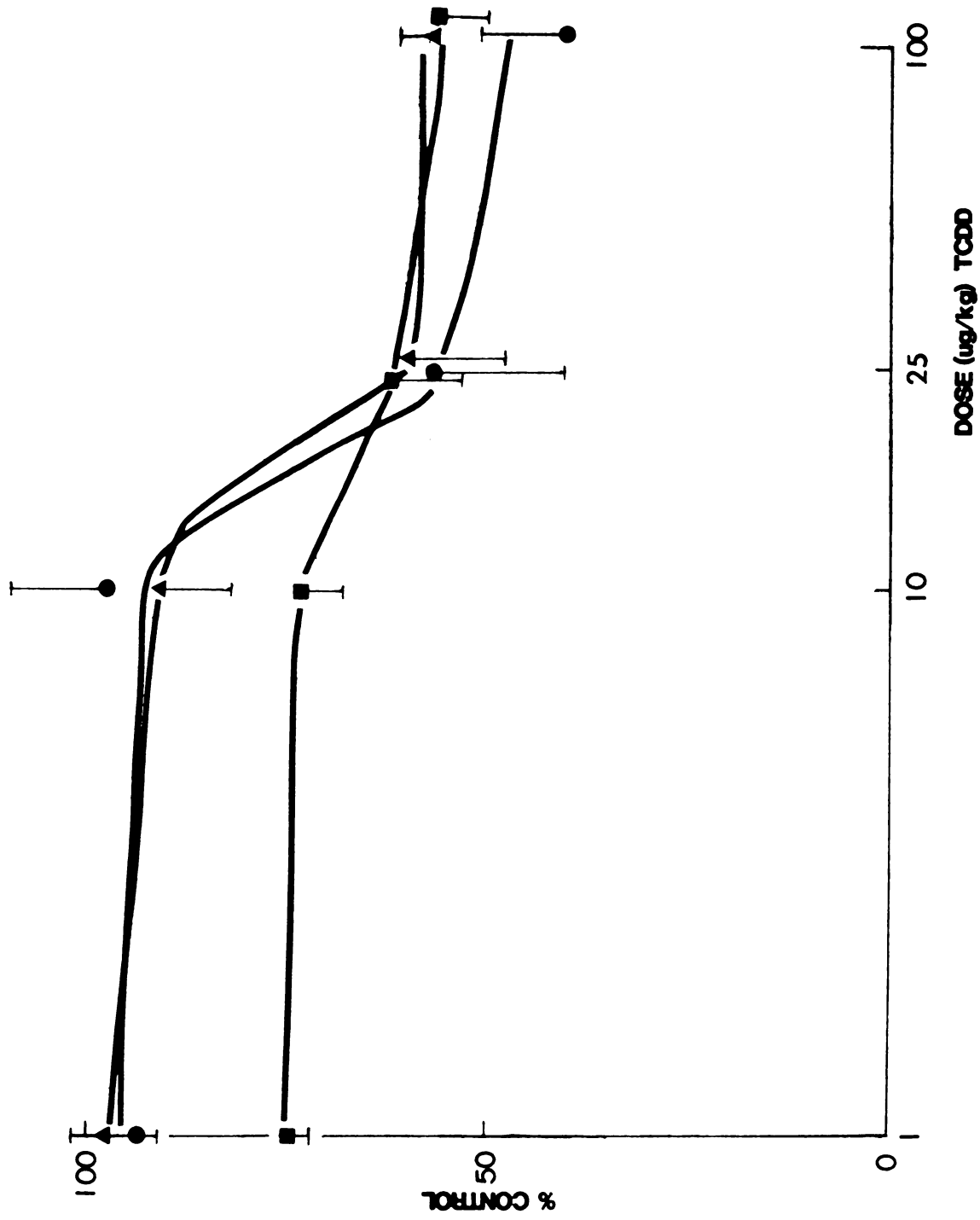
TCDD caused many functional changes in the rat hepatic plasma membrane. Na-K, Mg, and Ca ATPase were decreased 41, 39, and 44% respectively, 10 days after a single dose of 25 ug/kg. Con A binding declined 23% and EGF binding 88%. However, insulin binding showed an initial increase of 44% before becoming 60% of control levels at 20 days after treatment. The largest change produced by this chemical was in protein kinase activity which increased 274% and 308% in

Table 9. Enzyme activity of control guinea pig, rat, or hamster hepatic plasma membrane, 10 days after treatment with 1, 25, or 6000 ug/kg TCDD respectively.

Enzymatic Activity[a]	Species					
	Guinea Pig		Rat		Hamster	
	Control	TCDD	Control	TCDD	Control	TCDD
Na-K ATPase	864 ± 303(5)[b]	1199 ± 234(3)	1496 ± 142(3)	890 ± 178(3)*	1865 ± 130(4)	2085 ± 64(4)
Mg ATPase	566 ± 60(4)	1255 ± 157(4)*	1820 ± 10(2)	1110 ± 149(2)	1594 ± 167(3)	1732 ± 8(3)
Ca ATPase	477 ± 102(4)	513 ± 53(3)	609 ± 65(4)	341 ± 140(8)	1014 ± 259(4)	1078 ± 153(3)
Protein Kinase						
- c-AMP	177 ± 56(5)	174 ± 74(3)	36 ± 12(4)	147 ± 67(7)*	ND[c]	ND
+ c-AMP	182 ± 37(4)	165 ± 42(4)	58 ± 25(4)	217 ± 110(7)*	ND	ND

[a] Activity expressed as in Table 6.
[b] Results expressed as mean ± SD for the number of animals in parenthesis. Data were analyzed with two tailed student's "t" test.
*Statistically different from respective control with $P<.05$.
[c] ND - not determined.

Figure 15. Dose response of rat hepatic plasma membrane Na-K (▲), Mg (■), and Ca ATPase (●).


100
50
0
% CONTROL
1
10
25
100
DOSE (ug/kg) TCDD

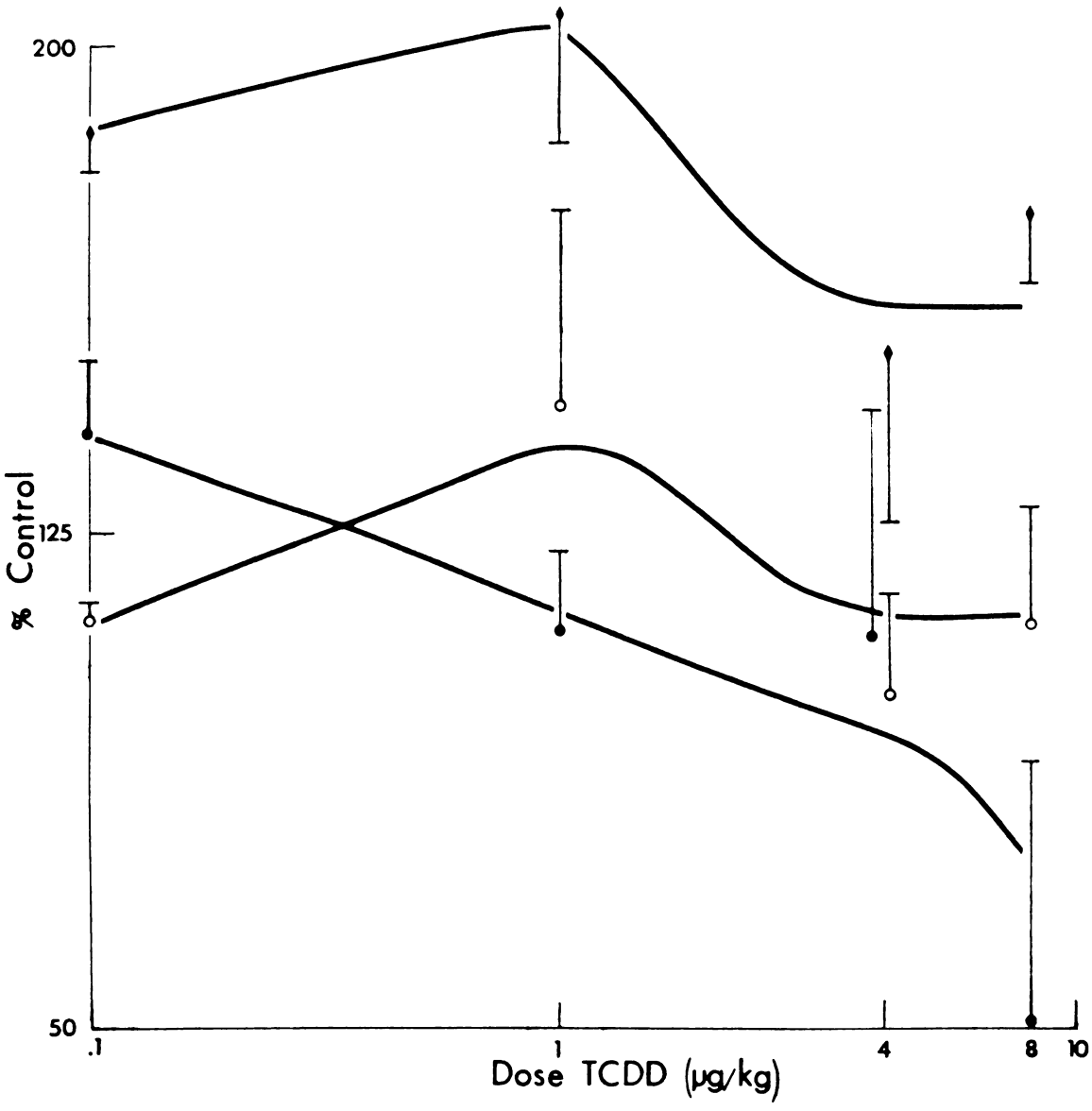


Figure 16. Dose response of guinea pig hepatic plasma membrane Na-K (o), Mg (♦), and Ca ATPase (●).

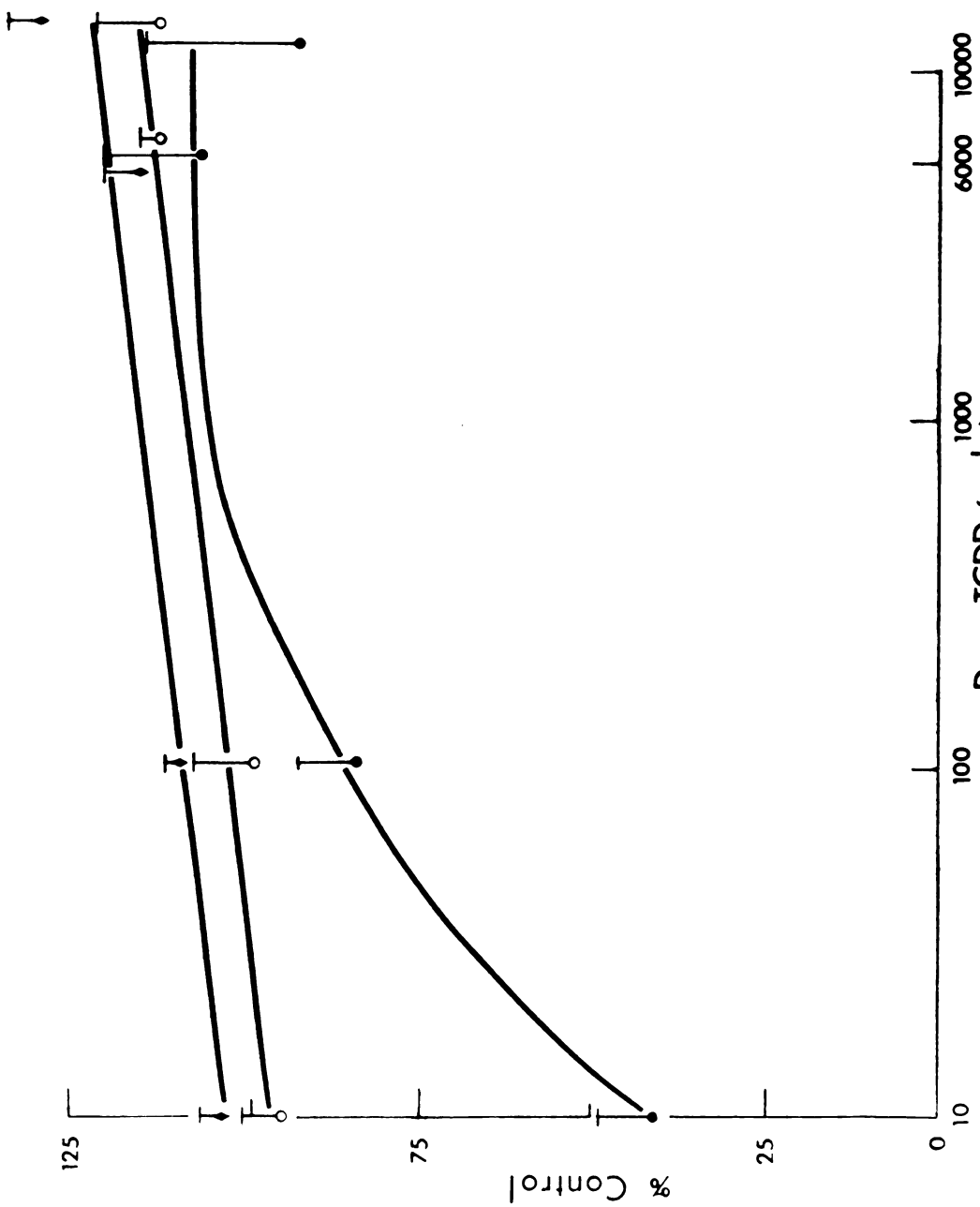


Figure 17. Dose response of hamster hepatic plasma membrane Na-K (○), Mg (♦), and Ca ATPase (●).

the presence and absence of c-AMP respectively. At day 10 only gamma-glutamyl transpeptidase activity was not significantly different from control levels.

All of these differences were noted at 2 days, reached their maximum at 20 days post treatment then appeared to begin a return to normal levels as mirrored by protein bands in the electrophoretogram in Figure 7. This may or may not be a true reversal since at 40 days after administration only a fraction of the treated population survived. An approximate LD_{50} dose was used in these experiments, therefore these animals could be a subgroup of rats more resistant to the effects of dioxin. Rats exposed to higher doses should be used to answer this question in future studies.

To answer the question of whether these changes in plasma membrane function are associated with any toxicant which causes general stress, the effects of 5 other toxic chemicals on these membrane parameters were examined by D.W. Bombick. Rats were treated i.p. daily, for 10 days with 9:1 corn oil/acetone, 3-methylcholanthrene (20 mg/kg), Aroclor 1242 (50 mg/kg), phenobarbital (120 mg/kg), DDT (0.3 mg/kg) or Firemaster B-6 (i.e., PBB, 50 mg/kg). At the end of the in vivo treatment, hepatic plasma membrane was isolated and the extent of Con A binding was measured. Levels of specific binding as expressed in terms of ng/50 ug plasma membrane protein were: control 17.0 $\pm$ 2.0,

3-methylcholanthrene 21.0 ± 4.9, Aroclor 1242 18.4 ± 2.1, phenobarbital 10.6 ± 1.8, DDT 13.9 ± 1.2 and Firemaster B-6 19.5 ± 6.6 (mean ± standard deviation of 3 animals except for DDT and Firemaster experiments where 2 animals were used). Only phenobarbitol significantly reduced Con A binding from control levels ($P \geq 0.05$).

Therefore, these results are not due to general stress or microsomal induction in these animals. Moreover, they are not due to severe losses of body weight since membrane preparations from starved animals show enzymatic activity and receptor binding similar to those from ad lib controls.

The purpose of this investigation was to survey how widely such TCDD-induced changes occur, following an observation where the levels of many protein constituents of the rat liver plasma membrane were altered as a result of TCDD exposure (Brewster et al. 1982). It is difficult to correlate many of the in vitro changes observed here with the in vivo symptoms of dioxin toxicity. It is postulated that many other changes are occurring that have not been elucidated in this study which may result in toxicological consequences if they occur at critical biochemical sites.

Although the results of this investigation do not indicate the cause of these changes or which of the changes are causally related to TCDD's toxic action, the phenomenon of the alterations in plasma membrane functions as a result of TCDD exposure merits further consideration. For

instance, some of the altered enzymes and receptors found in the current investigation are known to carry out very important physiological and biochemical functions. Ca-ATPase and Na-K ATPase are involved in the transport of Ca^{2+} and Na^{+} across the plasma membrane, respectively. Insulin receptor certainly is a vital system controlling carbohydrate and lipid metabolism and body weight homeostasis.

The observed large sustained membrane phosphorylation induced by TCDD could be critical since the effect of protein kinase activity on cellular biochemistry is well known. In addition to the regulation of many critical pathways, kinase activity is important in the cellular response to growth factors and hormones (Schlessinger et al. 1982; Hayden and Severson 1983). Some of the kinase actions regulate surface receptors which in turn control total cellular homeostasis.

The change in EGF binding is thought to be due to down regulation of the receptor as a result of increased receptor phosphorylation within the cell after TCDD treatment (see Madhukar et al. 1984). Therefore some of the symptoms of TCDD toxicity may be associated with changes in the EGF receptor which is critical in the hyperplastic response and in the inhibition of terminal keratinocyte differentiation as shown by Rheinwald and Green (1977) and Knutson and Poland (1980). Other symptoms of TCDD toxicity which are

associated with EGF include: fatty invasion of the liver (Heimberg et al. 1965; Gupta et al. 1973), promotion of skin cancer (Rose et al. 1976, Poland et al. 1982), conjunctival cell proliferation (Cohen and Elliott 1963, Norback and Allen 1973), inhibition of gastric secretion (Todaro and DeLarco 1978, Norback and Allen 1973), and serum hypertriglyceridemia (Heimberg et al. 1965, Albro et al. 1978).

No explanation is offered at this time for the cause of the plasma membrane alterations. TCDD is known to bind with a cytosolic receptor (Poland and Glover 1976; Poland and Kende 1976; Nebert 1979) and transported into the nucleus. Therefore, it may be reasonable to assume that the changes in the plasma membrane enzyme and receptor activities are also triggered by such induced DNA pleiotropic responses (or altered gene expression) rather than the result of TCDD's direct interaction with the plasma membrane. At least, there is evidence that the reduction of Na-K ATPase activity, as a result of in vivo administration of TCDD, is not due to a direct interaction of this chemical with the enzyme itself (Peterson et al. 1979a) since the amount of TCDD found in the plasma membrane after in vivo administration is not sufficient to cause inhibition of this enzyme in vitro.

Conclusions

In conclusion, many physiological homeostatic mechanisms are dependent upon cellular surface functions; therefore, alterations in the cell surface could conceivably lead to some toxic manifestations of TCDD. Because cell surface membranes are functionally different in different tissues, species, and sex, it is reasonable to expect differential manifestations of toxicity in different organs and animals.

This conclusion is at least partially confirmed with the results of the enzyme assays in the guinea pig and hamster, where very few changes were seen nor was a dose response evident. This is in stark contrast to the rat where a good dose response relationship was observed and all the enzymes were reduced. The guinea pig and the hamster liver did not show many signs of toxicity after TCDD administration, therefore these results are not totally unexpected.

CHAPTER IV

EFFECTS OF 2,3,7,8 TETRACHLORODIBENZO-P-DIOXIN ON THE THYMUS

INTRODUCTION

Thymic involution is one of the most consistent pathological changes observed in different species after administration of TCDD (Gupta et al. 1973, Harris et al. 1973, Vos et al. 1973, Kociba et al. 1976, Neal et al. 1979, Van Logten et al. 1980). Zinkl et al. (1973) noticed a loss of lymphocyte numbers in the thymus and theorized this to occur from a decrease in precursor cells or a direct cytotoxic effect on mature cells. They further hypothesized that reduced precursor cells could be the result of depressed stem cell division or an inhibition and blockage of the maturation steps leading to mature T lymphocytes. However, Kociba et al. (1976) concluded that involution resulted from increased lymphocytic migration to the thymic medulla followed by secretion of cortical thymocytes into the cardiovascular system.

Faith and Moore (1977) observed that TCDD caused an impairment of the thymus dependent immune system through differential suppression of T cell subpopulations. No effect was found on the humoral system. This confirmed the results of earlier studies suggesting that animals

administered TCDD showed increased sensitivity to bacterial infections (Vos et al. 1973, Thigpen et al. 1975, Vos et al. 1978). However, this depression of cellular immunity is not responsible for lethality since Greig et al. (1973) found that germ free environments for TCDD treated animals did not prevent death; nor did death occur from massive bacterial infection in animals exposed to the normal atmosphere.

In vitro evidence does not suggest TCDD to act directly on lymphocytes since cultured cell lines showed no effect when exposed to this dioxin (Knutson and Poland 1980). In vitro metabolic activation appears unnecessary since TCDD is capable of producing cytotoxicity in several other mammalian cell types (Niwa et al. 1975, Milstone and LaVigne 1984) without the addition of metabolic activators. Furthermore, there is little evidence in the literature to suggest that TCDD needs activation in vivo in order to produce toxicity.

The mechanism for this thymic involution has not yet been elucidated. Adrenalectomized animals treated with TCDD did not reverse this atrophy, therefore increased levels of serum corticosteroids do not seem to be involved (Neal et al. 1979, Van Logten et al. 1980). Van Logten et al. (1980) also present evidence eliminating the pituitary and thyroid glands.

Because of the marked sensitivity of the thymus to TCDD a brief examination of the thymocyte membrane was undertaken

in an effort to understand how dioxin may act in this organ. Perhaps the thymus could then be used as a model to discern the biochemical mode of action of this chemical in other organs showing manifestations of toxicity.

MATERIALS AND METHODS

Thymocyte Isolation and Plasma Membrane Preparation

Male Sprague-Dawley Rats were housed and treated with TCDD as before. Thymocyte isolation and plasma membrane fractionation were performed according to Koizumi et al. (1980). Procedures for Scanning and Transmission Electron Microscopy are outlined by Hooper et al. (1979) and were performed by Dr. Stan Flegler and Dr. Karen Klomparens-Baker respectively, from the Center for Electron Optics at Michigan State University.

Biochemical Assays

Determinations of Na-K, Mg, and Ca ATPase activity, EGF and insulin binding were as before. Glucagon binding was performed as described by Matsumura et al. (1984). Glucose uptake was determined by adding 0.5 ml of 1 mM D-glucose (in reaction buffer) containing approximately 0.1 uCi ^{14}C-D-glucose to 100 ug protein in 4.5 ml of saline-bicarbonate-acetate buffer pH 7.0. After 15 minutes at 37^{o}C the reaction was stopped by filtration with Millipore HAWP (0.45 u) filters. The filters were then washed with 2-10 ml portions of ice cold buffer, dried, and quantitated by

liquid scintillation counting. Final salt concentrations for reaction buffer were: NaCl 115 mM; KCl 5 mM; $CH_3COO^-Na^+$ 10 mM; NaH_2PO_4 1.2 mM; $CaCl_2$ 1.9 mM; $MgSO_4$ 1.2 mM; and $NaHCO_3$ 25 mM.

RESULTS

Electron Microscopy

The thymocyte surface was markedly affected by TCDD 10 days after dosing as shown by the scanning electron micrographs in Figure 18. TCDD caused a reduction in surface pits in all samples examined and increased bleb formation or cellular extensions. Bleb formation resulted in an obvious ruffling of the thymocyte as opposed to the relatively smooth surface of cells from control animals. Transmission electron microscopy revealed a loss of cytoplasmic material and an obvious sloughing off of the outer surface of the cells (Figure 19). Preparations from four different control or treated animals were examined.

Biochemistry

Two days after treatment, there was no significant change in total thymus weight or thymic weight relative to overall body weight in TCDD treated animals compared to controls (Table 10). After 10 days, thymic weight had been reduced 80% by TCDD.

Data shown in Table 11 indicated little effect of TCDD on these biochemical parameters of the thymocyte membrane.

Figure 18. Scanning electron micrographs of rat thymocytes isolated 10 days after administration of either acetone:corn oil (A) or 25 ug/kg TCDD (B). Note the pits (arrowheads) and lack of cellular extensions in the control cells compared to the treated.

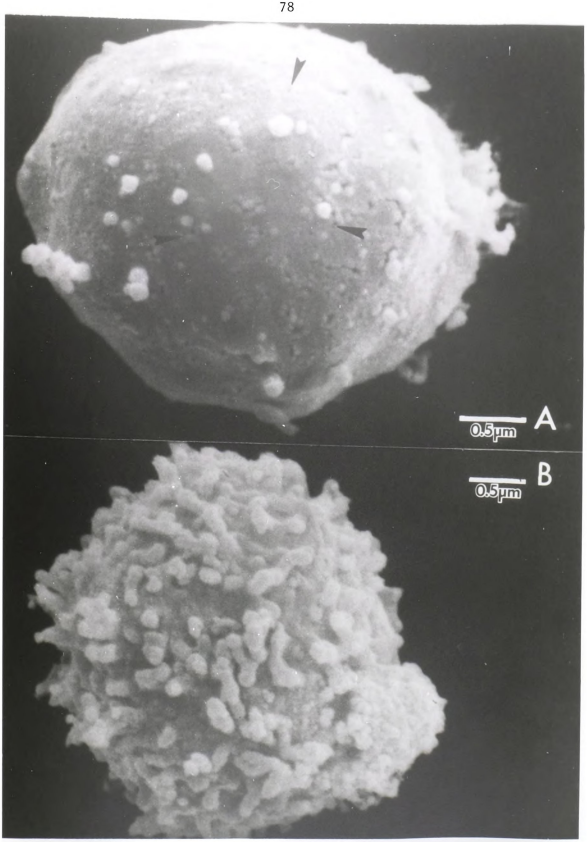

0.5μm
A
0.5μm
B

Figure 19. Transmission electron micrographs of rat thymocytes 10 days after administration of either acetone:corn oil (A) or TCDD (B). Note the reduction in cytoplasmic material, the tendency for increased cellular projections, and the sloughing off of the plasma membrane (arrowheads) in cells from a treated animal compared to control.

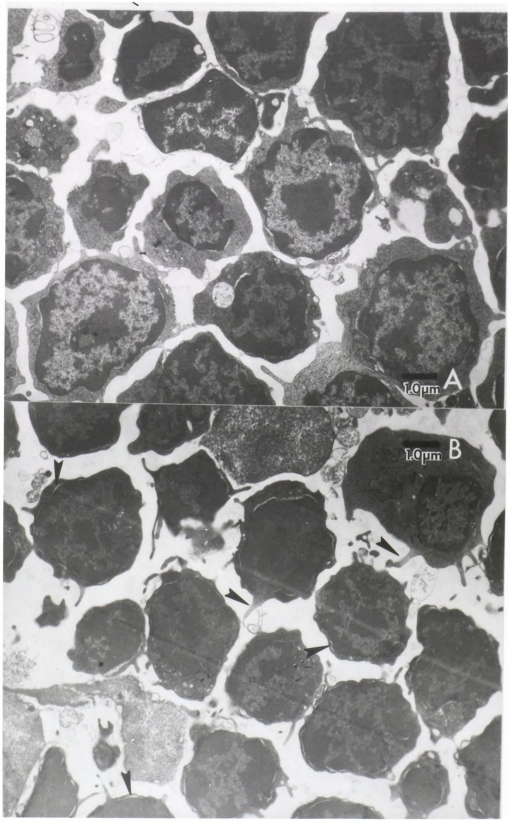

1.0μm A
1.0μm B

Table 10. Effects of 25 ug/Kg TCDD on thymus weight at 2 and 10 days after administration.

	Control		TCDD	
	2 Days	10 Days	2 Days	10 Days
Thymus weight (g)	0.40 ± 0.05(7)[a]	0.53 ± 0.08(6)	0.37 ± 0.01(3)	0.10 ± 0.06(17)*
Thymus as % body weight	0.16 ± 0.03(7)	0.24 ± 0.06(6)	0.14 ± 0.01(3)	0.05 ± 0.03(17)*

[a]Results expressed as mean ± SD for the number of animals in parenthesis. Data analyzed using two tailed students "t" test.
*Significantly different from respective controls at P<.001.

Table 11. The effect of TCDD on various biochemical parameters of thymocytes or thymocyte plasma membrane isolated 10 days after treatment with acetone:corn oil or 25 ug/Kg TCDD.

	Thymocyte		Membrane	
	Control	TCDD	Control	TCDD
Na-K ATPase (nM P_i liberated/mg)	305	385	1575 ± 106(2)[a]	1784 ± 150(2)
Mg ATPase (nM P_i liberated/mg)	410	429	1592 ± 73(2)	1780 ± 143(2)
Ca ATPase (nM P_i liberated/mg)	125	90	ND[b]	ND
EGF binding[c]	115	314	28.2	33.8
Insulin binding[c]	ND	ND	36.5 ± 5(2)	33.1 ± 27(2)
Glucagon binding[c]	ND	ND	13.28	1.33
Glucose uptake (cpm)	5618	3382	ND	ND
Yield of plasma membrane protein (mg)	--	--	4.46 ± 2.88(4)	2.44 ± 1.43(4)

[a]Mean ± SD of the number of animals in parenthesis.
[b]Not determined.
[c]Pg of ^{125}I-EGF, insulin, or glucagon bound/50 ug protein/20 min.

The increased ATPase activity of the plasma membrane compared to whole cells indicate that membrane was indeed isolated. The lack of replicates and large variability makes other conclusions difficult.

DISCUSSION

The thymocyte surface is visibly altered 10 days after treatment with TCDD. The loss of pits in cells from treated animals may indicate changes in gap junctional areas or alterations in membrane regions important for nutrient uptake. If gap junctions are affected, these cells have lost the ability to communicate with each other; if nutrient uptake occurs through these pores, these cells would be nutritionally starved. In either case drastic influences throughout the body may be observed since one of the roles of the thymus is postulated to be trephocytic (Metcalf 1966). Metcalf presents convincing evidence that thymic lymphopoiesis occurs to provide cellular building blocks to tissues throughout the body. Metcalf (1966) and Fisher (1968) explain that under stress and infection T cell derived lymphocytes are released into the bloodstream via thymic dissolution, they are then transported to peripheral areas of the body where disrupted ion occurs and their cellular contents reutilized by tissues in need. Tracing radiolabeled lymphocytes has confirmed this to occur (Metcalf 1966, Russkanen and Kouvalainen 1973, Ernstrom et al. 1973). Therefore, if cellular communication is

inhibited and thymocytes are energy deficient, the severe wasting syndrome produced by TCDD is implicated. It is interesting to note that neonatal thymectomy results in severe body weight loss similar to that produced by TCDD (Parrott 1962, Hess 1968, Faith and Moore 1977).

The bleb formation and pit disappearance induced by TCDD may be due to disturbances in intracellular calcium regulation. Jewell et al. (1982) report the formation of hepatic cellular extensions as a response to changes in extramitochondrial calcium ions and theorize an inability of the hepatocyte cytoskeleton to maintain surface morphology. Increased levels of cellular Ca also obliterate gap junctions and inhibit cellular communication in hepatocytes as shown by Peracchia (1978). Conceivably, these same mechanisms could be occurring in the thymus.

Data presented here indicate a significant loss of thymic tissue after 10 days of treatment but not after 2 days. Biochemical studies are difficult to perform on isolated plasma membrane from this organ after TCDD treatment as little material is available for study. Perhaps lower doses or earlier time points should be used to ascertain biochemical disturbances in membrane parameters related to involution.

Glucocorticoid transport and glucose uptake should be examined in the thymus. Glucocorticoid binding is regulated by Ca and causes the induction of a protein which inhibits

glucose transport and thereby results in thymic involution (Foley et al. 1980, Homo et al. 1980). Perhaps dioxin acts in a similar way; either by altering intracellular Ca, inhibiting glucose transport, or inducing the glucose transport inhibitory protein. Since the thymus contains a very high concentration of the TCDD cytosolic receptor (Carlstedt-Duke 1979, Mason and Okey 1982) effects of TCDD should be able to be demonstrated in this tissue.

CHAPTER V

2,3,7,8-TETRACHLORODIBENZO-P-DIOXIN INHIBITION OF GUINEA PIG ADIPOSE LIPOPROTEIN LIPASE ACTIVITY AS CAUSE FOR SERUM HYPERTRIGLYCERIDEMIA

INTRODUCTION

TCDD is unique in that it produces differential responses in different animals and different tissues. The guinea pig has been shown to be the most sensitive mammalian species to this dioxin (Gupta et al. 1973), but its response is unusual because the liver shows very little pathology. Data presented in Chapter II confirm this observation. Gupta et al. (1973) could find neither, proliferation of endoplasmic reticulum, increased fat deposition, nor edema formation as had been observed in rats. No induction of microsomal enzymes, arylhydrocarbon hydroxylase, or ALA synthetase has been reported (Neal et al. 1979). Therefore, these hepatic biochemical pathways appear not to perform a major role in TCDD's toxicity in this species.

Gasiewicz and Neal (1979) and Swift et al. (1981) observed one of the most significant toxic manifestations in guinea pigs dosed with TCDD to be serum hyperlipedemia. Serum triglyceride and cholesterol concentrations began to increase as soon as 3 days after treatment. Hypertriglyceridemia is a major factor in coronary heart disease and

atherosclerosis (Sirtori et al. 1975, Hulley et al. 1980, Cabin and Roberts 1982) and may therefore have a great impact on TCDD treated laboratory animals. In fact, preatherosclerotic lesions appear in aortic vessels of rabbits as soon as 20 days after treatment with TCDD (D. Bombick, unpublished observation).

Injections of heparin containing blood were found to abolish severe lipemia in dogs (Hahn 1943). This observation and other studies (Weld 1944, Anderson and Fawcett 1951) implied that lipemia is reversed by the release of some "factor" into the serum upon administration of heparin (Hamosh and Hamosh 1983). Korn (1955) demonstrated this factor to be an enzyme which hydrolyzed serum triglycerides. Subsequent studies showed this enzyme to be synthesized in extrahepatic tissue (especially adipose), secreted into the blood, and attached to capillary endothelial cells where its function is the catabolism of triglyceride carrying lipoproteins (see Robinson 1963a, Garfinkel et al. 1967, Hamosh and Hamosh 1983). For a comprehensive review of lipid metabolism see Nilsson-Ehle et al. 1980, Brown et al. 1981, Oscai and Palmer 1983, and Brown and Goldstein 1984.

Because adipose LPL has a critical role in controlling serum triglyceride concentration and adipose uptake of free fatty acids the effect of TCDD on this enzyme was examined in the guinea pig. This is the most sensitive animal to

TCDD in terms of lethality, body weight loss, and serum hypertriglyceridemia.

MATERIALS AND METHODS

Young (200-225g) male Albino English Shorthair Guinea Pigs (Strain MDH:SR(A)) were purchased from the Michigan Department of Health and male Sprague Dawley Rats were obtained from Harlan Animals, Haslett, Michigan. Animals were dosed once intraperitoneally (ip) with either TCDD (obtained as a gift from Dow Chemical Company, Midland, Michigan -- purity >99.99%) dissolved in acetone:corn oil (1:9) or vehicle alone. Animals were housed in suspended stainless steel cages and given food (Purina Guinea Pig or Rodent Chow) and water (supplemented with ascorbic acid) ad libitum. Where pair-fed studies were conducted the control animals received only the amount of food consumed by the TCDD treated animals. At the indicated time periods animals were anesthetized with CO_2 and blood was collected via cardiac puncture. The blood was allowed to coagulate, then serum was collected after spinning 15' at 8000 g and stored at -70°C until use.

Preparation of Acetone/Ether Fat Powders

Abdominal and perirenal fat tissue was removed, washed with cold 0.85% NaCl, and extracted with acetone and ether according to Robinson (1963b) except, the tissue was ground in 20 volumes of cold acetone then extracted with 5 volumes

of room temperature acetone followed by 2 volumes of ether. The parchment like filtrate was air dried and stored in a dessicator at -20°C until use.

LPL Assay

The enzyme source was extracted out of the fat powder, and lipoprotein lipase activity was measured with the procedure described by Nilsson-Ehle and Schotz (1976). Serum triglyceride concentrations were determined with the colorimetric procedure set forth in Sigma Chemical Company Bulletin #405.

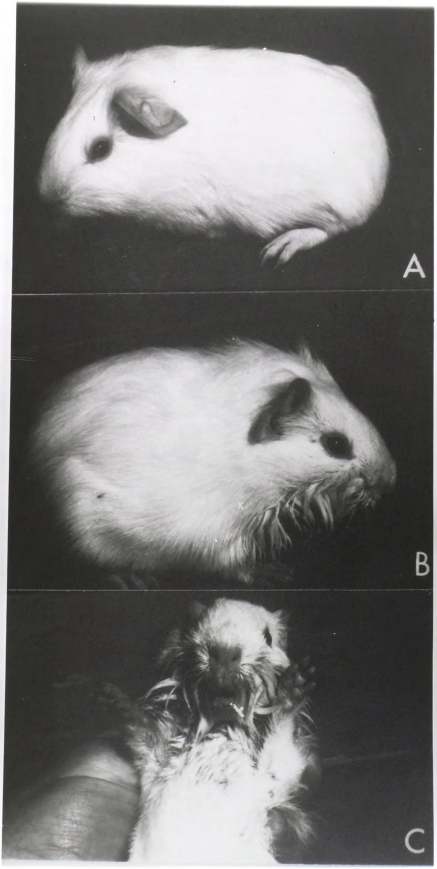
Glycerol tri [9,10(N)-^{3}H] oleate (1 Ci/mol) was purchased from Amersham, Arlington Heights, Illinois. All other reagents were of analytical grade and purchased from Sigma Chemical Corporation, St. Louis, Missouri. Data are expressed as mean $\pm$ SD for at least 5 animals or as indicated. Statistical analysis was performed using either 1 way or factorial ANOVA and means compared with either Dunnett's or Tukey's test for unconfounded comparisons. In all cases $P<.01$.

RESULTS

Wasting and Slobbering

TCDD caused the typical wasting syndrome and loss of body weight as described previously. As seen in Figure 20

Figure 20. A. Typical appearance of a control and (B) a 1 ug/kg TCDD treated guinea pig 10 days after administration. C. Severe slobbering effect induced by TCDD.


A
B
C

these animals also exhibited abnormal posture, pilo-erection, hair loss, vasodilation of the extremities, and a severe drooling or slobbering effect.

Time and Dose Changes of LPL Activity and Serum Triglyceride Concentration

Ten days after treatment with 1 ug/kg TCDD, adipose LPL activity was only 18% that of control animals (Table 12). Concurrently, serum triglyceride levels were almost tripled. Body weight and adipose weight were 67 and 31% of control weights respectively. After 20 days of treatment very little adipose tissue remained in the TCDD treated animals compared to the pair-fed control (Figure 21).

These changes were both dose and time dependent. Doses greater than 0.5 ug/kg caused significant losses in LPL activity resulting in an ED_{50} of 0.68 ± 0.06 ug/kg at 10 days after treatment (Figure 22, Table 13). The ED_{50} for loss of adipose weight was approximately 0.5 ug/kg, however for loss of body weight it was 3.27 ± 0.27 ug/kg. Enzymatic activity was suppressed as soon as 1 day after treatment when body weight gain began to decline and serum triglyceride levels began to rise (Figures 23-25).

As shown in Figure 24 TCDD caused a severe loss of body weight beginning one day after a single i.p. administration. Because adipose LPL activity decreases upon starvation (Nilsson-Ehle et al. 1980, Hamosh and Hamosh

Table 12. Adipose LPL activity, serum triglyceride concentration, body weight, and adipose weight in *ad lib* control, TCDD treated, and pair-fed control guinea pigs 10 days after treatment with either acetone: corn oil or 1 ug/kg TCDD.

	Treatment		
	Ad lib Control	TCDD	Pair-fed Control
LPL Activity[a]	2189.8 ± 259.1(8)[c,d]	391.9 ± 251.4(11)*	615.5 ± 123.9(5)*
+ Oral glucose[b]	ND	376.9 ± 71.5(4)**	1635.8 ± 558.6(4)
Serum Triglyceride (mg/dl)	84 ± 38(4)	232 ± 79(6)*,**	29 ± 14(9)*
Body Weight (% initial)	142 ± 5(10)	95 ± 8(11)*,**	105 ± 3(5)*
Adipose Weight (g)	3.0 ± 1.2(20)	1.1 ± 0.7(29)*	1.0 ± 0.6(15)*

[a] nmoles ^{3}H oleic acid/mg extracted fat powder/hr.
[b] Glucose was given orally (2.7 ml of 75% solution) then animals sacrificed 2 hours later.
[c] LPL activity at 0 days was 1975.8 ± 113.9(5) nmoles free fatty acid/mg extracted fat powder/hr.
[d] Results are expressed as the mean ± standard deviation for (n) animals. Data was analyzed with Tukey's test for unconfounded comparisons after factorial ANOVA.
* Statistically different from *ad lib* control, $P < .01$.
** Statistically different from pair-fed control, $P < .01$.

Figure 21. Abdominal dissection of (A) pair-fed control and (B) TCDD treated guinea pig, (20 days after 1 ug/kg single i.p. dose). Note severe loss of perirenal and abdominal body fat (arrowhead) in the treated animal. C-descending colon, F-fat, K-kidney.

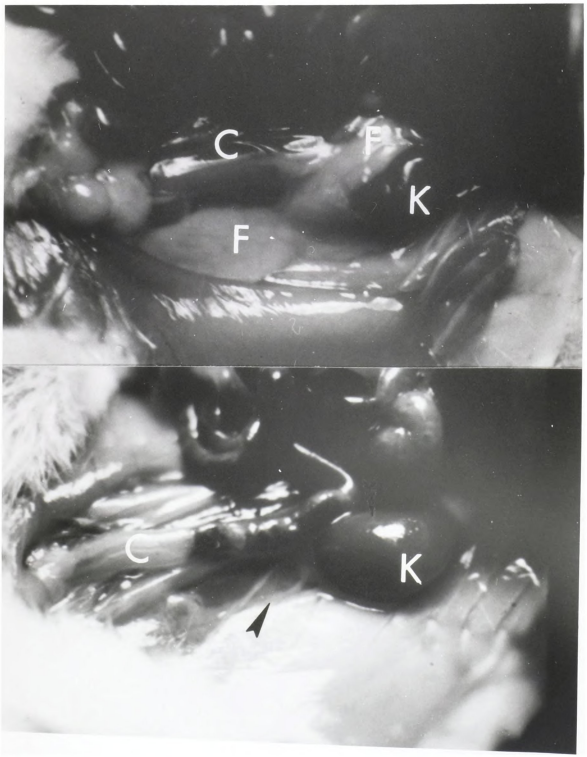

C
F
K
F
C
K

Figure 22. Dose response relationship of LPL activity (●) and body weight (▲) (as % control) 10 days after a single i.p. dose of TCDD. [a]Statistically different from control LPL activity or [b]control body weight at P <.01.

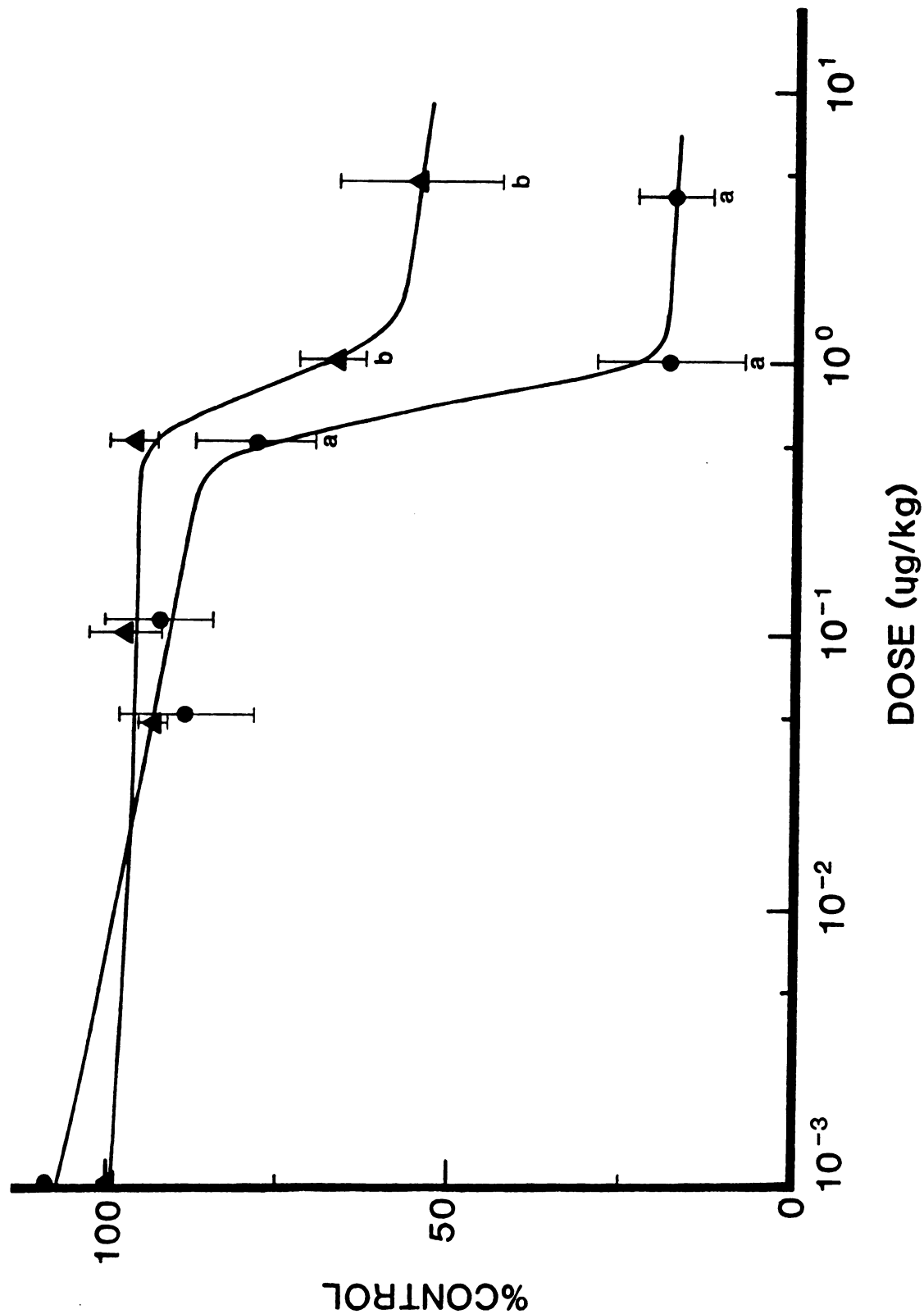

%CONTROL
100
50
0
10^{-3}
10^{-2}
10^{-1}
10^{0}
10^{1}
DOSE (ug/kg)
a
b
a
b

Table 13. Dose response relationship of LPL activity, serum triglyceride concentration, body weight, and adipose weight 10 days after TCDD administration.

Dose (ug/kg)	LPL Activity (nmole FFA/mg extracted fat/hr)	Serum Triglyceride (mg/dl)	Body Weight (% initial)	Adipose Weight (grams)
0	2189.8 ± 259.1(8)[a]	84 ± 38(4)	142 ± 5(10)	3.15 ± 1.3(16)
0.001	2367.0 ± 44.9(4)	ND[b]	136 ± 6(4)	3.6 ± 0.7(4)
0.05	1927.6 ± 219.5(3)	ND	129 ± 14(3)	3.6 ± 1.4(3)
0.10	2005.5 ± 169.5(4)	58 ± 10(5)	133 ± 6(4)	2.8 ± 0.9(4)
0.5	1698.5 ± 195.8(8)*	71 ± 6(3)	132 ± 5(8)	1.6 ± 0.1(6)*
1.0	391.9 ± 251.4(11)*	232 ± 79(6)*	93 ± 12(11)*	1.0 ± 0.8(25)*
4.0	369.9 ± 141.6(4)*	ND	74 ± 15(4)*	0.9 ± 0.8(4)*

[a]Variability expressed as mean ± standard deviation for (n) animals. Data analyzed by Dunnetts test after ANOVA.
*Statistically different from control (0 ug/kg) at $P < .01$.
[b]Not determined.

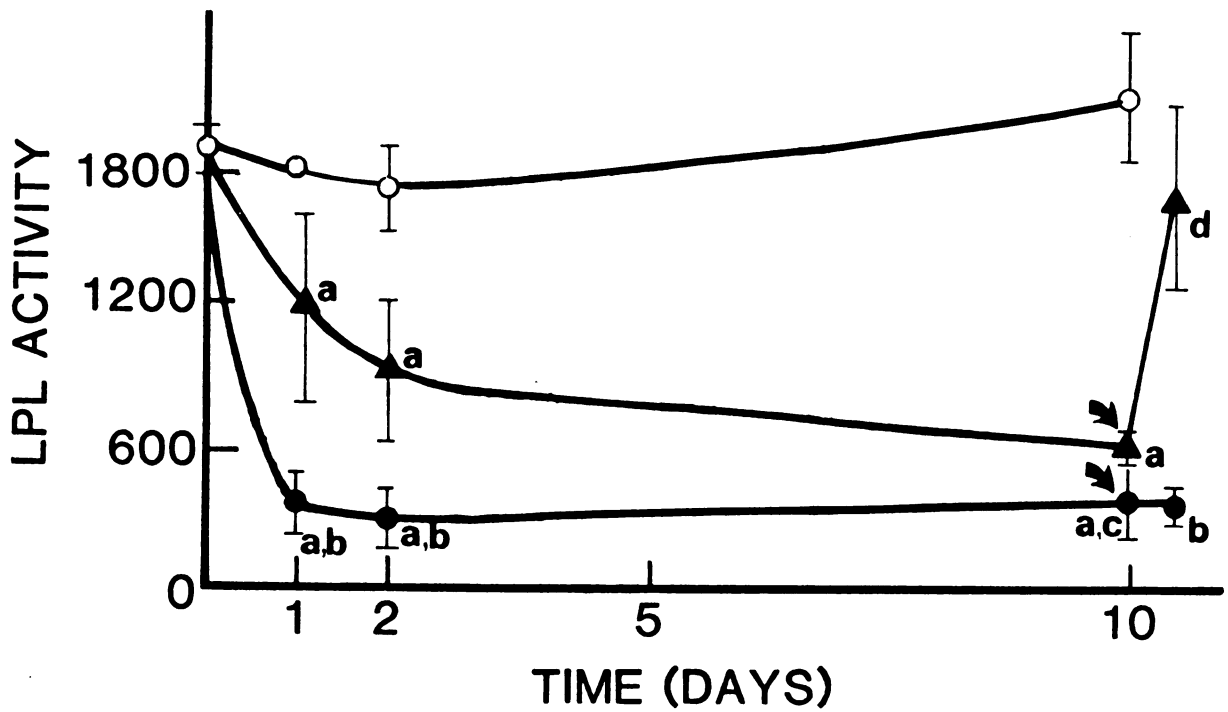
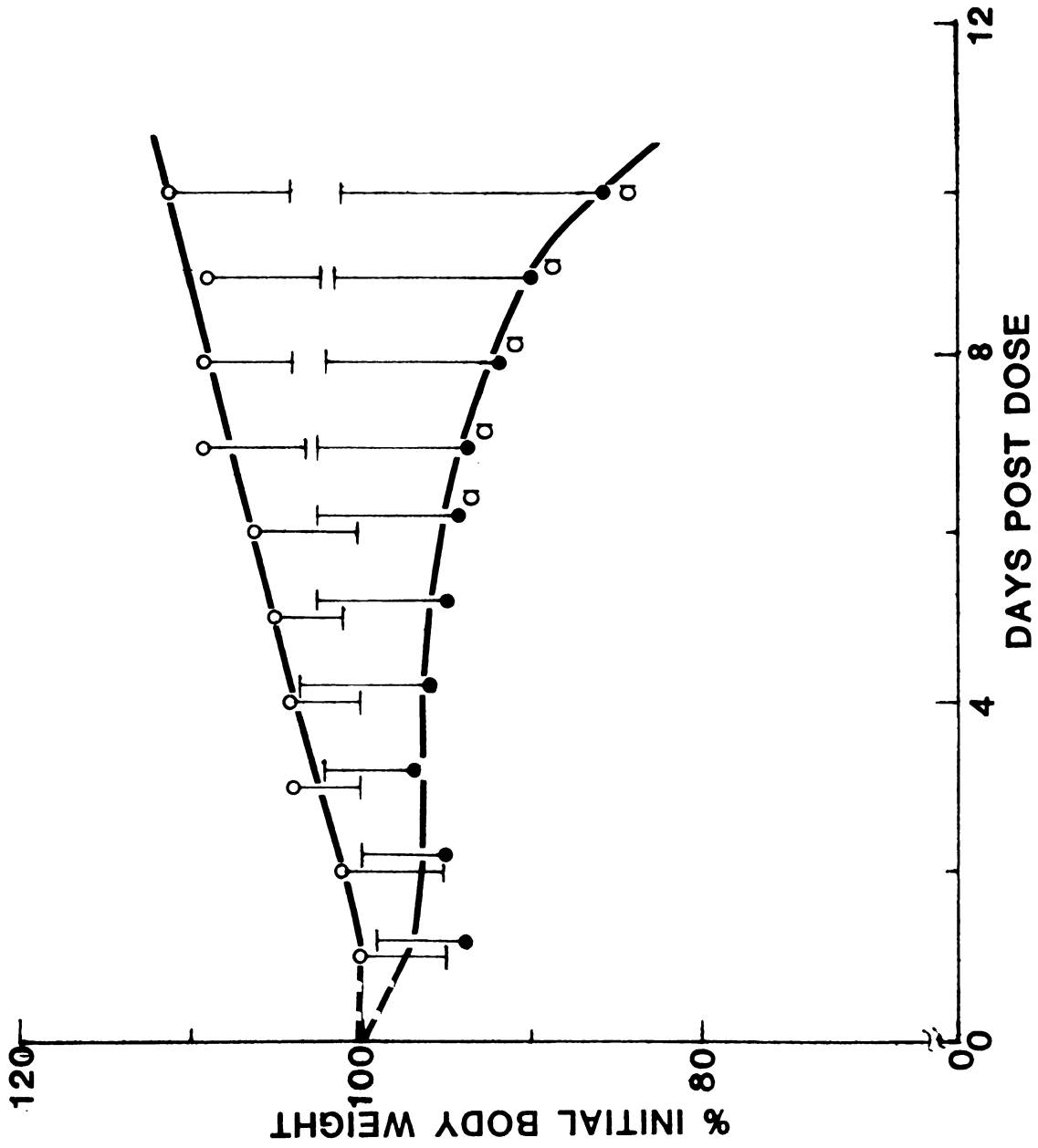


Figure 23. Adipose LPL activity (nM ^{3}H oleic acid released/mg extracted powder/hr) at 0,1,2, and 10 days in TCDD treated (●), ad lib. control (○), or pair fed control (▲) guinea pigs. On day 10, 2.7 ml of a 75% glucose was orally administered and enzyme activity measured 2 hours later (see arrows). [a]Significantly different from ad lib controls; [b]Significantly different from pair-fed control; [c]Not significantly different from pair-fed control; [d]Significantly different from 10 day pair-fed control, using Tukey's test for unconfounded comparisons (P =.01).

Figure 24. Time course of changes in body weight (as % initial) TCDD treated (●), or pair-fed control guinea pigs. [a]Significantly different from pair-fed control, after ANOVA and Dunnett's test.


% INITIAL BODY WEIGHT
120
100
80
0
4
8
12
DAYS POST DOSE
a
a
a
a
a

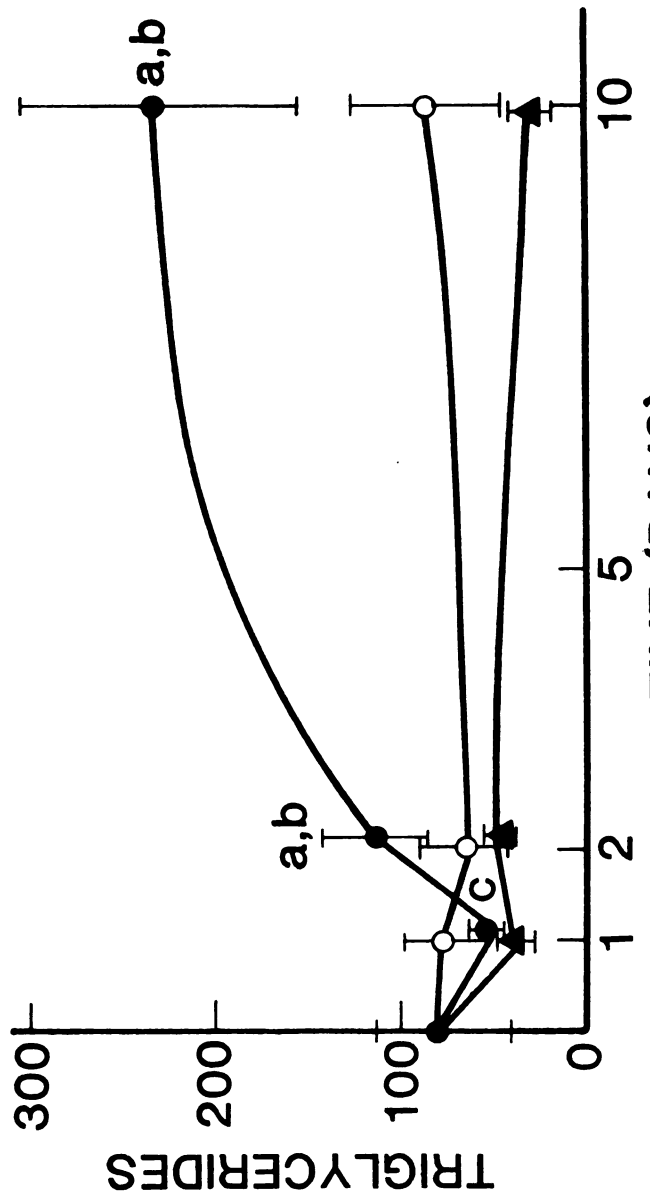


Figure 25. Time course of changes in serum triglyceride concentration (mg/dl) of ad lib (○), TCDD treated (●), or pair-fed control guinea pigs. Designations are as in Figure 23.

1983) the effect of pair-feeding on these parameters was assessed. After 10 days, enzymatic activity in the pair-fed animals was significantly less than ad lib animals as expected, and no difference existed between TCDD treated and pair-fed guinea pigs (Table 12, Figure 23). Serum triglyceride levels had increased 8 fold and body weight had decreased by 10% (Figure 25) compared to pair-fed animals. It was observed that pair-fed control LPL activity decreased at a much slower rate than did the TCDD treated (Figure 23), since after 1 day the treated animals had already fallen 83% while the pair-fed only 39% relative to 0 day control levels. At the same time serum triglyceride levels began to rise dramatically in the TCDD animals but not in the pair-fed controls.

Glucose Effect on LPL Activity

Large doses of glucose in vivo are known to reverse the starvation induced depression of adipose LPL by stimulating synthesis within the cell (Cryer et al. 1974, 1976). Therefore, if reduced food intake was causing the decline of activity in the TCDD treated animals, the effect should be able to be reversed by the administration of glucose. No such effect was seen. Although the pair-fed animals were greatly stimulated to near ad lib control levels, no change was noted in the treated animals (Table 12, Figure 23).

Serum Apoprotein Effect on LPL

It is well known that LPL is stimulated by some serum apolipoproteins (Apo C-II in rats) and inhibited by others (Brown et al. 1981, Hamosh and Hamosh 1983). To assess whether the TCDD induced decline of LPL resulted from alterations in these serum factors, enzymatic activity was measured using various combinations of control, TCDD treated, and pair-fed control guinea pig or rat serum as the activation source (Table 14). LPL is defined as an enzyme activated by APO-CII and inhibited by high ionic concentrations (Hamosh and Hamosh 1983). Data in Table 14 clearly indicated that adipose LPL was being measured since enzyme from control, TCDD, or pair-fed animals was markedly increased by the addition of rat serum (compare lines 1 and 2 of Table 14) but significantly inhibited by 1.0 M NaCl. Guinea pig serum alone contained no stimulatory factor in agreement with Fitzharris et al. (1981). Serum from TCDD treated guinea pigs (10 day 1 ug/kg) stimulated activity from control, and pair fed control but not from treated guinea pigs. Because serum from treated animals has high levels of triglyceride carrying lipoproteins such as VLDL and chylomicrons, this stimulation was not unexpected since the apoproteins of these lipoproteins can activate LPL. Enzymatic activity of fat powder from TCDD treated animals could not be stimulated using any serum combination, (compared to both controls) nor was control activity

Table 14. Effects of serum factors from ad lib control, TCDD treated, and pair-fed control guinea pigs and rats on triglyceride hydrolyzing capability of control, TCDD treated, and pair-fed control guinea pig LPL.

Activator[b]	Guinea Pig LPL Activity[a] (nM ^{3}H oleic acid/mg extracted fat powder/hr)		
	Ad lib Control	TCDD	Pair-fed Control
CGP (basal level)	151.0 ± 34.8(15)[c]	63.7 ± 23.6(11)	53.1 ± 31.9(10)
CGP + CR	2362.3 ± 263.1(8)	444.1 ± 245.5(12)	664.1 ± 92.8(4)
CGP + CR + NaCl[d]	54.4 ± 20.8(8)	13.2 ± 4.6(3)	42.5 ± 21.9(6)
CGP + TR	2802.7 ± 214.7(4)	508.9 ± 288.8(5)	638.2 ± 60.9(3)
CGP + PFR	2894.5 ± 183.0(4)	354.8 ± 110.3(5)	821.6 ± 52.1(3)
TGP	1026.9 ± 272.7(4)	119.9 ± 91.3(7)	314.9 ± 53.1(3)
TGP + CR	2371.9 ± 225.0(4)	657.4 ± 138.0(4)	820.0 ± 141.0(3)
TGP + TR	2436.2 ± 263.1(4)	145.7 ± 13.1(3)	570.2 ± 68.9(3)
TGP + PFR	2658.9 ± 148.9(4)	236.6 ± 90.4(3)	ND
PFGP	292.5 ± 47.7(4)	ND[e]	ND
PFGP + CR	2376.6 ± 125.2(4)	ND	ND
CR	2378.6 ± 125.2(4)	592.5 ± 126.1(6)	858.4 ± 81.0(3)
TR	2458.7 ± 138.7(4)	470.2 ± 198.3(4)	554.2 ± 47.7(3)

[a] Ad lib fed control, 1 ug/kg TCDD treated, or pair-fed controls 10 days after a single ip dose.
[b] CGP = control guinea pig serum; CR = control rat serum; TR = 10 day 25 ug/kg TCDD rat serum; PFR = 10 day pair-fed (to TR) rat serum; TGP = 10 day 1 ug/kg guinea pig serum; PFGP = 10 day pair-fed guinea pig serum (to TGP).
[c] Results expressed as the mean ± standard deviation for (n) animals.
[d] Final concentration 1.0 M NaCl.
[e] Not determined.

inhibited by treated serum. Therefore the changes in LPL activity are not due to alterations in serum factors.

Regression analysis of LPL activity on body weight revealed a coefficient of determination of 0.82 (Figure 26). Therefore 82% of the variance in body weight is explained by variance in LPL activity.

DISCUSSION

TCDD administration to guinea pigs produced the typical wasting effect described for other laboratory animals. It aso caused a severe drooling or slobbering effect. This slobbering effect, or "slobbers" as discussed by Navia and Hunt (1976) is normally due to abnormal wearing of the incisor teeth thereby allowing the teeth to overgrow and prevent normal eating and swallowing functions. As a result the animal usually starves to death. If TCDD does indeed act through the epidermal growth factor receptor as discussed by Madhukar et al. (1984) quite possibly alterations are occurring in dental growth to cause abnormalities in swallowing and drinking. TCDD significantly increases the time to incisor eruption in mouse neonates thereby mirroring the same effect caused by exogenous administration of EGF (Madhukar et al. 1984). Quite possibly, this dioxin also has toxic manifestations leading to "slobbers". TCDD also caused a time and dose dependent depression of adipose LPL activity resulting in

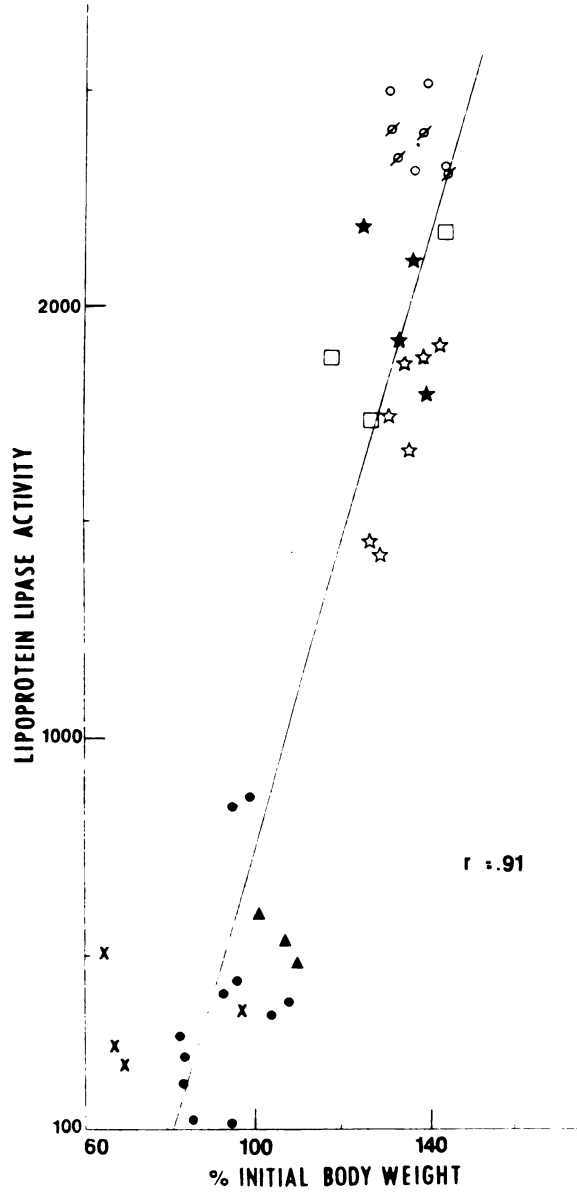


Figure 26. Relationship of body weight and adipose LPL activity. 10 day ad lib control (o), 10 day control pair-fed to 1 ug/kg TCDD (▲), 10 day TCDD treated at 0.001 (ø), 0.05 (□), 0.1 (★), 0.5 (☆), 1.0 (●), and 4.0 (x) ug/kg TCDD.

the severe serum hypertriglceridemia seen in this species. Associated with this loss of enzymatic activity was inhibition of body weight gain and loss of adipose tissue. Pair-feeding had similar effects on body weight gain and adipose tissue loss. Restricted food intake however, was not entirely responsible for loss of LPL activity since pair-fed animals showed a slower decline of activity compared to TCDD treated and were able to be reversed after 10 days by a large oral administration of glucose. Serum from ad lib, pair-fed, and TCDD treated rats showed little difference in their abilities to activate enzymatic activity, therefore inhibitory serum factors (i.e. apoproteins) seemed not to be involved. Neal et al. (1979) concluded that there was no generalized impairment of glucose absorption from the intestine of guinea pigs 7 days after treatment with 2 ug/kg TCDD. Seefeld and Peterson (1984) also concluded that TCDD had little influence on intestinal absorption. Therefore it seems likely serum levels in the treated animals contained adequate glucose concentration to induce LPL activity.

Serum triglycerides did not increase in the pair-fed animals because functional enzyme was still being produced by the adipocyte. The pair-fed control activity as measured by this assay was low because only the active pool within the adipocyte was assayed. LPL is synthesized as an inactive proenzyme then activated via post translational

modifications before or during secretion from the cell (Nilsson-Ehle et al. 1976, Ashby et al. 1978, Borensztajn 1979). This inactive pool can be activated by glycosylation (Hamosh and Hamosh 1983) as seen with the 10 day pair-fed control animals (glucose also increases de novo synthesis of enzyme). Apparently no reserve enzyme pool is present in the treated animals.

Cause for Loss of Adipose Tissue

At least 5 possibilities exist for the cause of adipose tissue loss: (a) increased energy expenditure (b) decreased intestinal nutrient absorption (c) decreased production of hepatic and intestinal serum triglyceride-carrying lipoproteins, (d) increased hormone sensitive lipase (HSL) activity leading to lipolysis, and (e) decreased LPL activity and failure to deliver free fatty acids from the endothelial cells to the adipocytes.

Neal et al. (1979) and Cunningham and Williams (1972) have indicated no affect of TCDD on overall energy production or consumption in experimental animals. TCDD did not alter $^{14}CO_2$ production from radiolabelled glucose, oleate, or alanine, nor did it change fatty acid compositions, ATP levels, pyridine nucleotides, or their ratios in guinea pigs. Lucier et al. (1973) also found hepatic mitochondrial respiratory activity and subsequent ATP synthesis to be the same as controls. Consequently increased energy expenditure can not account for fat

losses. Seefeld and Peterson (1984) concluded as did Neal et al. (1979) malabsorption of intestinal nutrients did not occur upon treatment with TCDD, because fecal energy loss (as a percentage of food intake) was no different between control and treated animals. It appears likely then, that nutrient depletion is not the cause for loss of adipose. Since TCDD increases serum concentrations of very low density lipoproteins and triglycerides, fat deposition should increase as more storage is necessary, therefore the third possibility can be eliminated. Increased lipolysis could account for the loss of adipose tissue since serum free fatty acid concentration is controlled by HSL through a feedback system coupled to LPL activity (Patten 1970). However, Swift et al. (1981) found no difference in serum fatty acid concentration between pair-fed control and treated guinea pigs. As a result, only decreased LPL activity and failure to deliver free fatty acids from endothelial cells to adipocytes can explain the loss of adipose tissue exhibited by TCDD treated guinea pigs.

LPL and Serum Triglycerides

There is little doubt that LPL controls serum triglyceride concentration thereby affecting fat storage (Robinson 1963a, Garfinkel et al. 1967, Robinson 1970, Borensztajn 1979, Nilsson-Ehle et al. 1980). Injection of antiserum against LPL totally blocks serum triglyceride removal from chickens in vivo (Kompiang et al. 1976). There

is also good evidence that reduction of LPL levels is directly correlated to hyperlipidemia in humans, e.g. among untreated diabetes and Type I familial hyperlipoproteinemia patients (Huttunen and Lindquist 1973, Fredrickson et al. 1978, Walker and Martin 1979). Therefore, reduction in adipose LPL activity upon TCDD administration is responsible for serum hypertriglyceridemia and the loss of fat storage in adipose tissue. TCDD-induced "wasting syndrome" has always been observed to accompany loss of adipose tissue, and present data indicate that in these animals very little adipose tissue remained 20 days after 1 ug/kg TCDD. This loss of fat confirms results found by Swift et al. (1981).

LPL is known to be synthesized in adipocytes and delivered to the luminal surface of the endothelial cells, where it is attached through a receptor (heparan sulfate). The quantity of LPL at this site is determined by a feedback system with serum insulin levels being the main determinant of the rate of de novo synthesis of LPL in adipocytes (Nilsson-Ehle et al. 1980, Hamosh and Hamosh 1983). The failure of LPL from the TCDD-treated animals to respond to high levels of serum triglycerides or to exogenously added glucose in vivo, as contrasted to LPL from pair-fed animals, indicates that its de novo synthetic process may be inhibited. This could be from an impairment of the above feedback system. The reduction of LPL is not likely to be

caused by the direct interaction of TCDD with the enzyme itself as judged by the qualitative and quantitative similarities of LPL from treated and pair-fed animals (both at day 10, Table 14) and by analogy with the well studied case of the TCDD-caused reductions of membrane ATPases (Matsumura 1983, Matsumura et al. 1984). Since LPL at the endothelial site has a very short halflife (Nilsson-Ehle et al. 1980, Hamosh and Hamosh 1983), the inability of the adipocyte to supply fresh LPL in the treated animals makes them incapable of adapting to nutritional changes and needs.

Two major questions arising from this study are whether this conclusion is applicable to other animals and whether this phenomenon is causally related to the lethal action of TCDD. It is well known that patterns of serum lipoprotein metabolism are very different among mammalian species (Brown et al. 1981). Therefore, one must be cautious in drawing a direct analogy to other animal species. However, wherever serum hypertriglyceridemia is observed as a result of TCDD administration, one could at least suspect a reduction in adipose LPL activity as one of the causes.

For instance, elevated serum triglycerides, phospholipids, and cholesterol have been frequently reported in humans (particularly among industrial workers) with a history of exposure to chlorinated dioxin-containing products (Oliver 1975, Walker and Martin 1979, Zack and

Suskind 1980, Pazderova-Vejlupkova et al. 1981). While such epidemiological data are often difficult to interpret, results of our current study and the similarities of human hypertriglyceridemia to that of the guinea pig (Huttunen and Lindquist 1973, Fredrickson et al. 1978) suggest the possibility of LPL reduction being the cause for these problems.

It is premature to conclude that loss of LPL per se causes death in the guinea pig as starvation itself is not expected to lead to death under these experimental conditions. However, there are additional factors to be considered. First, adipose LPL of treated animals is irreversibly reduced, as judged by the lack of effect of glucose, indicating that the affected animals have lost the ability to adapt to nutritional changes. Second, the serum of the treated animals contained very high titers of triglyceride carrying lipoproteins, while starved animals did not. Third, there was a concomitant increase in cholesteryl ester carrying lipoproteins in the treated guinea pigs probably as a result of reduction in LDL receptor activities on the plasma membrane of the hepatocyte (Bombick et al. 1984). The combined actions of these lesions could conceivably cause various secondary effects such as arteriosclerosis, hemorrhages, xanthoma and lipemia (Greten et al. 1976) which can be detrimental to the affected animals. In fact, in the above glucose test we

have observed that 4g of glucose/animal (instead of 2g/animal as shown in Figure 23) killed all TCDD-treated guinea pigs (4 out of 4) within 2 hrs. None of the pair-fed animals died. The cause of death was probably glucose shock. Such an effect illustrates the overall inability of the treated guinea pigs to readjust to nutritional changes.

Conclusions

The following mechanism of action is proposed: TCDD causes inhibition of adipose LPL, by some as of yet unknown mechanism, which results in decreased serum triglyceride breakdown (from both dietary sources via chylomicra and hepatic synthesized sources via very low density lipoproteins). Consequently, serum levels of triglycerides increase and levels of free fatty acids being stored by fat decrease. Lipolysis proceeds normally via HSL utilizing previously stored fatty acids and resulting in loss of adipose stores. In the meantime, since serum levels of triglycerides and fatty acids are elevated, normal feedback mechanisms may signal a reduction of food intake thereby contributing to body weight loss. After 20-25 days when adipose stores are exhausted protein catabolism begins, which coupled with secondary effects from hypertriglyceride-mia and hypercholesteronemia could conceivably alter critical biochemical mechanisms and lead to death. This energy depletion is similar to that proposed by McConnell et al. (1978b). LPL is important in providing fatty acids to

muscle cells for energy production (Hamosh and Hamosh 1983). If this inhibition by TCDD occurs throughout the body one could then hypothesize severe muscular effects, such as protein catabolism and energy loss taking place. Future research should focus on the long term effects of TCDD with respect to both adipose and muscle LPL.

Serum concentrations of insulin and glucose will be examined in an effort to determine why LPL activity is decreased by TCDD. Secretion of active enzyme will be decreased if serum insulin is depressed, the adipocyte insulin receptor is altered, or glucose transport across the adipocyte plasma membrane is inhibited.

CHAPTER VI

DIFFERENTIAL SPECIES RESPONSE OF ADIPOSE LIPOPROTEIN LIPASE TO 2,3,7,8 TETRACHLORODIBENZO-P-DIOXIN

INTRODUCTION

Serum triglyceride concentrations have been reported to be unchanged in the rat (Albro et al. 1978) and hamster (Olson et al. 1980), but significantly increased in the rabbit (Lovati et al. 1984) after acute administration of TCDD. If adipose LPL is responsible for controlling serum triglyceride levels, these changes should then reflect alterations in this enzyme's activity. Dioxin is unique in having a wide differential species response (both in lethality and symptomology) therefore it would not be surprising if the response of fat LPL was different in these animals. It has already been demonstrated that TCDD causes a tremendous differential species response of hepatic plasma membrane bound receptors and enzymes, in terms of binding and enzymatic activity.

Little data exists on serum parameters in TCDD treated mice. Poland and Glover (1980) have clearly shown that TCDD toxicity (i.e. cleft palate formation and thymic involution) segregates with the genetic locus responsible for the aryl hydrocarbon hydroxylase (AHH) activity. Nebert et al. (1972) had previously demonstrated that exposure to

polycyclic aromatic hydrocarbons caused induction of AHH activity in certain inbred strains of mice but not others. These strains are termed responsive and nonresponsive, respectively. Poland et al. (1974) showed both classes of mice to be inducible by TCDD but variable in their sensitivity of induction. This induction was found to result from TCDD binding to either a high affinity cytosolic receptor in the responsive mice or a low affinity receptor in the nonresponsive mice strains (Poland and Glover 1976). They postulated the low affinity receptor to occur from a mutation which produced an altered receptor with diminished binding for polycyclic aromatic hydrocarbons. Therefore one of the aims of this study was to ascertain whether serum hyperlipedemia follows this same pattern in these species.

MATERIALS AND METHODS

Male Sprague-Dawley Rats (100-125 or; 225-250g) were obtained from Harlan Animals, Haslett, MI; mice (18-25g) from the Jackson Laboratory, Bar Harbor, Maine; English shorthair Guinea Pigs (200-250g), White Albino Rabbits (1500-3500g), and Golden Syrian Hamsters (75-100g) from the Michigan Department of Health. Mice strains termed AHH responsive (high affinity TCDD receptor) were: C57BL/6J, A/J, BALB/c, SEC/1ReJ, and CBA/J. Non-responsive strains (low affinity receptor) included: AKR/J, RF/J, DBA/2J, SWR/J, and 129/J. Animals were housed as described

previously, given a single dose i.p. of TCDD or vehicle alone, and sacrificed 2 or 10 days later by anesthesia.

The methods of serum and fat collection, preparation of fat powder, measurement of LPL activity and serum triglyceride concentrations were as before. Serum cholesterol and protein were measured using the methods of Carr and Drekter (1956) and Lowrey et al. (1951), respectively. Data are presented as the mean $\pm$ standard deviation for the number of animals indicated. Statistical analysis was performed with the 2 tailed student's "t" test or ANOVA; P was always $<.05$.

RESULTS

Rabbit

Adipose LPL activity was reduced and concurrent serum triglyceride concentrations increased, in the guinea pig and rabbit, in a dose dependent fashion 10 days after TCDD treatment (Table 15). These data indicate the rabbit to be even more sensitive than the guinea pig in terms of LPL inhibition since the rabbit at 1 ug/kg (115 x less than the reported LD_{50} by Schwetz et al. 1973) showed a 44% decline compared to pair-fed controls. The guinea pig showed no such inhibition until an approximate LD_{50} dose was administered (see Table 15). One can conclude that, at least at 1 ug/kg, the rabbit is as sensitive to TCDD as the guinea pig, if not more so. Rabbit serum triglyceride levels

Table 15. Adipose LPL activity and serum triglyceride concentration (TG) of the guinea pig, rat, rabbit, and hamster 10 days after the indicated dose of TCDD.

Species	Dose (ug/kg)	Adipose LPL (nM free fatty acid/mg/hr)	TG (mg/dl)
Guinea Pig	0	2189.8 ± 259.1(8)[a]	84 ± 38(4)
	0 (PF to 1)[b]	376.9 ± 71.5(4)*	29 ± 14(9)*
	1	391.9 ± 251.4(11)*	232 ± 14(9)*,**
Rat (young)[b]	0	1204.8 ± 43.6(4)	59 ± 19(6)
	25	1665.7 ± 92.0(4)*	64 ± 32(6)
Rat (old)[c]	0	1694.2 ± 388.9(4)	83 ± 28(3)
	25	1626.4 ± 231.1(4)	90 ± 53(2)
Rabbit	0	463.2 ± 92.2(3)	92 ± 56(5)
	0 (PF to 1)	400.1 ± 124.0(3)	90 ± 54(3)
	1	224.5 ± 197.3(3)*	203 ± 30(4)*,**
	0 (PF to 50)	242.4 ± 168.9(8)*	77 ± 12(5)
	50	52.8 ± 19.0(3)*,**	240 ± 101(6)*,**
Hamster	0	1643.9 ± 181.2(3)	126 ± 63(4)
	25	1444.9 ± 294.5(4)	92 ± 45(3)
	0 (PF to 1157)	1684.0 ± 178.2(3)	85 ± 51(5)
	1157	780.9 ± 366.1(4)*,**	220 ± 112(4)

[a]Data expressed as mean ± standard deviation of (n) animals and analyzed with Dunnetts test after ANOVA.
* Statistically different from control, P <.01.
** Statistically different from pair-fed control, P <.01.
[b]PF = pair-fed to indicated dose.
[c]Young rats, 108-121g.
[d]Old rats, 222-243g.

increased 125% at 1 ug/kg and 211% at 50 ug/kg compared to the respective pair-fed control levels (Table 15).

Serum from the higher dosed rabbits was yellow in color and very milky. This is indicative of high lipid concentrations. Serum cholesterol and triglyceride was increased 1.7x and 3.1x respectively but no change was observed in serum protein concentration (Table 16).

Hamster

LPL activity of the hamster declined 12% at the lowest dose tested but was 46% of pair-fed control levels at the LD_{50} dose. The decline of activity in both the hamster and rabbit is not due to depressed food intake as treated animals in both species showed activity significantly below that of pair-fed control levels. Pair-feeding had no effect on hamster LPL compared to ad lib controls.

Hamster serum triglycerides were significantly increased above pair-fed control levels. High variability in the hamster triglyceride levels is believed to be due to the peculiar food eating pattern of these animals. The excess skin around their mouth and necks allows them to stuff their cheeks with large quantities of food, thereby, providing a constant nutritional source.

Rat

Little change in either LPL activity or serum triglyceride levels were noticed in older rats. However

Table 16. Serum triglyceride, cholesterol, and protein concentrations 10 days after administration of 1 or 50 ug/kg TCDD or acetone:corn oil and pair-fed to treated rabbits.

	1 ug/kg TCDD	PFC	50 ug/kg TCDD	PFC
Triglyceride (mg/dl)	203 ± 30(4)[a]*	90 ± 54(4)	240 ± 101(6)*	77 ± 12(5)
Cholesterol (mg/dl)	105 ± 28(4)*	68 ± 39(4)	103 ± 15(5)*	60 ± 27(5)
Protein	13.4 ± 1.2(4)	13.4 ± 1.3(4)	13.0 ± 0.8(5)	13.5 ± 0.9(5)

[a]Results are mean ± standard deviation for (n) animals. Data analyzed with Tukey's test after random factorial ANOVA.
* Significantly different from PFC, $P < .01$.

there was a significant increase in LPL activity from younger rats (100-125 g) (Table 15).

Mice

Serum analysis of 5 AHH responsive and 5 AHH nonresponsive mice strains 2 days after treatment indicated 3 of the responsive (BALB/c, A/J, and CBA/J) strains to have significantly increased levels of triglycerides while all 5 of the nonresponsive strains remained unchanged (Table 17). One responsive (SEC/1ReJ) and one nonresponsive (SWR/J) strain each showed nonsignificant increases in triglycerides. C57BL/6J, the other sensitive strain, revealed lower but nonsignificant levels of serum triglycerides. None of the strains tested indicated any significant change in serum cholesterol concentrations except 129/J. This result is believed to be due to an artifact since the control samples were highly hemolyzed thereby influencing the colorimetric assay. Furthermore it appears that the normal cholesterol range for these strains is between 38 and 49 mg/dl; values far below that obtained for the 129/J control samples. These triglyceride and cholesterol assays were done by myself and Ms. Laura Anderson.

DISCUSSION

The rabbit seems to be as sensitive to TCDD as the guinea pig in terms of a hyperlipedemic response.

Table 17. Serum triglyceride and cholesterol concentrations (mg/dl) in responsive and nonresponsive mice strains 2 days after dosing with either 30 ug/kg TCDD or acetone:corn oil.

Strain	Triglyceride (mg/dl) C	Triglyceride (mg/dl) T	Cholesterol (mg/dl) C	Cholesterol (mg/dl) T
Responsive				
BALB/C	132 ± 16(3)[a]	275 ± 18(4)*	58 ± 3(4)	51 ± 6(4)
A/J	116 ± 37(5)	232 ± 49(4)*	35 ± 8(3)	41 ± 3(4)
SEC/1ReJ	90 ± 23(4)	126 ± 52(5)	51 ± 7(4)	47 ± 2(4)
CBA/J	252 ± 31(5)	428 ± 72(4)*	44 ± 7(4)	59 ± 5(2)
C57BL/6J	127 ± 42(6)	109 ± 31(4)	34 ± 6(4)	38 ± 4(2)
Nonresponsive				
AKR/J	122 ± 13(4)	148 ± 49(6)	39 ± 3(6)	39 ± 4(5)
RF/J	99 ± 38(3)	97 ± 35(3)	38 ± 5(3)	48 ± 5(3)
SWR/J	100 ± 21(3)	199 ± 52(3)	49 ± 13(3)[b]	49 ± 1(4)
129/J	56 ± 19(3)	60 ± 14(4)	87 ± 2(3)	49 ± 9(3)*
DBA/2J	106 ± 32(4)	87 ± 9(4)	39 ± 3(3)	39 ± 6(3)

[a] Results expressed as the mean ± standard deviation for (n) animals. Data analyzed with 2 tailed students "t" test.
* Statistically different from control at P <.01.
[b] Highly hemolyzed serum, see text.

Significant inhibition of LPL activity as well as serum triglyceride elevation occurred in the rabbit at doses far below the LD_{50} value. These responses were not seen to the same degree in the guinea pig until the LD_{50} dose was attained or exceeded. Furthermore treated rabbits began to die sooner than did treated guinea pigs. Schwetz et al. (1973) reported rabbits died beginning the sixth day after a single lethal intraperitoneal dose of TCDD. Studies in this laboratory support this finding, thus the reason for having LPL data from 8 pair-fed control rabbits but only 3 animals at 50 ug/kg 10 days after treatment. The earliest any treated guinea pig died was 18 days after injection.

As reported previously (Brewster and Matsumura 1984) adipose LPL activity does correlate with serum triglyceride levels in the guinea pig. As demonstrated here the same is true for the rabbit and the hamster. No change was observed in either enzymatic activity or triglyceride concentrations in old rats. Why TCDD caused an increased activity in the younger animals is not known. It appears as if the activity of control animals is underestimated since LPL is not age dependent and since little difference was observed between the 2 treated groups and old control group. LPL activity does follow a diurnal pattern in rats according to when feeding occurs (Hamosh and Hamosh 1983), therefore, it is possible these control rats were sacrificed after active feeding had ceased. This however offers no explanation as

to why, after 10 days, treated animals which have reduced their food intake still have enzyme levels approaching that of ad lib controls. In conclusion TCDD does not inhibit rat adipose LPL thereby causing serum hypertriglyceridemia. This confirms the observations of Albro et al. (1978).

Olson et al. (1980) observed no change in hamster serum triglyceride levels 10 days after treatment but did see a 47% decrease in levels 20 days after treatment with TCDD. These were compared to ad lib control animals. Present observations indicated a significant increase in serum triglyceride concentration with a severe inhibition of LPL activity 10 days after an LD_{50} dose of TCDD compared to pair-fed control animals. In addition, pair-feeding had no effect on hamster LPL. This may be attributed to the fact that lipid metabolism in the hamster is vastly different from the rat, guinea pig and rabbit and that serum triglycerides are regulated by other controlling mechanisms. In fact, hamster metabolism is quite different from other animals which undergo hibernation since serum glucose concentrations increase rather than decrease during dormancy (Orr 1976). This animal must waken every few days and consume food in order to prevent starvation. Therefore, in order to remove endogenously produced triglycerides (via VLDL) this animal must have other mechanisms available during dormant periods in order to prevent adipose LPL decline due to lack of food intake. One such mechanism

could be increased serum glucose which in turn increases pancreatic insulin output and results in stimulation of LPL synthesis. Present results seem to support this since no change was seen in pair-fed control levels.

The milky blood serum obtained from the 50 ug/kg dosed rabbits was indicative of the high lipid concentrations produced by inhibition of adipose LPL (present study) and hepatic LDL binding (D. Bombick, personal communication). Low density lipoprotein (LDL) is the major cholesterol carrying lipoprotein in the blood. It is produced by the stripping off of triglycerides from VLDL particles by LPL and by direct hepatic synthesis and is efficiently removed by binding to its hepatic receptor (Brown et al. 1981). Both processes are inhibited by TCDD, in the guinea pig (Bombick and Matsumura 1984, Brewster and Matsumura 1984) and the rabbit, thereby causing hypercholesteronemia and hypertriglyceridemia. The yellow color imparted to this serum may be due to increased levels of bilirubin since in contrast to the guinea pig, rabbit liver is markedly affected by TCDD. Fatty infiltration, hepatomegaly, discoloration, and brittleness or loss of elasticity of blood vessels were seen in livers of rabbits exposed to 50 ug/kg TCDD.

Significantly increased blood serum triglycerides were noted in 3 of the 5 AHH responsive strains of mice and none of the nonresponsive strains. If toxicity segregates with

the AH locus as suggested by Poland and Glover (1980) this is not surprising. However, the question arises as to why C57BL/6J and to a lesser extent SEC/1ReJ (both responsive strains) and SWR/J (a nonresponsive strain) do not show the expected toxic response. These data suggest that these mice are genetically different from the other strains. Knutson and Poland (1982) suggest that TCDD and other halogenated hydrocarbons produce 2 distinct pleiotropic responses: The first is associated with the AHH gene and involves microsomal induction and enzymes involved in drug metabolism, while the second involves an additional gene complex manifesting other toxic lesions. These authors conclude this battery of genes to be present but unexpressed in some tissues or species (i.e. in the present study perhaps C57BL/6J or SEC/1REJ). It is simplistic to think that all the pleiotypical responses evoked by TCDD are due to interactions at the Ah locus. This would mean that this locus controls lipid metabolism, microsomal induction, cellular proliferation, epithelial hyperplasia, thymic involution, body weight, and all other toxic symptoms. It appears more logical if other loci are involved. Therefore to understand how TCDD manifests its toxicity at the subcellular level, one must understand how it regulates and deregulates gene expression in different tissues. The same genes are present in all tissues of a given species but their expression depends upon the particular cell type and

vice-versa. TCDD in some, as of yet unknown way, must alter this gene expression.

No increases in serum cholesterol levels were seen in any mouse strain. Perhaps longer exposure to TCDD or higher doses would result in hypercholesterolemia in the AHH sensitive mice. Previous research with guinea pigs (Brewster and Matsumura 1984, Bombick et al. 1984) indicated serum triglyceride levels to increase at lower doses and after shorter exposures to TCDD than serum cholesterol.

Conclusions

In conclusion, TCDD reduced adipose LPL activity in the guinea pig, rabbit, and hamster, thereby causing the serum hypertriglyceridemia seen after dioxin administration to these species. No depression occurred in the rat nor did serum triglycerides increase. Hamster adipose LPL was unchanged in pair-fed animals (compared to ad lib controls) but other mechanisms peculiar to this hibernating species probably prevent excess increased triglyceride buildup. Finally hypertriglyceridemia as a toxic response appeared to segregate with the Ah locus in several responsive and nonresponsive mice strains.

The hyperlipedemia observed in the rabbit is of special interest as this species has been extensively used in atherosclerotic studies. Since lipid metabolism in the white rabbit is similar to that of man's this animal has

proved to be a good model to investigate defects in lipid metabolism leading to arteriosclerosis. Further research should be conducted to examine the role of TCDD in serum hyperlipidemia and the atherogenic process.

For example, recent evidence indicates that LPL may be important in the formation of active HDL (high density lipoprotein) by stripping off the surface components of VLDL and chylomicra triglycerides during lipolysis to form active HDL (Dieplinger et al. 1985). HDL is a serum cholesterol scavenger and has a protective role in preventing atherosclerosis (Dieplinger et al. 1985, Brown et al. 1981). Since TCDD was seen to inhibit rabbit LPL in the present study it is suggested that this action may play a role in the formation of the preatherosclerotic lesions (along with the hepatic inhibition of LDL binding). Although Swift et al. (1981) found no changes in serum levels of HDL, between control and TCDD treated animals, it is not known whether these particles are active in removing serum cholesterol i.e. it is not known whether this HDL was activated or not.

It is not surprising that TCDD produces different effects in different animals as regards to lipid metabolism. Their biochemical pathways are quite different. For example the size and composition of the lipoprotein moities and the apoprotein composition varies with species (Chapman et al. 1975, 1980). Linoleic acid is

the major fatty acid in the guinea pig while oleic acid is primary in the rat. Furthermore, guinea pigs lack apoprotein C-II but still have efficient triglyceride clearance mechanisms and are normally resistant to hypertriglyceridemia. Guinea pigs have low serum levels of hepatic lipase compared to rats and therefore have only one mechanism to remove serum triglycerides as opposed to two in the rat. Rabbits typically have low levels of VLDL and HDL. Finally, as previously discussed, hamsters hibernate and therefore have completely different lipid biochemical pathways compared to guinea pigs, rats, and rabbits.

Since LPL is known to be controlled by insulin (Hamosh and Hamosh 1983) quite possibly what may be occurring in these species in regard to TCDD is either an inhibition of serum glucose regulation, an inhibition of pancreatic insulin production or secretion, or an inhibition of insulin action at the fat cell. Any of these would decrease LPL activity resulting in increased blood triglycerides in the guinea pig or rabbit.

CHAPTER VII

EFFECTS OF 2,3,7,8 TETRACHLORODIBENZO-P-DIOXIN UPON THE GUINEA PIG HEART

INTRODUCTION

Lipoprotein lipase activity (LPL) has been found to be present in a variety of extrahepatic tissues other than adipose such as lung, skeletal muscle, lactating mammary gland, milk, aorta, corpus luteum, brain, and heart. (Hamosh and Hamosh 1983). To achieve a constant energy source, cardiac LPL is regulated differently than adipose LPL. Fielding (1976) has presented evidence for a high affinity heart lipase enzyme and a lower affinity fat lipoprotein lipase enzyme. Although these enzymes differ in structure and in kinetics their function is to hydrolyze circulating triglyceride-rich lipoproteins, thereby providing fatty acids to either the heart for energy or to fat for storage. Both enzymes are activated by apo-C II and are inhibited by high ionic concentrations. It has been postulated (see Hamosh and Hamosh 1983) that these lipase enzymes have a critical physiological function since the high affinity cardiac lipase provides energy to the heart even when serum triglyceride concentrations are low, such as during fasting periods. Likewise, adipose LPL will provide storage materials to fat only when blood triglyceride

concentrations are high, such as post prandial. As a result, shunting of potential energy occurs between fat and other active tissues with increased metabolic requirements. Therefore, cardiac LPL is less nutritionally dependent than adipose LPL, and in fact, cardiac activity may rise during fasting to insure adequate nutrition to the muscle.

Known Cardiotoxicity of Dioxin

Since guinea pig adipose LPL activity has been shown to be by TCDD (Brewster and Matsumura 1984) it was of interest to know of what effects this dioxin would have on heart LPL activity. If lipase activity in this muscle is reduced by dioxin, one would expect severe effects on heart function to occur. This coupled with the observed high serum lipid concentrations could be a contributing factor to death of the animal. Although chlorinated dioxins were shown to cause chick edema disease (characterized as edema of the pericardial sac) in the late 1960's (see Firestone 1973), virtually no investigators have focused their attention on the effects of TCDD concerning heart function. Three reports are found in the literature describing pathological or histological lesions in the heart after dioxin treatment, but these effects are seen only after very high doses of TCDD or after chronic exposure. None has dealt with heart function and all have been described only for the rat (see Table 18).

TABLE 18. Reported cardiac lesions after treatment with TCDD.

Lesion	Dose; Administration	Exposure Time	Reference
Valvulitis; endocardial erosion of valve faces; fibrinoid thrombi and valvular edema; degeneration of myofibrillae	1 or 10 mg/kg; single, i.p.	10 days	Buu-Hoi et al. 1972
Hemorrhage; organized thrombi	10 ug/kg; daily, oral	18 days	Gupta et al. 1973
Myocardial degenerative changes	0.1 ug/kg	2 years	Kociba et al. 1979

MATERIALS AND METHODS

Cardiac LPL Assay

Guinea pigs were obtained and housed as described before. After 10 days, ad lib control, TCDD treated, or pair-fed control animals were anesthetized with ether and their hearts removed after which, cardiac LPL activity was estimated using the same procedure previously described for adipose tissue LPL. In a subsequent experiment TCDD treated or pair-fed animals were given 2.7 ml of a 75% glucose solution 2 hours before sacrifice.

Atrial Isolation and Heart Function Tests

Heart function was evaluated by measuring the inotropic (force of contraction) and the chronotropic (rate of contraction) responses in isolated atrial muscle. Animals were administered a single i.p. dose of TCDD (1 ug/kg) or acetone:corn oil (1:9) and either pair-fed or allowed to feed ad libitum. After 10 or 20 days the guinea pigs were stunned with a blow to the head, the hearts rapidly removed and immersed in room temerpature Krebs-Henseleit bicarbonate buffer (118.0 mM NaCl, 27.2 mM $NaHCO_3$, 4.8 mM KCl, 1.2 mM $MgSO_4$, 1.0 mM KH_2PO_4, 1.4 mM $CaCl_2$, 11.1 mM glucose). After retrograde perfusion with aerated buffer the left atrium was excised and hung vertically in a jacketted tissue bath (containing buffer at 30°C and aerated with 95% O_2-5% CO_2 at a minimal rate) in order to measure the inotropic response. The chronotropic response was determined using the right

atrium hung in a similar manner in an adjacent bath. All four atria, from pair-fed control and TCDD treated animals, were hung at the same time and exposed to the same experimental conditions. Platinum electrodes were used for electrical stimulation of the left atria at 1.5 Hz of 5 ms duration approximately 100% above threshold (Grass S9 Stimulator, Grass Instrument Company, Quincy, MA). Resting tension of all samples was adjusted to 1 g whereupon the force of contraction of the left atria, in response to the electrical stimulation and the inherent rate of contraction of the right atria (no electrical stimulation) were continuously recorded on a physiograph recorder (Grass Model 7B Polygraph). The atria were attached to a force displacement transducer (Grass Instrument Company, FT-03C). Every 15 minutes throughout a 60-70 minute equilibration period the buffer in the baths was changed to prevent excess foaming. The resting tension was maintained at 1 gram throughout equilibration after which isoproterenol (in ethanol) was added to the bathing medium at 5 minute intervals so that final concentrations were $3x10^{-10}M$, $1x10^{-9}M$, $3x10^{-9}M$, $1x10^{-8}M$, $3x10^{-8}M$, $1x10^{-7}M$, and $3x10^{-7}M$.

All chemicals used were of the highest quality available and were purchased from Sigma Chemical Company, St. Louis, MO. Statistical analyses were performed by random factorial or one way analysis of variance where indicated

followed by the appropriate multiple comparison test as noted.

RESULTS

TCDD had little effect on cardiac weight 2 days after a single i.p. administration (Table 19). Heart weight was significantly depressed from pair-fed and ad lib controls 10 days but not 20 days after treatment. However, no differences were noted when weight was expressed as a percentage of body weight, thus indicating no significant loss of cardiac tissue after TCDD treatment.

LPL

Cardiac LPL activity was not significantly altered by dioxin treatment (Table 20). No changes were noted after 2 days or 10 days. Glucose administration 2 hours prior to sacrifice significantly increased activity in both TCDD treated and pair-fed control animals. Finally, administration of 4 ug/kg dioxin did not cause a statistically significant depression of lipase activity compared to pair-fed animals, 10 days after treatment.

Atrial Function

Cardiac atrial force of contraction was significantly depressed 20 days after a single administration of TCDD (Table 21). No effect was noted at 10 days after treatment and little effect was observed on basal rates of contraction in the absence of isoproterenol after 10 or 20 days of

TABLE 19. Guinea pig absolute heart weight or heart weight as a function of body weight (mean± S.D., n), at 2, 10, or 20 days after a single i.p. administration of 1 ug/kg TCDD or acetone:corn oil.

Exposure Time	HEART WEIGHT					
	Grams			% Body Weight[a]		
	Ad lib	TCDD	Pair-fed	Ad lib	TCDD	Pair-fed
2 Day	0.96±0.07(3)	0.91±0.25(7)	0.95±0.22(7)	0.37±0.02(3)	0.34±0.03(7)	0.34±0.03(7)
10 Day	1.28±0.04(2)	0.63±0.08(6)[b]*,**	0.83±0.13(6)*	0.42±0.02(2)	0.34±0.03(6)	0.34±0.03(6)
10 Day + glucose[c]	ND[d]	0.63±0.04(4)**	0.83±0.06(4)	ND	0.32±0.06(4)	0.30±0.01(4)
20 Day						
young[e]	1.27±0.17(3)	1.04±0.30(5)	1.13±0.23(5)	0.43±0.04(3)	0.42±0.08(5)	0.37±0.03(5)
old[f]	2.00±0.48(3)	ND	ND	0.37±0.06(3)	ND	ND

[a] No significant differences noted.
[b] Data analyzed with Dunnetts test after ANOVA.
* Significantly different from ad lib control.
** Significantly different from pair-fed control.
[c] After 10 days animals received a single oral dose of glucose before sacrifice as described in materials and methods.
[d] Not determined.
[e] Body weight 250-300 g at time of dosing.
[f] Body weight 500-600 g at time of dosing.

TABLE 20. Cardiac LPL activity 2 or 10 days after single i.p. treatment with 1 or 4 ug/kg TCDD or vehicle alone (mean ± S.D., n).

Exposure Time	Ad lib	TCDD	Pair-fed
2 Day (1 ug)	ND[a]	355.9±260.1(5)	303.8±55.4(6)
10 Day (1ug)	127.2±23.3(2)	292.9±122.0(9)	320.9±160.5(6)
10 Day (1 ug + glucose)[c]	ND	532.3±74.7(4)[b]*	655.7±137.9(4)*
10 Day (4 ug)	ND	131.1±115.0(3)[d]	ND
20 Day (1 ug)	1068.2±120.4(3)	1204.4±189.0(3)	781.5±132.8(2)

[a] Not determined.
[b] Data analyzed with Dunnetts test or Tukey's test for unconfounded comparisons after factorial ANOVA.
*Significantly different from respective 10 day animals, $P < .05$.
[c] Animals administered 2.7 ml of 75% glucose, 2 hours before sacrifice.
[d] There was no change in body weight gain or food consumption between animals administered 1 or 4 ug/kg TCDD.

TABLE 21. Force and rate of contraction of guinea pig atria 20 days after exposure to 1 ug/kg TCDD or corn oil only and fed ad libitum or pair-fed to the TCDD treated animals (mean ± S.D., n animals).

	Contraction Force[a]			Contraction Rate[b]		
	Ad lib	TCDD	PFC	Ad lib	TCDD	PFC
10 Day	ND[c]	0.7±0.4(4)	0.9±0.2(4)	ND	144±15(4)	157±5(4)
20 Day	0.3±0.1(3)	0.1±0.1(6)[d]*	0.6±0.2(6)	153±9(3)	136±12(6)	140±14(0)

[a]Grams of developed tension (left atria).
[b]Contraction number/minute (right atria).
[c]ND - not determined.
[d]Data analyzed with Dunnett's test after ANOVA.
* Significantly different from pair-fed control at P <.001.

treatment. Little change was noted in the inotropic and chronotropic responses of isolated atria to isoproterenol from 10 day TCDD treated or pair-fed control animals (Figure 27). After 20 days of treatment obvious differences were noted in the atrial contraction response to the drug but not in the chronotropic response (Figure 28). Although atria from pair-fed control and TCDD treated animals both developed a maximal tension of 2-2.5g, those from dioxin treated animals needed higher doses of isoproterenol to reach that response. To correct for atrial size differences the % of maximum developed tension as a function of isoproterenol dose was plotted in figure 29. The response of pair-fed control animals was the same as young ad lib control animals; TCDD treated animals followed the response of old ad lib control animals. The % of maximum developed rate, plotted against isoproterenol dose, is significantly altered by TCDD (Figure 30).

Isoproterenol Dose Response

Since atrial size influences contractile force, the % of maximum response as a function of isoproterenol concentration was plotted and analyzed (Figure 31). The ED_{50} values are presented in Table 22. After 10 days of treatment, 2.7X more isoproterenol was needed to obtain 50% of maximum contractibility in this muscle preparation from TCDD treated animals. After 20 days this difference was 3.1X that of pair-fed control levels.

Figure 27. Chronotropic (A) and inotropic (B) responses of isolated guinea pig heart atria to isoproterenol. Atria were isolated from TCDD treated (●) (1 ug/kg, i.p.) or pair-fed control (○) animals 10 days after administration. Each point represents the mean ± S.D. from 5 animals. Split-plot ANOVA revealed no significant differences between treatments.

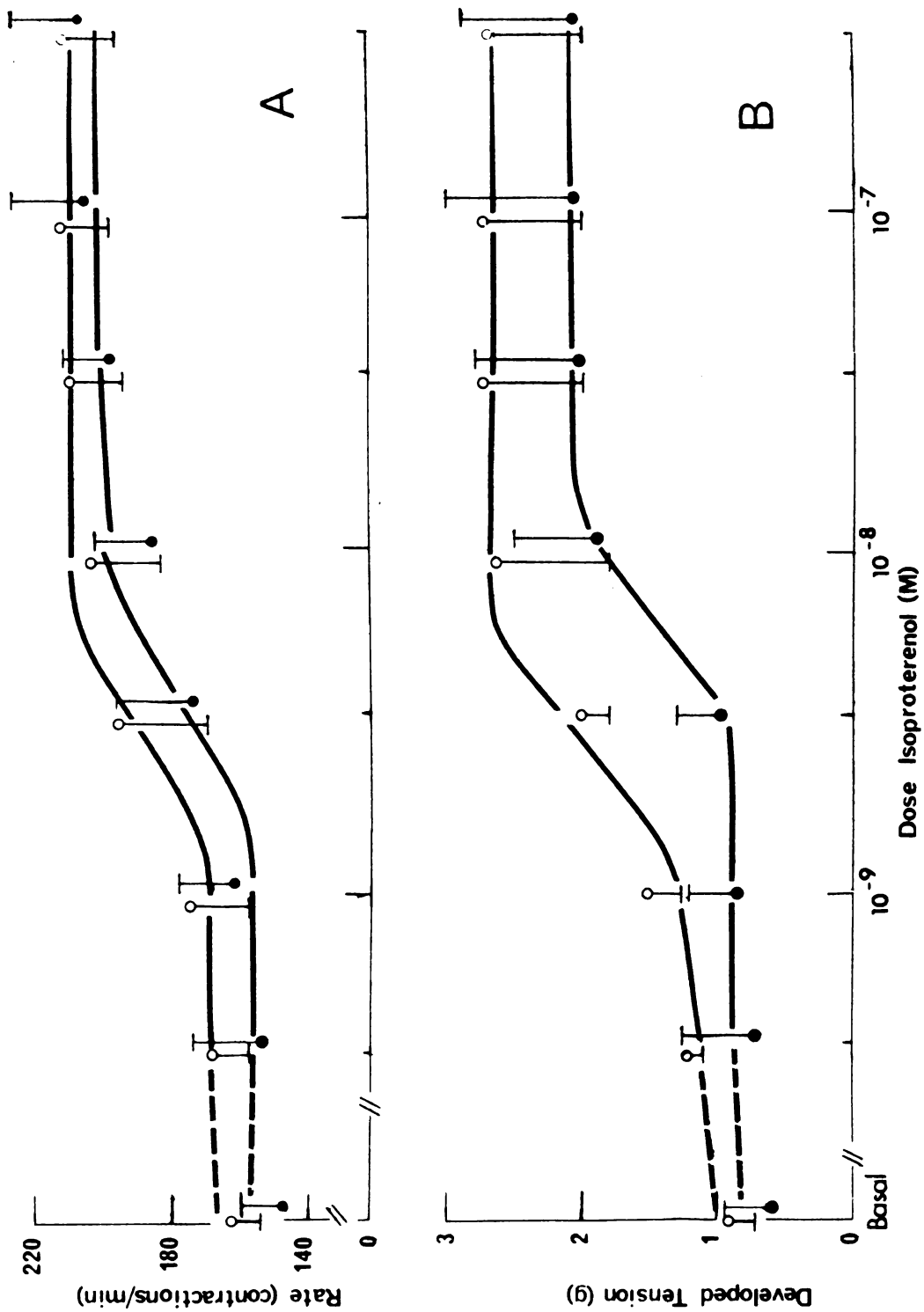

A
B
Rate (contractions/min)
Developed Tension (g)
220
180
140
0
3
2
1
0
Basal
10^{-9}
10^{-8}
10^{-7}
Dose Isoproterenol (M)

Figure 28. Chronotropic (A) and inotropic (B) responses of isolated guinea pig atria, to isoproterenol, 20 days after treatment with either 1 ug/kg (i.p.) TCDD (●) or acetone:corn oil (○) and pair-fed. Each point represents the mean ± S.D. of 5 animals and were analyzed with split-plot ANOVA followed by Tukeys Test for Unconfounded Comparisons with P = .01. *Significantly different from pair-fed control.

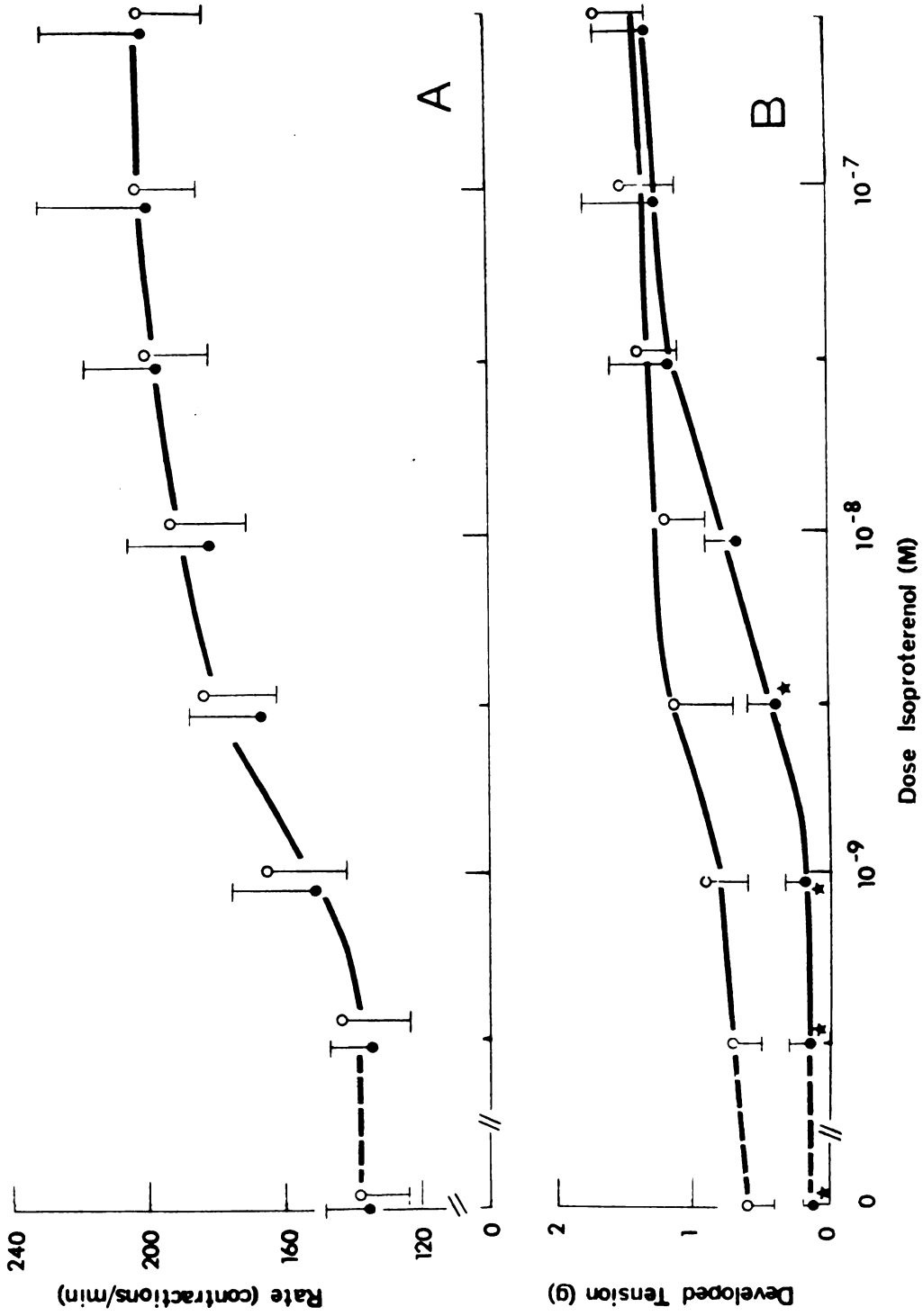
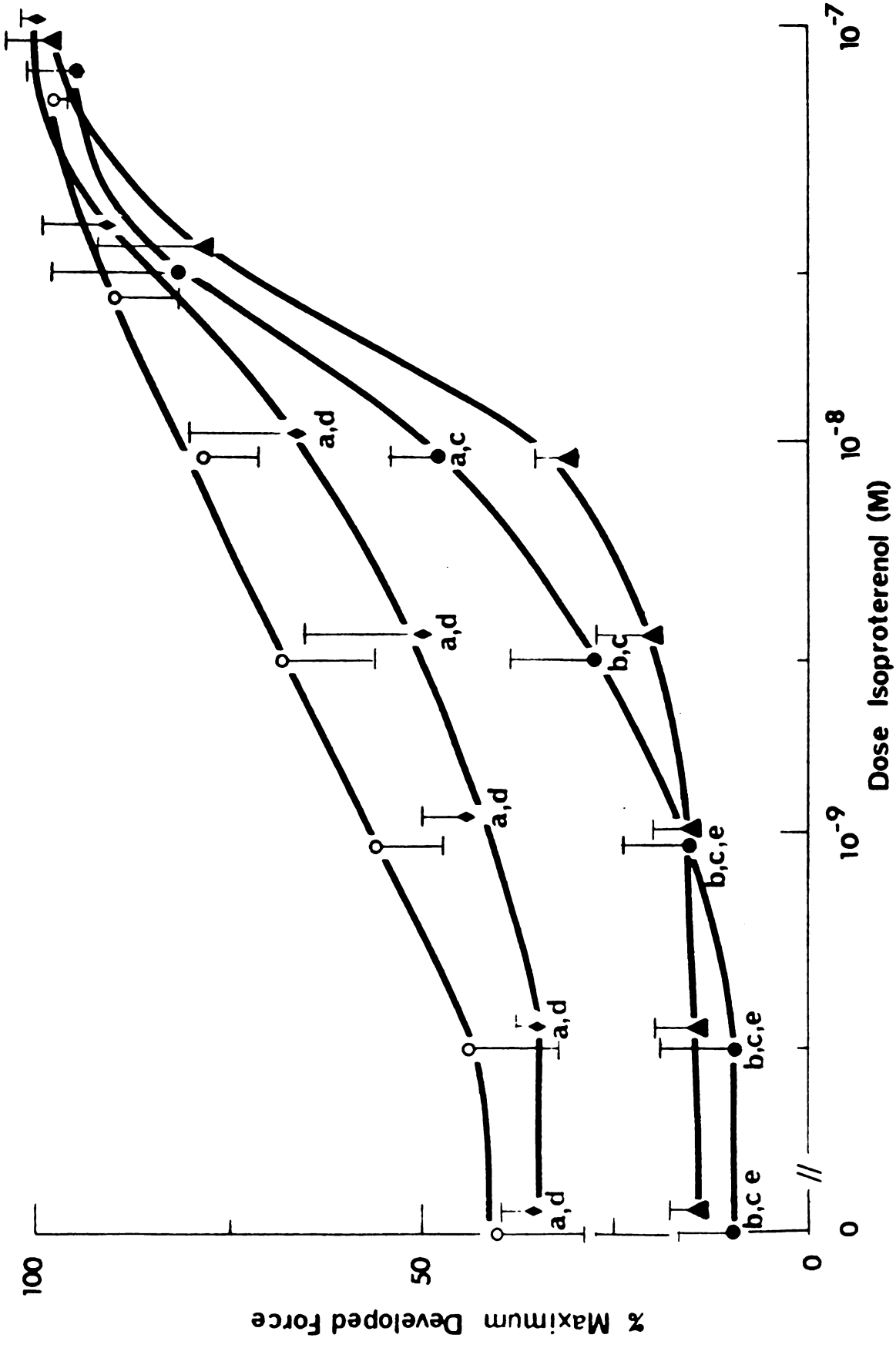

A
B
Rate (contractions/min)
Developed Tension (g)
Dose Isoproterenol (M)
240
200
160
120
0
2
1
0
10^{-9}
10^{-8}
10^{-7}

Figure 29. Inotropic response (as a percent of the maximum developed force) of isolated guinea pig atria (from young animals 250-300 g) 20 days after treatment with 1.0 ug/kg TCDD (●) or acetone:corn oil and pair-fed (○), or fed ad libitum (♦). The response was also measured in atria isolated from old animals (500-600 g) allowed to feed ad libitum(▲) for 20 days after treatment with vehicle only. Each point represents the mean ± S.D. from 3-6 animals. The data was transformed and analyzed with one way random factorial ANOVA followed by Scheffes test for multiple comparisons at P = .05. [a]Significantly different from old ad libitum control animals. [b]Not significantly different from old ad libitum control animals. [c]Significantly different from pair-fed control. [d]Not significantly different from pair-fed control. [e]Significantly different from young ad libitum control.


% Maximum Developed Force
100
50
0
Dose Isoproterenol (M)
0
10-9
10-8
10-7
a,d
a,c
a,d
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a,d
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b,c,e
b,c,e
b,c e

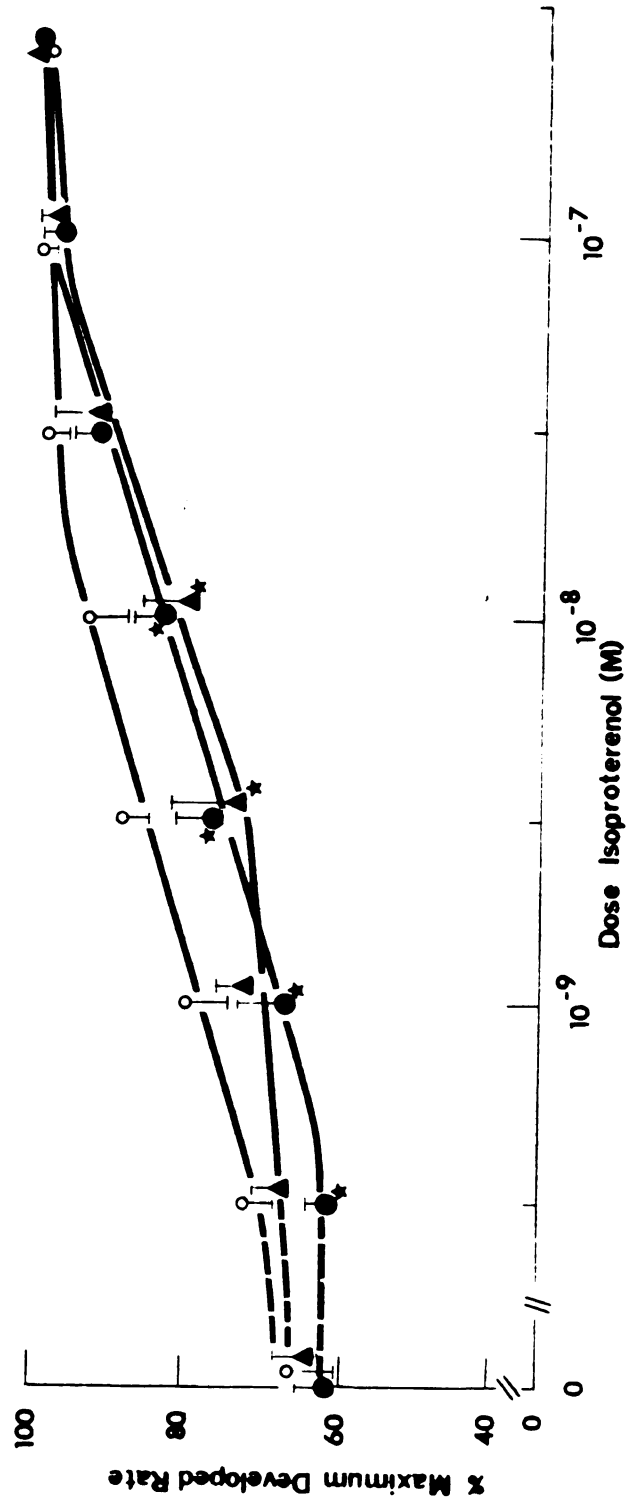


Figure 30. Chronotropic response (as a percentage of maximum developed rate) of isolated guinea pig atria 20 days after administration of 1 ug/kg TCDD (●) and pair-fed (○) or fed ad libitum (▲). Each point represents the mean + S.D. of 5 animals and were analyzed, as in Figure 26, using Tukeys Test for Unconfounded Comparisons at P =.01. *Significantly different from pair-fed control.

Figure 31. Isolated guinea pig atrial inotropic dose response to isoproterenol 10 or 20 days after treatment. (⌀) pair-fed control, 10 day; (⌀) TCDD, 10 day; (○) pair-fed control, 20 day; (●) TCDD, 20 day; (♦)young ad lib control, 20 day; (▲)old ad lib control, 20 day. (Error bars omitted for clarity).

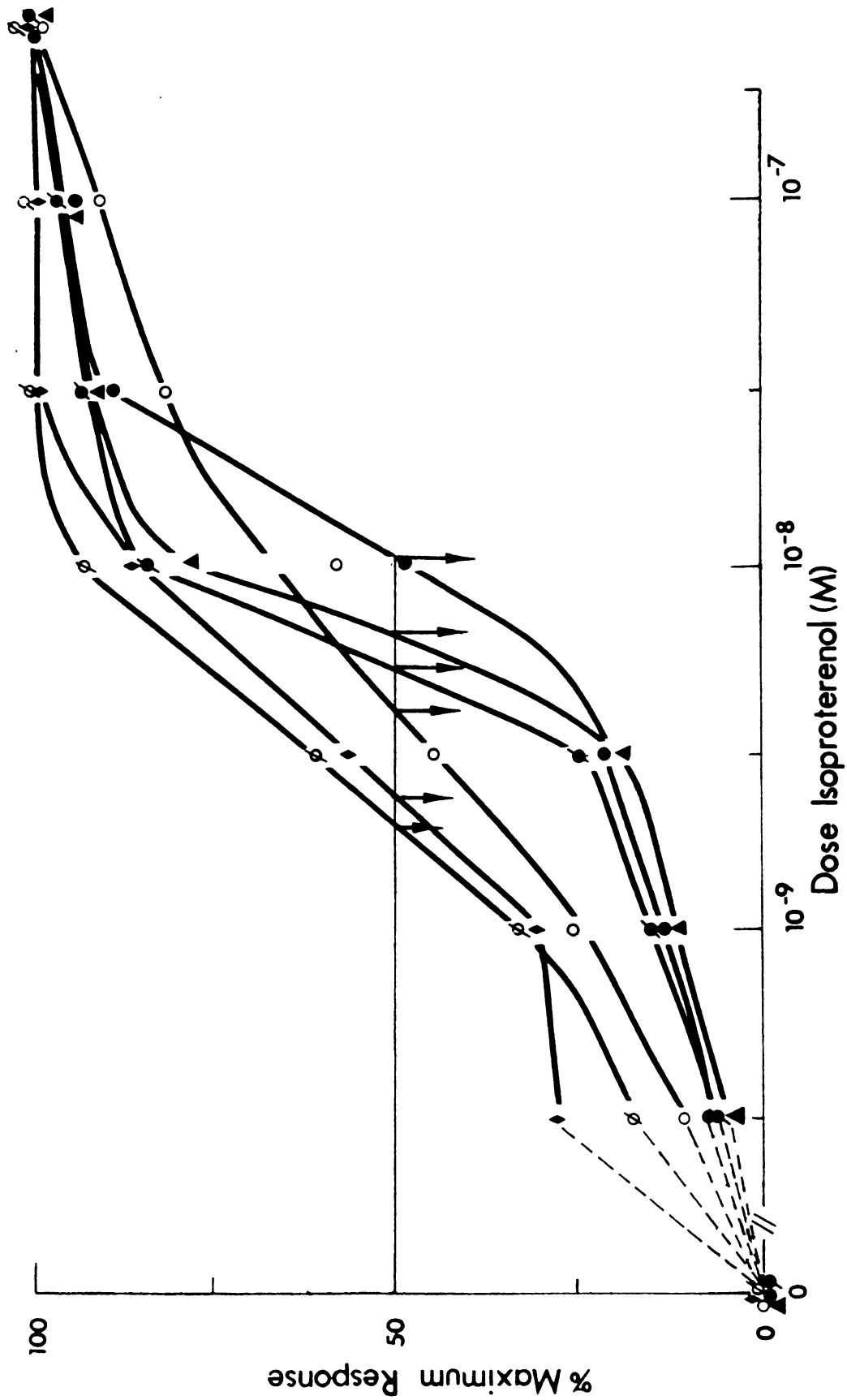

% Maximum Response
100
50
0
0
10^{-9}
10^{-8}
10^{-7}
Dose Isoproterenol (M)

TABLE 22. Effective concentration of isoproterenol to produce 50% of maximal contractile force in isolated guinea pig atria 10 or 20 days after i.p. administration of 1 ug/kg or acetone:corn oil and pair-fed or fed ad libitum.

	Ad lib control		TCDD	PFC
	young	old		
10 Day ED_{50}	ND[a]	ND	5.2×10^{-9} M	1.9×10^{-9} M
20 Day ED_{50}	2.3×10^{-9} M	6.8×10^{-9} M	1.1×10^{-8} M	3.5×10^{-9} M

[a]Not determined.

The 20 day PFC ED_{50} was 1.5X that of the young ad lib control but only half of the old ad lib control ED_{50} and as stated above about 1/3 of the preparations from TCDD treated animals (Table 23). The ED_{50} from treated animals was almost 5X that of young ad lib control but slightly more than 1 1/2X the old ad lib control levels (Table 23). Statistical analysis indicates significant differences between TCDD and PFC, PFC and old ad lib, TCDD and young ad lib but not between PFC and young ad lib or TCDD and old ad lib.

DISCUSSION

TCDD produced a 51 and 24% decrease in heart weight compared to ad lib control and pair-fed control animals respectively, 10 days after treatment. No change in weight was seen after 2 days. Since TCDD causes body weight loss or inhibition of body weight gain, heart weight was expressed as a percentage of body weight and was no different between TCDD treated and control guinea pigs. It is concluded, that this apparent loss of heart weight is a secondary response resulting from body weight loss. Why the absolute weight appears to return to normal levels after 20 days of TCDD treatment is not known at this time.

Fielding and Havel (1977) proposed that the high affinity heart LPL allows a constant lipid uptake even when serum triglyceride concentrations are low. Hamosh and Hamosh (1983) indicate that heart LPL increases after fasting to provide sufficient nutrient uptake. Data

TABLE 23. Twenty day "dioxin" factor for atrial contraction ED_{50} as a function of treatment.

	Young Ad lib	Old Ad lib	TCDD	PFC
Young Ad lib	--	2.96[a]	4.78	1.52
Old Ad lib	.34	--	1.62	.51
TCDD	.21	.62	--	.32
PFC	.66	1.94	3.14	--

[a]"Dioxin" factor calculated by dividing the ED_{50} value from each 20 day treatment group by each other.

presented here indicate that high doses of TCDD may inhibit this effect. After 10 days pair-fed and TCDD treated animals showed increases in LPL activity as expected, however those animals given 4 ug/kg TCDD exhibited a 59% decrease in activity compared to pair-fed controls. The difference was not statistically significant, possibly because of the large variability in the assay. Clearly more research needs to be completed before this question can be answered in its entirety. If TCDD does indeed inhibit cardiac LPL activity then one would expect heart function to be compromised thereby contributing to death of the animal. Studies are currently in progress to assess cardiac LPL activity after longer exposure periods.

Linder et al. (1976) have shown that heart LPL responds very little to insulin. It is therefore possible that the inhibition in adipose tissue shown by Brewster and Matsumura (1984) is not entirely due to alterations in hormonal insulin production or secretion. Experiments with cultured cells and in vitro TCDD exposures are being considered to assess whether inhibition of LPL activity is a direct or indirect effect upon adipose tissue.

TCDD produced significant alterations in cardiac function after a prolonged exposure to this chemical. Contraction force was 1/6 that of pair-fed controls 20 days after TCDD administration, and the response to isoproterenol was much less than that of pair-fed controls. Furthermore

it appears as if TCDD causes an age related change since treated animals exhibited a response similar to that of old guinea pigs, while pair-fed and young animals were similar. Further evidence for this invoked aging effect is discussed in Chapter VIII, where no change was noted in adipose lipolytic activity although the lipogenic actions were severely depressed characteristic of older animals.

Conclusions

Whether the decreased force of contraction is a direct effect of TCDD or not is not known at this time. The compromised ability of this organ to respond to isoproterenol suggests an alteration in the beta-adrenergic system. Future studies concerned with cardiac membrane binding should be considered to assess whether this effect is due to changes in receptor affinity or receptor number or due to changes post receptor binding. Isoproterenol is known to increase adenylate cyclase and c-AMP levels within the cell. Possibly receptor binding is changed only marginally and the changes in contraction are due to alterations in cyclic nucleotide levels. TCDD is known to influence c-AMP levels in adipose tissue (Brewster - unpublished observation). Contraction changes may also be due to alterations in ionic transport, another manifestation of toxicity which was seen in liver plasma membrane functions (Matsumura et al. 1984). This possibility could be investigated by studying the action potential and

observing changes in different areas of the spike. In this way one could infer whether Na, Mg, or Ca ions are involved. Whatever the underlying mechanism, the depression in heart function coupled with the many and varied other toxic lesions produced by this chemical (such as hyperlipedemia) could very conceivably result in lethality.

CHAPTER VIII

STUDIES ON THE CELLULAR MECHANISM OF LPL INHIBITION CAUSED BY IN VIVO ADMINISTRATION OF 2,3,7,8 TETRACHLORODIBENZO-P-DIOXIN

INTRODUCTION

With the elucidation of the cause for the severe serum hypertriglyceridemia, produced by TCDD in the guinea pig, came a much more complex and difficult problem -- the biochemical mechanism for the inhibition of the enzyme lipoprotein lipase (LPL).

As discussed by Patten (1970) the two main lipid metabolic pathways in the adipocyte are reciprocally regulated. Lipogenesis, controlled mainly by insulin, and lipolysis controlled mainly by catecholamines, can be said to be opposing pathways and thereby have opposite physiological functions (see Figure 32). During periods of fasting, epinephrine and norepinephrine stimulate the fat enzyme, Hormone Sensitive Lipase (HSL) responsible for the breakdown of stored triglycerides to free fatty acids. These fatty acid are then transported throughout the body and used where needed for energy via fatty acid oxidation. However when actively eating, insulin induces the synthesis of LPL the function of which is to remove circulating triglycerides from the blood for storage in the adipose tissue. Agents responsible for stimulating HSL activity

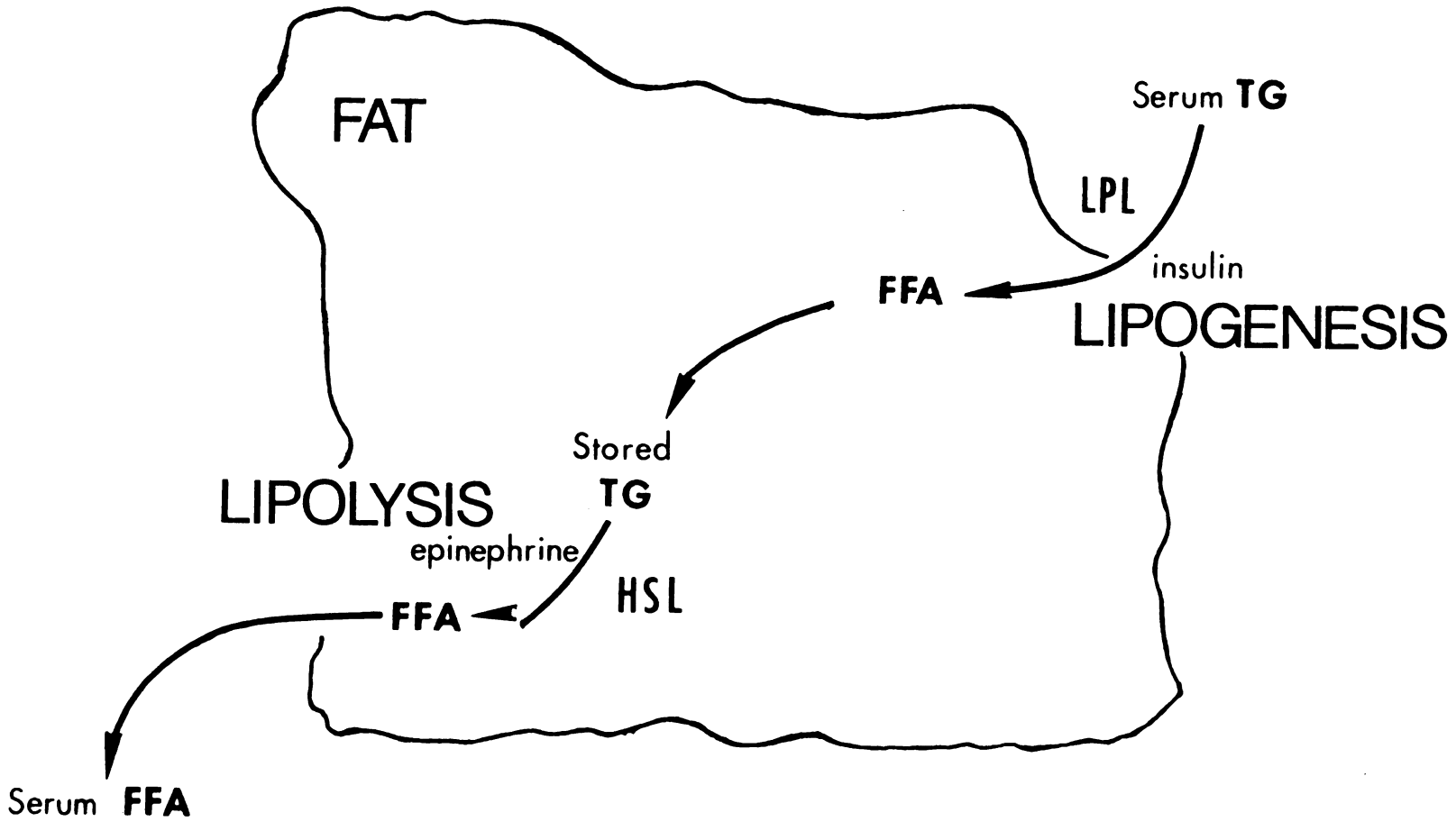


Figure 32. Simplistic schematic of lipogenesis and lipolysis in adipose tissue; TG-triglyceride, LPL-lipoprotein lipase, FFA-free fatty acids, HSL-hormone sensitive lipase.

inhibit LPL and those increasing LPL activity reduce HSL. It is understandable why this reciprocity was developed, and there is good evidence indicating LPL and HSL to be separate enzymes (Steinberg 1976).

LPL inhibition by TCDD could occur by two different mechanisms; either by affecting any of the steps shown in Figure 33 thereby influencing lipogenesis and insulin action through inhibition, or by altering any of the lipolytic pathways shown in Figure 34 and stimulating HSL which somehow causes a reduction in LPL activity. The first possiblity can be subdivided further into direct effects on insulin secretion by the pancreas or hormonal inhibition of LPL by other regulatory mechanisms such as increased serum thyroxine (T_4) levels (Hamosh and Hamosh 1983).

The second possibility is intriguing because TCDD has been shown to cause sustained cellular phosphorylation in the liver (Matsumura et al. 1984, Madhukar et al. 1984, Bombick et al. 1985). Along with the observed stimulation of cellular phosphorylation there occur alterations in epidermal growth factor (EGF) binding due to endogenous phosphorylation of the EGF receptor by TCDD (see Appendix A and Madhukar et al. 1984). Changes in EGF binding have been associated with serum hypertriglyceridemia (Heimberg et al. 1965).

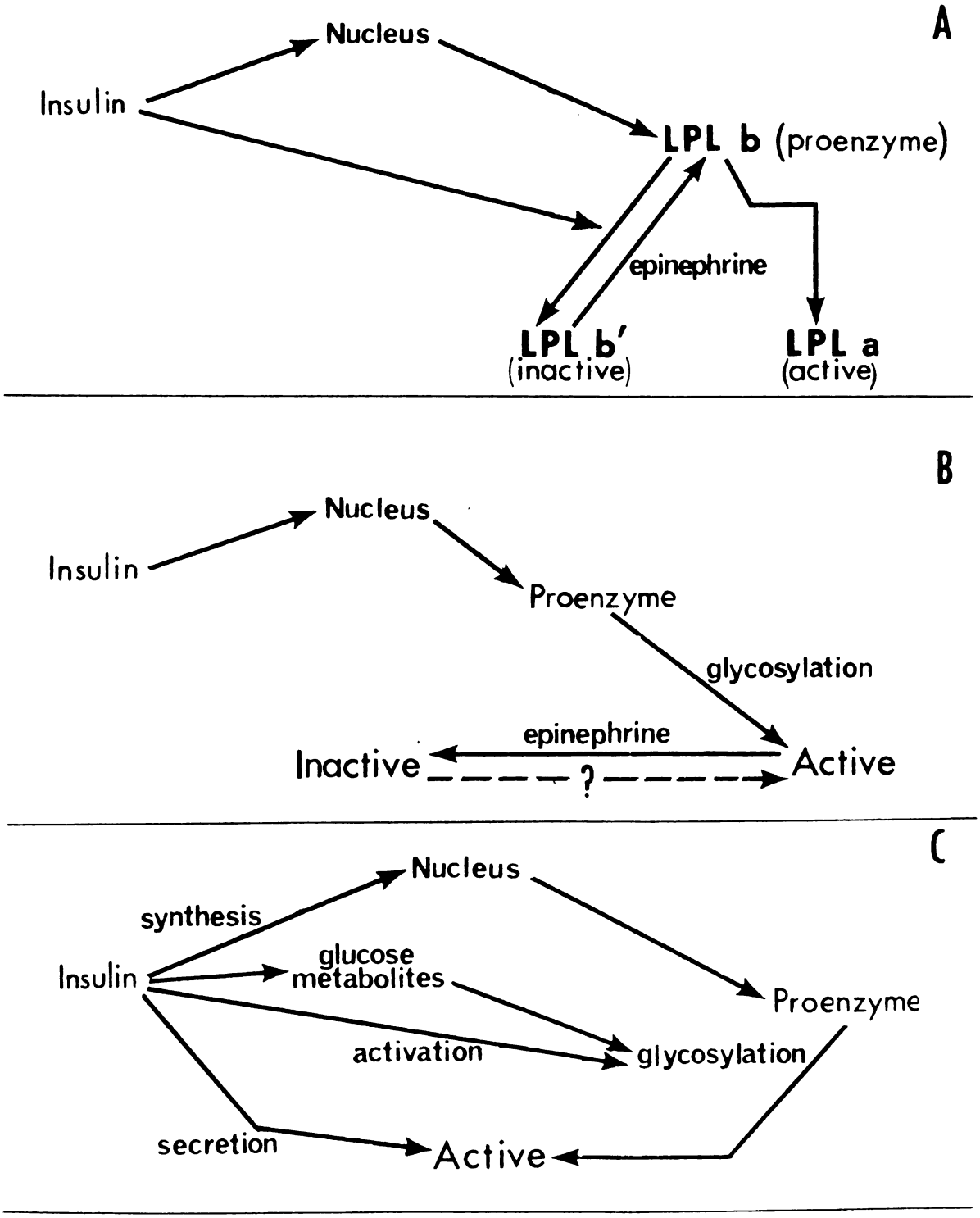


Figure 33. Three possible mechanisms for the production and cellular regulation of lipoprotein lipase. Panel A was taken from Cryer et al. (1975), panel B from Ashby et al. (1978), and panel C from Spooner et al. (1979).

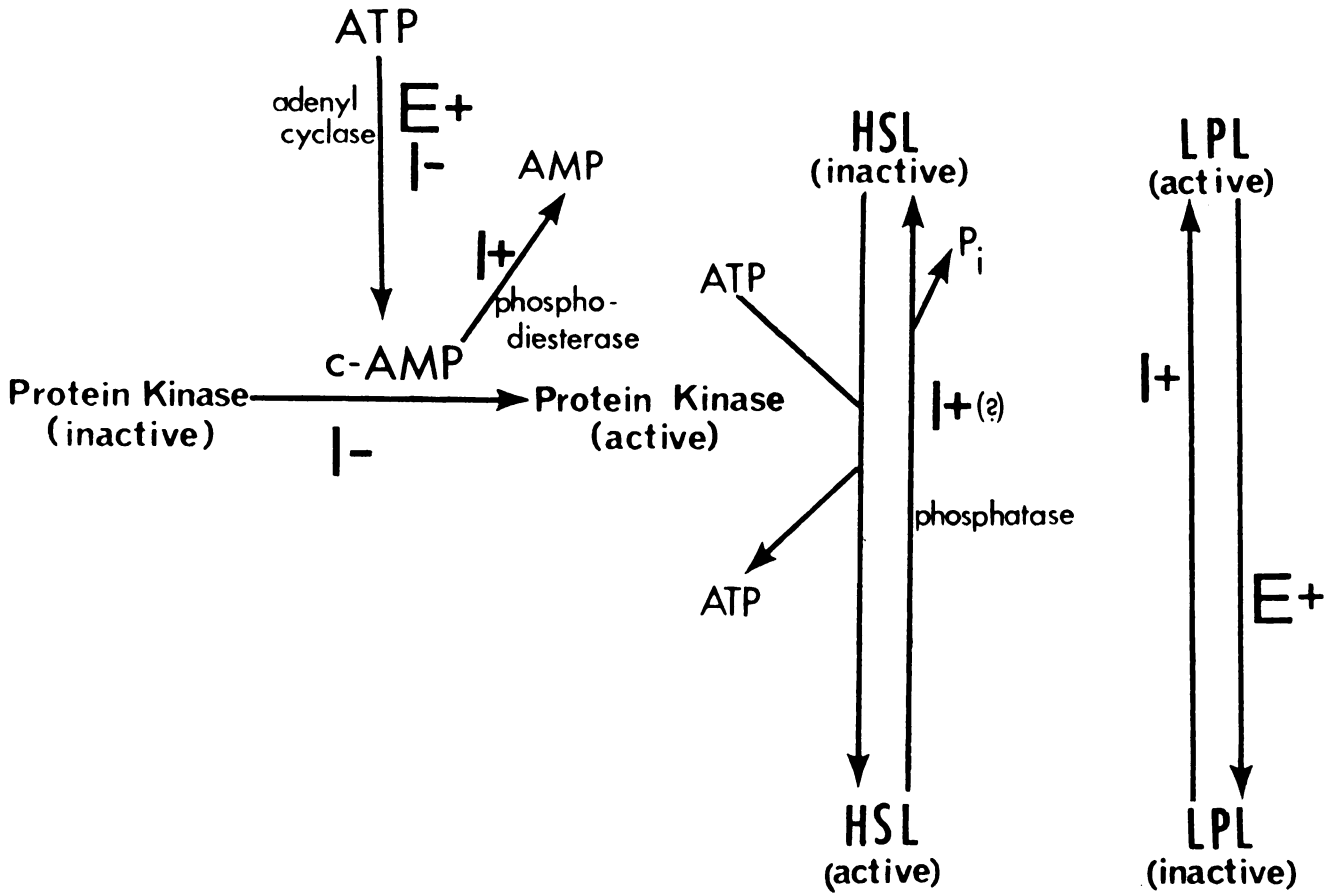


Figure 34. Cellular control and kinase regulation of adipose HSL and the reciprocal regulation of LPL. +/- indicate positive and negative hormonal regulation of key enzymes; E-epinephrine, I-insulin (in part from Steinberg 1976).

Lipogenesis

There are at least three mechanisms proposed for the synthesis and regulation of LPL (Figure 33). Cryer et al. (1975) suggested that insulin caused both an independent and dependent protein synthetic process which activates LPL. Insulin was shown to produce newly synthesized but inactive LPLb which gained full activation (LPLa) upon post translational modification just prior to being secreted from the cell. However investigators also noted another form of the enzyme, termed LPLb', which could not undergo this modification and therefore could not be activated to LPLa. This "storage form of the enzyme" could be acted upon by insulin to form LPLb. Conversely, adrenalin changed more of the proenzyme to the storage form (LPLb -- LPLb'). Ashby et al. (1978) concluded the proenzyme to be activated, probably through glycosylation, either immediately before or during secretion. These workers also noted an inactive LPL within the adipocyte but determined that adrenalin caused deactivation of the active form, not of the proenzyme as proposed by Cryer et al. (1975). In addition, they demonstrated little reversibility of the deactivation process. Finally, Spooner et al. (1979) ascertained that insulin regulates adipose LPL in three ways: (1) through the normal protein synthetic process, (2) through an energy independent process promoting increased secretion of the active form by either decreasing its binding to the plasma

membrane and other storage sites or by activating an inactive precursor and, (3) through stimulation of the hexose transport system and the formation of regulatory glycolytic intermediates. In conclusion, insulin causes the production of an inactive LPL precursor (probably through protein dependent and independent processes) which must undergo activation (probably through glycosylation) before secretion and that catecholamines (specifically epinephrine) inhibit this process. Glycosylation is thought to be responsible for activation of the proenzyme because (1) tunicamycin (a specific inhibitor of glycosylation) inhibits enzyme secretion, (2) LPL is glycosylated, and (3) glycosylation of proteins is known to be required for secretion in many cases (see Hamosh and Hamosh 1983). There are at least three regulatory sites involved with the production of viable LPL: stimulation of gene expression and energy dependent processes, activation of an inactive precursor, and inactivation of the active enzyme before secretion. Because the serum half life of LPL is only 7-25 min. (Hamosh and Hamosh 1983) hormonal control would be necessary to insure an adequate enzyme pool in the adipose tissue. This pool can be readily regulated under different nutritional conditions at the 2nd and 3rd regulatory steps. As discussed by Ashby et al. (1978), adrenergic stimuli could quickly and efficiently change LPL activity thereby influencing triglyceride uptake. Such responses would be

vital under conditions of stress where triglyceride uptake would need to be shunted to working muscles. An alteration in protein synthesis would delay this response. Along with inhibition of LPL, the catecholamines would activate HSL. This once again illustrates the reciprocity between these two systems in the adipocyte.

Lipolysis

Hormone sensitive lipase has been shown to be regulated by c-AMP dependent protein kinases in both in vivo (Steinberg 1976) and in vitro (Hirsch and Rosen 1984) systems. Epinephrine and ACTH are positive effectors of the system and stimulate membrane bound adenylate cyclase. The resulting c-AMP binds to the regulatory unit of c-AMP dependent protein kinase allowing phosphorylation of HSL to occur. Epinephrine also stimulates fatty acid transport out of the cell (Abumrad et al. 1985). As seen in Figure 34 insulin can inhibit the accumulation of active HSL at any of these points. However the converse is also true. If any of the steps in this pathway are stimulated by TCDD and increase HSL, then LPL would be depressed.

The purpose of these investigations was to examine the intracellular events responsible for the activation of LPL and HSL and ascertain what affect if any TCDD would have upon these parameters. Perhaps then the biochemical mechanism for LPL inhibition by this dioxin could be explained.

Adipose $pp60^{src}$

In the past 10 years many investigators have focused their attention upon oncogenes and the effect of oncogenic products on normal cellular metabolism. Proteins encoded by oncogenes were thought to always cause cellular transformation but within the last few years the normal physiological functions of these proteins in the absence of tumors have been investigated.

Since TCDD does promote tumor formation in laboratory animals (Kociba 1978) the effect of this dioxin on tumor promoting oncogenic activity was investigated. TCDD was found to increase $pp60^{SRC}$ activity in liver homogenates from rats (D. Bombick, personal communication). Since $pp60^{SRC}$ is a tyrosine specific protein kinase (see Hunter 1984 for review) as is the insulin receptor, it was of interest to know what effect TCDD would have upon $pp60^{SRC}$ in adipose tissue. Levels of $pp60^{SRC}$ in adipose tissue were measured in two ways; binding to a specific iodine labeled monoclonal antibody and measurement of its autophosphorylating capability.

MATERIALS AND METHODS

Shorthair male albino guinea pigs were obtained, housed, and administered 1 ug/kg TCDD or vehicle alone, as before. Adipose acetone:ether powders and blood serum were prepared and stored as previously noted until needed.

Adipose Plasma Membrane Isolation

Fat cell plasma membrane was isolated according to the method of Jarett (1974) utilizing a 9% and 15% discontinuous ficoll gradient (in 0.25 M sucrose). Briefly, perirenal and abdominal fat tissue was homogenized in 3.5 volumes of 10 mM TRIS-HCl (pH 7.4), 1 mM EDTA, .25 M sucrose (Thomas "B" glass-teflon homogenizer, 10 strokes, 1800-2600 rpm). The resulting pellet (16000 g, 15') was resuspended in 4 ml buffer (6 strokes, 1000-1250 rpm, Thomas "A" homogenizer) and centrifuged over the ficoll gradient 15-18 hours. The band formed on top of the 9% ficoll was precipitated with 10 mM TRIS-HCl, 1 mM EDTA, pH 7.4 (20 min., 10,000 g) then resuspended with either the same buffer or 50 mM phosphate buffer and frozen at -80^{o}C until needed.

Intestinal Plasma Membrane Isolation

Intestinal plasma membrane was isolated by a modification of Miller and Crane's original 1961 procedure: The proximal 2/3 of the small intestine was excised and flushed with cold saline or homogenizing buffer (0.25 M sucrose, 0.01 M triethanolamine-HCl, 0.5 mM EDTA, pH 7.5), then everted and the mucosal layer removed by scraping with a glass slide. All subsequent steps were at 4^{o}C. The mucosa was collected, homogenized in 50 ml buffer (25 strokes, loose fitting glass-teflon homogenizer, 1200 rpm), and the cellular debris precipitated by centrifugation (2600 g - 15 min). Twice, the supernatant was spun at 10,000 g - 20' and

the white fluffy layer on top of the pellet was resuspended by homogenization (50 ml, 5 strokes at 1200 rpm). The resulting homogenate was centrifuged at 20,000 g - 10 min., the white fluffy layer resuspended (10 strokes 12000 rpm), and centrifuged again (20,000 g - 20 min.). The resulting crude membrane pellet consisting of both basolateral and brush border membrane was resuspended in homogenizing buffer and stored at -80^{o}C until assayed for ATPase activity. Both fat and intestinal membrane preparations were monitored by electron microscopy for contamination by other cellular organelles.

Epinephrine Binding

Epinephrine binding to fat membrane was as follows: to 50 ug protein in 0.25 M sucrose-10 mM Tris (pH 7.4) was added reaction buffer (50 mM TRIS, 1% BSA, pH 7.4) to a final volume of 500 ul. After a 10 min. preincubation at 30^{o}C ^{3}H-Epinephrine was added (final concentration 10^{-7}M) and the tubes incubated an additional 20 min. at 30^{o}C. Cold reaction (3 ml) buffer was added to stop the reaction. The mixture was then quickly filtered over a 0.45 u cellulose-nitrate membrane filter (HAWP-Millipore) and washed with 2 - 5 ml aliquots of chilled buffer. The filters were allowed to air dry and quantified via liquid scintillation counting. Non-specific binding was measured by the addition of 10 ul cold epinephrine (final concentration 10^{-4} M) to alternate tubes prior to

preincubation and specific binding was calculated by subtracting the non-specific from the total amount bound.

Phosphodiesterase Assay

Fat tissue was prepared for the determination of c-AMP and c-GMP phosphodiesterase activity as follows: Tissue in 5 volumes of homogenizing buffer (0.25 M sucrose, 25 mM TRIS-HCl -- pH 7.4, 0.1 mM EDTA, 5 mM $MgCl_2:6H_2O$, 5 mM KCl, 1 mM phenylmethylsulfonyl fluoride -- PMSF, and 100 units/ml Aprotinin) -- was homogenized (6 strokes, medium speed) with a tight fitting glass-teflon homogenizer. The homogenate was then centrifuged at 1000 g (5 min. 4^oC) to separate the lipid after which the infranatant was subjected to 2500 g (20 min. 4^oC). The supernatant was frozen at -80^oC until use. The assay for phosphodiesterase activity was modified from that of Wolff et al. 1977: 50 ug of protein were added to 20 mM imidazole (pH 7.4), 100 uM $CaCl_2$ (final volume 300 ul) containing 25 uM ^3H-c-GMP or ^3H-c-AMP (New England Nuclear). After a 3 min. incubation at 37^oC the reaction was stopped by placing the tubes in a boiling water bath for 2-3 min, and 0.5 units 5' nucleotidase (Sigma Chemical) was added to hydrolyze the non-hydrolyzed cyclic nucleotide. After 30 min. (37^oC) 1 ml AG 1-X8 ion exchange resin (Bio Rad, Cl form, in isopropanol:H_2O:resin -- 2:2:1) was added, the mixture was vortexed, centrifuged (10 min, 3000 rpm, IEC Clinical Centrifuge), and samples of the supernatant taken

for liquid scintillation counting. The assay was performed by Mr. Yoshiro Kanemoto.

Hormone Sensitive Lipase Assay

Hormone sensitive lipase (HSL) was assayed according to the procedures set forth by Khoo and Steinberg (1975) and Khoo et al. (1976) with the following modifications: 100 ug protein in 50 ul 10 mM TRIS-1 mM EDTA (pH 7.4) was added to 50 ul of 10 mM Mg Acetate, 1 mM TRIS-ATP, 0.02 mM c-AMP, and 200 ug/ml c-AMP dependent protein kinase and incubated for 5 min at 30°C in order to activate the enzyme. The addition of 0.1 ml of a ^{3}H triolein substrate composed of 1 volume of the concentrated substrate used for the LPL assay (see Nilsson-Ehle and Schotz 1976) and 5 volumes 125 mM phosphate buffer containing 2.5 M NaCl and 3% BSA was followed by a second incubation at 30°C (30 min) after which the reaction was stopped with a methanol:chloroform:heptane mixture (1.43:1.25:1.0). K_2CO_3-$K_2B_4O_7$ (0.05 M, pH 10.5, 1.05 ml) was added and the aqueous and organic layers separated by centrifugation at 3000 rpm (IEC Clinical Centrifuge), 15 min. Aliquots of the aqueous (top) layer were sampled for labeled hydrolyzed oleic acid.

Serum Analyses

Serum glucose concentrations were measured using an enzymatic ultraviolet procedure (Sigma Technical Bulletin No. 15 - UV Sigma Chemical Co., St. Louis, MO), and serum

insulin, pancreatic insulin, and serum thyroxine concentrations were estimated via radioimmunoassay (Cambridge Medical Diagnostics, Inc., Billerica, MA). Intercellular c-AMP concentrations were also assessed by radioimmunoassay (Biomedical Technologies Inc., Cambridge, MA). The pellet formed during the final step of all radioimmunoassay procedures was dissolved in 0.5 ml NaOH (0.2 N) of which a 0.4 ml aliquot was sampled for beta emissions. Pancreatic insulin was extracted by the method of Potter et al. (1983).

The LPL, protein kinase, ATPase, and insulin binding assays were all performed as discussed previously, on either plasma membrane fractions or the dried acetone:ether powders as noted. Protein concentrations were determined according to Lowry et al. (1951).

SRC Gene Product, pp^{60} Assay

To get some indication of whether or not the SRC gene product pp^{60}, was present in fat tissue, 1 g of tissue (or 25 mg acetone:ether fat powder) was homogenized in 2 ml (1 ml) RIPA buffer in a glass-glass Tenbroek homogenizer. RIPA (Radioimmunoassay Precipitation Buffer) consisted of 1% Triton X-100, 1% sodium deoxycholate, 100 Kallikrein units/ml of aprotinin, 0.1% sodium dodecyl sulfate, 0.15 M NaCl, in 0.05 M TRIS-HCl at pH 7.2. Cellular debris was discarded after centrifugation for 5 min at 1500 g and the supernatant was recentrifuged for 30 min at 100,000 g. To

500 ug protein, (in 500 ul 0.25 M sucrose, 1 mM EDTA) obtained from the second supernatant fraction was added 3 ul iodinated SRC-Ab mixture. The SRC-Ab was obtained from Oncor, Inc. and iodinated with $Na^{125}I$ using the enzymobead and lactoperoxidase method suggested by Bio-Rad, Inc. The mixture was allowed to react 30' at 25°C then stopped with 5 ml chilled 50 mM TRIS-HCl, 1% BSA, quickly filtered with 0.45 u cellulose-nitrate membrane filters (HAWP-Millipore), and washed with 3 - 5 ml aliquots of cold TRIS-BSA buffer. After air drying the filters were dissolved in scintillation cocktail and monitored for residual activity.

Immunoprecipitation and quantification of pp60 was as follows: 1 gram of fat tissue (or 25 mg of fat acetone:ether powder) was homogenized in 2 ml (1 ml) RIPA buffer in a glass-glass Tenbroek homogenizer. The homogenate was centrifuged 2X as before and 500 ug of protein from the second supernatant fraction was added to 10 ul of the SRC-Ab mixture (in distilled H_2O). After allowing the mixture to sit for 30 min (4°C), 25 mg of protein A-sepharose (Sigma, Inc.), in 200 ul 50 mM TRIS-150 mM NaCl (pH 7.4), was added and the mixture was vortexed. After waiting 3 min the mixture was centrifuged at 1500 g (2 min), the supernatant discarded, and the bead complex resuspended with 200 ul RIPA. The procedure was repeated 4X with RIPA then once with 400 ul of TRIS-NaCl buffer. Autophosphorylation of the protein was accomplished by resuspending the protein

A-sepharose bead complex (containing the attached sarc Ab which was bound with the sarc gene product) in 50 ul kinase buffer (20 mM TRIS-HCl, 5 mM $MgCl_2$, pH 7.2) and adding 20 uCi gamma labelled ^{32}P-ATP (Amersham). After incubating 10 min (30°C) the reaction was stopped with 100 ul 0.125 M TRIS-HCl (pH 6.8), 4% sodium dodecyl sulfate, 20% glycerol, 10% 2-mercaptoethanol and heated for 1 min at 95°C to separate the Ab-Ag from the bead-Protein A complex. The complex was then precipitated by centrifugation (2 min, 1500 g). To 50 ul of the supernatant was added 3 ml 10% TCA plus 0.1 ml BSA solution (100 mg BSA, 136 mg KH_2PO_4/10 ml H_2O) to effect coprecipitation. The mixture was centrifuged 4 min, 3000 g and the pellet washed twice by resuspension with 0.2 N NaOH (0.5 ml) followed by reprecipitation with 1 ml 10% TCA and centrifugation. The final pellet was resuspended with 1 ml 0.2 N NaOH and aliquots sampled for phosphorylated protein using liquid scintillation counting.

All biochemicals were purchased from Sigma Chemical Co. except where noted. All other reagents were of the highest purity possible.

RESULTS

Glucose Reversal of LPL

Previous results indicated orally administered glucose to have no effect in stimulating adipose LPL activity when given 10 days after TCDD exposure. In an effort to further understand TCDD's toxicity, animals were given glucose at 1,

2, and 5 days after dioxin administration and LPL activity was monitored. As can be seen in Figure 35, glucose given 1 day after TCDD restored LPL activity to the same extent as when given to pair-fed animals, however after 2 days glucose reversed LPL activity of TCDD treated animals was 89% of pair-fed controls. After 5 days it was 83% of pair-fed controls and after 10 days it was 23% of pair-fed controls as noted before. TCDD seemed to produce a time dependent inability to provide active LPL upon adequate nutritional stimulation. LPL activity was decreased as soon as 1 hour after dosing with TCDD. The average activity of 1221.4 $\pm$ 394.9 nM ^{3}H- oleic acid released/mg extracted acetone:ether powder/hour in 5 guinea pigs examined. Serum triglyceride concentration was 88 $\pm$ 35 mg/dl.

The inability of glucose to reverse TCDD induced LPL depression, after 10 days, was not due to abnormal intestinal glucose transport or uptake. No change was noted in serum glucose concentrations from either TCDD treated, pair-fed control or ad lib control animals 2 days after treatment (Table 24). Although animals starved for 2 days exhibited levels slightly less than the other groups, they were not statistically significant. Nor was any difference seen between pair-fed control and TCDD treated animals after glucose intubation, however there was a considerable increase in serum glucose compared to the non-administered animals as was expected. The same was true for the 10 day

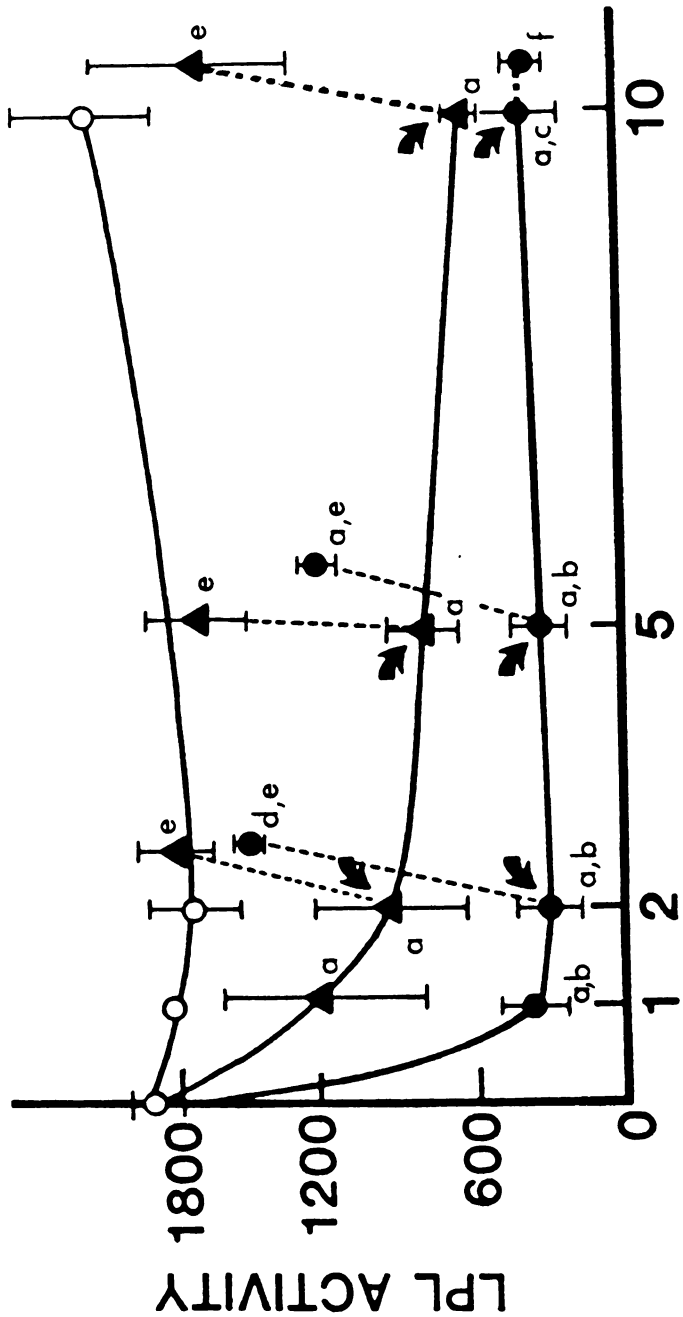


Figure 35. Time course effect of orally administered glucose (arrows) on adipose LPL activity of TCDD treated (●), pair-fed control (▲) or ad lib control (○) guinea pigs. [a]Significantly different from ad lib control; [b]Significantly different from pair-fed control; [c]Not significantly different from pair-fed control; [d]Not significantly different from pair-fed control + glucose; [e]Significantly different from respective treatment without glucose; [f]Not significantly different from 10 day TCDD without glucose; using Tukey's test for unconfounded comparisons (P =.01).

exposed animals (Table 24). Intestinal transport enzymes were not affected 10 days after dioxin exposure as no difference was noted in Na-K, Mg, or Ca ATPase activities between control and TCDD treated animals (Table 25).

Since fat LPL is strongly regulated by insulin and since glucose was being absorbed into the animal, it was of interest to know what effect TCDD had on serum insulin levels. As shown in Figure 36 LPL activity correlated very well with the serum insulin concentration which reflected the nutritional state of the animal. As serum insulin decreased, due to food deprivation, LPL activity also declined. Actual serum insulin concentrations are presented in Table 26 for 8 pairs of animals. Although the variability between animals was large, insulin concentrations were between 5 and 56% lower in the TCDD treated animals. As evidenced with the paired "t" test these differences were highly significant ($P < .001$) with the treated animals having an average serum insulin concentration approximately 35% lower than pair-fed controls. The synthetic capability of the pancreas to produce insulin, in response to orally administered glucose, was reduced by TCDD 2 days after treatment (Table 27). The insulin concentration of extracted pancreatic protein was 6.6X lower in animals previously treated with TCDD than in control animals and may explain the lower serum insulin values.

TABLE 24. Serum glucose concentrations (mg/dl) 2 or 10 days after treatment with acetone:corn oil or TCDD, with and without oral glucose supplement (mean ± S.D., n).

	PFC[a]	TCDD
2 days	156 ± 18 (6)	164 ± 18 (6)[b]
2 days + glucose	457 ± 36 (3)*	435 ± 11 (3)[b,c]*
10 days	164 ± 33 (4)	191 ± 62 (4)[b]
10 days + glucose	500 ± 35 (2)*	588 ± 15 (2)[b]*

[a]Values for animals fed ad libitum or starved for 2 days were 171 ± 43 (6) and 142 ± 19 (5) mg/dl respectively.

[b]Not significantly different from pair-fed control with Tukey's test for unconfounded comparisons after factorial ANOVA.
*P <.01.

[c]Significantly different from respective treatment without glucose administration. P <.01.

TABLE 25. Na-K, Mg, and Ca ATPase activity (nM P_i hydrolyzed/mg protein/hr) in isolated guinea pig intestinal plasma membrane 10 days after treatment with either 1 ug/kg TCDD or vehicle alone and pair-fed to the exposed animals.

	PFC	TCDD
Na-K	1189.6 ± 257.9	1196.3 ± 137.6[a]
Mg	1300.3 ± 225.6	1330.6 ± 66.3
Ca	825.9 ± 197.3	729.4 ± 81.6

[a]No significant differences with paired "t" test.
Mean ± SD for n = 3 pairs of animals.

Figure 36. Adipose LPL activity as a function of serum insulin concentration. (○)PFC, (●) TCDD, (◆) starved. Actual insulin values for 2 day starved 11.4 ± 6.1 (5) and for 2 day ad lib 25.2 ± 9.3 (11) uU/ml.

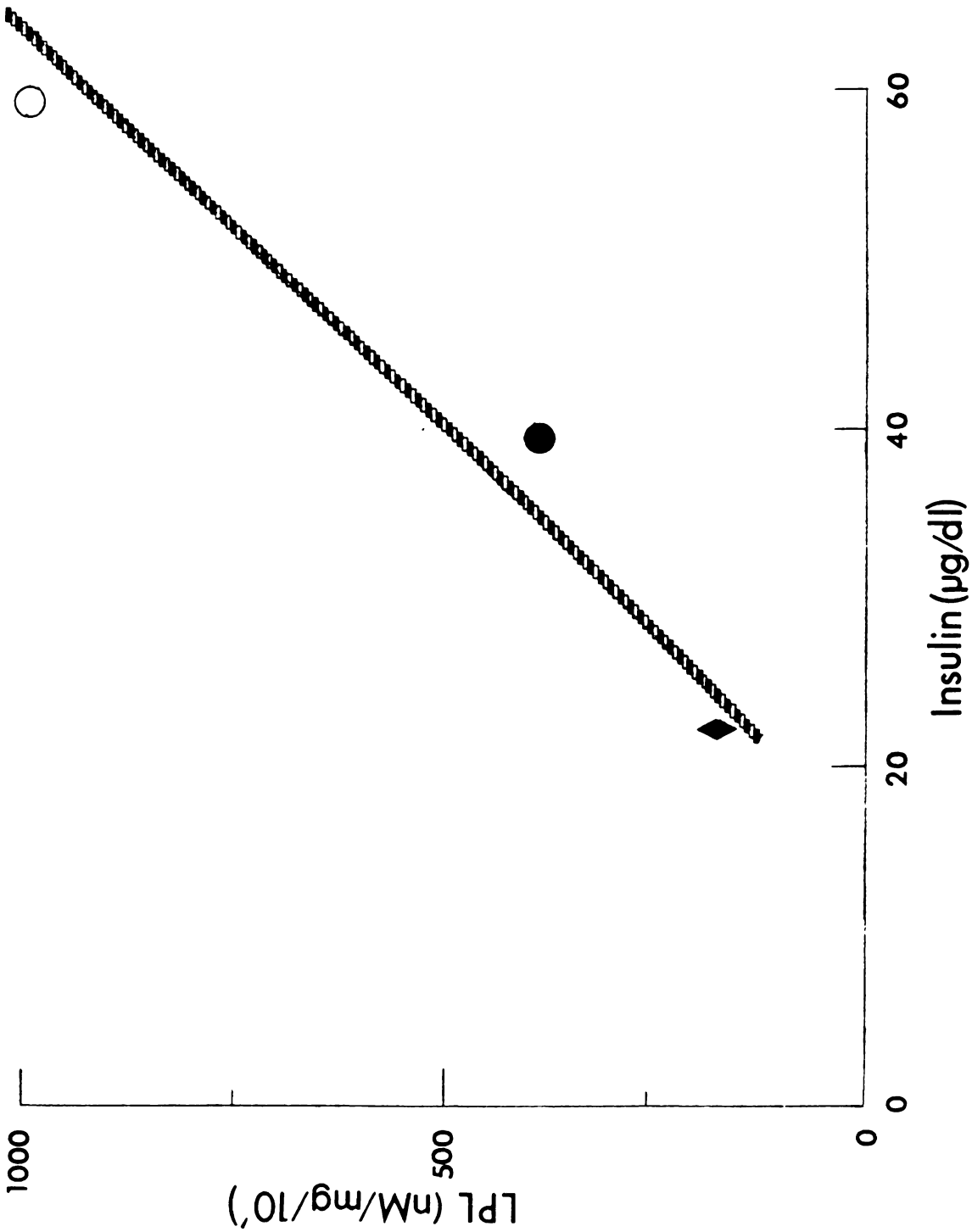

LPL (nM/mg/10')
1000
500
0
0
20
40
60
Insulin (µg/dl)

TABLE 26. Serum insulin (uU/ml) concentrations for 8 pairs of TCDD treated (1 ug/kg) or pair-fed control guinea pigs, 2 days after exposure.

	PFC	TCDD	% Decline
	72	60	17
	58	25	56
	35	19	46
	32	19	40
	98	66	33
	47	31	34
	40	38	5
	58	30	48
x	58 ± 21 uU/ml	39 ± 19 uU/ml	35%

TABLE 27. Effect of TCDD on various lipogenic and lipolytic parameters in blood serum and fat tissue of guinea pigs 2 days after treatment with either 1ug/kg TCDD or vehicle alone (mean ± SD for 3-8 pairs of animals).

	PFC	TCDD
Serum Insulin[a]	58 ± 21 (8)	39 ± 19 (8)*
Pancreatic Insulin[b]	51.5 ± 28.2 (3)	7.8 ± 4.8 (3)*
Insulin binding[c]	45.6 ± 8.0 (3)	154.2 ± 90.0 (3)*
Serum T_4[d]	1.1 ± 0.3 (6)	1.5 ± 0.5 (6)
Epinephrine binding[c]	665.23 ± 560.11 (3)	301.8 ± 164.19 (3)
Cellular cAMP[e]	329 ± 80 (3)	525 ± 69 (3)*
c-AMP phosphodiesterase[f]	226.1 ± 42.9 (4)	357.5 ± 17.6 (3)*
c-GMP phosphodiesterase[f]	1679.73 ± 333.37 (4)	1688.90 ± 273.49 (4)
Protein kinase		
Plasma Membrane[g]		
c-AMP dependent	111.47 ± 16.48 (3)	76.51 ± 13.29 (3)*
c-AMP independent	210.69 ± 25.20 (3)	188.13 ± 23.15 (3)
Cellular[h]		
c-AMP dependent	1756.08 ± 58.15 (3)	1709.62 ± 183.07 (3)
c-AMP independent	312.59 ± 61.45 (3)	302.93 ± 58.48 (5)
HSL[i]	43.4 ± 1.6 (3)	45.5 ± 3.3 (3)

[a] uU/dl
[b] uU/mg extracted pancreatic protein.
[c] pg ^{125}I-insulin or ^{3}H epinephrine specifically bound/mg adipocyte membrane/20 min.
[d] ug/dl
[e] pM c-AMP/g fat tissue.
[f] pM c-AMP or c-GMP hydrolysed/mg protein/3 min.
[g] pM Pi incorporated/mg plasma membrane protein/10min.
[h] pM Pi incorporated/mg acetone:ether fat powder/10 min.
[i] nM oleic acid/mg protein/hr.

*Significantly different from pair-fed control with paired "t" test, P <.05.

Insulin and Epinephrine Binding

Specific ^{125}I-insulin binding tended to be increased in isolated adipocyte membrane preparations from TCDD animals as opposed to pair-fed controls (Table 27). Although the variability was large, binding was increased approximately 3.4X over controls. This tendency for an increased binding and the wide variability in values is reasonable considering the decreased serum insulin concentration values and their variability.

Since thyroxine is also known to inhibit LPL activity (Hamosh and Hamosh 1983) it was of interest to know what effect TCDD would have upon serum thyroxine concentrations. Serum concentrations of T_4 from animals previously dosed with TCDD were not significantly different from control animals (Table 27). After 10 days exposure, concentrations were slightly increased above the 2 day values, but TCDD had no appreciable effect (1.4 $\pm$ 0.5 vs 1.7 $\pm$ 0.6 ug/dl -- control and TCDD treated respectively).

Dioxin reduced binding of ^{3}H-epinephrine to the adipocyte membrane by more than 30% after 2 days of exposure (Table 27). No effort was made to measure adenylate cyclase but the total cellular c-AMP concentration was increased 1.6X by TCDD. Levels of c-AMP phosphodiesterase activity were also increased 158% by dioxin exposure. This phosphodiesterase is one of the main catabolic pathways of cellular c-AMP and is under strong positive regulation by

insulin (see Figure 34). This promotes switching of a fat cell from lipolytic to lipogenic modes. No change was observed in c-GMP phosphodiesterase activity, total cellular protein kinase activity, total cellular c-AMP independent protein kinase, nor in total cellular c-AMP dependent protein kinase activity. Plasma membrane associated c-AMP independent protein kinase was reduced 11% by TCDD. Membrane bound c-AMP dependent protein kinase was significantly depressed 31% relative to control animals. This c-AMP dependent protein kinase is another key regulatory step in adipocyte biochemical pathways. High serum levels of insulin (such as postprandial when high serum levels of triglycerides also occur) shut off HSL activity by inhibiting activation of c-AMP dependent protein kinase and activating c-AMP phosphodiesterase thereby preventing lipolysis while activating lipogenesis. Cellular c-AMP dependent kinase activity was 15-20X higher than that found in the plasma membrane portion as would be expected (Table 27).

$pp60^{SRC}$ Estimation

There occurred 2.5X more $pp60^{SRC}$ in fat tissue from animals treated with TCDD than from pair-fed control animals (Table 28). Activity of the enzyme was estimated by autophosphorylation and found to be increased 114% over the pair-fed controls. It is noteworthy that although the variability was large TCDD treatment resulted in values

TABLE 28. Adipose pp^{60} activity of pair-fed control, TCDD treated guinea pigs (1 ug/kg), or T_4 treated (105 ug/kg) guinea pigs 2 days post-treatment.

	PFC	TCDD	T_4
Quantity via autophosphorylation[a]	3375 ± 547 (6)	3831 ± 788 (6)*	ND
Quality via filter binding[b]	1237 ± 959 (7)	3049 ± 689 (7)*	3325 ± 1334 (2)*

[a] CPM ^{125}I-IgG bound/500 ug protein/filter.
[b] CPM $^{32}P_i$ incorporated/500 ug protein.
*Statistically different from PFC with Signed Ranks Test, $P < .05$.

ranging from 1.5-18X that of control levels in the qualitative assay and from 1-2X that of controls in the quantitative assay. The TCDD treated value was always higher than the control animal in any single pair of animals examined. Administration of 105 ug/kg T_4 also significantly increased the amount of pp60 2.5X above control levels.

DISCUSSION

As concluded previously (Brewster and Matsumura 1984), TCDD reduced LPL activity thereby decreasing serum triglyceride clearance and causing hypertriglyceridemia. Considerable evidence exists for adipose LPL being the critical regulatory step in controlling serum triglyceride concentration (Robinson 1963a, 1970, Scow et al. 1972, Kompiang et al. 1976, Nilsson-Ehle et al. 1980). The present study indicated that reduction of the enzyme occurred as soon as one hour after dioxin administration, attained maximum depression after two days, and remained at that level throughout the 10 day observation period. Oral doses of glucose are known to reverse starvation induced depression of this enzyme (Cryer et al. 1974, 1975) and are correlated with rising serum concentrations of insulin. TCDD prevented this glucose reversal effect 10 days after administration but not after 1 and only marginally after 2 days. TCDD therefore seemed to produce a time dependent inability to provide active enzyme upon stimulation. The synthesis and/or activation of LPL seemed normal early but

lost this ability over time. This loss was not due to malabsorption of glucose into the blood but rather quite possibly to an inability of the pancreas to synthesize adequate amounts of insulin. Binding studies of the insulin receptor in the adipose plasma membrane at day 2 indicated it to be functioning normally and even up regulated in response to the lowered serum insulin levels.

One must be cautious in interpreting any of the insulin data reported here as the radioimmunoassay procedure used an antibody produced in the guinea pig against porcine insulin and as such would only bind to "foreign" insulin molecules. Guinea pig insulin is very different from that of other mammals (esp. rat, pig, and human) and therefore is of great use in current RIA procedures. About 1/3 of the guinea pig's insulin amino acid composition differs from other mammalian insulins and it does not bind Zn, dimerize at high concentrations, or form crystals (Smith 1966, Zimmerman and Yip 1974a, Jukes 1979). Unfortunately purified guinea pig insulin and especially anti-guinea pig insulin is not only very difficult to purchase but also very expensive. However, the guinea pig has been recently found to produce two insulins: classical guinea pig insulin found only in the pancreas and blood and a second insulin distinct from normal guinea pig insulin, produced in all tissues (including pancreas and brain) similar to typical mammalian insulin and at concentrations approximating those in non-pancreatic

tissues of other mammals (Rosenzweig 1980). It is this second "normal" insulin which was detected in the assays used here and discovered to be depressed by TCDD treatment. Furthermore the production or secretion of this normal insulin may increase upon food restriction since the ad libitum serum insulin values obtained here were always much less than either the pair-fed control or TCDD treated animals. Rosenzweig et al. (1980) concluded that the guinea pig has retained a typical mammalian insulin gene which is expressed in all tissues at low levels in like manner as other mammals.

Since the measurement of serum insulin correlated well with LPL activity (Figure 36) it is concluded from these data that at 2 days post treatment with TCDD pancreatic insulin output is decreased. This caused up regulation of the adipocyte membrane insulin receptor and loss of LPL activity. At 2 days oral glucose reversed this loss in activity, probably by de novo LPL synthesis, and activated an inactive cellular LPL pool which slowly declined over time. After 10 days this reversal could not occur; either glucose could not elicit the pancreatic insulin response or the adipocyte membrane insulin receptor was inoperable. In either case the inactive pool has been used by this time.

TCDD inhibited the lipolytic pathway in adipocytes: Epinephrine binding and c-AMP dependent protein kinase activity were both depressed and c-AMP phosphodiesterase

increased by TCDD treatment. Normally this lipolytic inhibition would cause LPL activity to increase, however LPL has been shown to be depressed by dioxin and HSL to be increased or at least unchanged (Brewster and Matsumura 1984, Swift et al. 1981). Preliminary experiments performed in this laboratory also indicate HSL not to be significantly changed. One may suggest this paradox to be explained by the large increases in c-AMP concentration observed here. However, there is evidence indicating that cellular c-AMP levels are compartmentalized and therefore not indicative of lipolysis (Steinberg 1976, Severson 1979). Severson concludes that changes in c-AMP levels can be dissociated from the antilipolytic action of insulin. The reduction of epinephrine binding was not from a downregulation of receptors in response to high levels of serum epinephrine. The treated animals did not display signs of hyperactivity, dermal vasoconstriction, increased cardiac force of contraction and tachycardia, or signs of central nervous system stimulation.

The question of the biochemical cause for the decline of LPL activity still remains. It is proposed that this effect is the result of aberrations at 2 different sites: A primary effect on the pancreas to decrease insulin synthesis and/or secretion and a secondary response in adipose tissue. After two days of treatment serum insulin concentrations are low (from decreased food intake if not

from pathological causes) which could account for the depressed LPL activity to some extent. Recent data (see Appendix D) suggests that more than decreased insulin levels are involved. Animals treated with TCDD and starved for 2 days had significantly lower levels of LPL activity than control animals starved for 2 days. These data indicate the cell functioned as if it had received increased insulin, since HSL activity was not changed. The absence of any change in HSL activity may be explained by a finely tuned regulatory system - the increase in activity from decreased food intake may be balanced by the antilipolytic actions occurring within the cell. There is precedence, at least within rat adipocytes, that starvation itself uncouples adenylate cyclase activation from lipolysis (Fain and Garcia-Sainz 1983). Although isolated adipocytes from starved animals were more sensitive to isoproterenol activation of adenylate cyclase than those from control animals, lipolysis was significantly reduced.

Conclusions

It is interesting that the adipocyte behaves like the hepatocyte in that the cell displays increased responsiveness to a substrate when that substrate to the cell is actually reduced (see Madhukar et al. 1984, Appendix I). It is proposed that as with liver cells and EGF, TCDD induces an internal phosphorylation, which then causes the antilipolytic actions discussed above. This

internal trigger could even be EGF itself, as it has been shown to promote intracellular lipogenic activities when administered in nanomolar concentrations to isolated adipocytes (Ng and Wong 1984). Although sustained cellular phosphorylation via protein kinase does not occur in fat tissue there is sustained phosphorylation via $pp60^{SRC}$ similar to that observed in the liver (D. Bombick - personal communication). This $pp60^{SRC}$ is an autophosphorylating kinase of 60,000 daltons which can be increased by EGF and whose cellular substrates are not fully realized. It is interesting to note that T_4 (also known to inhibit LPL) also increases pp60 activity. Administration of EGF in vivo has been observed to promote serum hypertriglyceridemia (Heimberg 1965). Therefore it is proposed that TCDD inhibits LPL activity by causing endogenous cellular phosphorylation (possibly through altered gene expression) which promotes the reesterification of free fatty acids to stored triglycerides. Therefore TCDD invokes an energy requiring futile cycle in adipocytes: HSL is normal and breaks down stored triglycerides to free fatty acids for export; the increased EGF like receptor activity and cellular phosphorylation reesterifies these fatty acids to stored triglycerides which require ATP consumption; some of the fatty acids are mobilized thus the reason for loss of adipose stores with time; consumption of ATP limits synthesis of LPL therefore no new fatty acids from serum

triglycerides enter the cell and hypertriglyceridemia develops. Patton (1970) concluded that LPL is regulated in just such a manner. Furthermore, if the same process is occurring in other cells, nutrient depletion and atrophy would be expected since the cell would reesterify any free fatty acids it obtains into stored triglycerides. This quite conceivably is the mechanism for the fatty liver observed in many species after dioxin administration. These cells are less likely to deplete their ATP stores because of the much greater concentration of mitochondria compared to adipocytes. The results presented here and in previous work are consistent with this possibility.

Further research concerning cellular substrates of the phosphorylated EGF receptor and its relationship to lipogenesis must be examined. In lieu of the similarity of the insulin and EGF receptors and their resulting kinase activities upon substrate interactions this proposal is attractive. An examination of tyrosine kinase activity, protein kinase C, and Ca (all of which influence EGF activity) must be undertaken after administration of TCDD.

It is also interesting that TCDD again, as discussed in Chapter VII, seems to provoke an aging response in these animals. Fain and Garcia-Sainz (1983) cite studies whereby the lipolytic sensitivity of adipocytes from older animals was markedly reduced from that of younger animals. In the present study no evidence of increased lipolytic activity

was noted after TCDD treatment even though LPL was significantly depressed.

CHAPTER IX

SUMMARY

These investigations have shown that TCDD does indeed alter the character of the plasma membrane in different tissues and species. Based on the inferences of earlier investigations it was prudent to investigate this cellular organelle further, and it was soon found that extensive time dependent alterations in protein composition of the cellular membrane occur after administration of TCDD. It was then hypothesized that changes occurring at vital physiological and biochemical sites could indeed be responsible for some of this agent's toxic manifestations.

In an effort to link these membrane alterations with specific biochemical pathways, a number of receptor and enzymatic interactions were examined for their response to TCDD treatment. The rat liver underwent time and dose dependent inhibitions in ion transport and regulation, growth factor binding, amino acid transport, and an inability to accumulate energy sources and nutrients such as glucose. There also occurred a sustained increase in cellular phosphorylation; a process critical in homeostatic cellular regulation to hormones, growth factors, and other critical biochemical pathways. Little change was seen in

the livers of guinea pigs and hamsters, two species showing very little hepatotoxicity in response to this chemical.

Next an effort was made to correlate some of these biochemical alterations with specific toxic lesions. One such pertubation examined was thymic atrophy. TCDD produced obvious morphological changes on the surface of the thymocyte which may prevent cellular communication and nutrient uptake and be involved in wasting and immunological incompetence. The biochemical mechanism responsible for the surface changes noted could quite conceivably be related to calcium transport and regulation, however this area needs to be examined further.

Investigative efforts were then focused upon the cause for the severe serum hypertriglyceridemia observed in the guinea pig after dioxin exposure. The mechanism for this anomaly was discovered to be almost complete inhibition of the enzyme lipoprotein lipase which is responsible for the removal of serum triglycerides. This depression was found to be time and dose dependent and responsible for the loss of fat stores. It also correlated with rising serum triglyceride concentrations and body weight loss; it could not be reversed with glucose after 10 days and it was not a result of changes in serum inhibitors or stimulators. Decreased LPL results in hypertriglyceridemia. This could contribute to secondary effects such as xanthoma, atherosclerosis, cardiac disfunction and eventually death.

In addition, the high serum titer of triglycerides could quite conceivably trigger normal feedback mechanisms to reduce food intake, contributing to weight loss and wasting.

The question was posed as to whether TCDD diminished LPL and caused serum hypertriglyceridemia in other animal species. Rabbits and hamsters resembled guinea pigs in their lipid response to TCDD and the similarity of rabbit and human lipid metabolism make rabbits a useful model to study cause and effect of lipid disorders. Rats had not been reported to have increased serum triglycerides and no change in LPL activity was noted. Mink showed a modest decrease in enzyme activity which did not correlate with serum levels of triglycerides probably because of their complex lipid metabolism. Mice hypertriglyceridemia, for the most part was observed in the sensitive strains and not in the less responsive strains. The size and small amount of adipose tissue available made LPL determination very difficult in this species.

Because of the high serum lipids and the reduction of adipose LPL it was of interest to know if heart function was compromised by TCDD. It soon became obvious that the heart is somewhat protected from nutritional regulation of LPL and showed little response to dioxin treatment. However, the membrane bound beta-adrenergic system was reduced in its response to isoproterenol after prolonged dioxin exposure.

The depressed rate and force of contraction (basal levels) of heart atria may have resulted from the increased serum lipid load and are indicative of an aging effect. This and the depressed adrenergic response could very well be linked with lethality.

Finally, the task was undertaken to elucidate the biochemical mechanism responsible for LPL inhibition. It can be definitely stated that the cause was not due to HSL activation and a reciprocal LPL depression. Enzymatic activity was decreased as soon as one day after treatment, and TCDD was shown to produce a time dependent inability to reverse this depression, independent of serum glucose levels. This finding implicated an inhibition of insulin synthesis or secretion. Although insulin serum levels and the pancreatic response to glucose are depressed the adipocyte functions as if excess insulin is present and prevents high rates of lipolysis. It is proposed that TCDD promotes phosphorylation of the EGF receptor which in turn causes sustained cellular phosphorylation of any number of substrates including $pp60^{SRC}$. It is this sustained phosphorylation which in turn blocks LPL synthesis and/or activation. This effect is secondary and probably occurs after the depression of pancreatic insulin synthesis and secretion.

As far as LPL depression is concerned there are two mechanisms by which the enzyme is not produced. First is

the reduction of pancreatic insulin. Data presented in Appendix D indicate that pancreatic EGF-like binding proteins are twofold greater in TCDD treated animals than pair-fed control, indicating a depression of insulin synthesis. However, since animals treated with TCDD and starved have significantly lower LPL activity than starved animals alone (see Appendix D) another mechanism must be occurring other than simply lack of insulin production (or secretion) by the pancreas. The second critical site is at the level of the adipocyte. Either the increased cellular phosphorylation shuts off synthesis directly, or feedback to the synthetic process prevents synthesis, or the cell simply runs out of energy. Patton (1970) theorized this third possibility to be the reason why enhanced HSL synthesis decreases LPL activity. HSL produces free fatty acids from stored triglycerides which in the presence of glucose and insulin are re-esterified to triglycerides. This process increases ATP consumption and causes a reduction in protein synthesis.

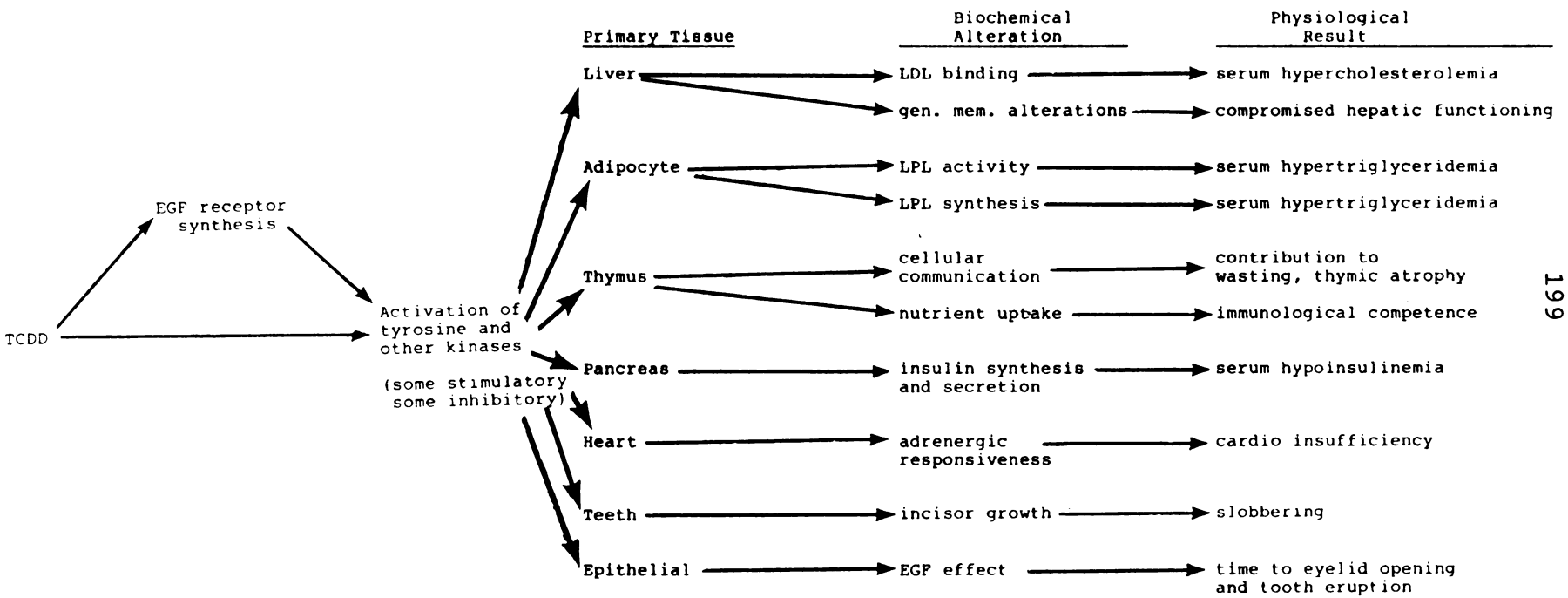
This study has elucidated some plasma membrane alterations responsible for a few of TCDD's toxic manifestations. However, the underlying mechanism of TCDD toxicity (how these alterations come about) is still a matter of conjecture. Direct interactions with the plasma membrane are not indicated as <u>in vitro</u> enzymatic inhibition does not occur, far more toxic lesions occur than the body

burden of TCDD allows (the number of dioxin molecules in the body is far less than the number of lesions) and TCDD is known to first interact with a cytoplasmic receptor, then is transferred to the nucleus to produce a pleotropic effect.

Proposed Unifying Theory for TCDD's Mode of Action

There is now some evidence that TCDD invokes an increased tyrosine kinase activity which is coupled with an increase in an activated EGF receptor synthesis (B.V. Madhukar - personal communicaton). This would account for the increased hepatic EGF receptor phosphorylation and depression of ligand binding (increased internal phosphorylation of the EGF receptor causes down regulation of the membrane receptor by a feedback mechanism - see Appendix A). Present results indicate that TCDD increased protein kinase and/or tyrosine kinase activity in the liver, adipocyte, and pancreas. Other studies in this laboratory have shown increased tyrosine kinase activity in the thymus, the liver (D. Bombick - personal communication), and in an in vitro system of XB cells (B.V. Madhukar - personal communication). In all tissues displaying toxic effects after TCDD administration, tyrosine kinase activity has been found to be increased. It is therefore postulated that activation of tyrosine and other kinases, ome stimulatory and others inhibitory, is responsible for the manifestations of toxicity in most of the tissues listed in Figure 37.

Figure 37. Proposed unifying hypothesis for TCDD's mode of action.


Primary Tissue
Biochemical Alteration
Physiological Result
TCDD
EGF receptor synthesis
Activation of tyrosine and other kinases
(some stimulatory some inhibitory)
Liver
LDL binding
serum hypercholesterolemia
gen. mem. alterations
compromised hepatic functioning
Adipocyte
LPL activity
serum hypertriglyceridemia
LPL synthesis
serum hypertriglyceridemia
Thymus
cellular communication
contribution to wasting, thymic atrophy
nutrient uptake
immunological competence
Pancreas
insulin synthesis and secretion
serum hypoinsulinemia
Heart
adrenergic responsiveness
cardio insufficiency
Teeth
incisor growth
slobbering
Epithelial
EGF effect
time to eyelid opening and tooth eruption

Activation of kinases in the liver system would be responsible for general membrane alterations and at least in some cases compromised hepatic function. Since the LDL receptor is a tyrosine kinase when bound with its ligand, endogenous activation of tyrosine kinase would cause down regulation of the LDL receptor and lead to serum hypercholesterolemia, a common response of TCDD exposure. Another example is the insulin receptor which is organized in a similar manner as the EGF receptor in that activation by a tyrosine kinase is expected to result in down regulation.

In the adipocyte, tyrosine kinase activation has also been demonstrated and may be responsible for the depressed LPL synthesis or activation described in the current study. This link has not been proven here but as evidenced by these data the reduction of LPL is responsible for the second major lipid defect produced by TCDD -- serum hypertriglyceridemia.

As for the thymus and pancreas, general growth hormone binding, nutrient uptake, and cellular communication are affected. Consequently thymic atrophy, wasting, depressed immunological competence, and serum hypoinsulinemia would be expected. An increase in the phosphorylation activity of the EGF receptor in the pancreas was shown in this study (see Appendix D). In view of recent evidence that increased EGF caused a decrease in insulin secretion from the pancreas

(Scott et al. 1985), it is reasonable to assume that the increased phosphorylation of the EGF receptor would cause the reduction of insulin synthesis or secretion by this tissue.

As for the heart, EGF can effect the beta-adrenergic system through alterations in GTP metabolism and the GTP binding protein. As discussed by Fain and Garcia-Sainz (1983) alpha and beta receptors can be modulated by guanine nucleotide binding protein which in turn is influenced by the phosphorylated EGF receptor and tyrosine kinase.

The effect of EGF on teeth and epithelial tissue is well known. It has been demonstrated in these studies that both EGF and TCDD administration cause early tooth eruption and eyelid opening in neonatal mice (Appendix A).

Significance

This research has elucidated some of the mechanisms responsible for TCDD's toxicity. An insight into the biochemistry of toxic symptoms has been established. TCDD is a unique chemical in that it provokes many different changes in different tissues and organs, and as a result it can be used as a probe to study the normal cellular physiology of those tissues. With this understanding one can gain an understanding of how other environmental pollutants cause toxicity and how to prevent these biochemical alterations from occurring.

APPENDICES

APPENDIX A

THE INFLUENCE OF 2,3,7,8-TETRACHLORODIBENZO-P-DIOXIN ON EPIDERMAL GROWTH FACTOR RECEPTOR BINDING IN THE HEPATIC PLASMA MEMBRANE OF THE RAT, GUINEA PIG, MOUSE, AND HAMSTER

It has recently been found that the plasma membrane protein composition and function of hepatocytes from TCDD-treated rats are quite different from those of control rats (Brewster et al. 1982, Matsumura et al. 1984). Therefore, the current investigation was undertaken with the following objectives: (i) find several biochemical parameters on the plasma membrane that are severely affected by TCDD, (ii) study whether any of the changes occur at low enough doses and at very early stages in susceptible species, and only at high doses in tolerant species, and (iii) make an attempt to relate such effects to some of the toxic manifestations in vivo. This study was done in collaboration with Dr. B.V. Madhukar and Mr. David Bombick of the Pesticide Research Center, MSU. Dr. Madhukar completed the dose response relationships, scatchard analyses, EGF binding to mouse plasma membrane and autoradiography, and Mr. Bombick the effect of various chemicals on EGF binding in the rat liver. I examined the time course of changes in specific binding of EGF in the rat, the changes of body and thymus weight after TCDD

treatment, and aided in ascribing the effect of TCDD on eyelid opening, incisor eruption, and hair growth in neonatal mice exposed to TCDD or EGF.

MATERIALS AND METHODS

Male Sprague-Dawley rats (150-200 g) and Golden Syrian hamsters (80-90 g) were obtained from Spartan Laboratory Animals (Haslett, MI). Male guinea pigs (200-250 g) were obtained from Michigan Department of Health, Lansing, MI and female BALB/c mice were purchased from Harlan Laboratories (Haslett, MI). Inbred mouse strains C57BL/6J, CBA/J, and AKR/J were obtained from The Jackson Laboratory. Food and water were provided ad lib. All chemicals used in vivo were administered to the animals intraperitoneally (i.p.) as solutions in either corn oil/acetone (9:1; TCDD), 0.85% NaCl (phenobarbital), or corn oil (all others). Control animals received an equivalent volume of the vehicle only.

TCDD was a gift from Dow and was >99% pure (GLC examined); 3,4,3',4'-tetrachloroazoxybenzene was generously given to us by M.T. Stephen Hsia (University of Wisconsin, Madison, WI) and was >99% pure; sodium phenobarbital was purchased from Mallinckrodt; 3-methylcholanthrene, and epidermal growth factor (EGF) were from Sigma; Aroclor-1242, a polychlorinated biphenyl mixture containing 42% chlorine, was a gift from Monsanto; 3,4,3',4'-tetrachlorobiphenyl (>99% pure) was obtained from Analabs, North Haven, CT; and 1,1,1-trichloro-2,2-bis (p-chlorophenyl) ethane >99% pure

was a gift from Montrose Chemical (Torrance, CA). Firemaster BP-6 was given to us by Matthew J. Zabik (Michigan State University).

^{125}I-labeled insulin (specific activity, 80-103 uCi/ug; 1 Ci = 37 GBq), ^{125}I-labeled EGF (specific activity, 161-174 uCi/ug), and ^{3}H-labeled Con A (specific activity, 25-50 Ci/mmol) were purchased from New England Nuclear. [^{32}P] ATP (Tris salt, specific activity, 3000 Ci/mmol) was obtained from Amersham, alpha-Methyl D-mannopyranoside and insulin (porcine) were purchased from Sigma. Receptor grade EGF was obtained from Collaborative Research (Waltham, MA). All other biochemicals and chemicals used were of the highest purity available.

Liver plasma membrane from normal or TCDD-treated animals was prepared as described by Peterson et al. (1979a) and preparations were periodically examined by electron microscopy. Binding of either ^{125}I-labeled EGF or ^{125}I-labeled insulin to liver plasma membrane was assayed according to the method of O'Keefe et al. (1974) and ^{3}H-labeled Con A binding was determined essentially as described by Chandramouli et al. (1977) with minor modifications as described by Brewster et al. (1982). Phosphorylation assay was done essentially as described by Rubin et al. (1982).

Females of BALB/c mice (15-18 days pregnant) were housed in plastic cages individually, and food and water

were provided ad lib. The time of delivery was closely followed and within 3 hr, the dams were treated i.p. with a single dose of either TCDD in corn oil/acetone (9:1) at 10 ug/kg or with the vehicle alone (0.5 ml per 100 g of body weight). Prior to treatment, two littermates from each litter were exchanged and marked to identify them from the original littermates. Body weights of the neonates were recorded daily and were checked for incisor eruption and eyelid opening twice daily (between 8 and 9 a.m. and between 5 and 6 p.m.). The day of delivery was considered as day 0. Hair diameter and length were measured on day 14 from samples taken from the mid-right dorsal region. They were mounted on glass slides with glycerol and measured under a microscope. EGF in 0.85% NaCl was injected subcutaneously to newborn mice daily at 2 ug/g of body weight. Body weights and other developmental parameters were measured as described above. All animals were sacrificed at 22 days of age and thymus weights were recorded.

RESULTS

We have examined the effects of in vivo-administered TCDD on three receptors of the rat hepatic plasma membrane at various doses to assess which of these is most sensitive. It is clear that the effect of TCDD on EGF binding was most pronounced, followed by that of Con A and insulin. It is significant that the effect on EGF binding

is observable at a dose as low as 0.1 ug/kg. It was also noted that insulin binding was significantly lower at the highest dose (115 ug/kg) and higher at low doses as compared to the corresponding control preparation at 10 days after treatment.

In view of the sensitivity of EGF binding to TCDD treatment, we examined the time course of TCDD effect after a single i.p. dose (25 ug/kg). As observed perviously (Figure 12) during the 40-day observation period, TCDD-treated rats gained consistently less body weight than did control rats. During the same period, the level of EGF binding was continuously suppressed. The decline was noticeable on the second day, reached a maximum on day 20, and by day 40 a trend of apparent recovery was observed. At this dose and treatment, this apparent recovery is believed to result from mortality of the susceptible population. The average mortality was 0 at day 20, but reached 20-30% by day 40; therefore, the data at this time point represent the value from surviving animals.

The nature of the changes in the EGF receptor was studied by Dr. Madhukar using Scatchard analysis of ligand-receptor binding. The results indicated that EGF binding generally showed a biphasic relationship, as shown by Ivanovic and Weinstein (1982) and that the number of high as well as low affinity receptors in the TCDD-treated rats

was reduced without any apparent changes in the receptor affinity.

To study whether such biochemical changes are also evoked by other toxic chemicals or only by TCDD, Mr. David Bombick assessed the effect of various xenobiotics *in vivo* on EGF binding *in vitro*. Among nine chemicals tested, only TCDD and Aroclor-1242 caused a significant decrease in EGF binding. In both cases, the effects appear to be dose related. It must be noted that rather high doses were used for other chemicals, resulting in the manifestation of toxicities in most cases. Yet, even under these conditions no reduction in EGF binding was observed.

To further understand the relationship between these biochemical changes and susceptibility of different species to TCDD, the dose response of EGF binding was examined by Dr. Madhukar. Ten days after single i.p. treatments, plasma membranes were isolated from each animal, and the levels of EGF binding were quantified. The results indicate that the guinea pig system was most sensitive, followed by that of the rat and the hamster. If one adopts the I_{50} (i.e., the dose of TCDD to suppress EGF binding to 50% of the control level) as a critical dose, the rat and the hamster may be considered to be ~14 and ~32 times less sensitive, respectively, than the guinea pig in this regard. At I_{75}, the species difference was much greater.

Inbred strains of mice (C57BL/6J, CBA/J, and AKR/J) known to show different degrees of tolerance to TCDD (Poland et al. 1974) were examined. The results (Table 29) clearly indicate that reduction in EGF binding was more severe in the two sensitive strains (C57BL/6J and CBA/J) than in the resistant strain (AKR/J). Although the CBA/J strain is known to possess a high affinity cytosolic TCDD receptor, it is tolerant to TCDD in terms of cleft palate formation (i.e., a teratogenic effect). It is interesting that this strain is sensitive to TCDD, as judged by EGF receptor assay as well as body weight loss and thymic involution.

To study whether some of the TCDD-caused toxic effects are similar to those produced by excess EGF (see Discussion), mouse neonates were treated postnatally with TCDD and various developmental parameters were examined.

The most recognized in vivo effects of EGF are early eye opening and tooth eruption in mouse neonates (Heimberg et al. 1965; Cohen and Elliot 1963). The action of TCDD in this regard was clear (Table 30) in that both events occur at an earlier age in treated animals than in controls. Other parameters examined also show that the lesions caused by TCDD are remarkably similar to those occurring in EGF-treated animals (Schlessinger et al. 1983).

When Dr. Madhukar isolated hepatic plasma membrane from treated and control rats, incubated it with [gamma-^{32}P]ATP, subjected it to gel electrophoresis, and autoradiography, it

TABLE 29. Changes in ^{125}I-labeled EGF binding to hepatic plasma membrane, body weight, and thymus weight in mouse strains 10 days after TCDD treatment.

Strain[a]	EGF bound, pg per 50 ug of protein		% initial body weight		Thymus, mg	
	Control (n)	Treated (n)	Control (n)	Treated (n)	Control (n)	Treated (n)
C57BL/6J	102.7± 5.8(7)	7.1±3.3(3)*	95.7±2.9(3)	89.8±4.6(3)	46.0±1.7(3)	10.7±1.2(3)*
CBA/J	122.0±14.0(7)	2.7± 0.1(3)*	103.1±2.8(3)	88.8±3.9(3)*	42.7±2.5(3)	11.0±2.7(3)*
AKR/J	102.8±11.9(4)	55.5±15.2(3)*	98.3±1.1(3)	96.8±5.7(3)	82.0±7.6(3)	23.0±0.9(3)

[a]Mice were treated with a single i.p. dose of TCDD in corn oil/acetone (9:1) at 115 ug/kg. Controls received an appropriate volume of the vehicle alone (0.5 ml per 100 g of body weight).
*Statistically different from control. P <.0005.

TABLE 30. Effect of TCDD on eyelid opening, incisor eruption, and hair growth on neonatal BALB/c mice.

	Treatment		
	Control (n)	TCDD (n)	EGF (n)
Development			
Eyelid opening, days	13.7± 0.5(14)	11.4±0.5(13)*	10.7±0.5(10)*
Tooth eruption, days			
Lower	9.9± 0.5(14)	9.0±0.4(13)*	7.5±0.5(10)*
Upper	11.0± 0.0(14)	10.2±0.4(13)*	8.2±0.1(10)*
Hair			
Length, mm	7.3± 0.9(24)	4.9±0.7(36)*	5.1±0.7(24)*
Diameter, um	17.5± 0.3(30)	12.3±0.2(30)*	8.2±0.5(25)*
Weight			
Body weight, g	10.0± 0.7(10)	7.9±0.8(10)*	8.2±0.5 (5)
Thymus, mg	74.0±12.6(10)	41.7±6.8(10)*	54.5±2.5 (5)

*Significantly different from control with students "t" test. P <.0005.

became evident that the band intensity, representing ^{32}P incorporation into the EGF receptor, increased in plasma membrane from animals sacrificed at days 1, 2, and 10. The results agree with the observation made by others (Schlessinger et al. 1983; Lee and Weinstein 1978) that agents affecting EGF receptors cause an increase in phosphorylation of the EGF receptor. It was also noted that there are other bands of which intensities were much higher in TCDD-treated preparations than in control.

DISCUSSION

In the current work we have established that TCDD, when administered in vivo, causes changes in receptor activities for EGF, insulin, and Con A on the rat hepatic plasma membrane, and that EGF receptor binding is the most sensitive parameter. Obviously, the most pressing question is how TCDD causes various toxic symptoms. Thus, the meaning of the above effect of TCDD on EGF receptor must be discussed.

In the past, several compounds have been identified to reduce EGF receptor binding. Examples are: phorbol acid esters such as 12-tetradecanoylphorbol 13-acetate (Lee and Weinstein 1978, 1979), Rous sarcoma and other viruses causing neoplastic transformation (Todaro et al. 1976; Erikson et al. 1981), hormones such as vasopressin (Rozengurt et al. 1981) and thyroxine (Hayden and Severson 1983), and the solvent dimethylsulfoxide (Rubin and Earp

1983). At least the first three groups of agents are known to cause or promote neoplastic development in various tissues. The most intriguing aspect of this phenomenon is that the tissues and the cells exhibiting loss of EGF receptor activities by these treatments elicit responses characteristic of excess EGF (Lee and Weinstein 1978, 1979). For instance, cells that have been treated with 12-tetradecanoylphorbol 13-acetate, showing decreased number of EGF receptors, exhibit an increase in ornithine decarboxylase, stimulation of plasminogen activator, enhancement of sugar transport, prostaglandin synthesis, and stimulation of mitogenic activities. All these cellular changes are observed in cells and tissues receiving excess EGF (Lee and Weinstein 1978, 1979). Also, Rose et al. (1976) have found that EGF promotes skin tumors in mice treated with methylcholanthrene, as in the case of 12-tetradecanoylphorbol 13-acetate. The tumor-promoting potential of TCDD in mouse skin has also been shown by Poland et al. (1982) in hairless mice.

The cause of this EGF-like effect by agents that decrease EGF receptor activity as proposed by DeLarco and Todaro (1978) and Weinstein and Lee (1978, 1979) appears to be due to the production of endogenous growth factor(s) by the affected cells and tissues. In the case of transforming viruses, such growth factors (termed TGF for "transformation growth factors") have actually been found, isolated, and

characterized (Todaro and DeLarco 1978; Todaro et al. 1979). An alternative explanation for these events may be that these agents or treatment ultimately cause phosphorylation of the EGF receptor, as shown in the case of 12-tetradecanoylphorbol 13-acetate (Schlessinger et al. 1982), dimethylsulfoxide (Rubin and Earp 1983), Rouse sarcoma virus (Erikson et al. 1981), partial hepatectomy (Rubin et al. 1982; Earp and O'Keefe 1981), etc., and that this phosphorylation action alone could activate a chain of events that is normally triggered by the binding of the ligand, EGF, to its receptor. In turn, these cells with phosphorylated receptors may no longer require external ligands to process the messages of transaction.

In the current study, we have found that TCDD does indeed increase phosphorylation of the EGF receptor in the rat liver plasma membrane, and that it has the same in vivo effects as exogenous EGF with regard to the early eyelid opening and tooth eruption in neonatal mice. The eyelid effect has been cited as the most specific biological index of EGF activity in vivo (Schlessinger et al. 1982).

With this evidence, we propose the hypothesis that some of the toxic manifestations of TCDD, particularly the hyperplastic response among epithelial tissues, is a result of the action of TCDD on the EGF receptor.

If one examines the toxic manifestations caused by TCDD from such a viewpoint, one cannot help but notice many

similarities between them and those caused by EGF administration *in vivo* and *in vitro*. They are, for example, fatty invasion of the liver (Heimberg et al. 1965; Gupta et al. 1973), inhibition of terminal differentiation of keratinocytes (Rheinwald and Green 1977; Knutson and Poland 1980), skin cancer promotion (Poland et al. 1982; Rose et al. 1976), proliferation of conjunctiva cells (Cohen and Elliot 1963; Norback and Allen 1973), inhibition of gastric secretion (Todaro and DeLarco 1978; Norback and Allen 1973; Bower et al. 1975), ornithine decarboxylase changes (Stastny and Cohen 1970; Potter et al. 1983), and serum hypertriglyceridemia (Heimberg et al. 1965; Albro et al. 1978), in addition to hair growth, early eye opening, etc. as shown in the current work. While in several cases the experimental conditions for TCDD studies differ from those of EGF and caution is needed to draw a direct analogy, these similarities are remarkable and therefore the relationship between TCDD and EGF effects warrants further attention.

APPENDIX B

CHARACTERIZATION OF THE SERUM HYPERLIPEDEMIA PRODUCED IN THE RABBIT UPON ADMINISTRATION OF 2,3,7,8 TETRACHLORODIBENZO-P-DIOXIN

INTRODUCTION

TCDD is one of the most toxic chemicals known, however the mechanism resulting in the manifestations of its toxicity have not yet been elucidated. It has recently been shown that TCDD causes large increases in the serum triglyceride and serum cholesterol levels in guinea pigs (Gasiewicz and Neal 1979, Brewster and Matsumura 1984, Bombick et al. 1984) and increased triglyceride concentrations in the plasma of the rabbit (Lovati et al. 1984). Since lipid metabolism in the rabbit is similar to that of man the white rabbit has proved to be a good model to study defects in lipid metabolism. Recent concern for human exposure to this compound has led to an intensive search for its biochemical actions.

Adipose lipoprotein lipase (LPL) is responsible for the removal of serum triglycerides for utilization in various tissues throughout the body and when this enzyme is inhibited serum concentrations increase (Hamosh and Hamosh 1983). In like manner hepatic LDL receptors are critical in

removing LDL particles from the serum. When LDL binding is inhibited serum cholesterol increases (Brown et al. 1981).

The purpose of the present study was to characterize the serum hyperlipedemia produced by TCDD in the rabbit. The mechanisms for this hyperlipedemia, acute results, and long term potential human health effects stemming from TCDD exposure are discussed.

This work was done in collaboration with Mr. David Bombick. He determined the LDL binding to isolated hepatocytes and prepared sections of aortic arches for electron microscopy. Determination of the concentration of various serum parameters (triglyceride, cholesterol, protein, insulin, and glucose) and LPL activity was done by myself.

MATERIALS AND METHODS

Outbred New Zealand White Rabbits (2-3 kg) were obtained from the Michigan Department of Health and housed in stainless steel cages on a 12 hour light - 12 hour dark cycle with constant temperature and humidity. Food (Purina Rabbit Chow) and tap water were provided ad libitum except where noted for pair-fed controls which received only as much food as consumed by the TCDD treated animals.

TCDD (>99.99% pure - Dow Chemical Co., Midland, MI) was dissolved in acetone:corn oil (1:9) and was administered intraperitoneally to the animals at doses of either 1 or 50 ug/kg. Ten days later animals were etherized, blood

collected via cardiac puncture, abdominal and perirenal adipose tissue collected, and the liver perfused. Serum triglyceride concentrations, adipose LPL activity and hepatic LDL binding were determined as before (Brewster and Matsumura 1984, Bombick et al. 1984). Serum cholesterol concentration was determined by the method of Carr and Drekter (1956), protein concentration via the procedure of Lowry et al. (1954), and insulin concentration via radioimmunoassay (Cambridge Medical Diagnostics, Inc., Billerica, MA).

Twenty days after treatment sections of aortic arches were prepared for transmission and scanning electron microscopy utilizing the methods put forth by Hooper et al. (1979).

All reagents used were of the highest quality available and were purchased from Sigma Chemical Corp., St. Louis, MO.

RESULTS

At these doses TCDD treated rabbits showed little of the wasting syndrome typically produced in other species after exposure to this compound for 10 days. Body weight at the end of the 10 day exposure was 89 $\pm$ 8% of initial body weight for animals dosed with 50 ug/kg TCDD and 94 $\pm$ 3% for the pair-fed controls to this dose. At 1 ug/kg treatment body weight was 96 $\pm$ 4% while pair-fed controls were 96 $\pm$ 3% of initial body weight. However, after 20 days at 50 ug/kg pilo erection and hair loss were evident and the animals

exhibited a significant decrease in cage movement preferring to remain huddled near the back of the cage. Food and water intake were qualitatively depressed. Abdominal and perirenal fat weight was approximately 12 g in the treated animals and 14 g in the pair-fed controls (Table 31).

Gross observation of the liver revealed hypertrophy, molted appearances, and occasional fatty infiltration in those animals with the higher dosage. An increased brittleness of the hepatic arteries was also apparent thereby making hepatic perfusion much more difficult in these animals. One liver from the 1 ug/kg group demonstrated severe fatty infiltration and hypertrophy. Others from this group resembled controls in all aspects. At the higher dose 6 of 9 animals died between days 7-10, therefore the reason for 9 pair-fed animals but only 3 TCDD treated. Serum from all treated animals was very cloudy, indicative of the high lipid concentration. A yellow to greenish pigment was observable in the serum from the higher dosed animals.

As seen by Table 32, LPL activity showed a dose dependent decrease being only 19% of pair-fed levels after 10 days of treatment. No significant change was observed in activity between pair-fed and ad lib control animals. Likewise, hepatic LDL binding was 50% of pair-fed control levels after 10 days and also showed a dose dependent depression.

TABLE 31. Various physiological parameters of rabbits 10 days after administering 1 or 50 ug/kg TCDD and pair-fed or fed ad libitum.

		1 ug/kg		50 ug/kg	
	Ad lib	TCDD	PFC	TCDD	PFC
Body Wt. (% initial)	ND	96 ± 4[a] (3)	96 ± 3 (4)	89 ± 8 (5)	94 ± 3 (6)
Fat Wt. (g)[b]	ND	12.1 ± 2.2 (3)	14.5 ± 3.0 (3)	12.0 ± 4.3 (3)	14.4 ± 6.9 (5)
Serum:					
Triglyceride (mg/dl)	92 ± 56 (5)	203 ± 30 (4)*	90 ± 54 (4)	240 ± 101 (6)*	77 ± 12 (5)
Cholesterol (mg/dl)	65 ± 20 (2)	105 ± 28 (4)*	68 ± 36 (3)	103 ± 15 (5)*	60 ± 27 (5)
Protein (mg/ml)	12 ± 1 (5)	13 ± 1 (4)	13 ± 1 (4)	13 ± 1 (5)	14 ± 1 (5)
Insulin (uU/ml)	26 ± 8 (4)	19 ± 8 (3)	19 ± 4 (3)	24 ± 9 (5)	18 ± 3 (4)
Glucose (mg/dl)	176 ± 34 (5)	123 ± 7 (3)*	156 ± 13 (3)	111 ± 19 (5)*	185 ± 28 (5)

[a] Mean ± standard deviation for (n) animals. Data analyzed with Random Factorial ANOVA followed by Tukey's test for unconfounded comparisons.
*Statistically different from respective pair-fed control with $P < .01$.

[b] Abdominal and perirenal fat.

TABLE 32. Adipose LPL activity (nM ^{3}H oleic acid/mg acetone:ether powder/hr) and hepatic LDL binding (ng ^{125}I-LDL bound/200,000 cells/hr) in rabbits 10 days after ad lib feeding or pair-fed to those dosed with 50 or 1 ug/kg TCDD.

	LPL Activity	LDL Binding
Ad Lib	463.2 ± 92.2 (3)	ND
1 ug/kg		
PFC	400.1 ± 124.0 (3)	134 ± 22 (4)
TCDD	224.5 ± 197.3 (3)	12 ± 22 (2)
50 ug/kg		
PFC	278.9 ± 191.9 (9)	138 ± 12 (2)
TCDD	52.8 ± 19.0 (3)	65 ± 23 (5)

Serum triglyceride and cholesterol concentrations were significantly increased by TCDD treatment 2.6 and 1.5 times above control levels respectively (Table 31). Little change was seen in serum protein concentration. Insulin was non-significantly increased over pair-fed control levels at the higher dosage but was no different from ad lib control levels. Serum glucose however was significantly lower in the treated animals.

Scanning electron microscopy revealed severe alterations in the surface morphology of aortic arches taken from treated animals 20 days after administration (D. Bombick - personal communication). Ruffling and sloughing off of the surfaces was evident. Transmission electron microscopy showed again a sloughing off of outer layers and loss of endothelial integrity.

DISCUSSION

TCDD produced dose and time dependent inhibitions in adipose lipoprotein lipase activity and hepatic LDL binding. Adipose LPL has been shown to be the key regulatory enzyme controlling removal of triglycerides from blood (Kompiang et al. 1976, Borensztajn 1979, Korn 1955, Robinson 1963a). It is synthesized by the adipocyte and secreted into the blood whereby it is bound by a glycosaminoglycon heparan sulfate receptor on the endothelial cell surface. Its main function is to hydrolyze triglycerides carried by dietary chylomicrons (produced by the intestine

and consisting of ~80% triglycerides) and endogenously produced very low density lipoproteins (60% triglyceride, 22% cholesterol and cholesteryl ester, produced from both the intestine and liver) thereby promoting storage of the resulting free fatty acids by adipose tissue or allowing beta oxidation of these energy sources by muscle tissue. Chylomicron remnants consisting primarily of cholesterol and cholesteryl ester are normally removed from circulation by hepatic uptake. Catabolism of VLDL also occurs via a reduction in triglyceride composition via LPL resulting in a lipoprotein consisting of 50% cholesteryl ester and 10% triglyceride termed low density lipoprotein (LDL), which functions to provide necessary cholesterol to tissues and organs throughout the body (Hamosh and Hamosh 1983). Therefore the function of chylomicra and VLDL is to transport dietary exogenous and endogenous triglycerides from the intestine and liver to various tissues for either storage or energy whereas that of LDL is to transport cholesterol, needed for membrane integrity and steroid synthesis.

Hepatic LDL binding was used as an indicator to measure removal of LDL particles from the serum. As discussed by Hamosh and Hamosh (1983) the "LDL pathway" consists of binding to a cellular surface receptor, followed by endocytosis and finally lysosomal degradation. The LDL receptor has been shown to be a glycoprotein and is

competitively inhibited by VLDL (Anderson et al. 1976, Goldstein and Brown 1974). Therefore it appears as if TCDD causes an alteration in surface membrane characteristics resulting in an inhibition of LDL binding and LPL binding to their respective receptors. LPL half life in the serum is controlled by its binding to the heparan sulfate receptor. The enzyme-receptor complex has a t 1/2 of 10-25 minutes whereas the free enzyme is rapidly removed from the liver and degraded within 1 minute. The surface alterations reported here and caused by TCDD administration are consistent with earlier reports from this laboratory suggesting changes in protein constituency to occur in the hepatic plasma membrane of the rat thereby resulting in pertubations of critical physiological enzymes and alterations in binding of important homeostatic ligands to their receptors such as insulin and epidermal growth factor (Brewster et al. 1982, Matsumura et al. 1984, Madhukar et al. 1984).

TCDD has also been shown to cause inhibition of LPL activity (Brewster and Matsumura 1984) and LDL binding (Bombick et al. 1984) in the guinea pig, resulting in the hyperlipedemia seen in this species. As demonstrated here, the same results occur in the rabbit.

The yellow imparted to the serum does not seem to be proteinaceous since no change in serum protein concentration was observed. It could be due to an excess production of

bile salts, bilirubin, or other products resulting from increased porphyrin synthesis since TCDD has been implicated to cause a large increase in delta-aminolevulinic acid synthetase activity in the chick embryo (Poland and Glover 1973a). This rate-limiting enzyme in the hemebiosynthetic pathway, is not significantly changed in rats (Woods 1973) upon exposure to TCDD. However, various mammalian species have a very wide variability in their response to porphyrogenic agents as discussed by Woods and Dixon (1972) and Woods (1973).

Since LPL synthesis is controlled to a large extent by insulin and to a lesser extent by thyroxine an effort was made to determine whether the serum concentration of these agents were altered by TCDD. No changes were seen in insulin, thyroxine, or triiodythyronine concentrations. Glucose was significantly below that of pair-fed control concentrations and may partially account for a decrease in active LPL levels since the enzyme is synthesized as an inactive precursor which needs to be glycosylated for activation before secretion (Hamosh and Hamosh 1983).

Recent evidence indicates that LPL may be important in forming HDL (high density lipoprotein) during the lipolysis of chylomicra and VLDL triglyceride (Dieplinger et al. 1985). Since HDL has been implicated in scavenging serum cholesterol and thereby protecting against atherosclerosis (Dieplinger et al. 1985, Brown et al. 1981) the inhibition

of LPL as well as the inhibition of LDL binding may play a role in the formation of the preatherosclerotic lesions observed in this study.

There is ample evidence to relate hyperlipedemia with atherosclerotic and arteriosclerotic processes. Stein and Stein (1979) cite several reports demonstrating the passage of lipoproteins and cholesterol into the intima, media, and adventitia of the endothelial wall. Results of this invasion include denudation, proliferation of smooth muscle cells, and platelet adhesion to the subendothelial surface. The earliest detectable lesion leading to atherosclerotic plaque formation is a deposition of cholesteryl ester in smooth muscle cells and macrophage foam cells of the intima and media as described by Goldstein et al. 1983. Similar results were produced in the present study with TCDD as observed with electron microscopy. Watanabe (1980) observed severe atherosclerosis within 2 months in rabbits deficient in LDL receptors therefore, it is feasible to notice preatherosclerotic lesions within 20 days after dioxin treatment as the present results indicate. After cholesterol deposition, eventually a necrotic cholesteryl ester filled core and a fibrous cap from the atherosclerotic plaque (Goldstein et al. 1983).

A potentially serious implication of this research is the production of similar types of effects in humans upon exposure to TCDD or other polyhalogenated aromatic

hydrocarbons. Oliver (1975) and Pazderova-Vejlupkova (1981) have reported hypercholesteronemia in humans exposed to either TCDD or 2,4,5-T. Industrial workers exhibiting chloracne, resulting from occupational exposure also exhibited hyperlipedemia (Walker and Martin 1979).

In conclusion, we have examined the mechanism by which TCDD produces 2 of its many toxic manifestations. We have studied the mechanism, the result, and long term effects of dioxin in the rabbit. Probably through membrane alterations, adipose LPL activity and hepatic LDL binding are depressed by this dioxin, resulting in increases in serum triglyceride and cholesterol concentrations. One serious direct effect of this hyperlipedemia is the changes in endothelial cells of the aortic arch reminiscent of pre-atherosclerotic lesions. Longer effects could conceivably include total arterial blockage, which along with high serum lipids could easily compromise heart function and contribute to lethality.

APPENDIX C

THE EFFECT OF TCDD ON ADIPOSE LPL AND SERUM LIPID PARAMETERS IN THE MINK

This study was done in collaboration with Drs. Aulerich and Bursian in the Department of Animal Science at MSU who had shown mink to be very sensitive to TCDD (LD_{50} ~7 ug/kg - personal communication). Because of the similarity in LD_{50} to that of the guinea pig it was of interest to know what effect TCDD had on various lipid parameters in this species.

These animals showed a dose response inhibition of LPL activity 28 and 43 days after treatment (Table 33). Changes in serum triglyceride concentrations in response to TCDD treatment were difficult to evaluate because of the high ad lib control value. If mink are nocturnal animals quite possibly these 4 individuals had just finished feeding before sacrifice, which occurred at approximately 8 a.m. Also these animals were sacrificed on day 28 not day 43 like the other groups. Overall, it appeared that serum triglycerides decreased with increasing dioxin doses. Little changes were noted in serum cholesterol or protein concentrations. Serum glucose was significantly elevated in the 28 day groups.

TABLE 33. Effects of orally administered TCDD on mink adipose LPL activity and various serum lipid components at 5, 28, and 43 days after administration.

Dose[a]	(#)	Exposure (days)	LPL nM ^{3}H-oleic acid/ mg fat extract/hr	Triglyceride mg/dl	Cholesterol mg/dl	Glucose mg/dl	Protein mg/dl
0 (Very old ad lib[b])	(4)	0	ND	145 ± 57	95 ± 11	185 ± 31	35.7 ± 0.71
0 (Ad lib)	(4)	28	1616.8 ± 272.3[d]	230 ± 23	83 ± 12	256 ± 18	33.9 ± 1.1
O(PFC)[c]	(1)	5	ND	109	63	186	ND
0.001	(2)	43	1291.0 ± 43.8	89 ± 8	78 ± 4	175 ± 60	33.0 ± 0.9
0.01	(2)	43	1222.5 ± 292.0	122 ± 63	73 ± 2	115 ± 16	31.0 ± 1.9
0.1	(2)	43	1048.0 ± 192.3	123 ± 4	64 ± 6	119 ± 14	33.6 ± 2.3
1.0	(2)	43	818	102 ± 20	80 ± 3	109 ± 1	33.1 ± 1.0
2.5	(4)	28	ND	124 ± 3	82 ± 8	224 ± 28	31.0 ± 1.4
5.0	(2)	28	ND	49	74	132	27.6
7.5[c]	(1)	5	ND	85	60	163	29.7

[a] All male single oral dose.
[b] Body wt. > 2000 g.
[c] Pastel mink, all others black.
[d] Mean ± standard deviation.

Because of the various exposure times, low number of replicates and different color variations of animals it is impossible to make any firm conclusions. The change in LPL and serum triglycerides along with the sensitivity to TCDD displayed in these animals does warrant further investigation.

In a second experiment animals were orally dosed with acetone:corn oil, or with 4 ug/kg TCDD, and pair-fed to the treated animals or allowed to feed ad libitum. After 4 days abdominal and perirenal adipose tissue was removed and serum collected for the determination of LPL activity and triglyceride concentration. Results displayed in Table 34 indicate serum triglyceride levels to be severely reduced in those animals exposed to TCDD compared to the controls. Unlike the other species investigated, this did not correlate with adipose LPL activity, since this was also lower than pair-fed control activity but marginally increased over the ad lib controls.

TABLE 34. Adipose LPL activity and serum triglyceride concentration of ad lib, 4 ug/kg TCDD or pair-fed mink after acute exposure.

	LPL (nM/mg extracted fat/hr)	TG (mg/dl)
Ad lib	1177.78 ± 222.45(5)	138 ± 64(7)
4 ug/kg TCDD	1467.43 ± 327.98(6)	58 ± 30(6)
Pair-fed	1917.75 ± 308.31(5)	138 ± 36(6)

Appendix D

ADDITIONAL DATA CONCERNING THE BIOCHEMICAL MECHANISM FOR LPL INHIBITION

In an effort to determine whether decreased serum insulin levels were the sole cause of depression of adipose LPL after dioxin treatment, five guinea pigs were administered 1 ug/kg TCDD and starved for two days prior to LPL determination. A second group of animals were administered acetone:corn oil, and also starved for two days as control animals. Food restriction depresses enzymatic activity by altering serum hormone levels. Therefore, if a synergistic effect occurs with dioxin, it must be due to an effect at the level of the adipocyte. Results indicated the TCDD treated group to have significantly lower activity (t test, $P < .01$) than the control (264.6 $\pm$ 72.8(5) and 411.5 $\pm$ 222.2(3) nM/mg extracted fat protein/hr, respectively). Therefore, it is concluded that TCDD not only lowered serum insulin levels but also had a direct effect on the adipocyte to impede LPL activity.

In a second experiment in order to ascertain the effects of TCDD on pancreatic EGF, a group of animals was sacrificed after a two day exposure to 1 ug/kg TCDD or acetone:corn oil. Pancreatic tissue was removed and assessed for ^{32}P incorporation into EGF-like binding

proteins. Results indicated TCDD treatment to increase this activity in the pancreas by two fold (PFC = 11.2 $\pm$ 4.1; TCDD 22.5 $\pm$ 3.2 nM ^{32}P incorporated/mg protein/3' -- 5 control and 5 TCDD treated animals).

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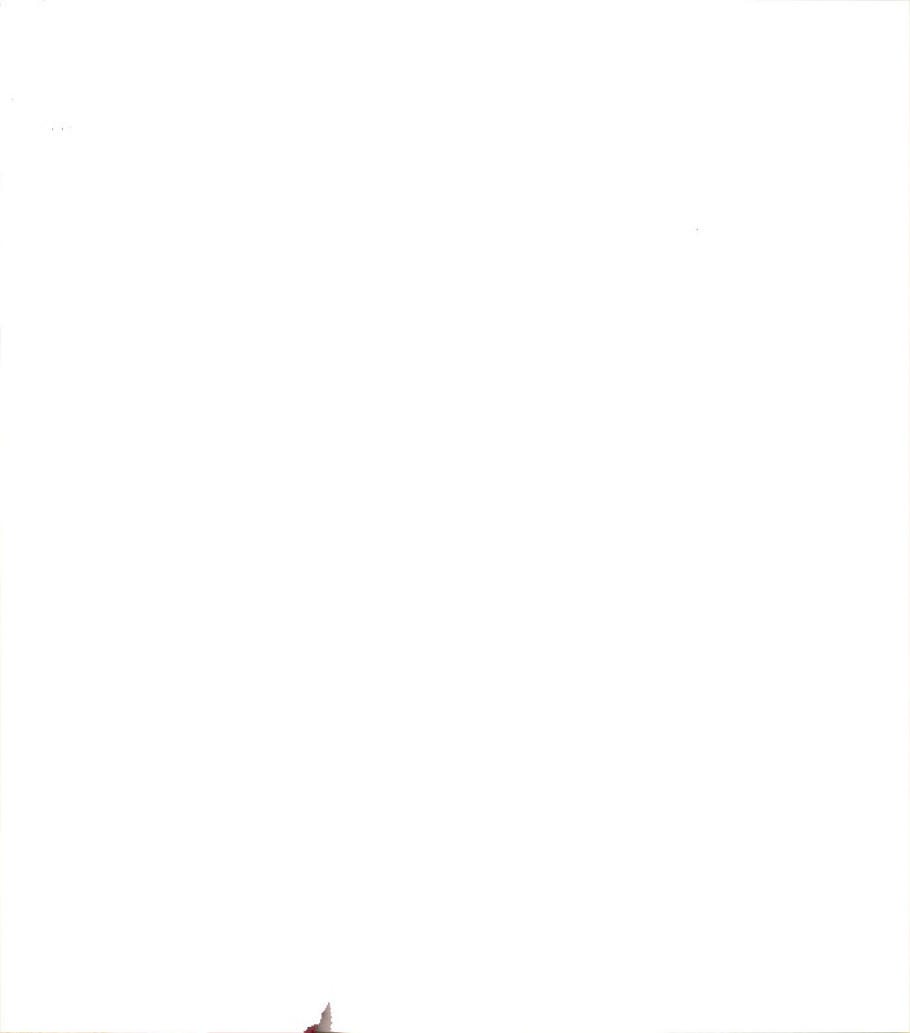

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