AN ABSTRACT OF THE THESIS OF

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Title: POTENTIATION OF ACUTE CARBON TETRACHLORIDE HEPATOTOXICITY BY DIETARY EXPOSURE TO CHLORO-PHENOTHANE (DDT) IN RATS

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There has been no toxicologic proof that the long-term, low-level exposure of man to chlorophenothane (DDT) is linked with an increased susceptibility to toxicity or disease. Acute pretreatment of rats with 75-100 mg/kg DDT has been demonstrated to potentiate CCl_4-induced hepatotoxicity in that species (McLean and McLean, 1966). Since the hepatotoxic response to CCl_4 is similar among mammalian species, it was believed man's response could also be altered by prior exposure to DDT. Chronic pretreatment of rats with DDT more closely duplicates man's exposure situation, which is mainly dietary. Therefore, this present investigation was undertaken to quantify the DDT-CCl_4 hepatotoxic interaction in rats from the standpoint of a chronic dietary exposure to DDT.

Chronic and acute pretreatments with DDT were compared for their relative effects on acute CCl_4 hepatotoxicity. Subgroups of rats

fed DDT at concentrations of 6-65 ppm for three weeks and 65 ppm for 24 weeks attained body burdens of DDT and its metabolites ranging from 6.1 to 30.6 ppm, compared to 0 ppm (no detectable) for controls. In the acute study, subgroups of rats were dosed with 35-150 mg/kg DDT by gavage 24 hours prior to CCl_4 exposure. Doses of CCl_4 ranging from 0.125 to 1.0 ml/kg were administered by gavage. All pretreatments with DDT, chronic or acute, resulted in a potentiated elevation of serum transaminase (SGPT and SGOT) activities following treatment with CCl_4. The degree of potentiation was dose-related to CCl_4 and to the total DDT body burden. In a temporal study, the onset of the potentiated response was noted six hours after CCl_4. All of the above-mentioned results were confirmed by histopathology.

Plasma BSP disappearance, dose-response and temporal studies were conducted in control and DDT-fed rat groups, utilizing the BSP test as an index of hepatic functional impairment. CCl_4-induced BSP retention was greatly potentiated by prior dietary exposure to DDT. This potentiation effect was obscured at a dose of 2.0 ml/kg CCl_4, due to the severe hepatic damage produced by this dose of CCl_4 given alone. The onset and development of the potentiated hepatic dysfunction paralleled that of the parenchymal cell destruction.

The potentiated CCl_4-induced increases in SGOT activity and centrilobularly-oriented coagulative necrosis in the DDT-fed animals were prevented by spinal cord transection at the level of the seventh

cervical vertebra. Chronic DDT pretreatment did not enhance CCl_4 uptake into the blood or livers of intact rats, and this was ruled out as a possible mechanism for the potentiation effect. Cord-sectioning did enhance tissue CCl_4 uptake, yet these animals were protected against CCl_4-induced central necrosis. Neither cord-sectioning nor the resultant hypothermia reduced the microsomal cytochrome P-450 content in control or DDT-fed rats. Hypothermia depressed the *in vitro* N-demethylation of ethylmorphine, so that oxidative drug metabolism was probably also decreased *in vivo* in the cordotomized animals.

Maximal blood and liver concentrations of CCl_4 were measured between 2-4 hours in the intact control and DDT-fed groups. These tissue CCl_4 concentrations decreased more rapidly between 4-12 hours in the DDT-fed rats, thus suggesting an increased rate of CCl_4 metabolism could have occurred at this time *in vivo* in these animals. Unlike the other indices of hepatic damage, the cytochrome P-450 response preceded rather than followed the hepatocellular destruction. About 75% of the induced cytochrome P-450 concentration in the DDT-fed rats was destroyed within six hours after CCl_4, at which time the onset of potentiated hepatic damage was detected. In the controls, the cytochrome P-450 concentration regenerated from a low value at 18 hours to normal by 48 hours after CCl_4. Hepatic regeneration was impaired in the DDT-fed rats, since the cytochrome P-450

concentration continued to decline between 24-48 hours. The amount of cytochrome P-450 destruction appeared to correlate with the degree of hepatic damage observed. Lower absolute concentrations of cytochrome P-450 were measured in controls 24 hours after dosing with 0.125-2.0 ml/kg CCl_4. More cytochrome P-450 was destroyed in the DDT-fed group, however, as a result of the induced microsomal concentrations of this cytochrome present initially before CCl_4 was given. Thus, the CCl_4-cytochrome P-450 interaction could play an integral role in the potentiation phenomenon, as McLean and McLean (1969) have suggested.

Liver weight increased in a dose-related manner to CCl_4 in both control and DDT-fed groups, but the response was greater in controls. In the control animals, the liver weight increased rapidly to a maximal value at 18 hours, but returned to normal by 48 hours after CCl_4. Although the onset of the response was slower in the DDT-fed rats, the liver weight continued to increase at 48 hours. Rectal temperatures declined between 6-24 hours only in the DDT-fed animals.

The pharmacologic agents SKF 525-A (drug metabolism inhibitor) and lead (porphyrin biosynthesis inhibitor) were utilized to assess the role of cytochrome P-450 in potentiation. SKF 525-A provided protection against the potentiated CCl_4-induced centrilobular necrosis at 11 hours after CCl_4. This protection could not be positively ascribed to SKF 525-A binding with cytochrome P-450 or

inhibiting CCl_4 metabolism, since SKF 525-A has been reported to alter the gastrointestinal absorption of CCl_4 (Marchand, McLean and Plaa, 1970). Lead significantly reduced the effect of prior DDT feeding in potentiating CCl_4 hepatotoxicity, but this protection did not result from a lowered microsomal cytochrome P-450 content. Thus, the role of cytochrome P-450 in the potentiation effect remains to be conclusively elucidated.

Mecamylamine pretreatment was used to determine the importance of the sympathomimetic properties of DDT in potentiation. This ganglionic blocking agent produced an insignificant tendency to decrease the potentiated CCl_4-induced BSP retention. However, the histologic evidence of protection against CCl_4-induced hepatic necrosis was not apparent in mecamylamine-pretreated animals. Thus, the central sympathetic nervous system stimulatory effects of DDT are probably not directly involved in potentiation.

The basis for extrapolation of these results from rat to man could be the total DDT body burden, which was similar in these rats (6.1-30.6 ppm) to that estimated for man, e.g., 10 ppm by Durham (1965). Since rats and man respond similarly to CCl_4, these results indicate that man's chronic exposure to DDT could render him highly susceptible to a secondary exposure to CCl_4.

Potentiation of Acute Carbon Tetrachloride Hepatotoxicity by Dietary Exposure to Chlorophenothane (DDT) in Rats

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I. GENERAL INTRODUCTION

> Environmental stress on human health involves a total exposure to a broad spectrum of agents, with the biologic challenge arising from three sources: inhalation, absorption, and ingestion, or any combination of these. These problems have provided a strong stimulus to exploratory research in the fields of toxicology and epidemiology in elucidating the mechanism of action of such environmental stresses. . . . The effects of repeated exposure and cumulative action of such exposure, whether to a pesticide or another environmental stress, must be considered in terms of man's performance or recuperative powers. With constant exposure of target organs and various tissues, there is the distinct possibility that there may be a threshold for metabolism of pesticides in a system, flooded today with other exogenous or endogenous agents under normal or debilitated conditions, which may potentiate induction of disease.
>
> H. F. Kraybill, 1969, p. 5

Recently the insecticide 1, 1, 1-trichloro-2, 2-bis-(p-chlorophenyl) ethane (Chlorophenothane, DDT) has received much publicity. National coverage in newspaper and magazine articles has been afforded, ranging from advocacy of total removal of DDT from use to persons claiming to have taken regular oral doses of the insecticide without experiencing noticeable toxicities or disagreeable side effects. The August 9, 1971 issue of Time Magazine contained an article entitled "The DDT Eaters and Other Eco-Centrics," which discussed the case of a California pesticide retailer and his wife, both

of whom had taken 10 mg DDT capsules daily for 93 days. Their purpose in so doing was simply to demonstrate there is no human health hazard associated with this well-known insecticide. However, a University of California chemist, Dr. William Westlake, was quick to point out that the experiment conducted by the DDT-Eaters was meaningless from a scientific standpoint, because the safety of high oral doses of DDT in man was already well established in the scientific literature (Corvallis, Oregon, Gazette-Times, March 13, 1971).

In a carefully conducted experiment utilizing human volunteers, Hayes, Dale and Pirkle (1971) showed that the ingestion of technical (p, p'-isomer) DDT at rates up to 35 mg per man per day for 21.5 months resulted in no definite clinical or laboratory evidence of injury. They concluded that a high degree of safety to DDT exposure exists for the general population. And according to Goodman and Gilman (1965), the lethal dose of DDT is sufficiently high that fatal acute poisoning by this agent is not a significant hazard. The average adult would have to ingest about 10-20 g of DDT in an oily solution to be poisoned severely; this represents approximately several hundred milliliters of common commercial insecticidal preparations. It was also pointed out that in the ingestion of such quantities of the insecticidal preparation, the vehicle would undoubtedly contribute substantially to the toxicity.

The acute exposure to DDT is not the primary concern of health scientists today. This is true because once the occasional accidental acute exposure to DDT occurs, the toxicity syndrome is readily recognized and can be treated, i. e., by central nervous system depressants and supportive therapy. In other words, acute exposure is not insidious, it can be perceived and treated.

With regard to chronic exposure, a great deal of information has been generated regarding the effects of long-term, low-level exposures to biologically persistent compounds that build up in the human body. To date, however, there has been no toxicologic proof linking the chronic human exposure to pesticides with an increased susceptibility to toxicity or disease. Because the organochlorine insecticide DDT is persistent, the chronic exposure of the general population is now receiving the focus of attention.

In 1969 a symposium was held in New York to assess the "Biological Effects of Pesticides in Mammalian Systems" (Kraybill, consulting editor, 1969). This meeting was sponsored by the New York Academy of Sciences, and included many prominent pharmacologists, toxicologists, epidemiologists, and other members of the health science team. These scientists were primarily concerned with the long-term effects of organochlorine residues in biological tissues. Dr. MacDougall summarized the purposes of this symposium:

> Basically we have two objectives in this conference. The first is to review possible evidence for undesirable side effects on mammalian systems of long-term, low-level exposure to pesticides, and the second is to determine better methods for assessing these. . . . The basic question, and the purpose for which this conference was

> called, is to determine whether there are undesirable or deleterious side effects attributable to the levels of pesticides to which the general public is exposed (p. 68-70).

The consulting editor of the conference, Dr. H. F. Kraybill, stressed the importance of chronic rather than acute exposures:

> Toxicologic research within the past decade has become sophisticated through an appreciation of cellular biochemistry, cytogenetics, and the potentialities of chronic responses to subthreshold toxic effects. Unfortunately, some clinical assessments in man are still made in terms of acute effects of pesticides rather than by investigating the potential of repeated episodes of exposure or prolonged exposure, with its induction of responses at the cellular level and ultimate pathologic alteration (p. 5).

With regard to epidemiological considerations, Drs. Hemphill and Goerke (1969, p. 62) emphasized the complexity of toxic interactions which may occur in the mammalian system due to multiple exposure, and stated that these toxic interactions are important research areas for the future:

> The presence of pesticides in the diet and environment results in recurrent exposure of the population. This circumstance constitutes one of the basic considerations for those who plan to evaluate long-term health effects of pesticides. Many different chemical compounds are used as pesticides; these chemicals are formulated with differing diluents, in differing concentrations, and often in combinations of more than one compound. . . . Eventually, these chemicals, and their degradation and metabolic products, together with those of other chemicals, drugs, alcohols, and pollutants of food, air, and water, enter the blood stream and the nutrient environment of the living human cell. The potential synergistic and antagonistic action of the pesticides with one another and with the other chemicals and pollutants are important problems for investigation. Consideration of these interacting effects on long-term health is a complexity added to that of the general diffusion of the pesticides themselves in the environment of man.

More recently, a Task Force including many prominent health scientists in toxicology, epidemiology, and other related fields, and directed by Dr. Norton A. Nelson of the New York University Medical Center, Department of Environmental Medicine, met at Oregon State University in Corvallis, Oregon, to conclude months of preparatory work. Their topic for discussion was "Man's Health and the Environment - Some Research Needs" (Nelson, 1971). This conference was sponsored by the Environmental Health Sciences Center at Oregon State University. The main purpose for meeting in Corvallis was to establish research goals for the United States health science community, on a priority basis. Research goals which were unanimously accepted as having high priority were given the status of "Recommendation." It is believed that several of these Recommendations are of special significance to this dissertation.

In the toxicology section of the published report of the meeting, it was emphasized that: "Toxic interactions should be thoroughly characterized in terms of their time and dose-response relationships. . . ." (Recommendation 8-13).

The following Recommendation, 8-14, states:

> It is recommended that greater effort be devoted to determining the quantitative relationships between effects of chemicals on enzymes that metabolize other foreign chemicals and the effective doses of these chemicals in intact organisms (p. 181).

Regarding the problem of acute or chronic interactions the claim is made that:

> . . . most current evidence of toxic interactions is based on acute responses. . . as an example, Garner and McLean (1969) have shown that phenobarbital pretreatment potentiates the hepatotoxicity of a single dose of CCl_4, and it would be of value to know if the development of chronic liver cirrhosis would be enhanced with repeated low doses of CCl_4 given with chronic administration of phenobarbital (p. 181).

Recommendation 8-15:

> The similarities or differences between acute and chronic injuries resulting from interactions of toxic agents should be evaluated (p. 181).

Now it seems clear from the Recommendations formulated by this Oregon State University Task Force that toxic interactions of chemical environmental contaminants are of singular importance. Further emphasis must be placed on time and dose-response relationships of these chemicals in laboratory animals, and special significance should be given to chronic studies, which more accurately duplicate the human exposure. Only in this way can the full environmental significance of these toxic interactions be predicted for man.

It is generally acknowledged that certain pesticides are capable of altering the responses of organisms to secondary chemical exposures, yet few of these biochemical interactions have actually been quantified. Pesticides which act as stimulators of liver microsomal drug-metabolizing enzymes were first studied by Hart and Fouts (1963) and Hart, Shultice and Fouts (1963). The prolonged feeding of

5 ppm DDT to rats in the diet produced elevations of liver microsomal enzymes that metabolize drugs (Hart and Fouts, 1965).

Treatment of rodents with the halogenated hydrocarbon insecticides increased the oxidative metabolism of a number of drugs, including hexobarbital, aminopyrine, and chlorpromazine (Fouts, 1963; Hart and Fouts, 1963, 1965; Hart, Shultice and Fouts, 1963), and protected against the lethality from the anticoagulant bishydroxycoumarin (Cucinell et al., 1965). After a single injection of 200 mg/kg DDT intraperitoneally, rats metabolize hexobarbital more rapidly for 65 to 90 days (Ghazal et al., 1964). The long action is attributed to the slow release of DDT from storage depots in body fat.

Other organochlorine insecticides, such as dieldrin (Ghazal et al., 1964) and chlordane (Burns et al., 1965; Welch, Harrison and Burns, 1966; Conney et al., 1967) have been found to stimulate various drug-metabolizing pathways, in rats and dogs, respectively. The organophosphorus insecticides are different, however, in that they inhibit rather than stimulate, the hydroxylation of drugs and steroids when given chronically (Rosenberg and Coon, 1958; Welch, Levin and Conney, 1967).

Several organochlorine insecticides, including DDT, have been found to affect the toxicity of other drugs and chemicals, in addition to lowering the duration and/or intensity of drug action. DDT-treatment of immature rats for several days increased the LD50 of

warfarin more than ten-fold, which was associated with decreased plasma levels of warfarin, and enhanced activity of liver microsomal enzymes that metabolize this drug (Ikeda, Sezesny and Barnes, 1966).

Enzyme inducing agents can effect the depletion of substances like insecticides, which accumulate in body fat and are metabolized by hepatic microsomal enzyme systems. DDT given to rats previously fed dieldrin or heptachlor markedly decreases the storage of the latter compounds in the fat (Street, 1964; Street and Blau, 1966; Street et al., 1966). DDT administered in the feed at 50 ppm also causes a 15-fold reduction in the amount of dieldrin in the fat of rats simultaneously fed 1 ppm of dieldrin. The enhanced depletion of dieldrin continues for weeks after DDT feeding has been discontinued.

Repeated administration of a drug sometimes results in a more rapid metabolism of the same agent upon subsequent administrations. Chronic treatment with the drug accelerates its metabolism, lowers its blood level, and decreases its effect. DDT was found to enhance its own subsequent metabolism in the rat (Morello, 1965).

DDT, like chlordane and phenobarbital, is a "broad spectrum" inducer which alters a variety of liver microsomal drug-metabolizing pathways, including oxidation and reduction, glucuronide formation, and de-esterification. The "narrow spectrum" inducers, such as the polycyclics 3-methylcholanthrene and 3,4-benzpyrene, stimulate the metabolism of a very limited number of drug metabolism pathways.

The two types of inducing agents differ in the course and intensity of their inducing effects. A single injection of DDT, 200 mg/kg intraperitoneally, in rats, elevated the metabolism of hexobarbital and acetophenetidin, in vitro, to a maximal two to three times control values in five to ten days (Ghazal et al., 1964). After the intraperitoneal injection of polycyclic hydrocarbons, the enzymatic activity is more than doubled within 3-6 hours, and maximal increases occurred after 24 hours (Conney, Miller and Miller, 1956, 1957).

McLean and McLean (1966) have recently shown a toxic hepatic interaction in rats, with high acute doses of DDT followed by high acute doses of CCl_4. These workers used doses of 75 or 100 mg/kg DDT to produce enzyme induction in rats, and then measured the increased sensitivity in hepatotoxic response to a challenging dose of CCl_4, which resulted in considerably greater damage to hepatocytes than is ordinarily observed in CCl_4-treated control rats. Although the implications of these experiments were of great importance, in terms of man's response to CCl_4, their immediate significance was masked because these results were based on a high acute exposure of the rats to DDT. Few people are exposed to high acute doses of DDT, and it is even more improbable that acute poisoning by DDT would be closely followed in the same individual by acute (or chronic) exposure to CCl_4.

To attain environmental toxicologic significance it was clear that this biochemical interaction of DDT and CCl_4 on the rat liver

would require quantification on the basis of chronic exposure to DDT. It seemed necessary to correlate the degree of sensitivity to CCl_4-induced hepatotoxicity with the exposure level of DDT, in terms of both feeding level and duration of DDT exposure. Chronic injections of DDT in rats would be the most accurate means of quantifying DDT exposure. However, the diet is the most logical means of exposure to DDT, if extrapolations to man are to be made, because man's primary exposure to DDT is via the diet.

The increased sensitivity to CCl_4-induced hepatotoxicity subsequent to chronic dietary exposure of rats to DDT could be related quantitatively to the total dose of DDT administered, or to the total body burden of DDT attained in the animals. If this proved to be the case, it could have special significance for certain subgroups of the general human population. Farmers who live in areas of high pesticide usage, formulators of insecticidal preparations containing DDT, commercial applicators of insecticides to crops, the heavily-spraying gardener or household user of insecticides could all receive a much larger exposure to DDT, possibly resulting in higher body burdens than are generally found in the population. Hayes et al. (1971) found that human volunteers ingesting DDT attained body burdens or storage of DDT and DDE which were proportional to dosage. In fact, the fat of those volunteers receiving the highest dosage of technical DDT contained 105 to 619 ppm, which was considerably higher than average

human levels estimated by Durham (1965) to be about 10 ppm for both DDT and DDE combined. Durham also demonstrated that occupationally-exposed persons had a higher storage and excretion rate for DDT-derived materials than the environmentally-exposed, and in turn, the environmentally-exposed had a higher rate than the general population. The DDT body burden appears to be a direct reflection of the level of insecticidal exposure. It is conceivable that the environmentally or occupationally-exposed subgroups of the general population could be most sensitive to a secondary exposure to CCl_4, if sensitivity to CCl_4 is directly related to the feeding level and body burden of DDT.

In the consideration of man's exposure to the chemical agent CCl_4, it may be either chronic, subacute, or acute. The professional cleaner exposed daily to a spot remover, the industry mechanic exposed to a degreasing agent, the housewife exposed occasionally to a commercial rug cleaner, a child who accidentally ingests a CCl_4-containing compound, the fireman who uses a Pyrene-containing fire extinguisher to control a blaze in a poorly-ventilated building--all of these exposure types indicate the extreme versatility of this agent, which can be absorbed by inhalation, skin, or ingestion. It is conceivable that the increased sensitivity to CCl_4 hepatotoxicity resulting from prior exposure to DDT could be related in a dose-response manner to the dosage level of CCl_4, or possibly to the mode of

exposure to CCl_4. The DDT - CCl_4 hepatotoxic interaction also requires quantification from this experimental viewpoint.

Because of its toxicity, CCl_4 may soon be removed from the consumer market entirely. CCl_4 must, however, be considered a prototypical agent. Other halogenated hydrocarbons will probably be used, at least in part, to replace CCl_4, and still others find use as general anesthetic agents. In time, all of these halogenated chemicals should be examined for the possibility that their toxicities might also be potentiated by enzyme-inducing agents such as DDT.

The main goal of this present study was to quantify the toxic hepatic interaction of DDT and CCl_4 from the standpoint of chronic exposure to DDT via the diet. It was believed chronic exposure more closely duplicated the conditions under which man is exposed to DDT, and that body burdens of DDT comparable to average human levels could be achieved in the experimental animals. Chronic and acute studies were compared, using the most sensitive tests of hepatic damage currently available. In all experiments, the temporal pattern of the developing lesion received special emphasis.

It is hoped this research project will serve as a small contribution toward the fulfillment of certain high-priority research goals recently established for the health science community. The rat liver exhibits a typical mammalian response to CCl_4 (Larson, 1971, personal communication); thus the quantification of any hepatic

interaction of dietary DDT and acute CCl_4 in rats could have direct toxicologic implications for man. The results of these studies could indicate the present chronic exposure to (and body burden of) DDT has deleterious effects on man's response to secondary chemical insults, notably to CCl_4, and perhaps to other halogenated hydrocarbons.

General Methods

Certain general procedures were used throughout the following experiments, regarding the maintenance and care of animals, obtaining blood and liver samples for analyses, and statistical considerations of data. The following statements briefly discuss these general methods.

Male rats weighing 250-350 g were used in these studies. The Sprague-Dawley strain of rat used was initially supplied by the Simonsen Laboratories, Inc. The animal room temperature was maintained at 70-72°F.

In preparing the DDT-containing rat diet, aliquots of 3000 g of Purina laboratory chow were weighed and subsequently placed in 4000 ml polyethylene beakers. To achieve the desired DDT concentrations in the diet, solutions of DDT (Technical Grade, Pennsylvania Salt Manufacturing Company; 83% p, p'-isomer, 16% o, p'-isomer, by analysis) dissolved in acetone were poured over the laboratory chow. The 3 kg of soaked pellets was then shaken approximately 200 times,

and all pellets appeared well-soaked in the solution. This beaker containing the soaked pellets was placed under a ventilation hood overnight, thus allowing the acetone to evaporate, while the DDT residue remained on the food. The dried DDT-containing laboratory chow was then substituted for the regular rat diet for animals receiving the DDT ration. In general, DDT-fed rats were given the same treatment as control animals: rats were housed five per cage (16" x 10" x 7"), and had free access to food and water. Control rats were given regular laboratory chow which had been soaked in acetone and dried, but containing no DDT.

Reagent grade CCl_4 (J. T. Baker, Inc.) was used throughout these experiments. The CCl_4 was dissolved in corn oil and administered by gastric intubation. In cases where low dosages of CCl_4 were employed, injection volumes were adjusted to 0.5 ml.

For analyses of plasma samples, blood was taken by cardiac puncture with the animals under a light ether anesthesia. Blood was placed in 5 ml culture tubes previously rinsed in 2.5% sodium oxalate. The culture tubes were centrifuged at 2000 rpm for 15-20 minutes, then the plasma was separated from the packed red blood cells and stored at $4^{\circ}C$ in sealed culture tubes.

Standard parametric and nonparametric statistics were used for analysis of the data obtained in these experiments.

II. ACUTE CARBON TETRACHLORIDE HEPATOTOXICITY IN RATS

Introduction

These preliminary studies were undertaken to establish the sensitivity of response of the enzymes serum glutamic pyruvic transaminase (SGPT) and serum glutamic oxaloacetic transaminase (SGOT) to acute carbon tetrachloride (CCl_4) hepatotoxicity in this laboratory. The establishment of a sensitive dose-response relationship to CCl_4 was deemed necessary for subsequent experimentation. The ultimate objective of this research project was to quantify the hepatotoxic interaction of dietary DDT and acutely-administered CCl_4. Thus, the acute hepatotoxic response of serum enzyme activities in control rats treated with CCl_4 served as a standard response in the DDT-CCl_4 interaction studies.

The serum transaminase activities were chosen as the indices of liver damage mainly because of their sensitivity. A comparison of several laboratory procedures has recently been made in mice (Klaassen and Plaa, 1966). They demonstrated that the activity of SGPT was the most sensitive indicator of all the tests employed, which was followed closely by the sensitivity of the sulfobromophthalein (BSP) retention test used to assess hepatic functional impairment. In the routine screening for liver damage in apparently healthy persons who may have been exposed to hepatitis or to a hepatotoxic agent, e. g.,

CCl_4, the test for elevation of SGPT is widely used (Netter, 1964). The impressive elevation of SGOT activity following CCl_4-induced hepatocellular damage, infectious hepatitis or homologous serum hepatitis was demonstrated by Wroblewski and LaDue (1955). Unlike SGOT, the concentration of SGPT is higher in the liver than in the myocardium or any other tissue. Therefore, it has been suggested that the SGPT activity may be a more specific index of hepatocellular injury than the SGOT activity. The values of the two enzymes are so closely correlated in most instances of liver disease, however, that the measurement of one serum enzyme appears to offer no distinct advantage over the other (Harrison et al., 1966). The measurement of the activities of other similarly-used enzymes, e.g., isocitric dehydrogenase, is thought to be a less sensitive indicator of hepatocellular damage than that of SGPT or SGOT (Netter, 1964). For these reasons the serum transaminase (SGPT and SGOT) activities were both utilized in these studies.

Methods

Serum Transaminase Activities

In the measurement of serum transaminase activities, a modification of the colorimetric determination of Reitman and Frankel (1957) for SGPT and SGOT activities was utilized. The determination of SGPT activity is based on the principle that this enzyme catalyzes the

conversion of alanine and alpha keto-glutaric acid to glutamic and pyruvic acids. In comparison, SGOT catalyzes the conversion of aspartic acid and alpha keto-glutaric acid to glutamic and oxaloacetic acids. The resulting keto acids, in both reactions, are reacted with dinitrophenylhydrazine to form their respective keto acid hydrazones which, when a solution of sodium hydroxide is added, yield an intense brownish color that can be read in a colorimeter. According to the method of Reitman et al., the change in optical density which occurs in these reactions is directly related to the amount of pyruvate (SGPT) or pyruvate and oxaloacetate (SGOT) which is formed. The concentration of these reaction products can then be directly related to the measured enzyme activities. In this way, the optical density is related to the serum transaminase activities. The change in optical density (delta O. D.) over a range of 0-0.5 is linear with respect to changes in enzyme activity corresponding to about 125 units of SGPT and 165 units of SGOT. When the measured serum enzyme activities are in excess of these values it is necessary to dilute the original plasma or serum samples, and to repeat the determination using these diluted samples. Reitman and Frankel recommend a 1:10 dilution of the original samples, and they found no elevation in control serum or plasma transaminase activities occurred when these measured activities were corrected for dilution. Thus, the serum enzyme activities can be measured in the linear portion of the

calibration curve regardless of how high these activities are in the original samples. According to Reitman, the serum and plasma show very little difference in transaminase activities. In the remainder of this dissertation, the discussion of SGPT and SGOT activities refers to the determination of enzyme activities in plasma rather than serum samples. Duplicate determinations and an individual blank were made simultaneously in the determination of SGPT or SGOT activity in each plasma sample. The optical density of the corresponding blank was subtracted from the average optical density of the duplicate determinations to obtain the optical density difference. This optical density difference was then used to determine the enzyme activity as described above. From the linear portion of the calibration curve it was determined that an optical density change of 0.1 was equivalent to an SGPT or SGOT activity of about 25 units. The concentration of SGOT is much higher in the red blood cells than in the serum or plasma, and for this reason hemolyzed blood samples were eliminated before the enzyme determinations were made.

Outline of the Experiment

In the preliminary studies the initial objective was to establish a relationship between the dose of CCl_4 employed and the serum transaminase activities attained in rats. Initially blood samples were taken by cardiac puncture 16 hours after CCl_4 was given. A series of

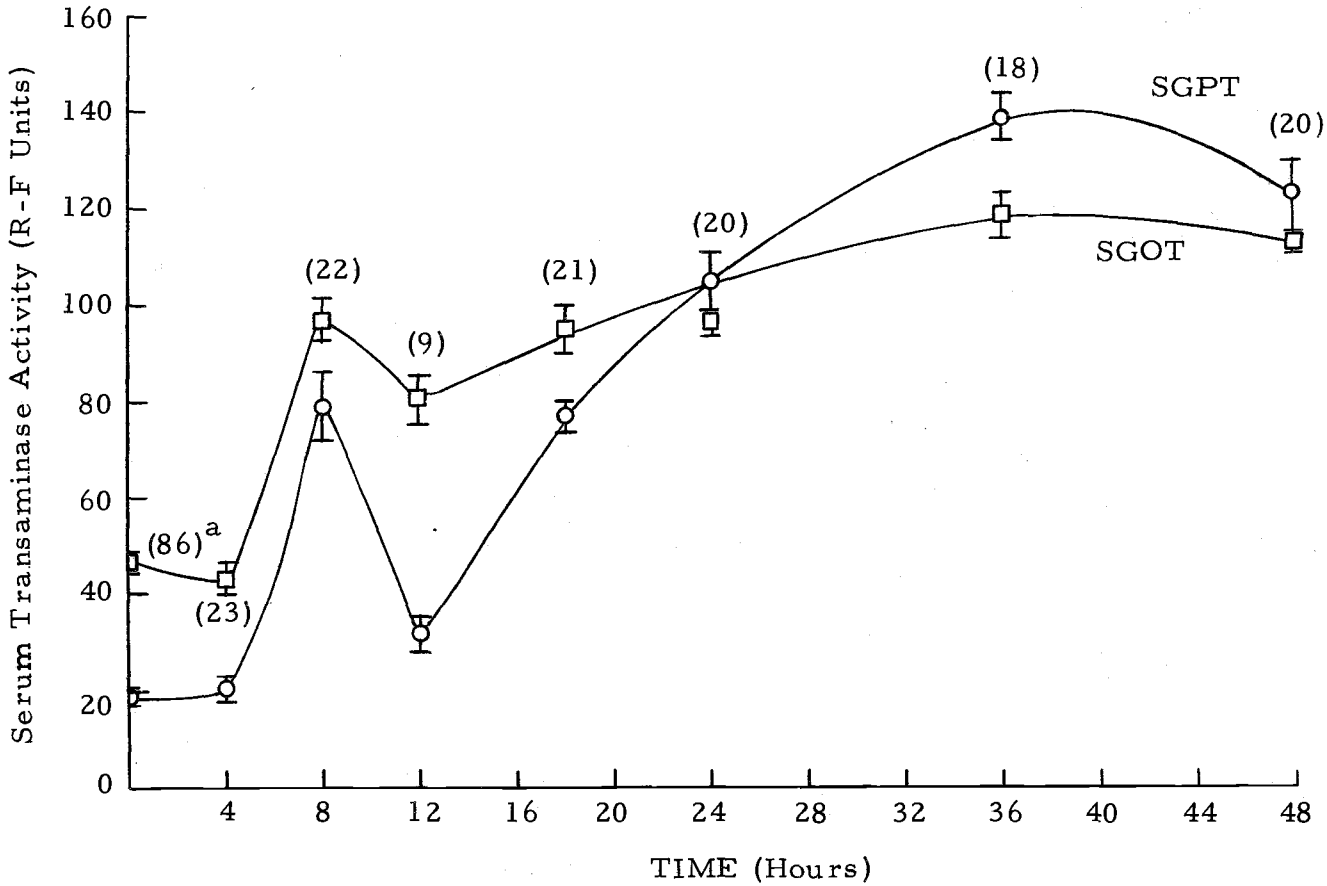
increasing dosages of CCl_4, ranging from 0.0075-1.25 ml/kg, was administered to groups of 5-21 rats and the resulting serum transaminase activities were compared to control enzyme activities. From this initial study, a dose of 1.25 ml/kg was selected for the present investigation, since this dose produced a moderate histologic degree of hepatic damage. The temporal pattern of serum transaminase activity was studied over a 48 hour period following CCl_4 administration. In this latter study, blood samples were taken at 0, 2, 4, 6, 8, 12, 17, 20, 24, 32, and 48 hours after CCl_4 in groups of nine or more rats, and the serum transaminase activities were then determined. From these results a sampling time of 24 hours post-CCl_4 exposure was chosen for subsequent experimentation. The dose-response relationship of CCl_4 to serum transaminase activities was reestablished at 24 hours for a CCl_4 dosage range of 0.125-5.0 ml/kg. The resulting activities, obtained in groups of six or more animals per dose of CCl_4, were compared to the control mean serum transaminase activities.

Results

The results of the temporal study are illustrated in Figure 2-1. The continuous curves were constructed between the discrete data points to aid in the visual representation of the results. Actually these serum enzyme activities probably do not increase or decrease

Figure 2-1. Temporal pattern of serum transaminase activities in male rats following the administration of 1.25 ml/kg CCl_4 by gavage. Each point represents the mean (± standard error) of at least 9 animals. Note the biphasic nature of the transaminase activity curves, which attained maximal values by 36 hr after CCl_4 administration.

[a]The number in parentheses represents the number of animals per group.


Serum Transaminase Activity (R-F Units)
160
140
120
100
80
60
40
20
0
4
8
12
16
20
24
28
32
36
40
44
48
TIME (Hours)
SGPT
SGOT
(86)a
(23)
(22)
(9)
(21)
(20)
(18)
(20)

in a smooth continuous fashion in vivo in response to a toxic agent. These temporal measurements of the serum transaminase (SGPT and SGOT) activities represent the combined data from two experiments. In the first experiment each animal served as its own control for comparison with subsequent observations in the same animal. In the second, subgroups of six animals or more given the same dose of CCl_4 were sampled at different intervals after CCl_4 was given. The resulting serum enzyme activities from the two experiments were compared to assess whether the elevated SGOT activity resulted from myocardial damage due to repeated cardiac punctures in the same animals. In addition to responding to hepatocellular damage, the SGOT activity is known to increase in cases of myocardial damage, such as in myocardial infarction, due to the rich supply of this enzyme in the heart. The results of the two experiments were essentially identical, which indicated the rise in SGOT activity was not in response to myocardial puncture. Thus, the data were combined to obtain greater numbers of observations at each interval after CCl_4 administration.

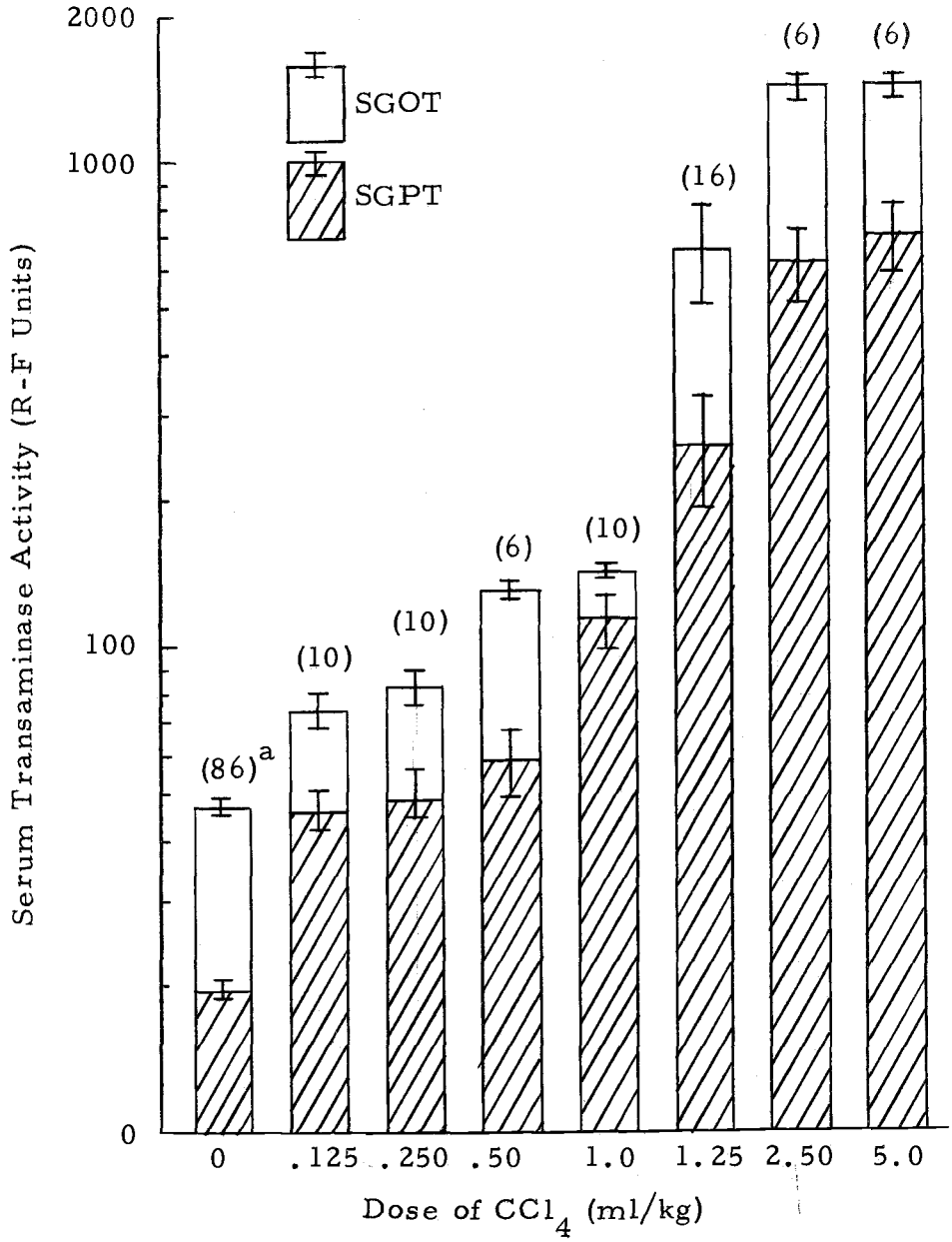
The response of the serum transaminase activities was essentially biphasic in that the continuous increase from 0-36 hours was interrupted by a brief decline between 8-12 hours after CCl_4. Maximal activities for both enzymes were attained at 36 hours (SGOT 120 units; SGPT 140 units), thereafter the activities declined between

36-48 hours. The greater response of SGPT indicated this enzyme could be the more sensitive indicator of hepatic damage. Near-maximal enzyme activities were attained by 24 hours, and this was chosen as a more convenient sampling time than 36 hours for subsequent studies. It was believed the results obtained at 24 hours would be consistent and reproducible from one experiment to the next. Schwetz and Plaa (1969) reported a maximal SGPT activity in mice 24 hours after CCl_4 with respect to the 48 hour mean, but they did not measure the serum enzyme activity at 36 hours.

The 24 hour dose-response relationship between CCl_4 and the serum transaminase activities is indicated in Figure 2-2. The activity of SGOT was generally higher than that of SGPT at this time after CCl_4. The control activity of SGOT is considered to be slightly higher than that of SGPT in mammals (Reitman et al., 1957). A dose-response relationship existed for both enzymes in a CCl_4 dosage range of 0.125-5.0 ml/kg. The mean activities for non-CCl_4-treated controls were 20 units for SGPT (86 samples) and 47 units for SGOT (82 samples). The degree of serum transaminase response correlated with the hepatic histologic damage. Doses of CCl_4 between 0.125-1.0 ml/kg produced small increases in serum transaminase activities and only mild-moderate damage histologically. When the dose of CCl_4 was increased to 1.25 and 2.0 ml/kg, moderate and severe increases in serum enzyme activities and centrilobular necrosis were noted.

Figure 2-2. Effect of increasing dosages of CCl_4 upon the serum transaminase activities measured 24 hr after CCl_4 administration. CCl_4 (0.125 - 5.0 ml/kg) was administered by gavage. Each bar represents the mean (± standard error) of at least 6 animals. Note the dosages of CCl_4 between 0.125 and 1.0 ml/kg produced a mild to moderate hepatic damage.

[a]The number in parentheses represents the number of animals per group.


2000
1000
100
0
Serum Transaminase Activity (R-F Units)
SGOT
SGPT
(86)a
(10)
(10)
(6)
(10)
(16)
(6)
(6)
0
.125
.250
.50
1.0
1.25
2.50
5.0
Dose of CCl4 (ml/kg)

Thus, the histopathologic findings confirmed that the increase in serum transaminase activities was based on an enhanced destruction of hepatic centrilobular parenchymal cells in vivo after CCl_4. The histologic analysis of damage in liver sections taken from CCl_4-treated controls has been deferred to Chapter III for comparison with livers taken from DDT-fed animals.

Discussion

The mechanism of carbon tetrachloride (CCl_4) hepatotoxicity has not been conclusively demonstrated. It can at least be assumed that CCl_4-induced hepatic necrosis is a principal manifestation of poisoning from this chlorinated aliphatic hydrocarbon solvent, which is uniformly concentrated and metabolized by the liver (de Reuck and Knight, 1964). In addition, the results of many studies have indicated that necrosis of the liver (or other tissues) is accompanied by the loss of intracellular enzymes to the blood (Abderhalden, 1961). Using a paper electrophoresis technique, Cornish (1962) demonstrated that the rise in serum esterase activity was due to the release of esterases from the necrotic liver cells. Zimmerman, Kodera and West (1965) have shown there is a direct relationship between the dose of the hepatotoxic agent administered and the magnitude of rise of the serum levels of a number of enzymes in experimental hepatic necrosis induced by CCl_4. More recent findings by the same author have dealt

with the loss of cellular enzymes into the surrounding medium as a measure of CCl_4 cytotoxicity in vitro (Zimmerman and Mao, 1965), and have confirmed their earlier hypothesis. The discovery of the transaminase enzymes was first made by Braunstein and Kritzmann (1937), who reported finding enzymes which catalyzed the transfer of amino groups from glutamic and aspartic acid to certain alpha keto-acids, such as alpha keto-glutaric acid.

Acute CCl_4 intoxication produces an increase in hepatic lipid peroxidation, cellular necrosis, and a decrease in the microsomal drug metabolizing enzyme activity, but it is apparent these impairments arise through diverse mechanisms (Sasame, Castro and Gillette, 1968). The elevated serum transaminase activities measured in the present study were probably a direct reflection of the degree of hepatic necrosis primarily, and did not necessarily correlate with the other signs of toxicity. The degree of damage histologically to the hepatic centrilobularly-oriented parenchymal cells correlated with the extent of increase in serum enzyme activities. Wirtschafter and Tsuyimura (1961) correlated the SGOT activity in rats with histological damage to the livers in 20 normal rats and in 48 rats exposed to a single injection of CCl_4, and the correlation failed in only 7/48 animals. They concluded that high SGOT activities can be correlated with marked hepatocellular damage, whereas low or normal activities cannot be used to exclude the presence of significant hepatic necrosis.

In general, the activities of SGPT and SGOT measured in this study were comparable to those reported in the scientific literature (Reitman et al., 1957; Schwetz and Plaa, 1969; Traiger and Plaa, 1971; Wei, Wong and Hine, 1971).

In the present investigation, an acute phase of CCl_4 intoxication was observed between 0-36 hours, when the serum transaminase activities increased to their maximal values. The diminishing serum enzyme activity between 36-48 hours marked the beginning of the regenerative phase. Similar temporal patterns of serum transaminase activity response to CCl_4 have been shown by Schwetz et al. (1969) in mice, and by Dinman and Bernstein (1968a, b) in rabbits that received 100 ppm CCl_4 by inhalation for six hours. Dinman et al. measured the activities of glutamic pyruvic transaminase (GPT) and glutamic oxaloacetic transaminase (GOT) along with several other enzymes, in the livers and sera at several intervals after CCl_4 exposure. The concentration of CCl_4 employed in their inhalation studies affected the temporal patterns of serum and hepatic enzyme activity response.

It could be argued that the elevation in serum transaminase activities measured in this study were due solely to an increased hepatic enzyme activity or to an increased release of intracellular enzyme from injured tissue in other organs. The fact that SGOT activity did not increase disproportionately with respect to SGPT

activity was an indication that myocardial damage was not contributing to the elevations of serum enzyme activities. And Dinman et al. (1968a, b) verified by autopsy that liver damage was the only contributor to the increase in serum enzyme. Perhaps the most important finding in the present study was that the histologic evidence of hepatic parenchymal cell disruption generally correlated well with the increases in serum transaminase activities. Dinman demonstrated that the rise in serum enzyme activities after CCl_4 was not due solely to the passive leakage of enzymes from the injured liver cells to the blood. Some enzyme activities in their study, including those of GPT and GOT, increased in the liver during the acute phase and again during the regenerative phase of CCl_4 intoxication. For many enzymes studies, e.g., isocitric dehydrogenase, normal or decreased hepatic activities were observed despite large increases in serum enzyme activities. Therefore, it appeared to Dinman and Bernstein that the increase in hepatic enzyme activities during the acute and regenerative phases of CCl_4 intoxication could have played a role in the elevations measured in the serum. Nevertheless, they also observed the histologic evidence of CCl_4-induced central necrosis correlated with the degree of increase in serum enzyme activities. They concluded that the role of cellular disruption and leakage of enzymes to the blood should not be minimized.

If the rise in serum transaminase activities after CCl_4 did

represent a combination of degenerative and homeostatic changes, then one could raise questions as to the cause and mechanism of the homeostatic response. Dinman et al. (1968a, b) observed the liver glycogen content is largely depleted early in CCl_4 intoxication. Therefore, the hepatic enzymes GPT and GOT could play an important role in increasing the gluconeogenic activity in the liver by providing new supplies of glucose precursors. Rubinstein (1962) observed the release of epinephrine after CCl_4 in the rat, which was supposedly a compensatory response to glycogen depletion. Indeed, epinephrine is known to be released from the adrenal medulla in both the rat and rabbit (Brody and Calvert, 1960). Epinephrine stimulates the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary. ACTH in turn stimulates the release of glucocorticosteroids from the adrenal cortex (Weber et al., 1964). According to Weber, the glucocorticoids cause the release of lactate, various amino acids and 3-carbon precursors from various tissues, e.g., muscle, which arrive to the liver via the blood stream. First the increased levels of metabolites saturate the pre-existing enzymes and thus a rise in pyruvate to glucose production can be achieved. Increases in the hepatic activities of a number of enzymes involved in gluconeogenesis, including those of the alanine alpha ketoglutarate (Rosen et al., 1958) and tyrosine alpha keto-glutarate (Kanney, 1962) transaminases have been reported following glucocorticoids. Rosen (1962) suggested there

was a correlation between glycogen synthesis and the response of alanine alpha ketoglutarate transaminase in the liver to cortisol. Further, the increase in glutamic-alanine transaminase is due to an increased rate of enzyme synthesis (Segal, Kim and Hopper, 1965) reflecting an increased hepatic protein synthesis (Kanney et al., 1965). In this context, the observation by Dinman et al. (1968a, b) that the hepatic activities of GPT and GOT increased after CCl_4 inhalation in rabbits could be explained on the basis of an adaptive response to glycogen depletion mediated by the pituitary-adrenal system. Thus, a conceivable mechanism for the CCl_4-induced stimulation of hepatic gluconeogenic enzyme activity is apparent, however, the role of this adaptive response in the contribution to serum enzyme remains to be conclusively elucidated.

Subsequent experimentation (Chapter V) revealed that the uptake of CCl_4 into the blood and livers of intact control rats occurred in a biphasic manner similar to the biphasic temporal serum transaminase response. Apparently the responses of SGPT and SGOT were, at least in part, a function of the concentration of CCl_4 attained in the livers at various intervals after CCl_4 treatment. These findings, along with the demonstration of a dose-related increase in serum enzyme activities, confirmed that the release of cellular enzymes into the surrounding medium was directly related to the dose of CCl_4 employed in vivo (Zimmerman, Kodera and West, 1965) and in vitro

(Zimmerman and Mao, 1965). In addition the evidence brought forward from other laboratories confirmed the present findings with respect to the temporal pattern of serum transaminase response and associated histopathologic changes in livers (Dinman et al., 1968a, b).

III. POTENTIATION OF ACUTE CCl_4 HEPATOTOXICITY BY DIETARY EXPOSURE TO DDT

Introduction

The chlorinated hydrocarbon solvents are known to interact at the hepatic level with a variety of physical and chemical agents. The hepatotoxicity of CCl_4 has been potentiated by pretreatment of rats with ethanol (Wei et al., 1971), phenobarbital (Garner and McLean, 1969; Stenger, Miller and Williamson, 1970), and acutely-administered DDT (McLean et al., 1966). Schwetz et al. (1969) demonstrated the potentiation of CCl_4 hepatotoxicity in mice by pretreatment with catecholamines. More recently, the physical stress of a cold exposure has been shown to potentiate the CCl_4-induced hepatic damage in mice (Adam and Thorpe, 1970) and rats (Wei et al., 1971).

The hepatotoxic interaction of acutely-administered DDT and acutely-administered CCl_4 was extensively studied (McLean et al., 1966; Seawright and McLean, 1967; McLean and McLean, 1969). McLean and coworkers demonstrated a potentiated response to CCl_4 in rats pretreated with 75-100 mg/kg DDT (s.c.) in terms of the accumulation of liver water and increases in plasma enzyme and bilirubin concentrations. This potentiated response overcame the hepatic protective effect afforded by feeding a protein-depleted diet. The above-mentioned evidence for hepatic damage was confirmed by histopathology.

In general, the potentiations of CCl_4-induced hepatotoxicity have resulted from acute pretreatments with the potentiating agent. In the cases where ethanol or phenobarbital were used as potentiators, the subacute or acute administration of these agents may come close to mimicking the human exposure. In fact, there are clinical warnings against the ingestion of ethanol prior to CCl_4 exposure (Drill, 1965). The pretreatment of rats with high acute doses of DDT does not relate directly to man's exposure situation, which is mainly dietary insofar as the general population is concerned.

The present studies were designed to assess the role of chronic dietary exposure of rats to DDT, in terms of their subsequent response to a challenging hepatotoxic dose of CCl_4. The total body burden of DDT-derived material was determined as a basis for the extrapolation of these results to the potential health hazard associated with the DDT body burden in man. Chronic and acute pretreatments with DDT were compared for the ability to potentiate CCl_4-induced hepatotoxicity. In addition, the temporal pattern of the potentiated response was determined in the DDT-fed animals.

Methods

GLC Analyses for DDT and Its Metabolites

The Environmental Health Sciences Center Pesticide Laboratory, Oregon State University, used a gas-liquid chromatographic procedure

(Porter, Young and Burke, 1970) to determine the concentrations of DDT and its metabolites in extracts of whole rats and laboratory chow. The method of Giuffrida, Bostwick and Ives (1966) was used in place of the hexane-acetonitrile extraction. The resulting concentrations of DDT-derived materials in whole rat extracts and the DDT-containing rat diet were expressed as ppm DDT and its metabolites. A QF-1/DC-11 column was used for the GLC analyses. On this column, p, p'-DDE, o, p-DDT, p, p'-TDE and p, p'-DDT can be equally separated.

Histopathology

Liver sections were taken at the time of blood sampling. Liver slices of 1/16" to 1/8" thickness were placed in 10% buffered formalin solution and refrigerated at 4°C for 24 hours. Fixed liver slices were cut and stained with a hematoxylin-eosin (H and E) stain by Dale Hays of the Veterinary Medicine Department, Oregon State University. Histopathologic examination of livers prepared in this manner was made by subjective evaluation of characteristic patterns of hepatocellular degeneration. These single-blind evaluations were used to confirm the results obtained from the measurements of serum transaminase activities.

Outline of the Experiment

Chronic Studies. In the initial chronic studies, four feeding schedules of DDT were employed. Three groups of male rats were exposed to DDT-containing feed at levels of 6, 16 or 65 ppm, respectively, for a three-week period. A fourth group received 65 ppm via the diet for 24 weeks.

Acute Studies. Three groups of rats were dosed with 35, 75 or 150 mg/kg DDT, respectively; these doses of DDT were chosen from the experiments of McLean et al. (1966), who used 75 and 100 mg/kg DDT, s.c., as pretreatment doses in their dietary studies with CCl_4. DDT was dissolved in corn oil and administered by gastric intubation. The 150 mg/kg dose of DDT represented an approximate LD10, but resulted in no spontaneous deaths in these studies.

Subgroups of 6-12 animals, representing each dose level (chronic and acute) of DDT were challenged with CCl_4 at doses ranging from 0.125 to 1.0 ml/kg. These doses of CCl_4 were chosen from the initial dose-response studies in controls (see Figure 2-2, Chapter II), where they produced only mild-moderate hepatic damage up to 48 hours after CCl_4. In the acute studies, CCl_4 was administered by gavage 24 hours after acute DDT administration. In both chronic and acute studies, blood and liver samples were taken 24 hours after CCl_4 administration for analyses of serum transaminase (SGPT and SGOT) activities and for histopathologic examination of liver tissues.

The temporal pattern of serum transaminase activities was determined for DDT-fed (16 ppm x 2 wks) rats dosed with 1.0 ml/kg CCl_4. Blood samples were taken at 0, 1, 2, 4, 8, 16 and 24 hours after CCl_4 and the plasma was analyzed for SGPT and SGOT activities.

Results

GLC Analyses for DDT and Its Metabolites

The results of the GLC analyses for DDT and its metabolites in whole rat extracts and samples of DDT-containing feed are summarized in column 2 of Table 3-1. The technical DDT used in the preparation of the DDT-containing rat diet was found by analysis to contain approximately 83% p, p'-DDT, with nearly all of the remainder being o, p'-DDT. The values for these two isomers were combined in establishing the total DDT content of the whole rat extracts and feed. The DDT-containing ration was calculated to contain 10, 25 or 100 ppm DDT. The GLC analyses revealed that the actual DDT content in these diet samples was 6, 16 and 65 ppm DDT, respectively. These results compared favorably with a value of zero ppm in regular Purina laboratory chow. The animals maintained on diets containing 0 (no detectable), 6, 16 or 65 ppm DDT for three weeks attained total body burdens of 0.6 (control animals), 6.1, 10.4 and 10.7 ppm DDT, respectively. A fourth group of rats exposed to 65 ppm for 24 weeks achieved total body burdens of 30.6 ppm.

Table 3-1. SGPT and SGOT Activity in Rats Treated with DDT and CCl_4.

Dose of DDT	Body Burden of DDT[a]	Dose of CCl_4 (ml/kg) 0	0.125	0.25	0.50	1.0
Chronic (ppm)		mean SGPT activity ± S. E.[b]				
0	0.6	20±1	46±5	49±4	58±9	114±15
6 x 3 wks	6.1	35±2	1920±142	2069±298	5350±725	---
16 x 3 wks	10.4	27±1	---	1437±433	2094±437	---
65 x 3 wks	10.7	29±3	2344±238	4463±606	4464±505	---
65 x 24 wks	30.6	33±2	3128±643	2963±622	4883±506	5675±868
Acute (mg/kg)						
35		26±2	---	130±81	---	---
75		35±1	---	---	3475±846	---
150		30±2	---	2935±501	---	---
		mean SGOT activity ± S. E.				
Chronic (ppm)						
0	0.6	47±2	74±6	83±7	132±5	143±3
6 x 3 wks	6.1	46±2	2086±214	2564±320	8043±681	---
16 x 3 wks	10.4	45±2	---	4288±515	6894±502	---
65 x 3 wks	10.7	63±1	4253±1267	6628±938	7321±846	---
65 x 24 wks	30.6	58±3	3890±630	4565±575	6146±560	6484±581
Acute (mg/kg)						
35		57±4	---	594±108	---	---
75		52±2	---	---	5095±454	---
150		54±3	---	3960±322	---	---

[a] Total DDT and metabolites in ppm.
[b] The mean values were obtained from groups of 6-19 rats.

Gross Observations

After CCl_4 administration, rats breathed rapidly at first, which was possibly due to excitement from handling. Respiration slowed markedly within a few minutes after the initial increase, and this could have been due to a slight CNS depression. These animals were sick and remained almost completely motionless. Rats given the corn oil vehicle only appeared more alert. From 8 to 24 hours after CCl_4 the condition of the animals deteriorated markedly. No longer did they attempt to maintain cleanliness, but rather they appeared filthy with their fur disheveled. A faint yellowish color developed in the skin, thus indicating the onset of a mild jaundice. Rats handled 24 hours after CCl_4 offered much less resistance to handling than did the controls. There appeared to be a slight amount of hematuria at the higher doses of CCl_4 employed, e.g., 1.0-5.0 ml/kg. Blood samples were more difficult to obtain by cardiac puncture from CCl_4-treated animals than from controls. Hepatic enlargement or hypertrophy was also noted after CCl_4 treatment. These gross observations, and in particular the onset of jaundice and the sickly appearance, were more readily apparent in the DDT-fed rats challenged with CCl_4.

Serum Transaminase Activity

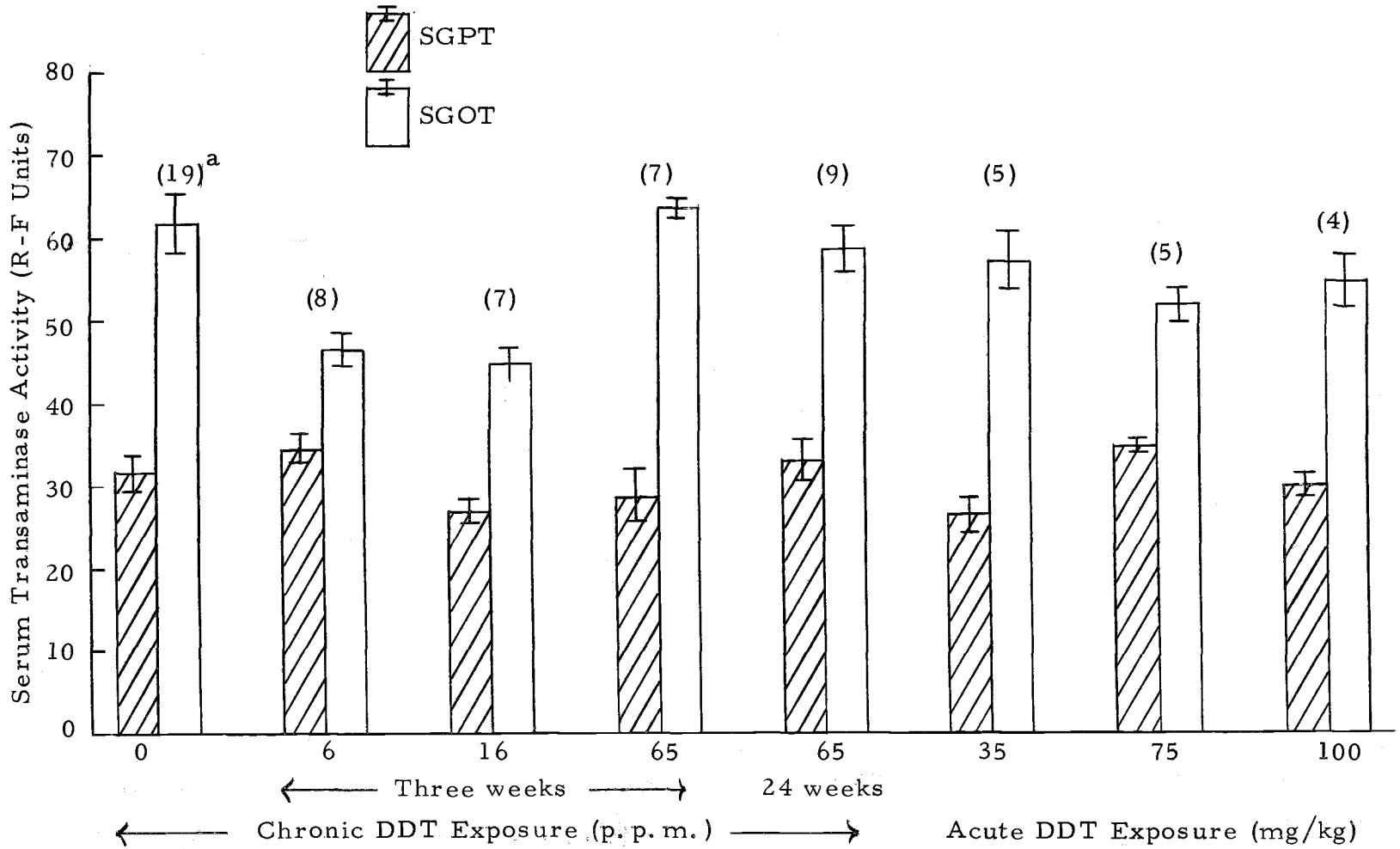
The control activities of SGPT and SGOT were 30 units and 60

units, respectively. It can be observed in column 3 of Table 3-1 and in Figure 3-1, that neither chronic nor acute DDT pretreatments produced an elevation in serum transaminase activities. The effect of low doses of CCl_4, e.g., 0.125-1.0 ml/kg, upon the serum transaminase response is summarized in lines 1 and 9 of Table 3-1. A dose of 1.0 ml/kg produced an increase in SGPT activity to 114 units, and in SGOT activity to 143 units, which corresponded to a moderate damage histologically. At high doses of CCl_4, e.g., 2-5 ml/kg, the serum enzyme activities were elevated in excess of 1000 units, suggesting marked hepatotoxicity.

In the main body of Table 3-1, the effect of CCl_4 treatment on the serum transaminase response in DDT-fed rats is summarized. Virtually every combination of DDT + CCl_4 treatments resulted in a marked potentiation of SGPT and SGOT activities. In fact, the lowest dose of CCl_4 employed, i.e., 0.125 ml/kg, administered to rats fed the lowest dietary level of DDT, i.e., 6 ppm x 3 weeks, produced a tremendous potentiation of serum enzyme activities; in fact, the SGOT activity was increased about 30-fold over the CCl_4-treated control value, to about 2000 units. The smallest degree of potentiation was noted when animals pretreated acutely with 35 mg/kg DDT were challenged with 0.25 ml/kg; the resulting SGOT activity was about 600 units, representing an approximate seven-fold potentiation. The degree of potentiation was dose-related to CCl_4 and to the total DDT

Figure 3-1. Effect of chronic or acute DDT treatment upon the serum transaminase activities in male rats. Acute DDT was dissolved in corn oil and administered by gavage at dosages of 35, 75, or 150 mg/kg. The SGPT and SGOT activities were determined 24 hours after the acute DDT administration. Each bar represents the mean (± standard error) of at least 4 animals. Note that neither chronic nor acute DDT treatment produced a significant elevation ($P > 0.05$, Student's t-test) of the serum transaminase activities.

[a]The number in parentheses represents the number of animals per group.


SGPT
SGOT
Serum Transaminase Activity (R-F Units)
80
70
60
50
40
30
20
10
0
(19)a
(8)
(7)
(7)
(9)
(5)
(5)
(4)
0
6
16
65
65
35
75
100
Three weeks
24 weeks
Chronic DDT Exposure (p.p.m.)
Acute DDT Exposure (mg/kg)

body burden. The potentiated effects of doses of CCl_4 ranging from 0.125-0.5 ml/kg on serum transaminase activities, when the CCl_4 challenge was superimposed upon dietary DDT (6 ppm x 3 wks) pretreatment, are illustrated in Figure 3-2. The SGOT activities were greatly potentiated at all doses of CCl_4 in comparison to the activities measured in animals treated with either DDT or CCl_4 alone. The effects of a single dose of 0.25 ml/kg CCl_4 in rats fed various dietary concentrations of DDT are shown in Figure 3-3. At all levels of DDT feeding, the serum transaminase activity was greatly potentiated in response to CCl_4. The acute pretreatment of rats with 35 or 150 mg/kg DDT prior to CCl_4 administration (0.25 ml/kg) had a similar potentiating effect (Figure 3-4). The higher acute dose of DDT resulted in a considerable potentiation of SGOT activity to about 4000 units. Thus, the potentiating effects of the dietary DDT concentrations employed in this study were comparable to the results obtained after acute DDT pretreatment. The temporal responses of SGPT and SGOT activities in DDT-fed rats for a 24 hour period following CCl_4 administration are shown in Figure 3-5. The activities of both enzymes increased rapidly from 8-24 hours. The activity of SGOT exceeded that of SGPT throughout the 24 hour study, however, the SGOT activity response was apparently curvilinear in comparison to the rectilinear response of SGPT activity. The reason for the different shapes for the two enzyme activity curves is not known, although

Figure 3-2. Effect of dietary DDT (6 ppm X 3 wks) upon the serum transaminase activity response to increasing dosages of CCl_4. CCl_4 (0.125 - 0.5 ml/kg) was administered by gavage. Each bar represents the mean (± standard error) of at least 7 animals. Note the SGPT and SGOT activities were potentiated by dietary DDT at all dosages of CCl_4 (see Figure 2-2).

[a]The number in parentheses represents the number of animals per group.

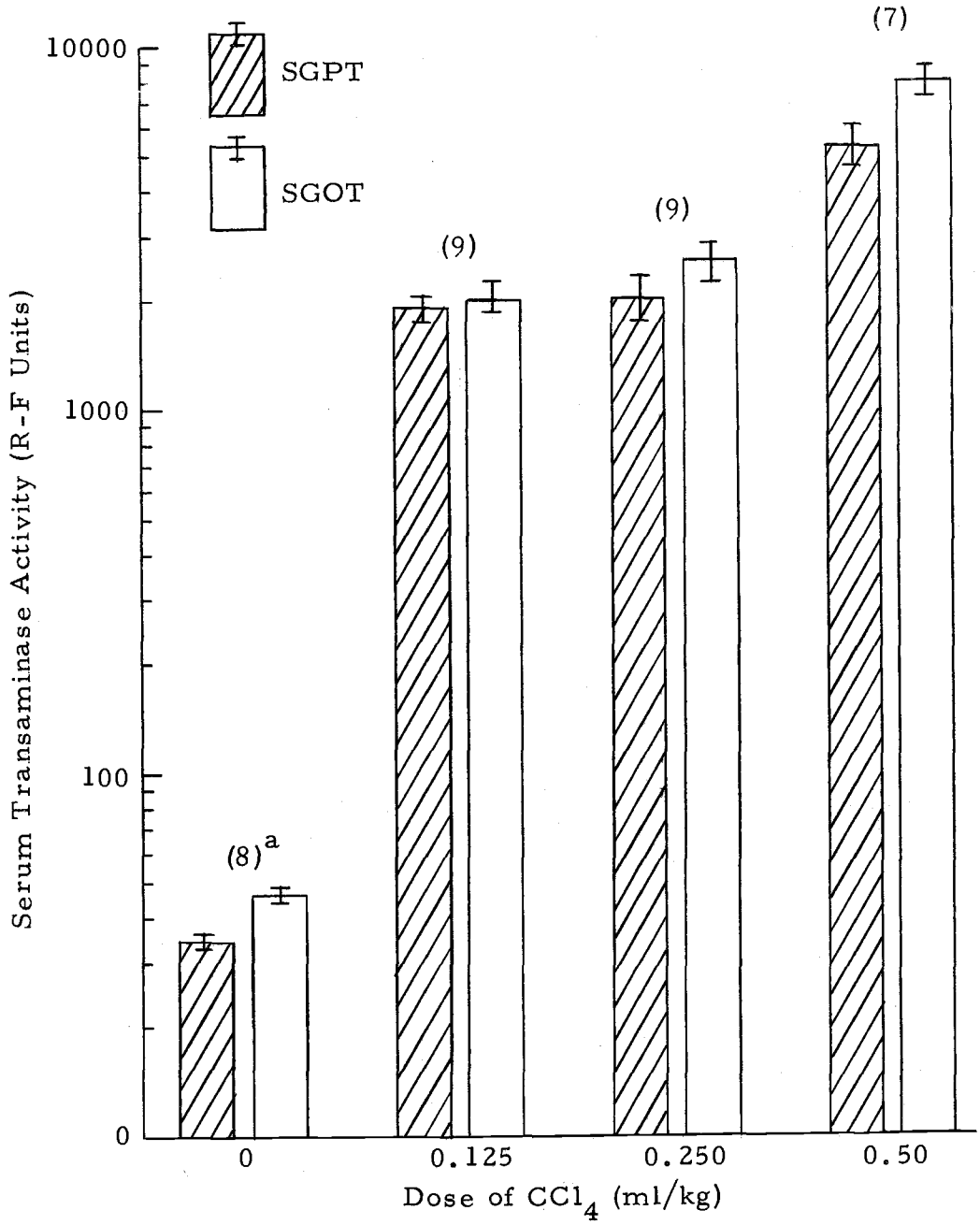

10000
1000
100
0
SGPT
SGOT
Serum Transaminase Activity (R-F Units)
(8)a
(9)
(9)
(7)
0
0.125
0.250
0.50
Dose of CCl4 (ml/kg)

Figure 3-3. Effect of increasing dietary concentrations of DDT upon the serum transaminase activity response to 0.25 ml/kg CCl_4. CCl_4 was administered by gavage. Each bar represents the mean (± standard error) of at least 8 rats. Note the response of the serum transaminase (SGOT) activity was potentiated by all of the dietary DDT concentrations employed.

[a]The number in parentheses represents the number of animals per group.

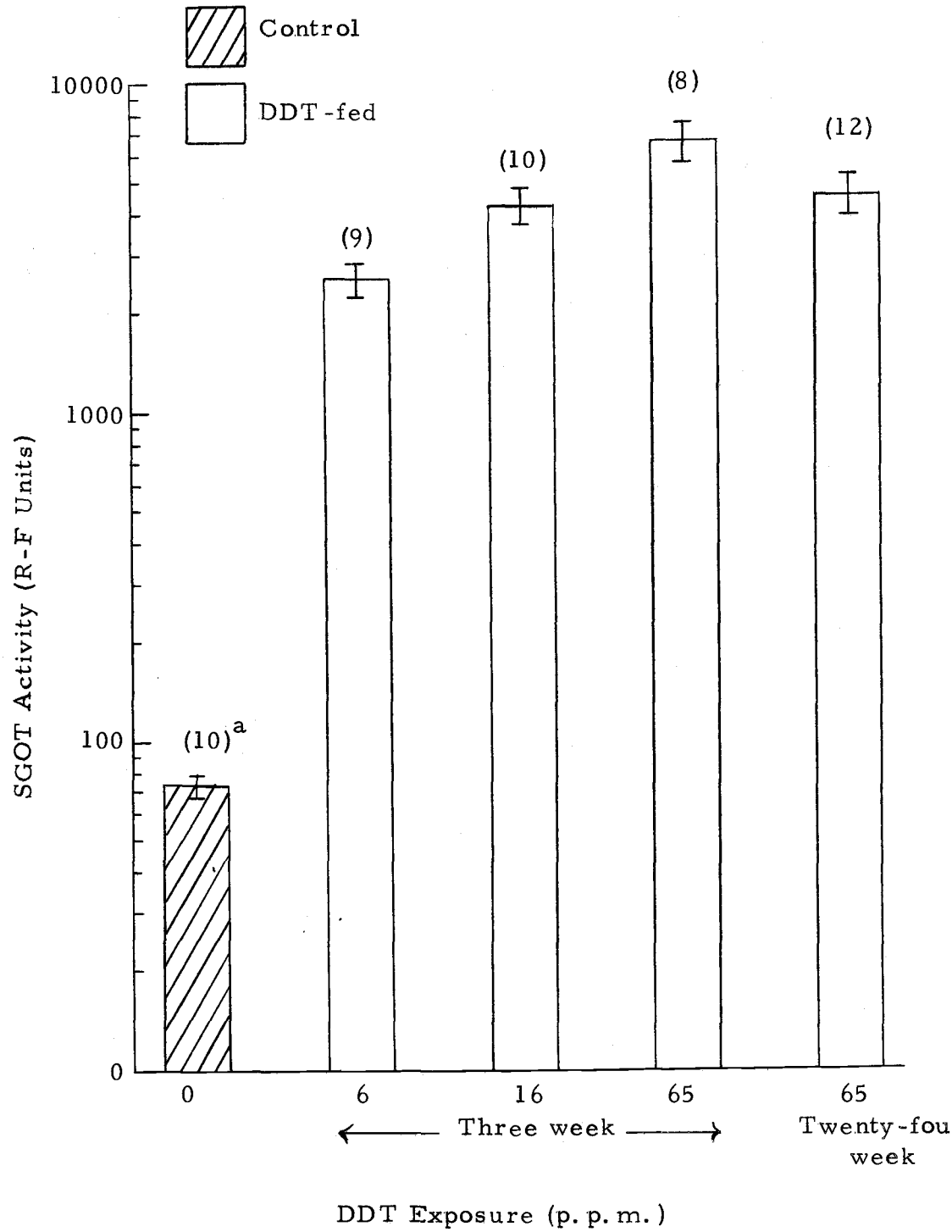

Control
DDT-fed
10000
1000
100
0
SGOT Activity (R-F Units)
(10)a
(9)
(10)
(8)
(12)
0
6
16
65
65
Three week
Twenty-four week
DDT Exposure (p. p. m.)

Figure 3-4. Potentiation of the serum transaminase activity response to 0.25 ml/kg CCl_4 by acute DDT pretreatment. DDT was dissolved in corn oil, and administered by gavage at dosages of 35 or 150 mg/kg. Twenty-four hours after DDT administration CCl_4 was administered by gavage, and 24 hr later the serum transaminase activities were determined. Each bar represents the mean (± standard error) of at least 4 animals. Note the potentiation of serum transaminase activities by 150 mg/kg DDT was comparable to the degree of potentiation achieved when dietary DDT pretreatment was employed.

[a]The number in parentheses represents the number of animals per group.

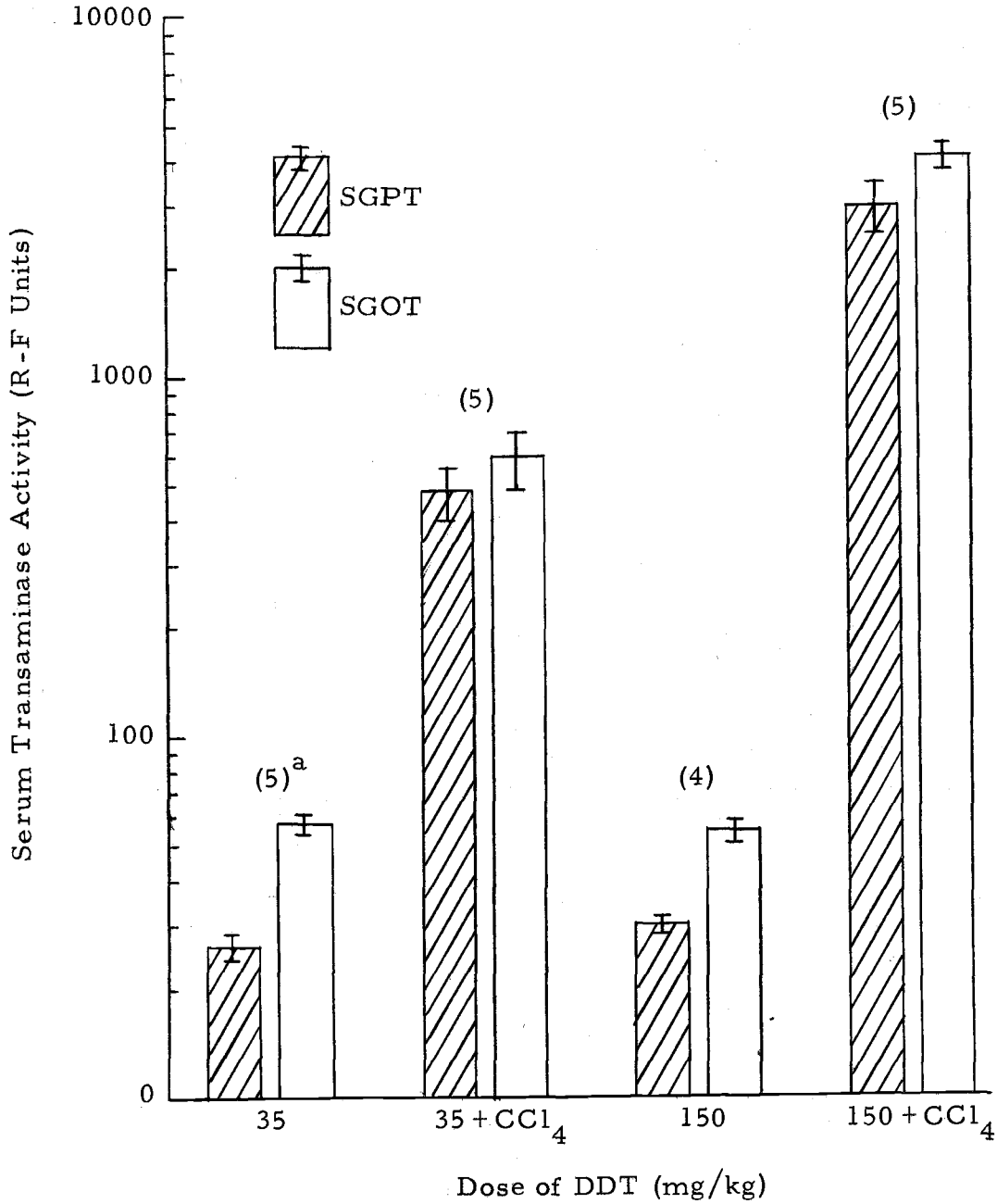
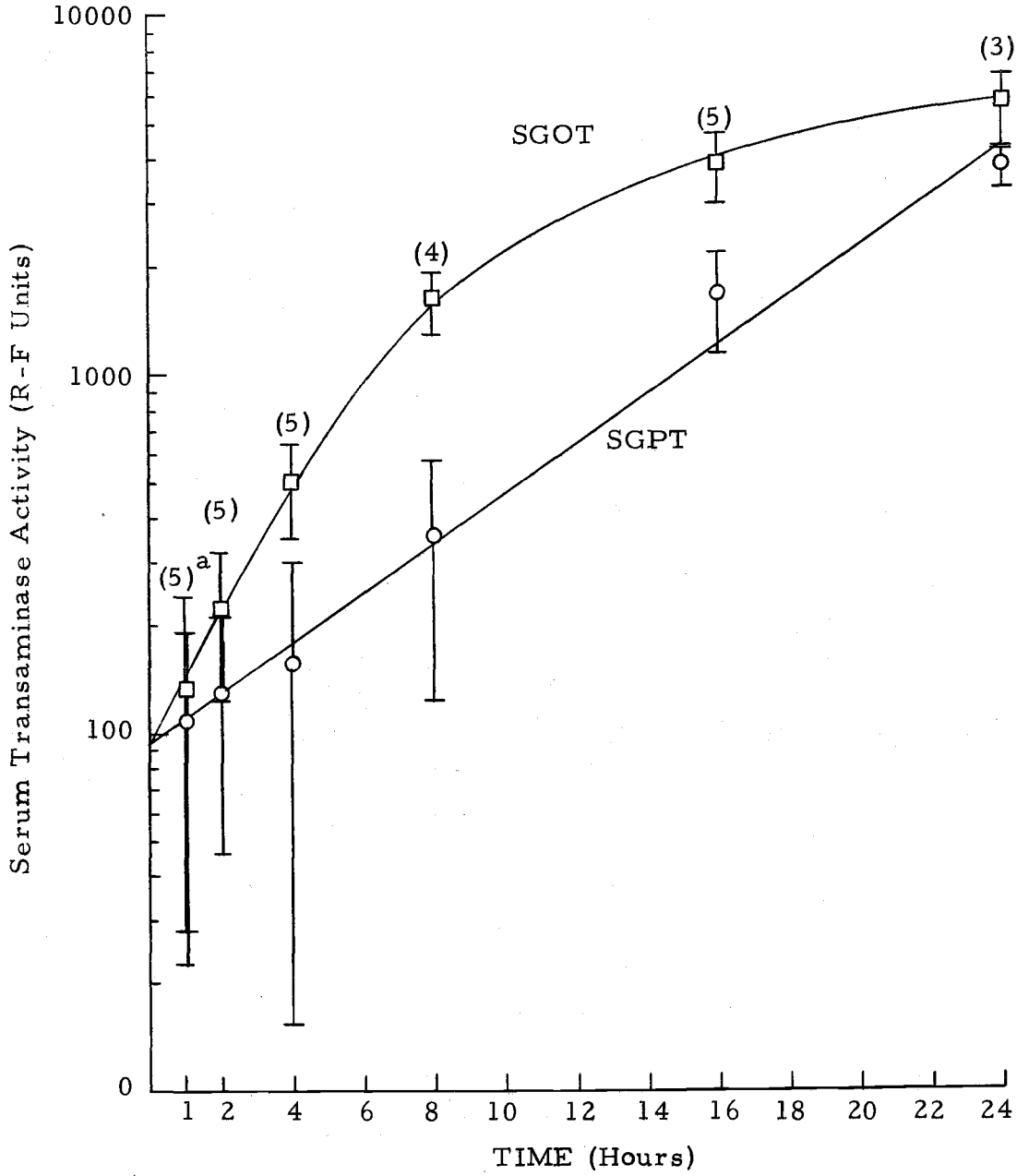

10000
1000
100
0
Serum Transaminase Activity (R-F Units)
SGPT
SGOT
(5)
(5)
(5)[a]
(4)
35
35 + CCl4
150
150 + CCl4
Dose of DDT (mg/kg)

Figure 3-5. Temporal pattern of the potentiated serum transaminase activities following the administration of 1.0 ml/kg CCl_4 to DDT-fed (16 ppm X 2 wks) rats. CCl_4 was administered by gavage. Each point represents the mean (± standard error) of at least 3 animals. Note the serum transaminase activities increased steadily for 24 hours after CCl_4 was given.

[a]The number in parentheses represents the number of animals per group.


10000
1000
100
0
Serum Transaminase Activity (R-F Units)
SGOT
SGPT
(5)a
(5)
(5)
(4)
(5)
(3)
1 2 4 6 8 10 12 14 16 18 20 22 24
TIME (Hours)

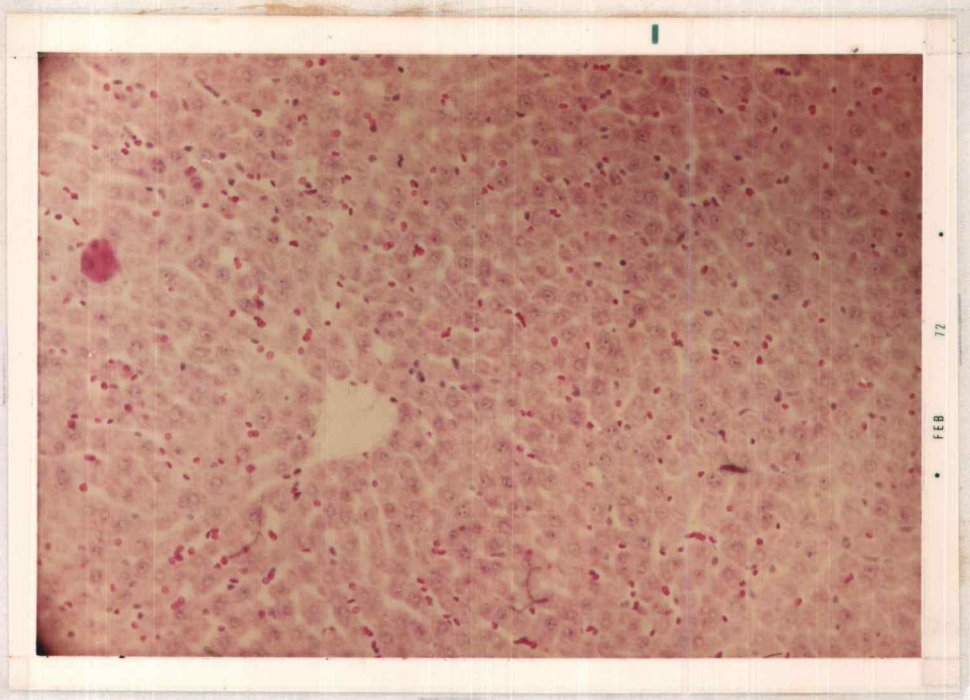
there could be differences in the relative supplies of the two enzymes in the hepatocytes, or differences in the time required for their intracellular regeneration.

Histopathology

The liver section taken from a rat treated with the corn oil vehicle only is shown in Figure 3-6. This liver has the characteristic lobular pattern of the mammalian liver. These lobular boundaries are distinct, and there is no evidence of lipid or water accumulation in the cells. The normal appearance of hepatocytes was also maintained in the following liver section (Figure 3-7), which was taken from an animal fed 65 ppm DDT for three weeks. Again, the cellular boundaries are distinct, and the plates of hepatic parenchymal cells are normally oriented with respect to the central veins and portal triads. There is no evidence of cloudy swelling or other degenerative changes. The cytoplasm of the hepatocytes appears somewhat denser than normal under light microscopy, possibly indicating an enhanced formation of intracellular membranous structures had taken place. A liver section taken from a non-DDT-fed rat treated with 0.25 ml/kg CCl_4 is shown in Figure 3-8. Areas of extensive cloudy swelling pervade the centrilobular zones, which are surrounded by rings of midzonal balloon cells having undergone hydropic degeneration. There is some evidence of centrilobular necrosis as well. A liver section

Figure 3-6. Liver section taken from a rat treated with corn oil, illustrating the normal hepatic architecture and hepatocellular appearance. H and E stain (400X)

Figure 3-7. Liver section taken from a rat fed 65 ppm DDT for 3 wks. Note the overall histologic appearance of this liver is comparable to that seen in the previous figure. The cytoplasm of hepatocytes seems dense by appearance, possibly indicating an enhanced formation of intracellular membranous structures. H and E stain (400X)


FEB 72

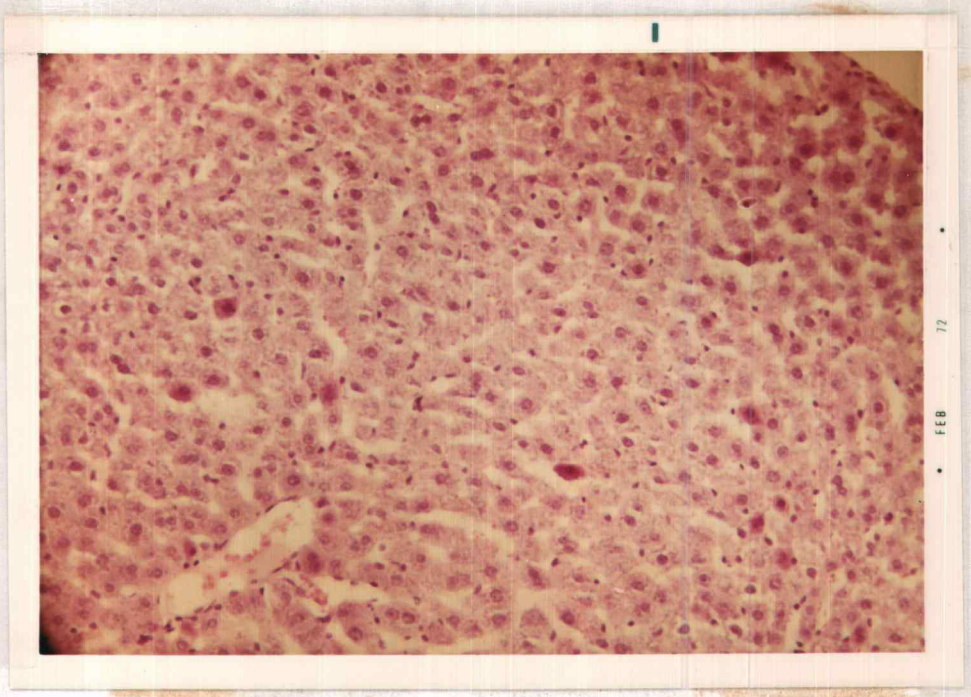
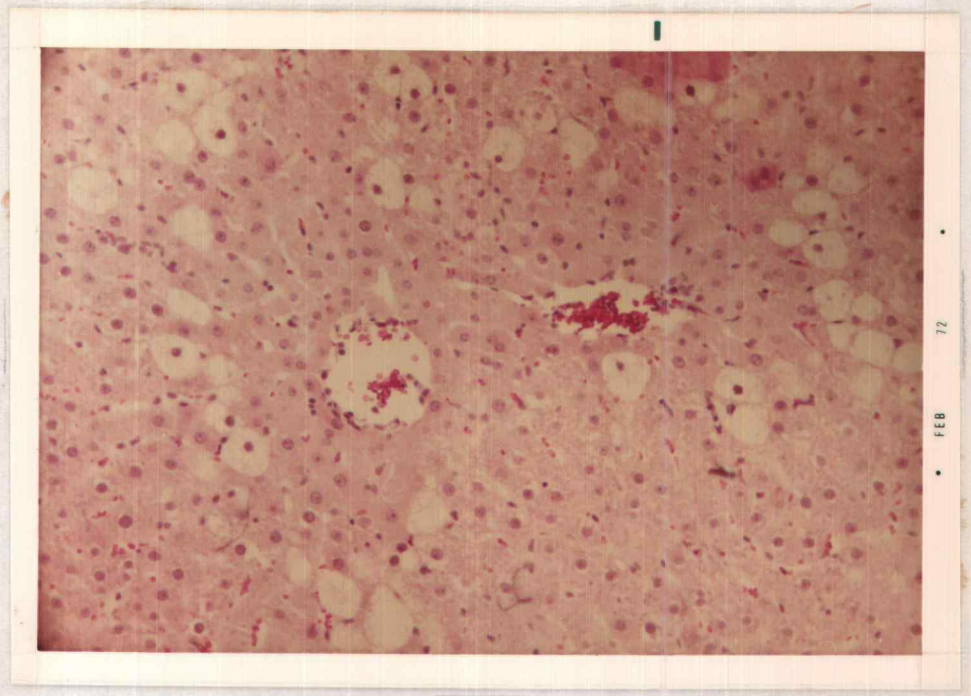

FEB 72

Figure 3-8. Liver section taken from a rat 24 hours after the administration of 0.25 ml/kg CCl_4 by gavage. Note the central cloudy swelling and mild degree of centrilobularly-oriented necrosis. A scattering of midzonal balloon cells surrounds the central zone. H and E stain (400X).

Figure 3-9. Liver section taken from a rat 24 hours after the administration of 1.0 ml/kg CCl_4 by gavage. Note the signs of CCl_4-induced hepatic damage are similar to those described above in Figure 3-8, but the centrilobular necrosis is manifested to a greater degree in this liver. H and E stain (400X).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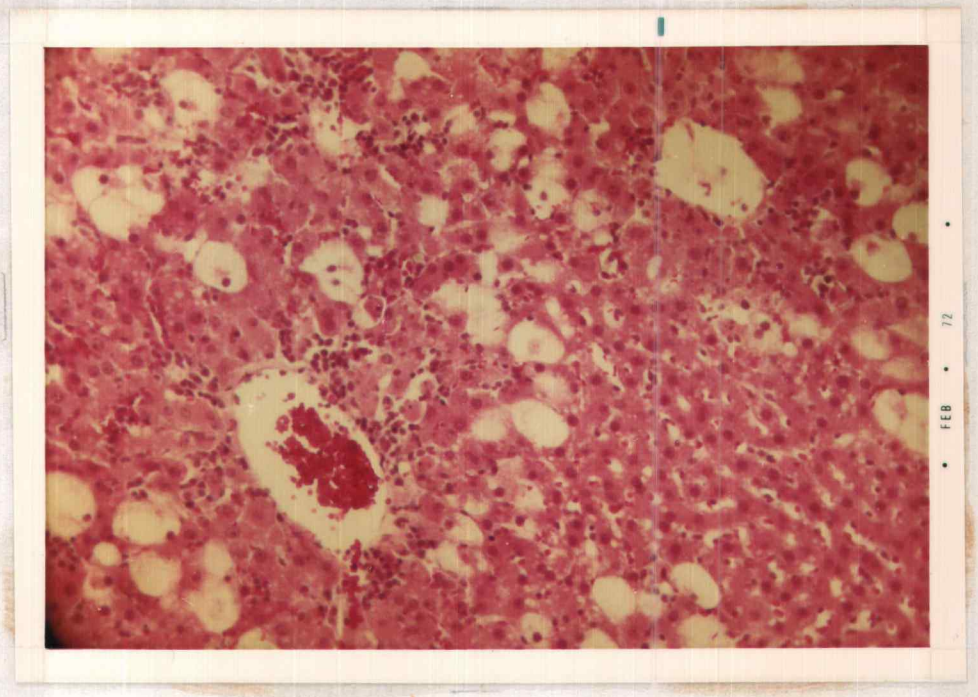
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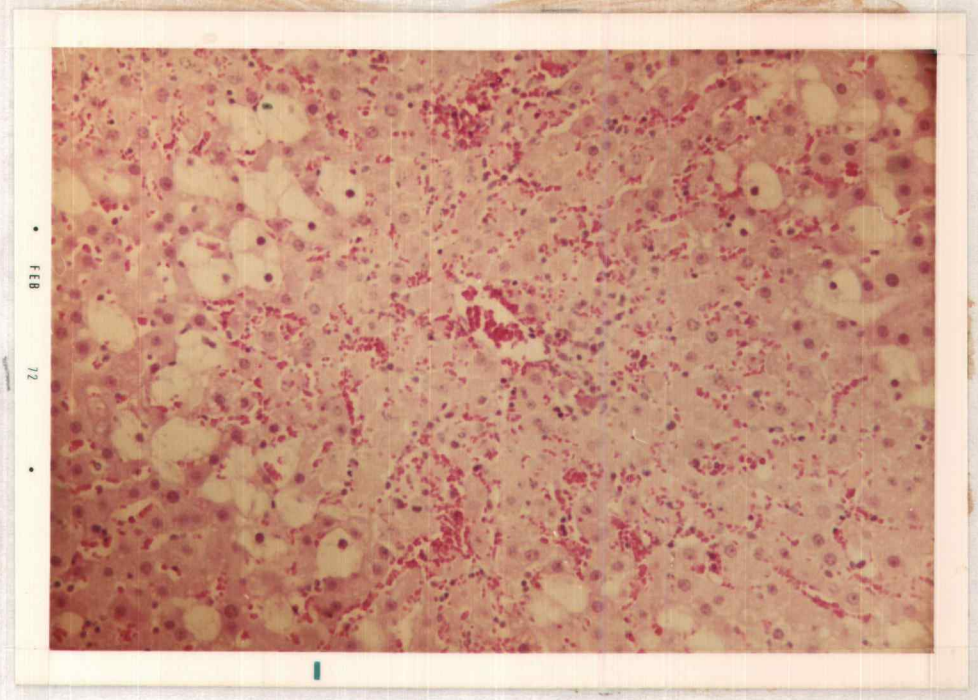
Figure 3-10. Liver section taken from a DDT-fed (6 ppm X 3wks) rat 24 hours after the administration of 0.25 ml/kg CCl_4 by gavage. Note the extensive cloudy swelling and centrilobularly-oriented coagulative necrosis, encircled by a midzonal scattering of balloon cells. The normal hepatic architecture was altered by sinusoidal congestion and necrosis, particularly in the central region. The hepatocellular destruction was more extensive than that observed in the previous figure (3-9), where a larger dose of CCl_4 was employed. It can be seen in this liver that the necrosis bridged interlobularly, and thus the degree of damage bordered on submassive necrosis. H and E stain (400X).


FEB 72

taken from a rat treated with 1.0 ml/kg CCl_4 is illustrated in Figure 3-9. Although the signs of damage are similar to those found in the previous liver section, the degree of damage is somewhat greater. A more severe centrilobularly-oriented coagulative necrosis is seen in Figure 3-10. This rat was fed 6 ppm DDT for 3 weeks and then dosed with 0.25 ml/kg CCl_4 by gavage. There was a loss of the characteristic basophilic stipling in the cytoplasm of the central parenchymal cells. There is a slight reduction in the number of midzonal balloon cells, in comparison to the liver sections taken from the CCl_4-treated controls. The most characteristic change from the control response was the finding that the centrilobular necrosis had bridged from lobule to lobule. Thus, the necrosis produced by CCl_4 encompassed a much larger proportion of the liver parenchyma in the DDT-fed animals. The damage in this latter group bordered on submassive necrosis.

Discussion

The influence of dietary exposure of rats to DDT on their subsequent response to a challenging dose of CCl_4 has been investigated and quantified. The degree of potentiated hepatic damage produced by the DDT-CCl_4 interaction was measured by the amount of increase in the serum transaminase activities. Dietary concentrations of DDT as low as 6 ppm fed to rats for three weeks produced a

hepatotoxic response to low doses of CCl_4.

The importance of dosage of pesticides in relation, not only to toxicity in a limited sense, but also to metabolism, storage, potentiation, and induction of microsomal enzymes has recently been discussed by Hayes (1967). This investigator also indicated that many other factors, including the schedule and duration of dosage, were important determinants of toxicity (Hayes, 1969). Durham (1969) showed how the body burden of DDT was attained in rats exposed to DDT, and discussed the relationship of DDT dosage to the body burden attained in rats and in man.

In the present investigation, the DDT body burdens attained in rats after feeding 6-65 ppm for three weeks ranged from 6.1 to 10.7 ppm. Thus, the dosage of DDT was indeed an important factor in influencing the body burden of this insecticide achieved. The duration of exposure to DDT was also an important factor here. When the dietary exposure to 65 ppm was increased from three to 24 weeks, the total DDT body burden increased nearly three-fold from 10.7 to 30.6 ppm. The body burdens of DDT measured in these rats were comparable to that estimated for man by Durham (1965), which is 10 ppm of DDT and DDE combined. According to Duggan (1969), the average pesticide residue content in FDA domestic food samples from 1963-66 was below 0.5 ppm for 95% of the samples, and below 0.03 ppm for 57.9% of the samples analyzed. These results confirm the

estimate by Gillett (personal communication, 1971) that the feeding levels of DDT used in the present studies were approximately 1-2 orders of magnitude higher than those concentrations estimated for the diet of man. The long-term duration of man's exposure to DDT probably compensates for the low dietary concentration, thus enabling a fairly high body burden to be established. It is known that certain subgroups of the general population are exposed to higher DDT concentrations and attain higher DDT body burdens than are generally found (Durham, 1969). One pesticide formulator had a body burden of 648 ppm DDT measured in his body fat. In the present investigation, the degree of potentiation of CCl_4-induced hepatic damage apparently increased as the body burden of DDT increased. This finding could be an indication that the person with an extremely high DDT body burden is extremely susceptible to the hepatic-destructive effects of CCl_4.

The potentiation of acute CCl_4 hepatotoxicity by dietary DDT exposure was successfully quantified for concentrations of 6-65 ppm DDT administered via the diet for 3 and 24 weeks. Every feeding schedule of DDT resulted in an increased susceptibility of rats to low dosages (0.125-1.0 ml/kg) of CCl_4. The supporting evidence derived from histopathology corroborated that the potentiation of SGPT and SGOT activities was due to an enhanced hepatocellular destruction by CCl_4. In the animals having potentiated damage, the livers had a

considerable centrilobularly-oriented coagulative necrosis with bridging, which bordered on submassive necrosis. The non-DDT-fed animals given a larger dose of CCl_4 (e.g., 1.0 ml/kg compared to 0.125 ml/kg) had a less severe central cloudy swelling and parenchymal cell necrosis. These results are in agreement with those of McLean et al. (1966), who found that acute pretreatment of rats with DDT produced an increased susceptibility to CCl_4-induced hepatic necrosis. Garner et al. (1969) discovered the hepatic histopathologic damage in phenobarbital-pretreated rats given 0.25 ml/kg was equivalent to that observed in non-pretreated controls dosed with 2.5 ml/kg CCl_4.

It was previously observed (Chapter II) that the increased hepatic activities of GPT and GOT after CCl_4 may have contributed to the rise in serum enzyme activities. Therefore, it could be argued that the potentiated serum enzyme levels in the DDT-fed rats after CCl_4 reflected a higher rate of hepatic enzyme synthesis, rather than an increased parenchymal cell injury. The histologic evidence of a greater degree of centrilobular necrosis in the induced animals negates this argument. Moreover, McLean et al. (1966) measured a potentiated isocitric dehydrogenase (ICD) activity in rats pretreated acutely with DDT or phenobarbital. Dinman et al. (1968a, b) demonstrated the hepatic activity of ICD did not increase significantly during the acute phase of CCl_4 hepatotoxicity (0-36 hours) after inhalation

exposure of rabbits to CCl_4. Therefore, the potentiated serum ICD activities measured by McLean must have largely reflected the degree of hepatic cell disruption. Thus, the increase in hepatic GPT and GOT activities arising through an adaptive mechanism could probably not account for the detected potentiation in serum transaminase activities.

The determinations for SGPT and SGOT activities are routinely employed for screening hepatic damage in man in clinics and hospitals throughout the world. If man's DDT body burden renders him more susceptible to a secondary exposure to CCl_4 or to some other halogenated hydrocarbon solvent or anesthetic, then clinicians should be made aware of this possibility. Prior exposure of man to enzyme-inducers like DDT or phenobarbital could result in a severe response to a low dose of CCl_4 normally considered harmless. It would be of clinical interest to know if the potentiated hepatotoxic response to CCl_4 is detectable using other hepatic function tests.

IV. POTENTIATION OF CARBON TETRACHLORIDE-INDUCED HEPATIC DYSFUNCTION BY DIETARY DDT

Introduction

A number of laboratory methods have been devised for the assessment of hepatic functional impairment resulting from exposure to the halogenated hydrocarbon solvents. These tests include the pentobarbital anesthesia or "sleeping time," lethality experiments (LD50), and verification by histopathology or serum transaminase (SGPT) activity. The sulfobromophthalein (BSP) retention test, recently modified by Kutob and Plaa (1962a), is an excellent one for assessing hepatic function. Various combinations of the above-mentioned tests have been used in comparative studies of the liver-damaging halogenated hydrocarbon solvents (Plaa, Evans and Hine, 1958; Kutob and Plaa, 1962b; Klaassen et al., 1966).

BSP is a weak organic acid indicator which is removed from its attachment to plasma proteins by the reticuloendothelial cells of the liver and excreted into the bile by the parenchymal cells. If the hepatic function is impaired, a larger portion of the dye remains in the circulation. The effects of CCl_4 upon the uptake, storage, metabolism and excretion of BSP have been investigated (Brauer and Pessotti, 1949; Brauer, Pessotti and Krebs, 1955; Plaa and Hine, 1960). The excretion patterns of BSP in the bile following hepatic BSP

metabolism were examined by Krebs and Brauer (1960) and CCl_4 was found to alter this excretion pattern. Maggio and Fujimoto (1966) measured the concentrations of BSP and its glutathione conjugate in the blood and livers of CCl_4-treated mice. More modern methods for the assessment of BSP storage and conjugation were utilized by Klaassen and Plaa (1968a) to distinguish factors affecting storage and excretion from those affecting conjugation in CCl_4-treated rats. Priestly and Plaa (1970) studied the temporal aspects of CCl_4-induced alteration of BSP metabolism and excretion.

The present investigation was designed to determine the effect of CCl_4 treatment upon hepatic function in DDT-fed rats. The main goal was to determine whether the potentiated hepatocellular damage (SGPT and SGOT activities, Chapter III) was paralleled by a similar potentiation in hepatic dysfunction. Moreover, the estimates of BSP retention are well-established and widely used in the clinical assessment of liver function in man. Histopathologic examination of livers is not routinely employed for diagnostic purposes. If the potentiation of CCl_4-induced hepatotoxicity could be detected in the BSP test, then the results of this test could be used to confirm the results of the SGPT test.

Methods

BSP Retention Test

The impairment of liver function was determined using a modification of the method employed by Kutob et al. (1962a) in mice. The adaptation of their method was utilized to determine the extent of plasma BSP retention in CCl_4-treated control and DDT-fed rats. BSP (50 mg/kg) was administered by injection into the dorsal tail vein (IV) and, except where indicated in the text, blood samples were taken by cardiac puncture 30 minutes later for the analyses of plasma BSP content. This dose of BSP, route of injection and sampling time were previously employed successfully for the assessment of BSP retention in rats in this laboratory by Thompson (1969). In the actual determination of plasma BSP content, duplicate determinations and an individual blank were made for each plasma sample. To 0.1 ml of plasma was added 1.0 ml of 0.9% saline and two drops of 10% NaOH (sample) or 10% HCl (blank). Microspace adapters were placed in the cuvettes to bring the volumes to sufficient levels to be recorded in a Bausch and Lomb Spectronic 20 colorimeter at 580 nm. Results were expressed as mg of BSP retained per 100 ml of plasma.

Outline of the Experiment

Plasma BSP Disappearance. BSP (50 mg/kg) was injected IV in

four treatment groups of rats, and blood samples were taken 10, 20 and 30 minutes later for analyses of plasma BSP concentrations. The four treatment groups were: No treatment, DDT-fed (16 ppm x 8 wks), CCl_4-treated (0.5 ml/kg) and DDT + CCl_4 treated. This feeding level of DDT was chosen as adequate to produce DDT body burdens of 10-30 ppm in the animals, on the basis of the body burden determinations discussed previously in Chapter III. Those DDT body burdens had produced potentiated responses to CCl_4. In this study, in the two groups of rats receiving CCl_4 treatment, the BSP test was performed 24 hours after CCl_4 administration.

Temporal Studies. Control and DDT-fed rats were dosed with 0.5 ml/kg CCl_4, and the BSP test was performed in subgroups of six or more rats at intervals of 0, 6, 12, 18, 24 and 48 hours after CCl_4. Blood samples were taken 30 minutes after IV BSP administration.

Dose-response Curves. Different groups of control and DDT-fed (16 ppm x 8 wks) rats were treated with increasing doses of CCl_4 (0.125-2.0 ml/kg), and 24 hours later BSP was injected IV. The plasma BSP content was determined in blood samples taken 30 minutes after BSP administration. The resulting values for BSP retention were compared to similar results obtained in control and DDT-fed rats not treated with CCl_4.

Results

BSP Retention Test

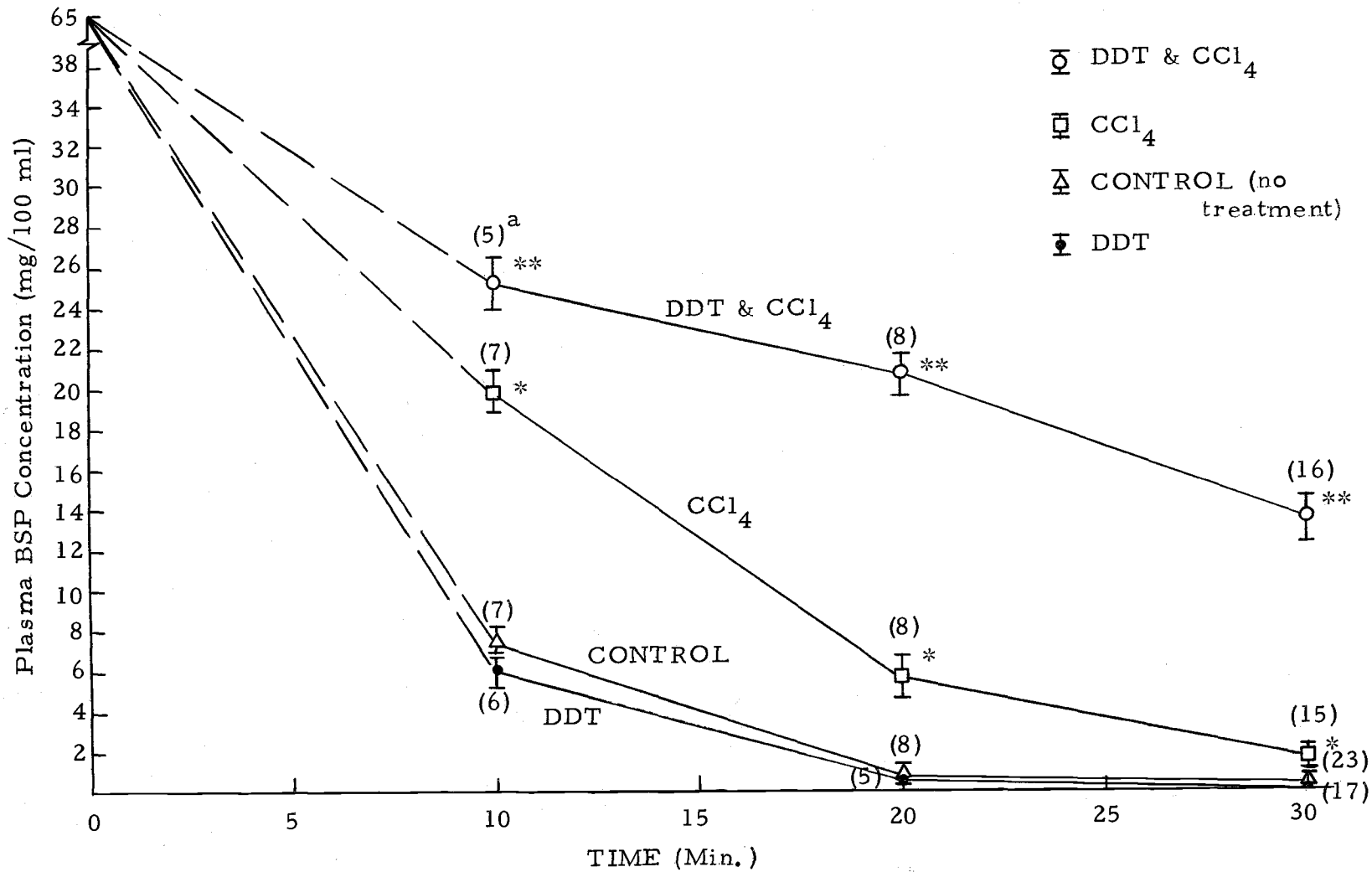
Plasma BSP Disappearance. The rate of BSP disappearance from control rat plasma in a 30 minute period after BSP injection is expressed as one curve in Figure 4-1. Ten minutes after BSP administration 7.6 mg/100 ml was retained. This value represented approximately 12% of the total administered dose, as calculated from the data of Priestly et al. (1970). Less than one mg/100 ml remained in the plasma at 20 minutes, and by 30 minutes the BSP concentration was approaching zero. The rate of plasma BSP disappearance in DDT-fed rats equaled, and tended to exceed, that observed in controls. These differences were significant ($P < 0.05$, Student's t-test) at 30 minutes, at which time the BSP retentions were 0.76 mg/100 ml (controls) and 0.53 mg/100 ml (DDT-fed). The CCl_4-treated controls cleared BSP from the plasma more slowly than their non-CCl_4-treated control counterparts. The CCl_4-treated group retained 5.7 mg/100 ml at 20 minutes ($P < 0.05$), however, at 30 minutes the mean BSP retention was nearly equivalent to that of controls ($P > 0.05$). When the DDT-fed rats were treated with 0.5 ml/kg CCl_4, there was a prolonged and potentiated BSP retention. Thirty minutes after BSP injection nearly 14 mg/100 ml was still retained. This latter value represented an approximate seven-fold increase ($P < 0.05$) in BSP

Figure 4-1. Effect of dietary DDT (16 ppm X 8 wks) upon the reduction of plasma disappearance of BSP resulting from acute CCl_4 treatment. CCl_4 (0.5 ml/kg) was administered by gavage, and 24 hr later the BSP test was performed. BSP (50 mg/kg) was given by tail vein injection and blood samples were taken by cardiac puncture 10, 20, and 30 min later for analysis of the plasma BSP concentrations. Each point represents the mean (± standard error) of from 5-23 animals. The four treatment groups are indicated in the figure. Note the CCl_4-induced decrease in plasma disappearance of BSP was potentiated by the prior dietary exposure to DDT.

[a]The number in parentheses represents the number of animals per group.

*Significantly different ($P < 0.05$, Student's t-test) from the respective control mean.

**Significantly different ($P < 0.05$) from the mean of the respective group receiving the CCl_4 treatment only.


Plasma BSP Concentration (mg/100 ml)
65
38
34
32
30
28
26
24
22
20
18
16
14
12
10
8
6
4
2
0
5
10
15
20
25
30
TIME (Min.)
DDT & CCl4
CCl4
CONTROL (no treatment)
DDT
(5)a
**
DDT & CCl4
(8)
**
(16)
**
(7)
*
CCl4
(8)
*
(15)
*
(7)
CONTROL
(8)
(23)
(6)
DDT
(5)
(17)

retention with respect to the 30 minute value in the CCl_4-treated control group. Thus, from the standpoint of the plasma BSP disappearance rates, the CCl_4-induced hepatic dysfunction was clearly potentiated by dietary DDT pretreatment.

Temporal Studies. It can be seen in Figure 4-2 that the response of controls to 0.5 ml/kg CCl_4 attained a maximal BSP retention in plasma 24 hours after CCl_4 (1.8 mg/100 ml). From 24 to 48 hours the hepatic function apparently improved toward the non-CCl_4-treated control mean of 0.76 mg/100 ml. In the DDT-fed animals, the degree of hepatic dysfunction was comparable to that of controls until six hours after CCl_4. Between 6 and 12 hours the amount of BSP retained in the plasma increased rapidly, thus indicating the onset of the enhanced hepatic functional impairment in this group. The difference in BSP retention between control and DDT-fed rats treated with CCl_4 was almost seven-fold by 12 hours ($P < 0.05$), and continued to increase through 48 hours. In the latter group, 17 mg/100 ml was retained by 48 hours, in comparison to 14 mg/100 ml at 24 hours. These results could give an indication of the relative prognoses for hepatic regeneration in the control and DDT-fed animals after CCl_4.

Dose-response Curves. The results of this study are indicated in Figure 4-3. Plasma BSP retention was only slightly increased in controls treated with increasing doses of CCl_4 in the range of 0.125 to 1.0 ml/kg. A sharp rise in BSP retention was noted when the dose

Figure 4-2. Effect of dietary DDT (16 ppm X 8 wks) upon the CCl_4-induced BSP retention at various intervals following the administration of 0.5 ml/kg CCl_4 by gavage. BSP (50 mg/kg) was administered by tail vein injection, and blood samples were taken 30 min later for analysis of plasma BSP content. Each point represents the mean (± standard error) of from 4-23 animals. Note the onset of the potentiation of the CCl_4-induced BSP retention at 6 hr following CCl_4 administration. The hepatic function impairment continued to worsen over the entire time period in the DDT-fed group, whereas there was noticeable improvement in the non-DDT-fed animals by 48 hr after CCl_4.

[a]The number in parentheses represents the number of animals per group.

*Significantly different ($P < 0.05$, Student's t-test) from the respective control mean.

**Significantly different ($P < 0.05$) from the mean of the respective group receiving the CCl_4-treatment only.

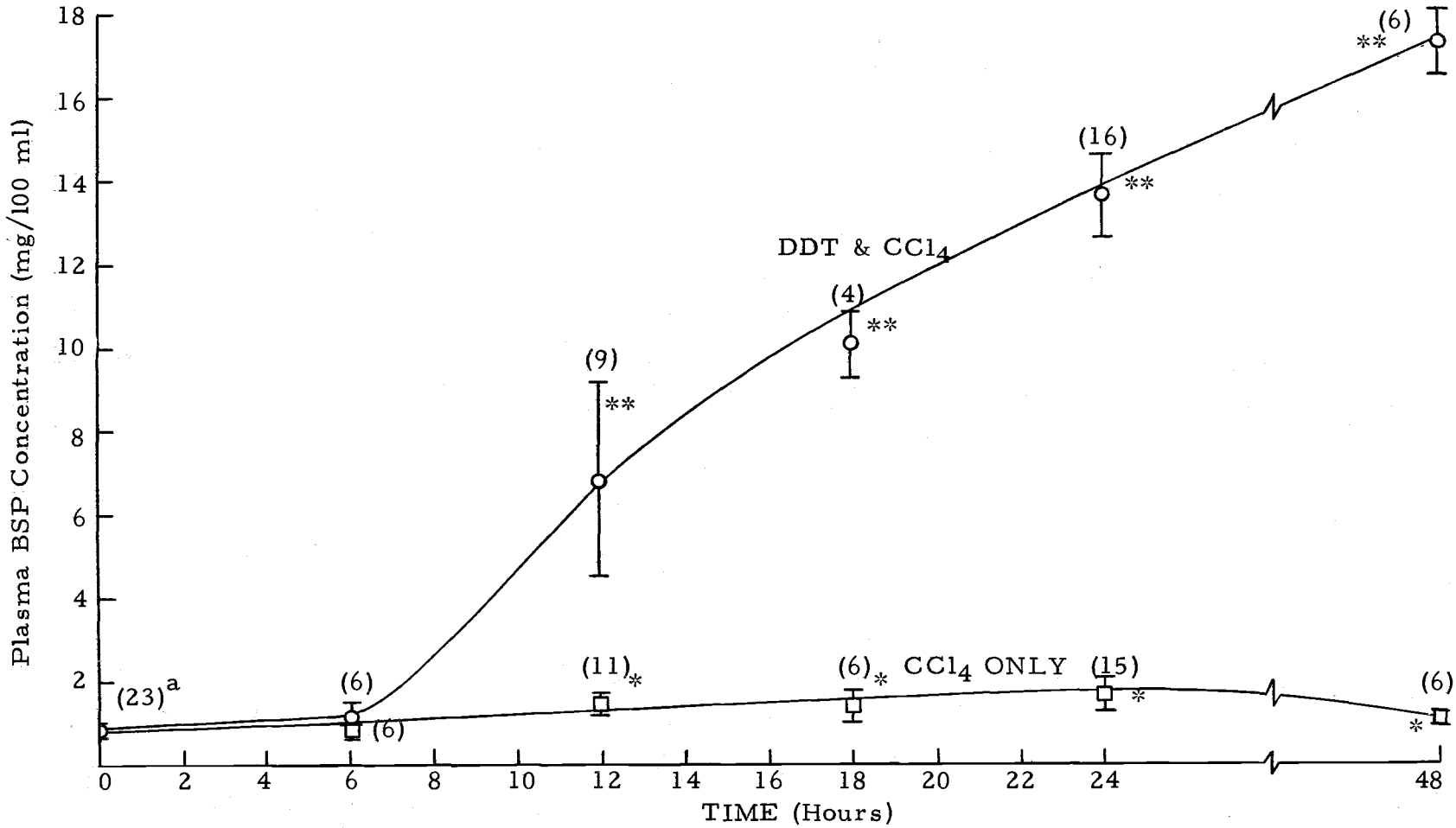
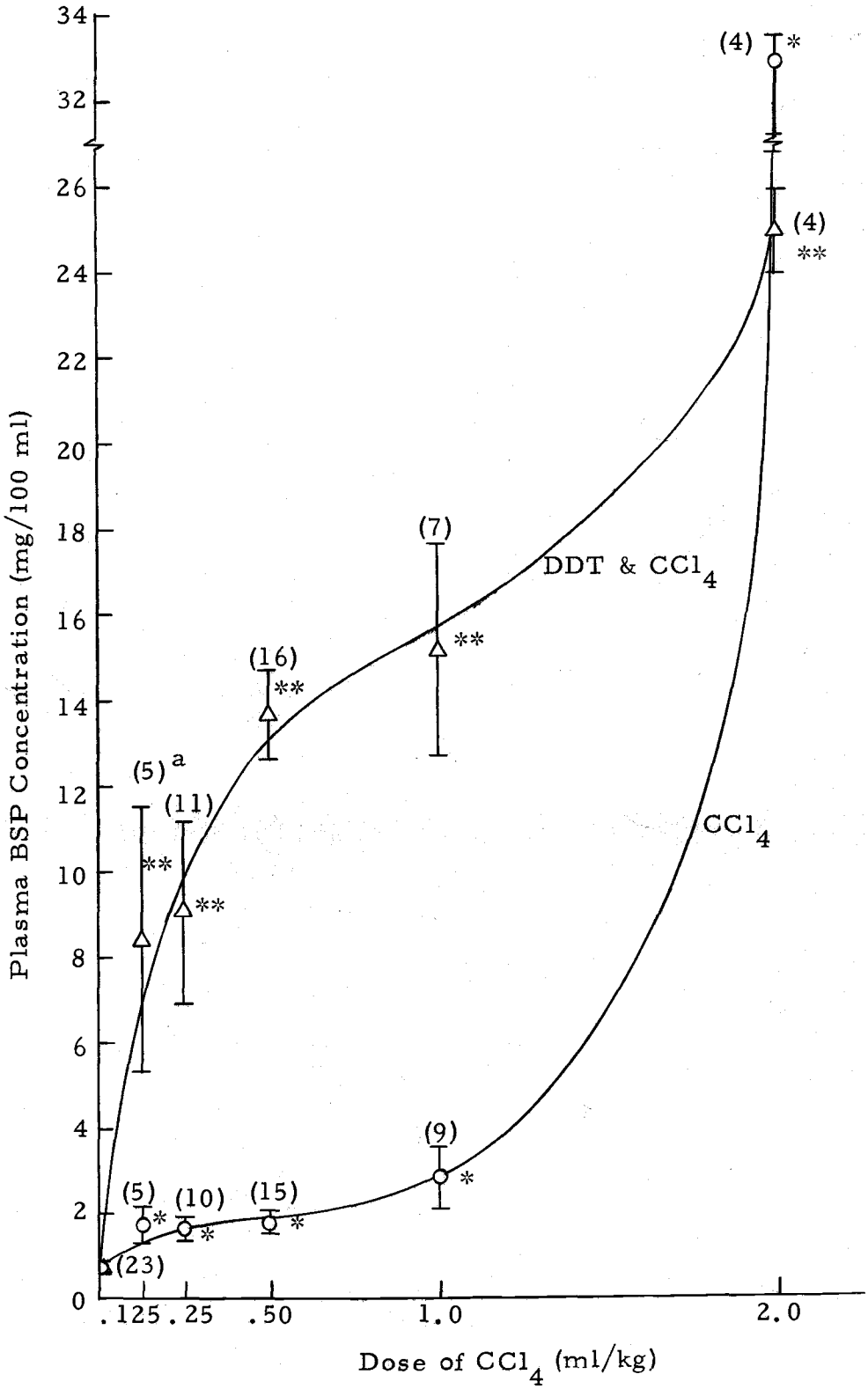

Plasma BSP Concentration (mg/100 ml)
18
16
14
12
10
8
6
4
2
0
2
4
6
8
10
12
14
16
18
20
22
24
48
TIME (Hours)
(23)a
(6)
(6)
(9)
**
(4)
**
DDT & CCl4
(16)
**
(6)
**
(11)*
(6)*
CCl4 ONLY
(15)
*
(6)
*

Figure 4-3. Effect of dietary DDT (16 ppm X 8 wks) upon the plasma BSP retention response to increasing dosages of CCl_4. CCl_4 (0.125 - 2.0 ml/kg) was administered by gavage. BSP (50 mg/kg) was given by tail vein injection 24 hr after CCl_4 administration, and blood samples were taken 30 min later for analysis of plasma BSP content. Each point represents the mean (± standard error) of at least 4 animals. Note the potentiation of the CCl_4-induced BSP retention by dietary DDT occurred at the lowest dosage of CCl_4 employed (0.125 ml/kg), and was dose-related to CCl_4.

[a]The number in parentheses represents the number of animals per group.

*Significantly different ($P < 0.05$, Student's t-test) from the control mean.

**Significantly different ($P < 0.05$) from the mean of the respective group receiving the CCl_4 treatment only.


Plasma BSP Concentration (mg/100 ml)
34
32
26
24
22
20
18
16
14
12
10
8
6
4
2
0
.125
.25
.50
1.0
2.0
Dose of CCl4 (ml/kg)
DDT & CCl4
CCl4
(4) *
(4) **
(7)
**
(16)
**
(5) a
**
(11)
**
(9)
*
(5)
*
(10)
*
(15)
*
(23)

of CCl_4 was increased from 1.0 to 2.0 ml/kg. At a dose of 1.0 ml/kg, 2.8 mg/100 ml was retained, whereas at 2.0 ml/kg 32.9 mg/100 ml was measured in the plasma. In the DDT-fed animals, a low dose of 0.125 ml/kg CCl_4 resulted in a potentiated BSP retention to 8.5 mg/100 ml, compared to a value of 1.75 mg/100 ml in the CCl_4-treated control group. Further, when the dose of CCl_4 was increased to 0.5 ml/kg, the difference in plasma BSP retention between the control and DDT-fed groups was more than seven-fold. At a dose of 2.0 ml/kg, however, no potentiation was observed. This phenomenon could be due to the severe degree of hepatic damage resulting from this high dose of CCl_4 given alone. Thus, it appeared the potentiation of CCl_4-induced hepatic dysfunction by dietary DDT could be demonstrated only at doses of CCl_4 below 2.0 ml/kg.

Histopathology

Microscopic examination of livers confirmed the results of the BSP test. Liver sections taken from DDT-fed rats subsequent to CCl_4 treatment had a greater amount of centrilobular parenchymal cell necrosis than did the livers from their control counterparts. These livers were comparable to those examined in Figures 3-8 through 3-10, and thus are not shown.

Discussion

Comparing the rates of disappearance of BSP from the plasma in control and DDT-fed groups, the rates tended to be faster after dietary DDT pretreatment. Phenobarbital, another enzyme-inducer, increased the rates of plasma disappearance and biliary excretion of BSP (Klaassen and Plaa, 1968b), and the latter effect was probably due to an increased rate of biliary flow in rats (Roberts and Plaa, 1967). Thus, the effect of dietary DDT was not surprising.

A dose-related increase in plasma BSP retention was observed at doses of CCl_4 ranging from 0.125 to 2.0 ml/kg. Klaassen et al. (1968b) also demonstrated a dose-related alteration in the rate of plasma BSP disappearance in rats after varying doses of CCl_4. They performed the BSP test (60 mg/kg, IV, 32 minutes) 24 hours after CCl_4, and showed an enhanced BSP retention at 1.0 ml/kg with respect to that measured after 0.1 ml/kg. In the present study, the results obtained in the range of dosage between 0.125 and 1.0 ml/kg were similar to those of Klaassen. An additional dose of CCl_4, i.e., 2.0 ml/kg, was employed here, and a striking increase in the amount of BSP retained was noted at this high dose. Retrospective analysis of the data in Figure 2-2 shows that the SGPT and SGOT activities also increased considerably between the doses of 1.0 and 2.0 ml/kg. Further, the largest fraction of this increased serum enzyme activity

occurred when the dose of CCl_4 was increased from 1.0 to 1.25 ml/kg. Since the extent of functional impairment in the hepatocytes apparently correlated with the degree of hepatic damage, it is probable the largest increase in BSP retention also occurred between 1.0 and 1.25 ml/kg.

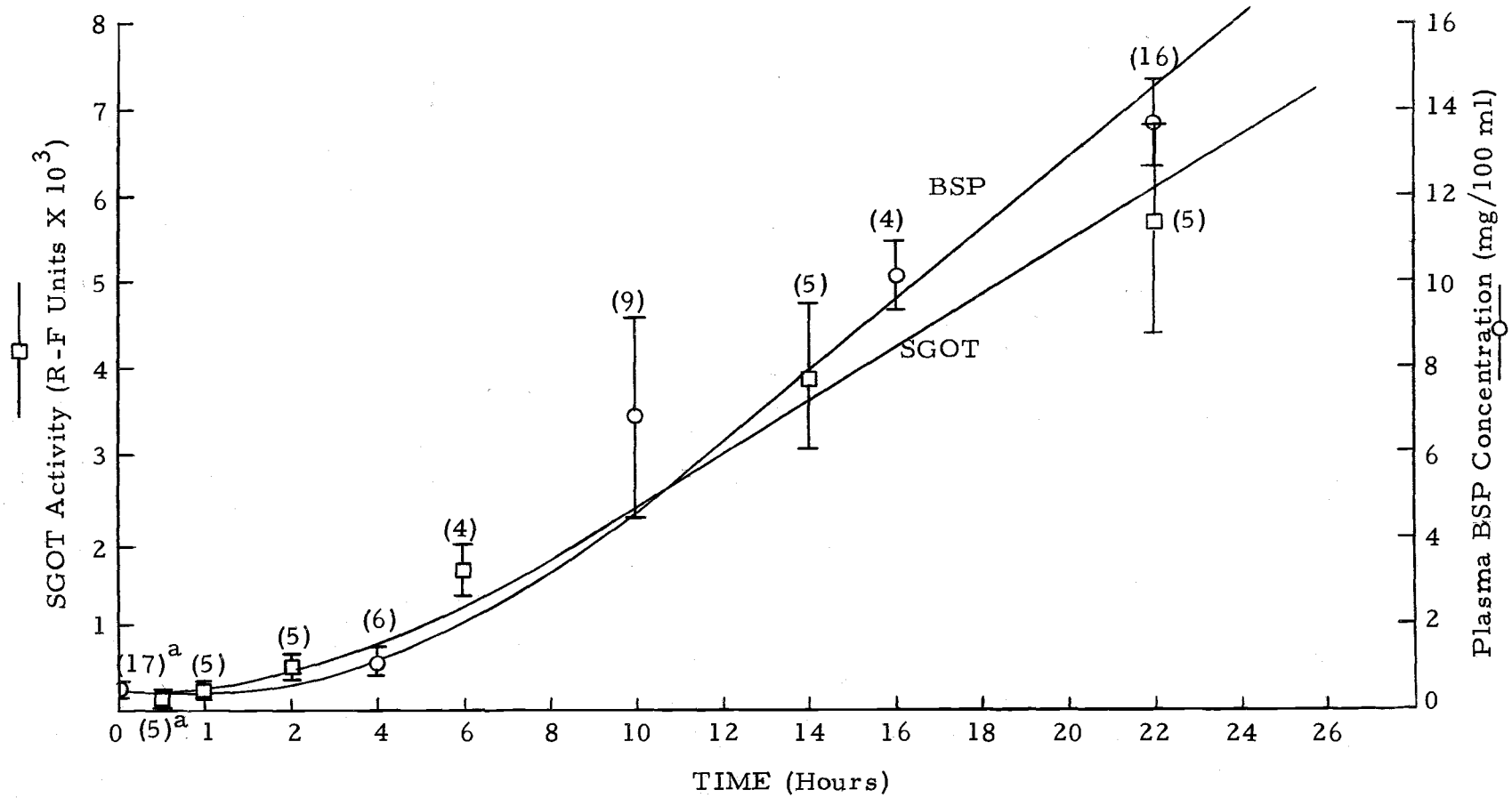
The temporal development of hepatic functional impairment in the CCl_4-treated controls was in accord with the findings of Priestly et al. (1970). The mechanisms operative in the CCl_4-induced BSP retention in rats are, however, better explained by their data and conclusions. They also demonstrated a marked plasma retention of BSP in rats 24 hours after 1.0 ml/kg CCl_4, and showed there was an altered hepatic excretory and metabolic function in those animals. Although alterations were measurable as early as three hours after CCl_4, the severity of the defect in hepatic excretory function increased with time. In their studies, as in the present investigation, the most severe defects were found at 12 and 24 hours after CCl_4. At 3, 12 and 24 hours Priestly observed the biliary concentrations of BSP were depressed, along with the biliary flow rates, to enhance the effect of a depressed biliary flow rate on lowering the transport maximum (T_m) capacity. According to them, the BSP must be conjugated with glutathione (GSH) in order to be secreted in high concentrations into the bile; when there is an impairment in conjugation, the total BSP and BSP-GSH biliary concentrations are reduced. An impaired

conjugation of BSP undoubtedly contributed to the general effect on BSP excretion seen in the later stages (12-24 hours), but during the early stages (3-6 hours) was less evident. Additional studies led Priestly to conclude the effect of CCl_4 on excretory function was probably most important, although an impaired conjugation of BSP could have contributed to the overall effect. CCl_4 did not alter the hepatic glutathione content or the BSP conjugation enzyme system. Therefore, Priestly believed the effect of CCl_4 on BSP conjugation was related to its degenerative effect on the hepatocyte. A decrease in the relative hepatic storage of BSP was noted after CCl_4, with a subsequent return toward normal after 24 hours. It was assumed the same mechanisms were operative in the present study, with respect to the CCl_4-induced BSP retention in control animals.

The results of the temporal study gave strong evidence that a potentiated hepatic dysfunction resulted from CCl_4 treatment in DDT-fed rats. These findings gave additional support to the conclusions made in the last chapter (III), which were based on the potentiated serum transaminase activities and histologic evidence of submassive central necrosis. In Figure 4-4, it can be observed that the temporal development of potentiated hepatic dysfunction was remarkably parallel to the potentiated hepatic parenchymal cell disruption. As Netter (1964) has stated, the degree of hepatic functional impairment is a function of the number of cells destroyed. Thus, the spread of the

Figure 4-4. Comparison of the temporal patterns of hepatocellular damage (SGOT activity) and hepatic function impairment (BSP retention) in DDT-fed rats challenged with CCl_4. In the SGOT activity study, rats fed 16 ppm DDT for 2 wks were treated with 1.0 ml/kg CCl_4 by gavage. In the BSP retention study, rats fed DDT for 8 wks were dosed with 0.5 ml/kg CCl_4 by gavage. Each point represents the mean of from 4-17 animals. Note the temporal development of hepatic function impairment parallels that of the hepatocellular damage.

[a]The number in parentheses represents the number of animals per group.


SGOT Activity (R-F Units X 10^3)
Plasma BSP Concentration (mg/100 ml)
TIME (Hours)
BSP
SGOT
(17)a
(5)
(5)
(6)
(4)
(9)
(5)
(4)
(16)
(5)
(5)a
0
1
2
4
6
8
10
12
14
16
18
20
22
24
26

CCl_4-induced hepatic lesion from lobule to lobule in the DDT-fed animals resulted in the destruction of a great number of parenchymal cells. The potentiated plasma BSP retention in this latter group was, in all probability, a direct reflection of this extensive necrosis.

Conceivably, the enzymes responsible for BSP conjugation are released from the damaged hepatocytes in concentrations reflecting the degree of cell membrane permeability. In this way, the degeneration of the hepatocytes could be related to the degree of plasma BSP retention. The contribution of an impaired conjugation of BSP was probably exaggerated in the DDT-fed animals, due to the extensive necrosis. The defect in hepatic excretory function also increased with time in the studies of Priestly, and undoubtedly this impairment was also potentiated in the DDT-fed animals. Thus, the various hepatic functions apparently hinge on the integrity of the cellular membranes. As the hepatic damage increases in response to CCl_4, all of these functions become increasingly curtailed.

In the temporal study, it was also noted the regeneration of hepatic function was severely impaired in the DDT-fed animals. Controls showed improved abilities to remove BSP from the plasma at 48 hours with respect to the 24 hour BSP retention. In contrast, the BSP retention in the DDT-fed animals, which was potentiated more than 10-fold over controls, continued to increase in severity between 24-48 hours. These results, which confirmed the finding of

a prolonged increase in the potentiated serum transaminase activities (Chapter III) in DDT-fed rats, could give further evidence for the relative prognoses for recovery in control and DDT-fed animals.

The potentiation of CCl_4-induced hepatotoxicity by dietary DDT can be detected through utilization of the BSP test as well as in the test for elevations in serum transaminase activity. Clinicians should be made aware such potentiated responses can occur. The results of the BSP test can therefore be used to verify the results of the SGPT test.

V. PROTECTION FROM CCl_4-INDUCED HEPATIC NECROSIS AFFORDED BY SPINAL CORD TRANSECTION AND TEMPORAL ASPECTS OF BLOOD AND LIVER CCl_4 UPTAKE IN DDT-FED RATS

Introduction

A number of investigators have studied the protective effect of spinal cord transection against CCl_4-induced hepatic necrosis. Calvert and Brody (1960) used cord-sectioning as a tool in their attempts to elucidate the mechanism of CCl_4-induced hepatotoxicity. They demonstrated that spinal cord transection at the level of the seventh cervical vertebra was the most effective of several techniques used to prevent certain characteristic biochemical and pathological changes from occurring in the livers of CCl_4-poisoned rats. Additional studies by Brody et al. (1960) attempted to correlate the concentrations of epinephrine and norepinephrine in the blood and adrenals, and adrenal gland weights, with CCl_4-induced liver damage, and with the protection afforded by spinal cord transection. Correlating the biochemical findings with the results of the protection studies, Brody, Calvert and Schneider (1961) concluded that the toxicity of CCl_4 on the liver was indirect, and mediated by the sympathetic nervous system. According to them, the cord-sectioning gave protection from hepatic necrosis as a result of the severence of the sympathetic nervous system pathways.

Larson and Plaa (1963) arrived at an alternative hypothesis to explain the protective effect of spinal cord transection in CCl_4-poisoned rats. They noted that the animals became hypothermic after cervical cord-sectioning, and in subsequent experiments showed that hypothermia itself provided the protective effect (Larson, Plaa and Crews, 1964; Larson and Plaa, 1965). Larson et al. (1965) also studied the temporal pattern of CCl_4 uptake into the blood and livers of spinal cord transected and non-transected rats. They found that the protective effect of cord-sectioning did not result from a reduced uptake of CCl_4 into the blood and livers of cordotomized animals.

In the present investigation, spinal cord transection was used as a tool to further clarify the mechanism for the potentiation of acute CCl_4 hepatotoxicity by chronic exposure of rats to DDT. It was unknown whether the protective effect of hypothermia or a reduced hepatic metabolism in cord-sectioned animals would be sufficient to overcome the CCl_4-potentiating effect of dietary DDT. The temporal patterns of CCl_4 uptake into the blood and livers were determined in spinal cord transected and non-transected control and DDT-fed rats. The temporal changes in tissue uptake of CCl_4 were compared to the temporal alterations in SGOT activity and hepatic histopathologic damage, in order to separately assess the contributions of DDT-feeding and cord-sectioning.

Methods

Spinal Cord Transection

Spinal cord transection was accomplished with the rat under a light ether anesthesia. A mid-dorsal incision of approximately 3/4" was made at the level of the seventh cervical vertebra. The incision having been made, sharp-pointed curved tissue forceps were used to sever the spinal cord. This level of cord-sectioning (C-7) was chosen from the experiments of Larson et al. (1964). They found that C-7 spinal cord transection gave the most dramatic degree of protection against CCl_4-induced hepatotoxicity. Following cord transection, the wound was thoroughly cleansed with 0.9% saline, and closed with a wound clip. In long-term experiments where spinal cord transected animals were to be maintained for 48 hours or longer, 4-5 ml of a normal saline-glucose solution was injected s. c. at intervals of 8-10 hours, to help maintain body fluids, electrolytes and a blood glucose supply in these animals. Rectal temperatures were recorded with an electronic thermometer and rectal probe. Post mortem examinations were made to validate the completeness of the cord-sectioning.

CCl_4 Determination

Conway microdiffusion cells were utilized for the extraction of CCl_4 from whole blood and homogenized livers into toluene. To the

inner well of each microdiffusion cell was added 1.0 ml reagent grade toluene (J. T. Baker, Inc.). For the extraction of whole blood, 2 ml of distilled water was added to the outer well to aid in spreading the blood. The top edges of the cells were coated with a tragacanth paste, which was prepared by adding tragacanth gum powder to distilled water. This paste was used to achieve an air tight seal when the ground glass cover was placed on top of the cell. Two ml of whole blood was taken from each rat by cardiac puncture with the animals under a light ether anesthesia. This blood was transferred immediately to the outer well of a prepared microdiffusion cell. The ground glass cover plate was quickly placed on top of the cell, and weights (300 g) were placed on top of the glass cover to ensure a tight seal with the tragacanth paste. Livers were quickly removed from the animals, and homogenized over ice (1 g/3 ml) as rapidly as possible. The homogenate was added to the outer well of a prepared microdiffusion cell, and sealed in the same manner as described above. The sealed microdiffusion cells were left at room temperature for from 2-6 hours. Extractions of standard concentrations of CCl_4 were made at two hour intervals for 12 hours after the cells had been sealed, in a preliminary experiment. Little change in the extraction efficiency was noted between two and six hours post-sealing. Maximal efficiencies ($66 \pm 4\%$ for blood; $46 \pm 6\%$ for liver) were obtained at the six hour extraction period. Similar efficiencies for the extraction of CCl_4

from tissues by microdiffusion into toluene have been obtained by other investigators (Recknagel and Litteria, 1960; Larson et al., 1965). Following the extraction of CCl_4 by microdiffusion into toluene, the CCl_4 concentrations were determined by the development of the Fujiwara chromogen, as described by Recknagel et al. (1960). For each sample, an incubation mixture was prepared in a 25 ml Erlenmeyer flask. This mixture included: 1 ml distilled water, 3 ml of 30% (w/w) KOH and 10 ml pyridine. At the end of the extraction period, 0.5 ml of reagent grade acetone (J. T. Baker, Inc.) and the remaining toluene (usually about 0.95 ml) in the center well of the microdiffusion cell were quickly added well below the surface of the incubation mixture. A glass stopper was then placed in the Erlenmeyer flask and securely fastened with a rubber band to prevent the stopper from coming out during the incubation step. These reaction mixtures were then incubated at 70°C in a Dubnoff Metabolic Shaking Incubator for 45 minutes. The reaction taking place during the incubation period depended upon the development of a red color in the pyridine layer following the addition of 1, 1, 1-trichloro compounds to the mixture containing aqueous caustic alkali and pyridine. Although this reaction is extremely sensitive and widely used in toxicological analyses, the mechanism of the reaction remains to be elucidated (Moss and Rylance, 1966). After incubation, the Erlenmeyer flasks were immediately placed in an ice bath to stop the reaction. After

cooling, 2 ml of the colored pyridine layer was added to a cuvette containing 0.5 ml of 0.01 N NaOH, and the color density of this solution was recorded at 530 nm in a Bausch and Lomb Spectronic 20 colorimeter. The concentrations of CCl_4 in the blood and livers were calculated by comparison with known standards. The results were corrected for extraction losses of both CCl_4 and toluene. The concentrations of CCl_4 were expressed as μg/ml of whole blood or μg/g of liver.

Outline of the Experiment

Control and DDT-fed rats (16 ppm x 8 wks) were each separated into two groups--spinal cord transected and non-transected. Rats in each of the four resulting subgroups were treated with 1.0 ml/kg CCl_4 by gavage. Blood and liver samples were taken from smaller subgroups of animals at intervals of 1/2, 1, 2, 4, 8, 12, 16, 24 and 48 hours after CCl_4 for analyses of SGOT activity, blood and liver CCl_4 concentrations and for histopathologic examination of liver tissues.

Results

Concentrations of CCl_4 in Blood and Livers of Intact Rats

The results of the temporal study of blood CCl_4 uptake in the

intact control and DDT-fed rats are illustrated in Figure 5-1. The curves representing the changing CCl_4 concentrations are similar in profile, and biphasic in that a steadily increasing concentration of CCl_4 was interrupted by a brief decline from 1-2 hours after CCl_4 was given. An initial maximal concentration was attained within the first one-half hour after CCl_4 (33 μg/ml, control; 23 μg/ml, DDT-fed); however, a secondary maximal concentration was noted in the DDT-fed group (26 μg/ml) at four hours. The concentrations of CCl_4 in the blood declined steadily after four hours in both groups, and by 48 hours essentially all of the CCl_4 had disappeared from the blood. In the DDT-fed animals, the blood CCl_4 concentrations were lower between 4-48 hours than in their control counterparts. Significant differences ($P < 0.05$) were observed at 12 and 48 hours after CCl_4.

In these same two groups, the concentrations of CCl_4 measured in the livers increased rapidly in the first one-half hour after CCl_4 (86 μg/g, controls; 32 μg/g, DDT-fed), as shown in Figure 5-2. Again, the curves representing the temporal uptake of CCl_4 into the livers had a biphasic profile, which was similar for the two groups compared. The steady rise in liver CCl_4 concentrations that culminated in maximal concentrations at 4-6 hours (138 μg/g, controls; 75 μg/g, DDT-fed) was interrupted by a brief decline at two hours, as was previously observed for blood. There was a continuous decline in the liver CCl_4 content in both groups beginning at 6-8 hours after

Figure 5-1. Concentration of CCl_4 in the blood as a function of time in control and DDT-fed (16 ppm X 7 wks) rats treated with 1.0 ml/kg CCl_4 by gavage. The CCl_4 content of the blood was estimated by the Fujiwara method following the extraction of whole blood by microdiffusion into toluene. Each point represents the mean ($\pm$ standard error) of from 3-15 animals. Note the DDT-fed rats tended to have lower concentrations of CCl_4 in their blood than did the corresponding control animals, although the differences in concentration were significant ($P < 0.05$) only at 12 and 48 hr after CCl_4.

[a]The number in parentheses represents the number of animals per group.

*Significantly different ($P < 0.05$ Student's t-test) from the mean of the respective DDT-fed group.

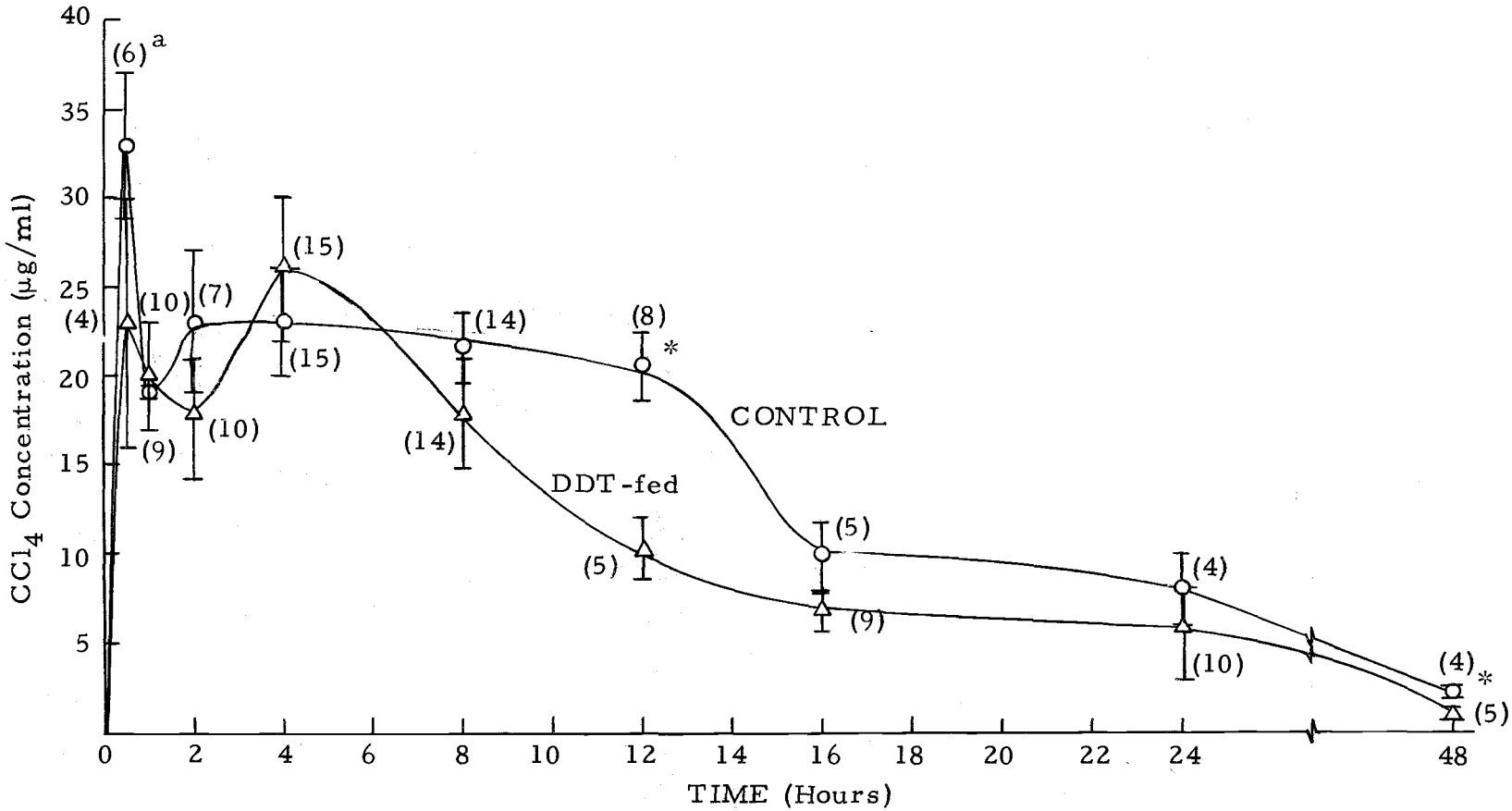
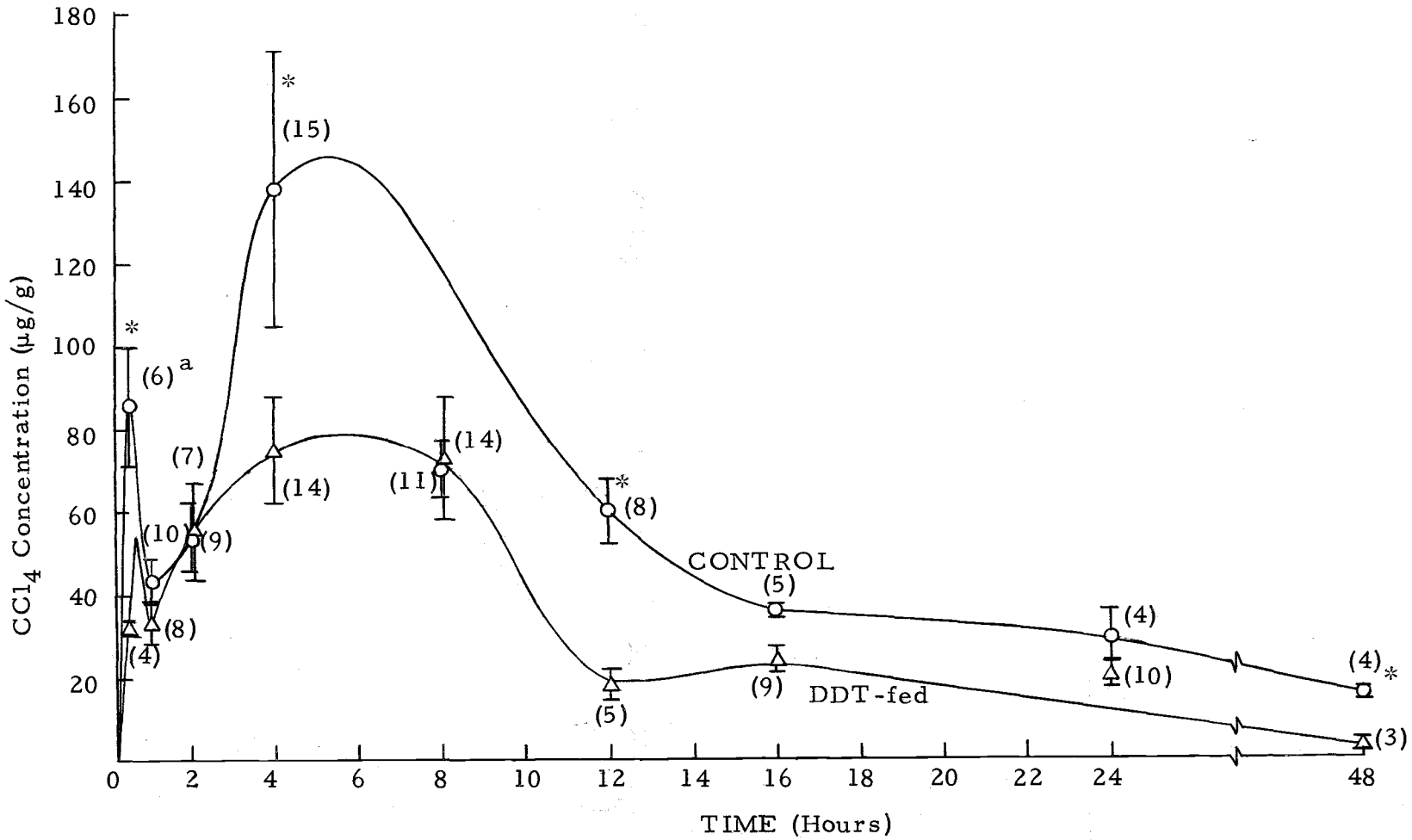

40
35
30
25
20
15
10
5
CCl_4 Concentration (µg/ml)
(6)a
(4)
(10)
(7)
(9)
(10)
(15)
(15)
(14)
(14)
(8)
*
CONTROL
DDT-fed
(5)
(5)
(9)
(4)
(10)
(4)*
(5)
0
2
4
6
8
10
12
14
16
18
20
22
24
48
TIME (Hours)

Figure 5-2. Concentration of CCl_4 in the livers as a function of time in control and DDT-fed (16 ppm x 7 wks) rats treated with 1.0 ml/kg CCl_4 by gavage. The CCl_4 content of the livers was estimated by the Fujiwara method after extraction of homogenized livers by microdiffusion into toluene. Each point represents the mean (± standard error) of from 3-15 animals. Note the concentrations of CCl_4 in the livers of DDT-fed rats are generally lower than the concentrations attained in the control rat livers. The differences are significant ($P < 0.05$) at 1/2, 4, 12, and 48 hr after CCl_4.

[a]The number in parentheses represents the number of animals per group.

*Significantly different ($P < 0.05$ Student's t-test) from the mean of the respective DDT-fed group.


180
160
140
120
100
80
60
40
20
CCl_4 Concentration (µg/g)
0
2
4
6
8
10
12
14
16
18
20
22
24
48
TIME (Hours)
CONTROL
DDT-fed
(6) a
(7)
(10)
(9)
(8)
(4)
(15)
(14)
(11)
(14)
(8)
(5)
(4)
(10)
(4)
(5)
(9)
(3)
*

CCl_4, and by 48 hours nearly all of the CCl_4 had disappeared. At all times the DDT-fed animals had lower liver concentrations of CCl_4 than did the controls, and these differences were significant ($P < 0.05$) at 1/2, 4, 12 and 48 hours. The liver CCl_4 concentrations were considerably greater in magnitude than the corresponding concentrations of CCl_4 measured in the blood. Nevertheless, the profiles of the CCl_4 uptake curves were similar for livers and blood.

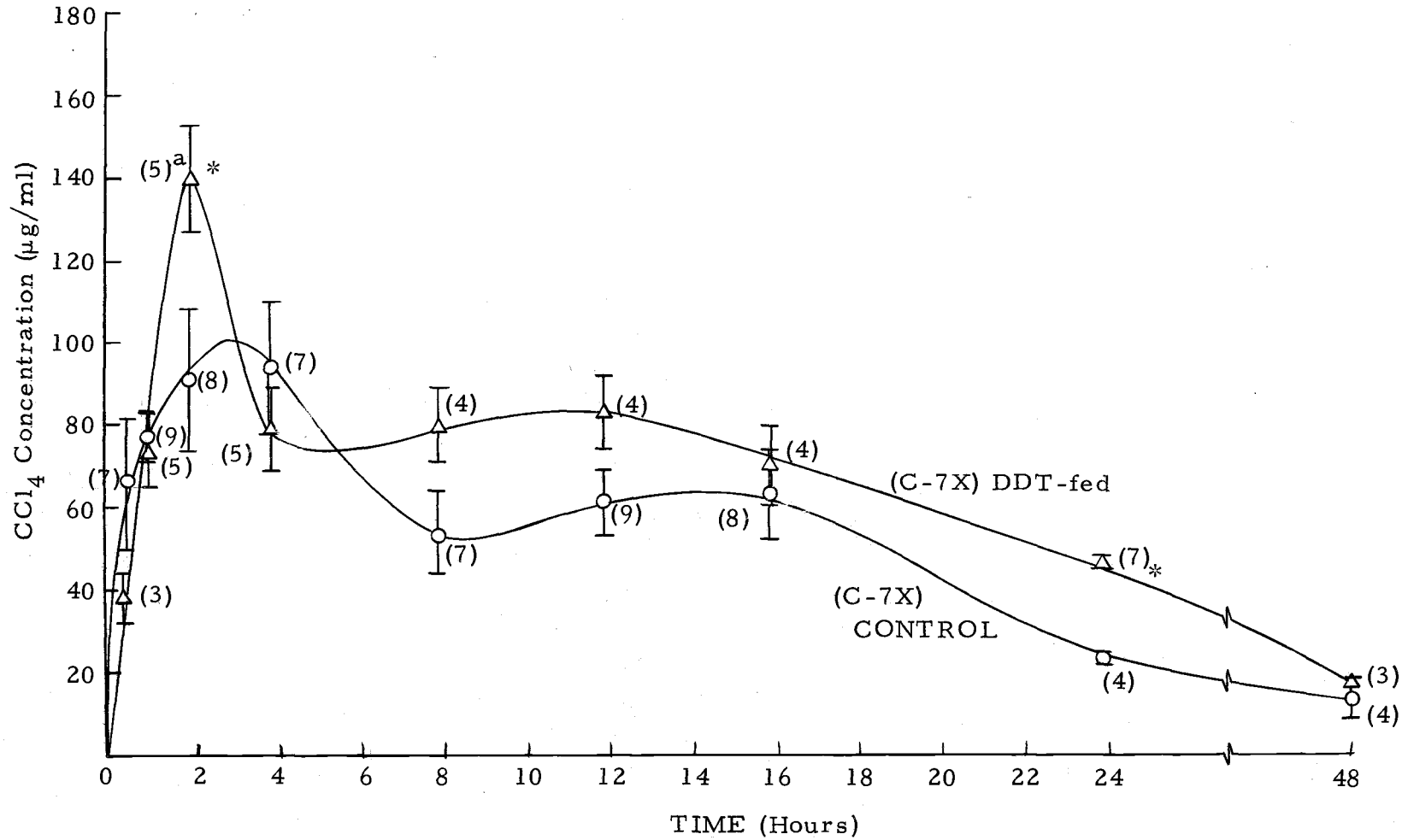
Concentrations of CCl_4 in Blood and Livers of Cord-sectioned Rats

The results of the temporal study of CCl_4 uptake into the blood of cord-sectioned control and DDT-fed animals are illustrated in Figure 5-3. Although the two curves are basically similar in profile, the blood concentrations of CCl_4 in the DDT-fed animals tended to be higher throughout the entire 48 hour study. These differences were significant ($P < 0.05$, Student's t-test) at 2 and 24 hours after CCl_4. Initial maximal concentrations of CCl_4 were attained at two hours (91 μg/ml, controls; 140 μg/ml, DDT-fed). After a brief decline to the four hour values, the blood CCl_4 concentrations varied little between 4-16 hours in the two groups; thereafter, they declined continuously to low values at 48 hours (13 μg/ml, controls; 17 μg/ml, DDT-fed). The concentrations of CCl_4 achieved in the blood of both cord-sectioned groups were markedly higher than the corresponding concentrations in the non-transected groups. The principal factor influencing

Figure 5-3. Concentration of CCl_4 in the blood as a function of time in spinal cord transected (C-7X) control and DDT-fed (16 ppm X 5 wks) rats treated with 1.0 ml/kg CCl_4 by gavage. The CCl_4 content was estimated by the Fujiwara method after extraction of whole blood by microdiffusion into toluene. Each point represents the mean (± standard error) of from 3-9 animals. Note the concentrations of CCl_4 in the blood of both groups are similar at all times, and that these values are generally higher than the blood CCl_4 concentrations attained in the non-transected groups (see figure 5-1). The profiles of the blood CCl_4 curves are similar for spinal cord transected and non-transected control and DDT-fed groups.

[a]The number in parentheses represents the number of animals per group.

*Significantly different ($P < 0.05$, Student's t-test) from the mean of the respective control (non-DDT-fed) group.


180
160
140
120
100
80
60
40
20
CCl_4 Concentration (µg/ml)
0
2
4
6
8
10
12
14
16
18
20
22
24
48
TIME (Hours)
(5)a *
(8)
(7)
(9)
(7)
(5)
(5)
(4)
(4)
(4)
(7)
(9)
(8)
(7)*
(3)
(4)
(3)
(4)
(C-7X) DDT-fed
(C-7X)
CONTROL

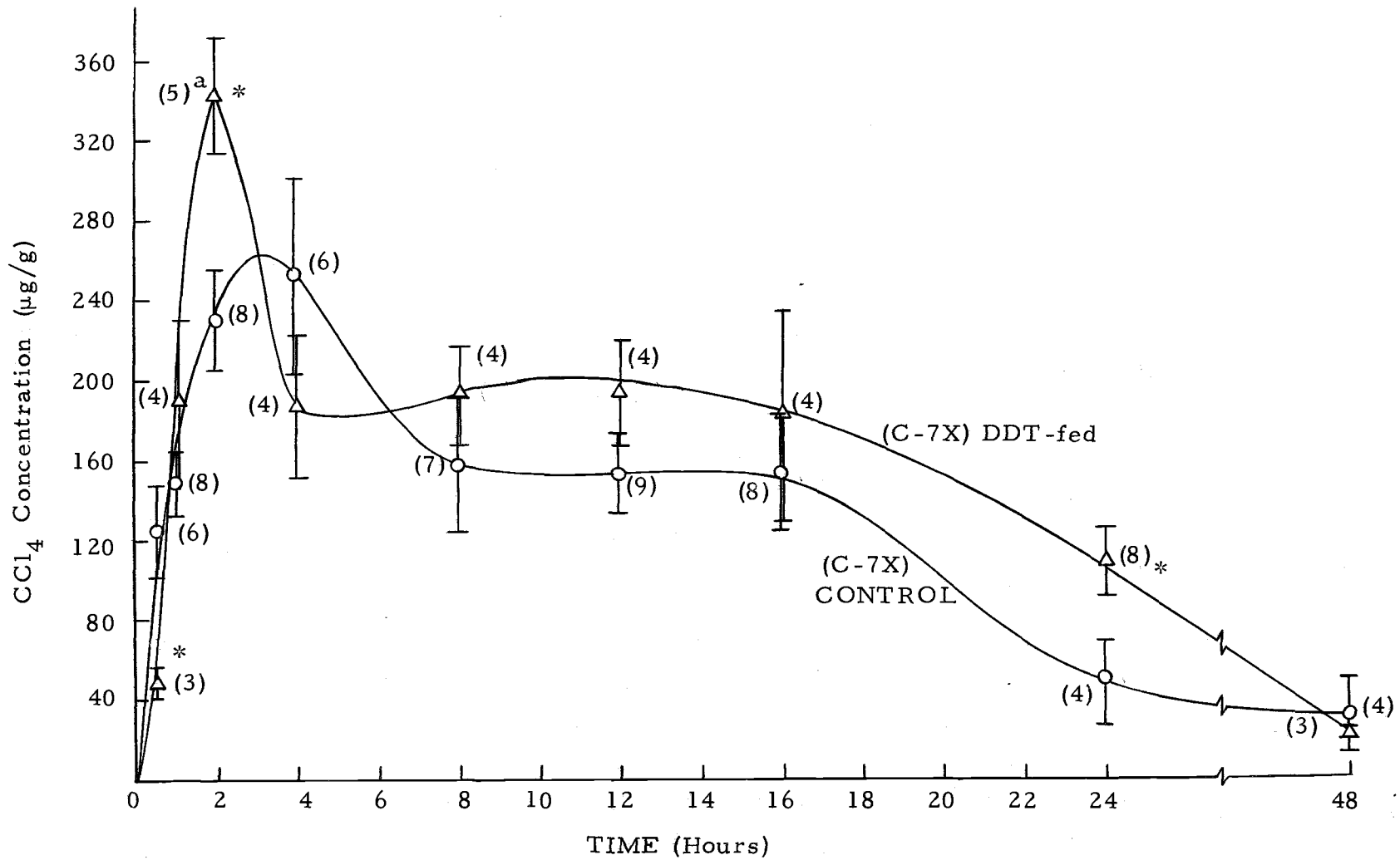
the enhanced CCl_4 uptake into the blood of cordotomized animals was undoubtedly the surgical severence of the spinal cord.

The temporal uptakes of CCl_4 into the livers of cord-sectioned control and DDT-fed rats are compared in Figure 5-4. The two curves are similar in profile to each other and to the curves representing CCl_4 uptake into the livers of non-transected groups. Again, as in the case of the blood CCl_4 concentrations, the concentrations of CCl_4 attained in the livers in the cord-sectioned groups were considerably greater in magnitude than the corresponding CCl_4 concentrations measured in the non-transected groups. In the cord-sectioned animals, the maximal liver CCl_4 concentrations were noted 2-4 hours after CCl_4 (252 μg/g, controls; 343 μg/g, DDT-fed). These liver concentrations declined by eight hours, but little change was observed between 8-16 hours; thereafter the liver CCl_4 content declined steadily to low concentrations at 48 hours (30 μg/g, controls; 20 μg/g, DDT-fed). The cord-sectioned DDT-fed rats had a more rapid initial CCl_4 uptake into their livers than did their control counterparts. Throughout the entire temporal study (with the exception of the 1/2 and 4 hour values) the liver CCl_4 concentrations were higher in the DDT-fed animals, and these differences were significant ($P < 0.05$) at 1/2, 2 and 24 hours.

Figure 5-4. Concentrations of CCl_4 in the livers as a function of time in spinal cord transected (C-7X) control and DDT-fed (16 ppm X 5 wks) rats treated with 1.0 ml/kg CCl_4 by gavage. The CCl_4 content of livers was estimated by the Fujiwara method after extraction of homogenized livers by microdiffusion into toluene. Each point represents the mean ($\pm$ standard error) of from 3-9 animals. Note the two curves have a similar profile to those from the non-transected groups (see figure 5-2). Also note the magnitude of the CCl_4 concentrations in livers of spinal cord transected groups was considerably greater than that of the respective values for the non-transected groups. Significant differences ($P < 0.05$) in CCl_4 concentrations were noted at 1/2, 2 and 24 hr after CCl_4.

[a]The number in parentheses represents the number of animals per group.

*Significantly different ($P < 0.05$, Student's t-test) from the mean of the respective control group.


CCl_4 Concentration (µg/g)
360
320
280
240
200
160
120
80
40
0
2
4
6
8
10
12
14
16
18
20
22
24
48
TIME (Hours)
(5)a
*
(4)
(8)
(6)
(3)
(6)
(4)
(4)
(7)
(4)
(9)
(4)
(8)
(4)
(C-7X) DDT-fed
(C-7X)
CONTROL
(8)*
(4)
(3)
(4)

Maximal Liver Concentrations of CCl_4

The maximal concentrations attained in the livers of the spinal cord transected and non-transected control and DDT-fed rats are compared in Figure 5-5. In the non-transected groups, the maximal concentrations were measured about four hours after CCl_4 (140 μg/g, controls; 75 μg/g, DDT-fed). These values are significantly different ($P < 0.05$). The dietary pretreatment with DDT did not result in an increased rate of absorption or maximal liver CCl_4 concentration in the intact animals. In the cordotomized groups, however, there was a tendency for the DDT-fed animals to absorb CCl_4 faster, and to achieve slightly higher maximal liver concentrations (252 μg/g, controls; 343 μg/g, DDT-fed). Thus, the effect of DDT in altering the tissue (blood or liver) uptake of CCl_4 was apparently negligible in comparison to the influence of cord-sectioning.

The concentrations of CCl_4 remaining in the livers of all groups 24 hours after CCl_4 are contrasted in the right hand portion of Figure 5-5. The interpretations given here in regard to the relation of one group to another are similar to those given above for the maximal liver CCl_4 concentrations. It seems worthy to note that the decline in liver CCl_4 concentrations which occurred after maximal concentrations were attained, was relatively uniform and consistent in all of the four groups studied. Thus, any differences noted in the 24 hour liver

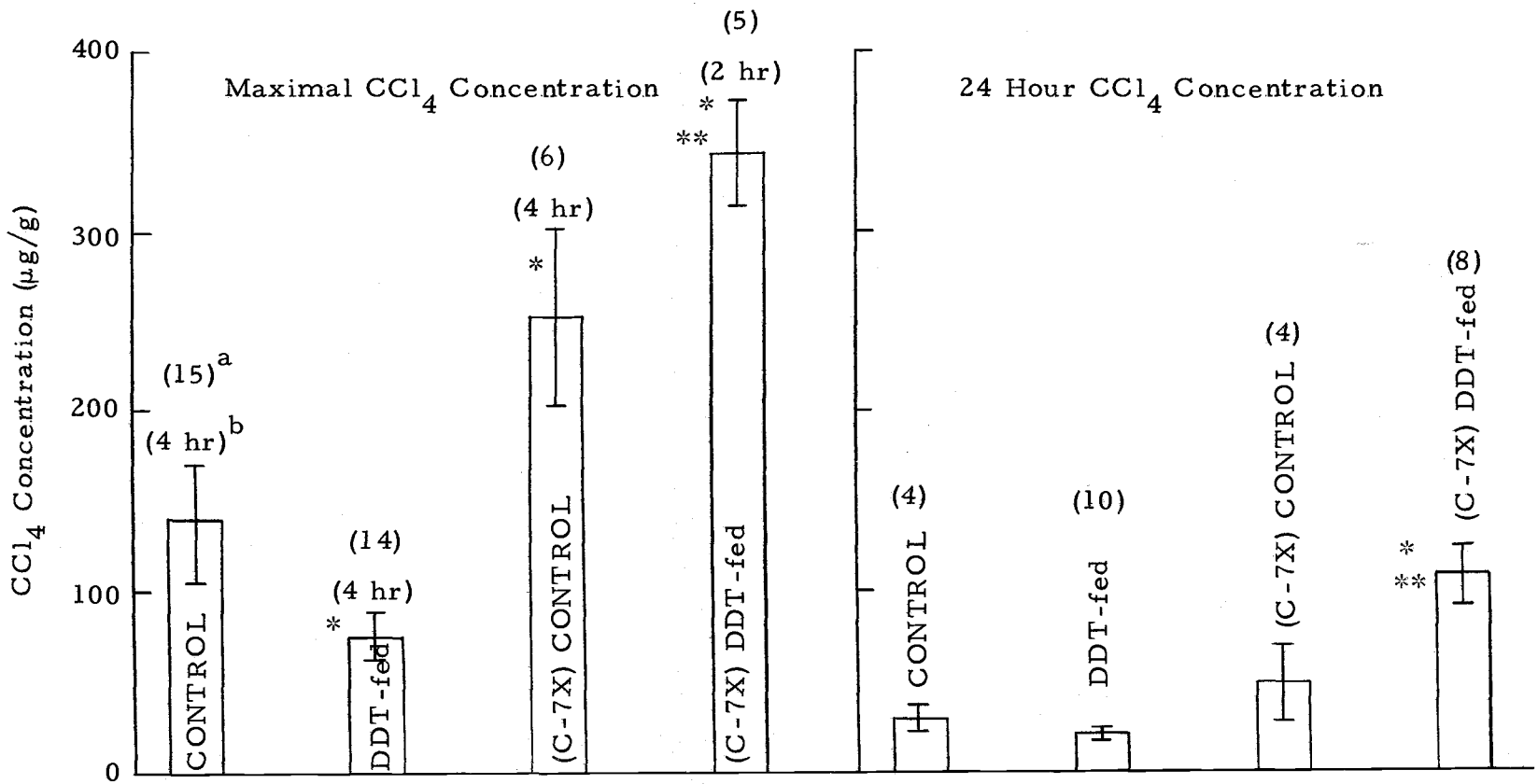
Figure 5-5. Maximal and 24 hr CCl_4 concentrations in livers of spinal cord transected (C-7X) and non-transected control and DDT-fed rats following the administration of 1.0 ml/kg CCl_4 by gavage. Each bar represents the mean (± standard error) of from 4-15 animals. Note the dietary DDT did not produce an increased uptake of CCl_4 into livers, whereas the spinal cord transection did significantly enhance ($P < 0.05$) the uptake of CCl_4 into livers of control and DDT-fed animals.

[a]The number in parentheses represents the number of animals per group.

[b]The number in parentheses represents the time of maximal CCl_4 concentration.

*Significantly different ($P < 0.05$ Student's t-test) from the mean of the non-transected non-DDT-fed group.

**Significantly different ($P < 0.05$) from the mean of the non-transected DDT-fed group.


400
300
200
100
0
CCl_4 Concentration (µg/g)
Maximal CCl_4 Concentration
24 Hour CCl_4 Concentration
(15)[a]
(4 hr)[b]
CONTROL
(14)
(4 hr)
*
DDT-fed
(6)
(4 hr)
*
(C-7X) CONTROL
(5)
(2 hr)
*
**
(C-7X) DDT-fed
(4)
CONTROL
(10)
DDT-fed
(4)
(C-7X) CONTROL
**
**
(8)
(C-7X) DDT-fed

CCl_4 concentrations undoubtedly reflected the maximal concentrations achieved, and probably did not reflect differences in hepatic absorption or elimination properties per se.

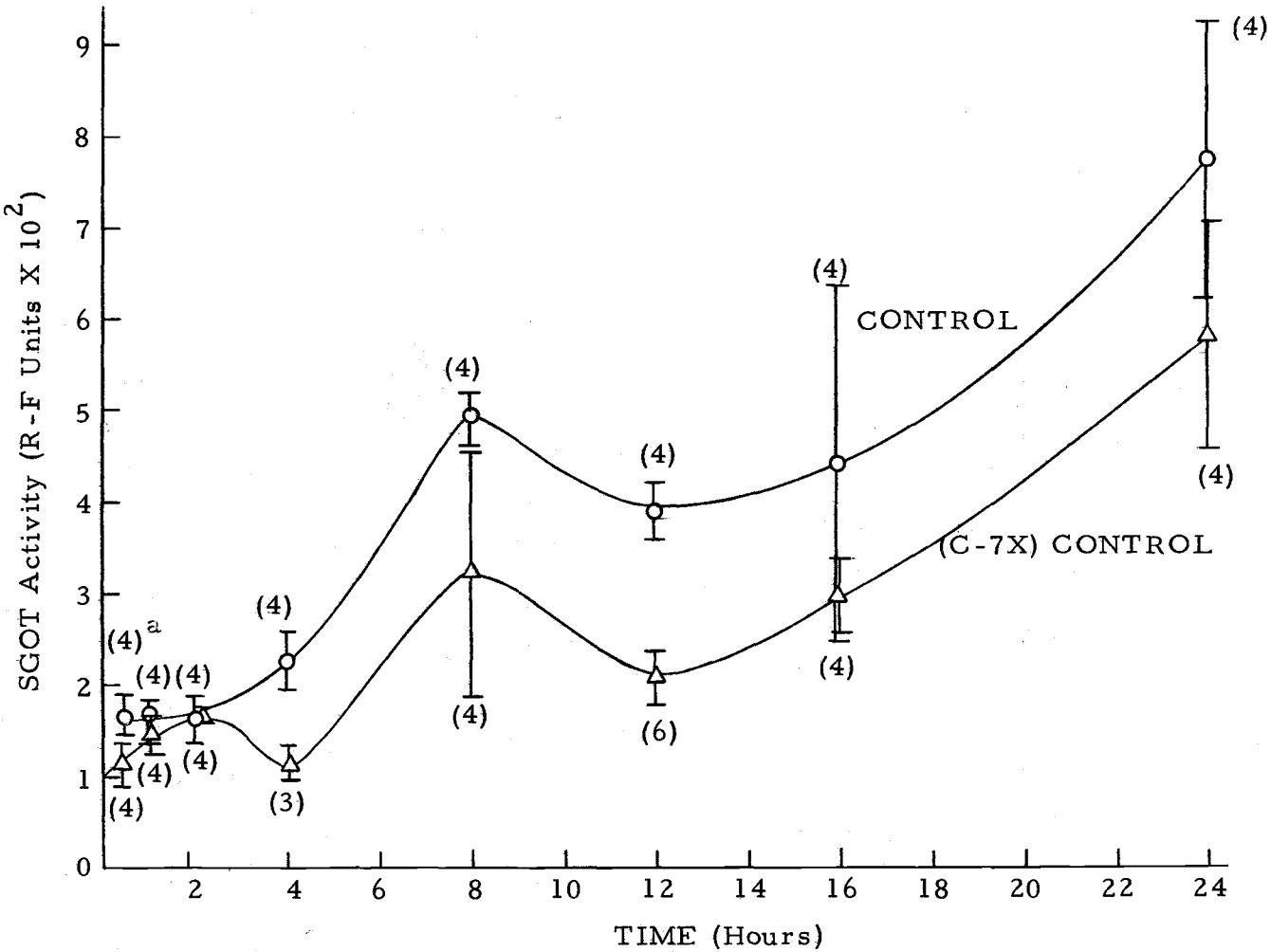
Serum Transaminase (SGOT) Activity

Intact and Cord-sectioned Controls. The temporal patterns of SGOT response after a dose of 1.0 ml/kg CCl_4 are shown in Figure 5-6. The curves representing the serum enzyme activity are similar and essentially biphasic. Cord-sectioning afforded little protection in terms of the SGOT response. Since a relatively complete protection was observed histopathologically in cordotomized animals up to 48 hours after CCl_4, it was a curious finding that this protection was not also manifested in a greatly reduced SGOT activity. Twenty-four hours after CCl_4, the cord-sectioned group had a lower SGOT activity (580 units) than did the non-transected group (710 units).

Intact and Cord-sectioned DDT-fed Rats. As indicated in Figure 5-7, the cordotomized animals were significantly protected ($P < 0.05$) in terms of SGOT activity from the potentiated hepatic damage usually seen 6-24 hours after CCl_4. In the non-transected group, the SGOT activity was potentiated to a maximal value of 6000 units measured 24 hours after CCl_4. In the former protected group, the mean SGOT activity at 24 hours was 700 units. This latter value was comparable to the SGOT activity in non-transected controls given the same dose of

Figure 5-6. Temporal pattern of SGOT activity in spinal cord transected (C-7X) and non-transected control rats following the administration of 1.0 ml/kg CCl_4 by gavage. Each point represents the mean (± standard error) of from 3-6 animals. Note the spinal cord transected rats were not significantly protected ($P > 0.05$, Student's t-test) in terms of SGOT activity 24 hours after CCl_4.

[a]The number in parentheses represents the number of animals per group.


SGOT Activity (R-F Units X 10^2)
TIME (Hours)
CONTROL
(C-7X) CONTROL
0
1
2
3
4
5
6
7
8
9
2
4
6
8
10
12
14
16
18
20
22
24
(4)
(4)
(4)
(4)
(4)
(4)
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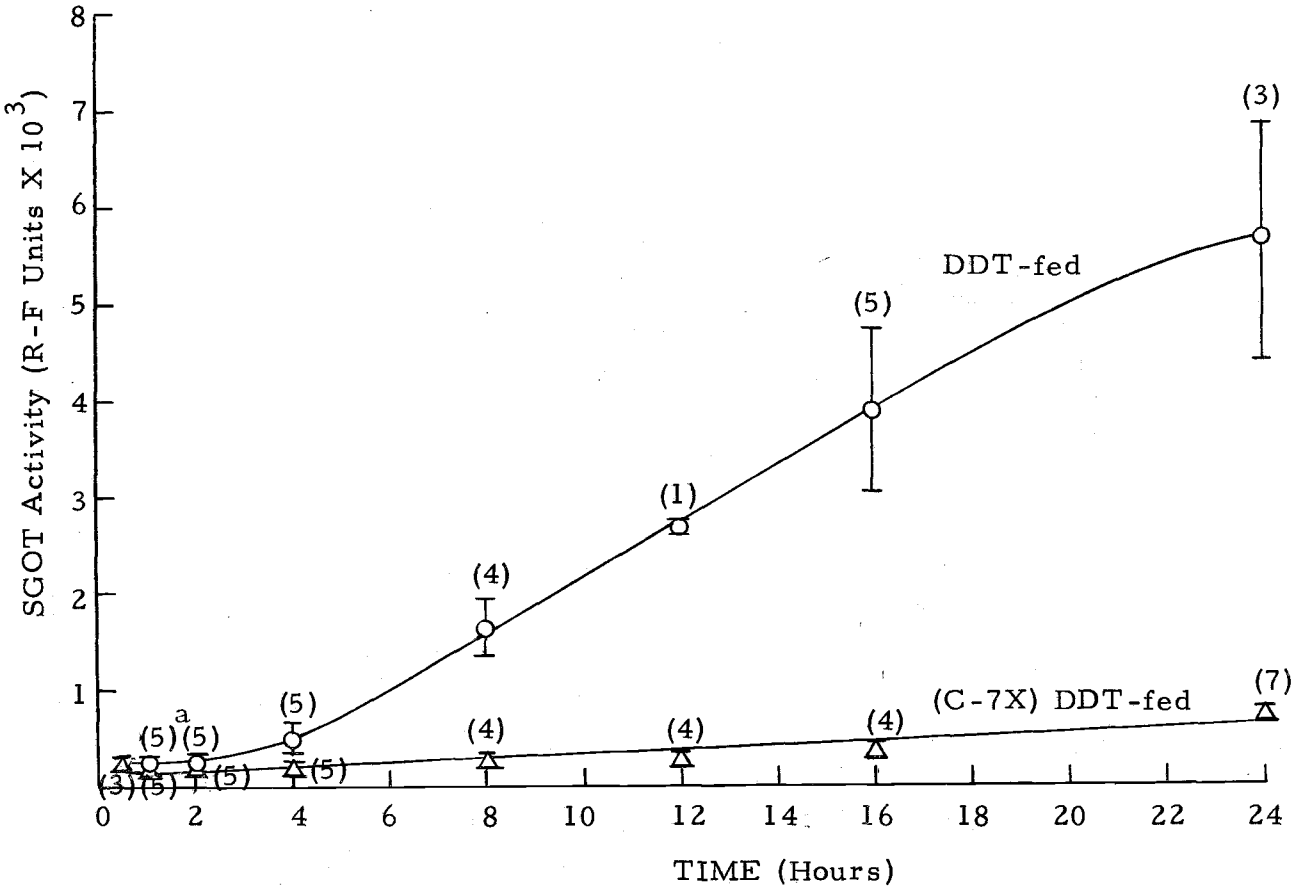
(4)
(4)
(4)
(4)
(4)
(4)
(3)
(4)
(6)
(4)
(4)
(4)
[a]

Figure 5-7. Temporal pattern of SGOT activity in spinal cord transected (C-7X) and non-transected DDT-fed (16 ppm X 5, 7 wks) rats following the administration of 1.0 ml/kg CCl_4 by gavage. Each point represents the mean (± standard error) of from 1-7 animals. Note the spinal cord transected DDT-fed rats were significantly protected ($P < 0.05$, Student's t-test) against the potentiation of SGOT activity by 8 hr after CCl_4.

[a]The number in parentheses represents the number of animals per group.


SGOT Activity (R-F Units X 10^3)
8
7
6
5
4
3
2
1
0
2
4
6
8
10
12
14
16
18
20
22
24
TIME (Hours)
DDT-fed
(C-7X) DDT-fed
(3)
(5)
(1)
(4)
(5)
(4)
(4)
(7)
(5)
(5)
(5)
(5)
(3)
(5)
a

CCl_4. This was a strange finding insofar as the latter group had the characteristic CCl_4-induced histopathologic lesion, whereas the former showed only extensive central cloudy swelling up to 48 hours after CCl_4. Thus, in the cord-sectioned group, a significant degree of protection from potentiation was recognized as early as 6-8 hours after CCl_4 intoxication, which correlated well with the observed histopathologic evidence of protection.

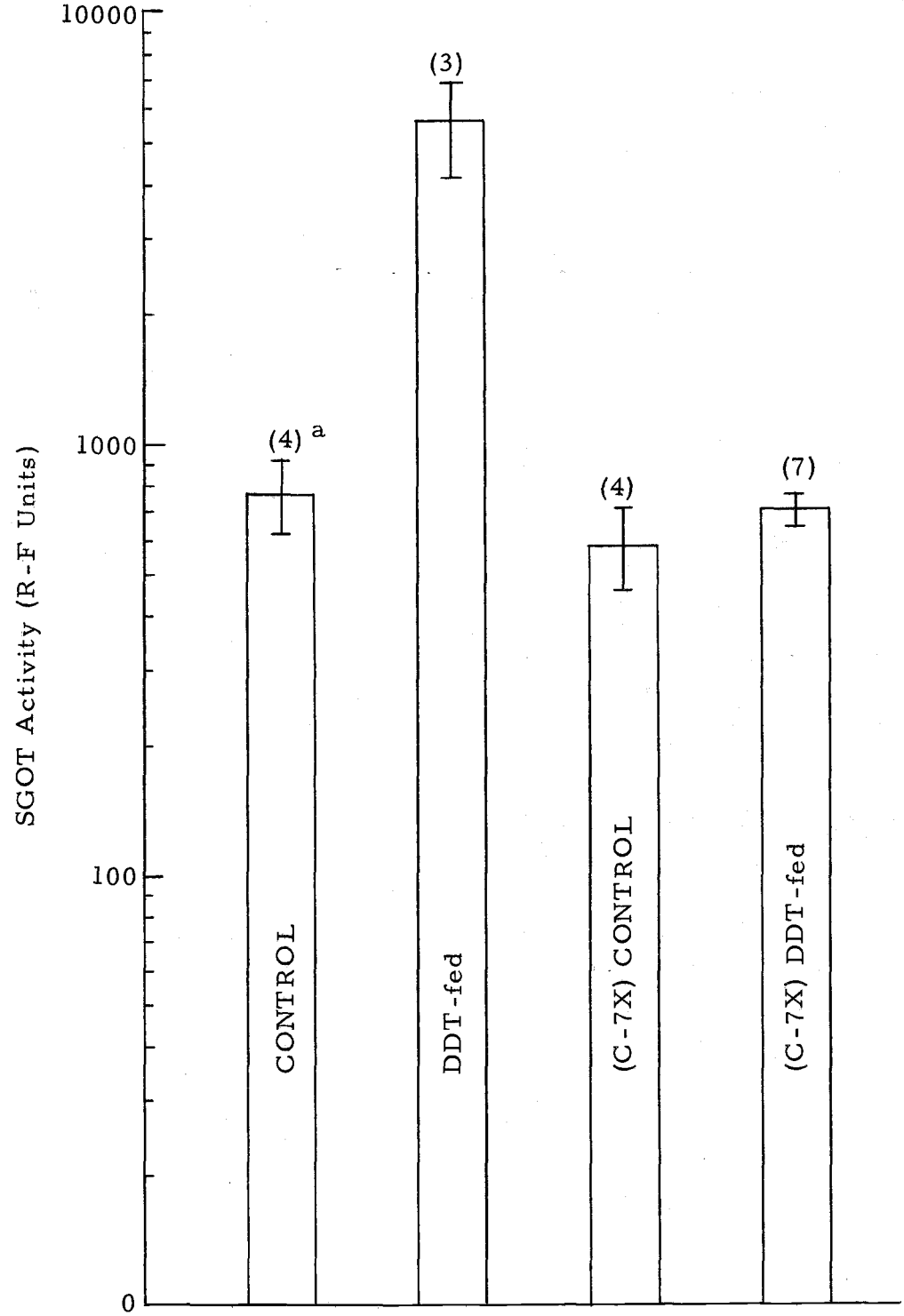
SGOT Activities 24 Hours after CCl_4. The SGOT activities measured in cord-sectioned and non-sectioned control and DDT-fed rats 24 hours after 1.0 ml/kg CCl_4 are compared in Figure 5-8. These results were discussed in detail above, however, they summarize nicely the findings in the SGOT activity studies. Thus, the extent of protection afforded by cord-sectioning against the potentiating action of dietary DDT can readily be visualized. In the DDT-fed animals, the potentiated serum enzyme activity of 6000 units was reduced to 700 units by cord-sectioning. In the controls, the protection afforded by spinal cord transection in terms of SGOT activity was not as readily apparent.

Histopathology

Non-transected Control and DDT-fed Rats. Liver sections taken from intact control and DDT-fed rats after CCl_4 treatment have been previously evaluated for histopathologic evidence of damage in

Figure 5-8. SGOT activity in spinal cord transected (C-7X) and non-transected control and DDT-fed (16 ppm X 5, 7 wks) rats 24 hr after the administration of 1.0 ml/kg CCl_4 by gavage. Each bar represents the mean ($\pm$ standard error) of from 3-7 animals. Note the spinal cord transected DDT-fed rats were significantly protected ($P < 0.05$, Student's t-test) against the potentiation of SGOT activity by dietary DDT.

[a]The number in parentheses represents the number of animals per group.


10000
1000
100
0
SGOT Activity (R-F Units)
(4) a
(3)
(4)
(7)
CONTROL
DDT-fed
(C-7X) CONTROL
(C-7X) DDT-fed

Chapter III of this dissertation. The reader is referred to that discussion.

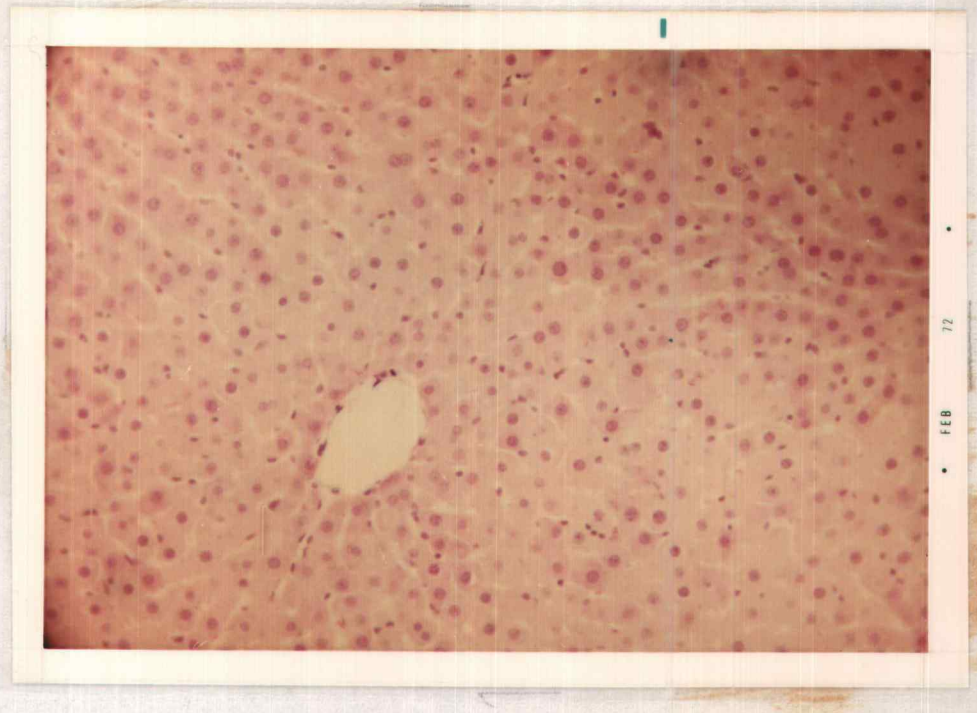
Cord-sectioned Control and DDT-fed Rats. The cord-sectioned controls were markedly protected histologically 24 hours after CCl_4 (Figure 5-9). There was considerable central cloudy swelling, and the characteristic basophilic stipling in the cytoplasm of the centrilobular parenchymal cells was greatly diminished. Nevertheless, there was little evidence of centrilobularly-oriented coagulative necrosis or midzonal balloon cell formation. Therefore, the degree of hydropic degeneration was reduced. The liver section taken from a cord-sectioned DDT-fed rat 24 hours after CCl_4 is shown in Figure 5-10. The degree of protection from hepatic necrosis observed was nearly comparable to that seen in the controls. This was an amazing finding in view of the potentiated CCl_4-induced central necrosis that was noted in the intact DDT-fed animals. The histopathologic evidence of protection observed in the cord-sectioned DDT-fed animals correlated well with the decreased transaminase activities measured 24 hours after CCl_4 in this group.

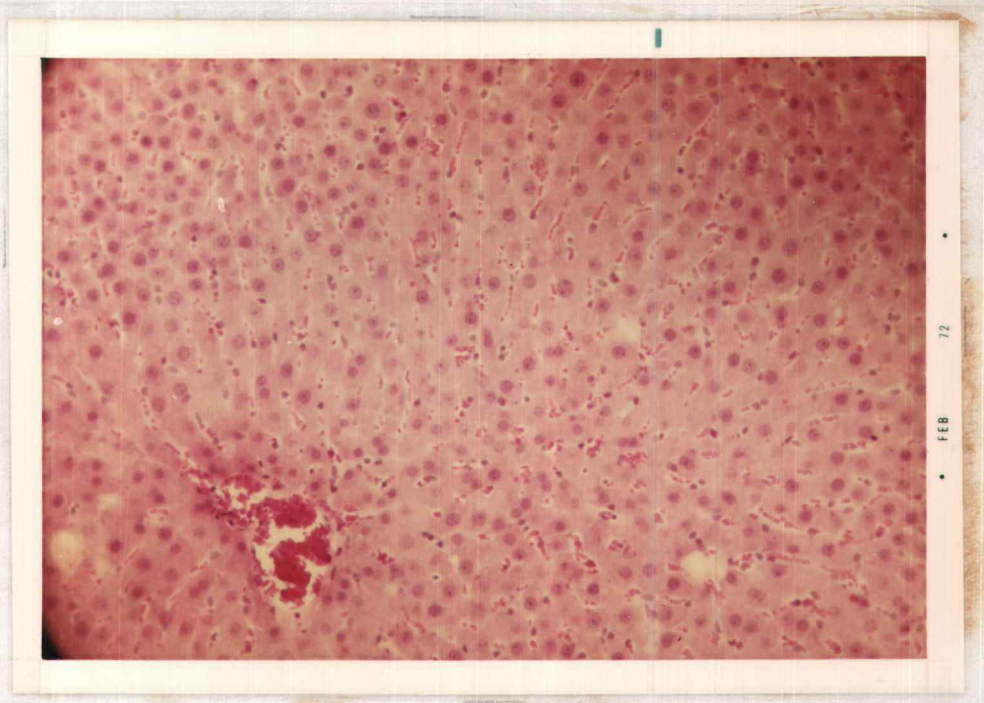
Discussion

In these temporal studies, the concentrations of CCl_4 attained in the blood and livers of spinal cord transected and non-transected control rats were similar in magnitude to the values reported by

Figure 5-9. Liver section taken from a spinal cord transected non-DDT-fed rat 24 hours after the administration of 1.0 ml/kg CCl_4 by gavage. Note the absence of centrilobularly-oriented coagulative necrosis and midzonal balloon cells. There was an apparent loss of the cytoplamic staining qualities in centrilobular cells. H and E stain (400X)

Figure 5-10. Liver section taken from a spinal cord transected DDT-fed (16 ppm X 5 wks) rat 24 hours after the administration of 1.0 ml/kg CCl_4 by gavage. Note the extensive cloudy swelling of cells in the central zone, with no evidence of centrilobularly-oriented coagulative necrosis and midzonal balloon cells. This liver was protected histopathologically against the potentiated CCl_4-induced hepatic damage subsequent to the dietary DDT pretreatment. H and E stain (400X)


FEB 72


FEB 72

Larson et al. (1965). The discrete measurements of CCl_4 concentrations were used as a basis for the construction of continuous curves representing the temporal pattern of tissue CCl_4 uptake. As noted previously for the temporal response of the serum transaminase activities to CCl_4, the representation of the data as continuous is probably an oversimplification of the actual situation in vivo. The minute to minute changes in absorption, distribution, metabolism and elimination of CCl_4, which factors may interact in vivo and may be influenced by changes in the external environment or the internal milieu, preclude the simple continuous uptake of CCl_4 into the blood and livers. Since the temporal pattern of tissue CCl_4 uptake was similar for all of the four treatment groups studied, it would appear that the continuous representation of the data was consistent.

The tissue concentrations of CCl_4 always increased biphasically, as a steadily increasing CCl_4 uptake was invariably interrupted by a brief decline, usually between 2-5 hours after CCl_4. In all groups, the profiles of the blood CCl_4 concentration curves were similar, and the same statement can be made for the livers. Another consistent finding was that the profile of the blood CCl_4 concentration curve for any one group corresponded rather closely to the profile of the liver curve for that group. For example, in the non-transected controls, the curves representing the blood and liver CCl_4 concentrations were nearly superimposable. Although these latter profiles

were similar, any increase or decrease in the liver CCl_4 concentrations was usually preceded in time by a corresponding alteration in the CCl_4 concentration of the blood. In addition, the measured concentrations of CCl_4 in the blood were generally considerably lower than those in the liver. McCollister et al. (1951) studied the distribution of total ^{14}C-radioactivity in a female monkey following inhalation of 46 ppm $^{14}CCl_4$ for 300 minutes. They reported a liver:blood ratio of 3:1, and this ratio was equaled or exceeded only by those of the bone marrow (3:1) or fat (8:1) in all tissues studied. The results of the present investigation and those of McCollister lend support to the statement of de Reuck et al. (1964), that the liver concentrates CCl_4.

Chronic DDT pretreatment did not result in an increased uptake of CCl_4 into the blood or livers of intact animals, with respect to controls. In fact the tissue concentrations of CCl_4 were generally lower in the DDT-fed animals. The maximal liver concentrations of CCl_4 measured in these groups were not significantly different ($P > 0.05$). Thus, an increased CCl_4 uptake into the blood and livers was apparently not related to the mechanism governing the potentiation of CCl_4-induced hepatotoxicity by dietary DDT. Furthermore, the concentrations of CCl_4 in the blood and livers in the intact DDT-fed rats declined more rapidly from the maximal concentrations attained to the 24 and 48 hour values than they did in the controls. This more rapid disappearance of CCl_4 from the tissues in DDT-fed animals was

suggestive of the more rapid disappearance of any drug undergoing metabolism in the liver, following the induction of hepatic microsomal drug-metabolizing enzyme systems that handle the agent. From the standpoint of CCl_4 clearance, then, a contribution of induced hepatic metabolism cannot be entirely eliminated.

The tissue concentrations of CCl_4 in the cord-sectioned groups were usually one to two orders of magnitude higher than the corresponding values in the non-transected groups. In Figure 5-5 for example, the liver CCl_4 concentrations in the cordotomized DDT-fed rats were significantly greater ($P < 0.05$) than the corresponding concentrations in the non-transected DDT-fed group. These results were in accord with those of Larson et al. (1965), who concluded that the concentrations of CCl_4 attained in the blood and livers of cord-sectioned rats were probably sufficient to produce hepatic damage. This conclusion of theirs was an apparent slight understatement, since in their study as well as in the present investigation, the tissue concentrations of CCl_4 in cordotomized rats exceeded those in non-transected animals. The elevated concentrations of CCl_4 in the tissues of cord-sectioned groups should have resulted in a degree of hepatic damage equivalent to, or in excess of, that observed in the non-transected animals given the same dose of CCl_4. This conclusion is valid if it is assumed that the measured concentrations of CCl_4 were at the hepatic sites of action where CCl_4 produces necrosis.

Further, the degree of hepatic damage observed had to be directly related to the amount of CCl_4 present at the locus of action. In this study, however, microscopic histopathologic examination of livers taken from cord-sectioned control and DDT-fed groups revealed that these animals were protected from the CCl_4-induced centrilobularly-oriented coagulative necrosis usually observed in non-transected groups. Thus, in the case of cordotomized rats, the degree of histopathologic damage observed did not correlate with the liver concentrations of CCl_4 attained. These findings appear to conflict with the conclusions of Zimmerman, Kodera and West (1965) and Zimmerman and Mao (1965), who believed that the degree of cytotoxicity after CCl_4, as measured by the leakage of cellular enzymes to the surrounding medium (e.g., blood) was directly related to the concentration of CCl_4 present both *in vivo* and *in vitro*. It is possible that the conditions of hypothermia and hypoxia in cord-sectioned rats (Larson *et al.*, 1965) inhibit the toxic mechanism of CCl_4.

Comparing the temporal uptake of CCl_4 into tissues with the serum transaminase response (Figures 2-1, 3-5), there was an apparent temporal relation between the blood and liver CCl_4 concentrations and the release of intracellular GPT and GOT to the blood. In both studies, the curves were similar in profile and biphasic. The temporal increase-decrease-increase pattern of liver CCl_4 uptake did, however, precede the similar development of serum enzyme

response by approximately six hours. The onset of hepatic damage (SGPT, SGOT) and functional impairment (BSP) in controls occurred about six hours after CCl_4, which was approximately two hours after maximal concentrations of CCl_4 were measured in the livers. Moreover, a similar situation was noted in the intact DDT-fed animals, where the onset of potentiated hepatic damage and dysfunction was also observed between 6-8 hours after CCl_4. Thus, the onset of rapidly accelerating hepatocellular destruction in the DDT-fed animals was apparently related directly, after a slight temporal delay, to the maximal liver CCl_4 concentrations attained. This temporal lag could be related to the time required for a sequence of events to occur in the hepatic parenchymal cells, leading to cell membrane disruption and a subsequent release of intracellular enzymes to the blood.

The onset and prolongation of potentiated hepatic damage in the DDT-fed rats cannot be explained merely on the basis of the blood and liver concentrations of CCl_4 achieved. Paul and Rubinstein (1963) estimated that by 18 hours after the intraduodenal administration of $^{14}CCl_4$ to rats about 85% of the administered dose had been excreted unchanged into the expired air and 1% had been converted to $^{14}CO_2$. In the present study, the concentrations of CCl_4 measured in the blood and livers of intact control and DDT-fed groups were nearly undetectable by 24-48 hours after CCl_4. In the controls, the serum enzyme response (Figure 2-1) began to diminish by 24 hours, although

maximal activities were measured 36 hours after CCl_4. The SGPT and SGOT activities declined toward normal control activities between 36-48 hours. In Chapter IV (Figure 4-3) it was observed that this same group had a slight improvement in functional capacity to remove BSP from the plasma 48 hours after CCl_4, with respect to the BSP retention at 24 hours. Therefore, there was an apparent relationship between the declining blood and liver CCl_4 concentrations and the regeneration toward normal hepatic conditions between 24-48 hours. In contrast, the potentiated hepatic damage (SGOT, Figure 3-5) and dysfunction (BSP, Figure 4-3) in the DDT-fed animals continued to worsen between 24-48 hours despite the negligible tissue CCl_4 concentrations present at this time. It was interesting that only the DDT-fed group had a potentiated CCl_4 lesion, although the measured tissue concentrations of CCl_4 were generally lower in this than in the control group. Although the concentration of CCl_4 attained in the livers undoubtedly has an important influence on the extent of damage (controls) or potentiated damage (DDT-fed), it is clear that the onset of potentiated damage at 6-8 hours after CCl_4 is related to some factor other than the absolute concentration of CCl_4 attained in the livers. The prolongation of the potentiated hepatotoxic response also appeared to be unrelated to the measurable tissue concentrations of CCl_4 between 24-48 hours after CCl_4. The mechanism responsible for the temporal differences in hepatotoxic response of control and

DDT-fed rats to CCl_4, in view of the similar tissue CCl_4 concentrations attained, remains to be conclusively elucidated.

According to the hypothesis of Brody et al. (1961), the action of CCl_4 was an indirect one, mediated primarily by the peripheral actions of norepinephrine. A secondary factor was the release of epinephrine and norepinephrine from the adrenal medulla into the systemic circulation. These hormones caused constriction of the circulatory supply to the liver, with a resultant tissue anoxia and subsequent hepatocellular necrosis. According to Brody et al., the severance of these sympathetic pathways by cord-sectioning disrupted the toxic action of CCl_4 and provided protection from hepatic necrosis in rats. Their hypothesis appeared to explain the present finding that relatively high liver CCl_4 concentrations did not produce centrilobular necrosis in cordotomized animals.

Larson et al. (1963) provided an alternative explanation for the protective action of spinal cord transection in CCl_4-poisoned rats. They observed that the animals became hypothermic after cord-sectioning; as the level of cord-sectioning was lowered from C-7, the measured rectal temperatures correlated with the degree of hepatic protection observed histologically (Larson, Plaa and Crews, 1964). Subsequent investigations were conducted to determine the mechanism of the protective effect (Larson, Plaa and Brody, 1964; Plaa and Larson, 1964; Larson et al., 1965). It is believed that a summary of

their findings is pertinent to this discussion: 1) Cord-sectioned rats maintained at normothermic temperatures were not protected. 2) Hypothermia produced by cold water immersion of rats also afforded protection. 3) The blood and liver CCl_4 concentrations were similarly elevated over control values in the cord-sectioned and water-immersed rats. 4) The degree of protection afforded by cord-sectioning was dose-dependent on CCl_4. 5) The route of CCl_4 administration was not a critical factor. 6) The hepatic histologic "protection" observed for 48 hours after CCl_4 in cordotomized animals was not a true protection, but rather a delay in the temporal development of the CCl_4 lesion. 7) Immunologic sympathectomy did not afford protection, thus a more subordinate role was indicated for the sympathetic nervous system in CCl_4 hepatotoxicity. 8) Direct infusion of catecholamines did not produce a hepatic lesion. 9) Studies on oxygen consumption in normothermic and hypothermic rats indicated a reduced hepatic metabolism was the operative mechanism in the protection afforded by spinal cord transection.

Cord-sectioning provided a powerful protection against the potentiated CCl_4-induced hepatic necrosis resulting from dietary pretreatment of rats with DDT. This histologic evidence of protection, which transpired despite high blood and liver CCl_4 concentrations, confirmed the finding of a vastly reduced SGOT activity between 6-24 hours in these animals.

Larson et al. (1965) apparently did not speculate on possible reasons for the return of the CCl_4-induced histologic lesion between 55-65 hours in "protected" animals. In view of the low (nearly undetectable) concentrations of CCl_4 measured 48 hours after CCl_4 in cord-sectioned rats in this study, it is reasonable to assume the liver CCl_4 concentrations at 55 hours in their study were insufficient to produce even mild hepatocellular destruction. Further, Paul et al. (1963) showed that most of the CCl_4 administered intraduodenally to rats had been excreted long before 55 hours. The transected animals were still hypothermic at 55 hours. There are at least two mechanisms which could be postulated to explain the delay and subsequent return of the CCl_4-induced histopathologic lesion during the period normally considered to be the regenerative phase of acute CCl_4 intoxication (Dinman et al., 1968a, b). First, the initial step in the damage process could be linked to the activation (Slater, 1966) or metabolism (McLean et al., 1969) of CCl_4 to a toxic form. Second, the extent of cellular disruption probably depends on the rate at which the active form of CCl_4 reacts with the chemical constituents of the membrane, i.e., in lipoperoxidation (Recknagel, 1967). Either of these steps could be greatly slowed by hypothermic and hypoxic conditions in the liver, and the most crucial rate limiting step would undoubtedly be the formation of an active form of CCl_4. If the second step was slowed, the damage could occur at a slower rate than

usual and still be manifest histologically say, by 55-65 hours after CCl_4. Further consideration should be given to these problems if there is to be a more complete understanding of the degenerative processes in the hepatocyte.

VI. THE INFLUENCE OF SPINAL CORD TRANSECTION AND THE DDT-CCl_4 HEPATOTOXIC INTERACTION ON CYTOCHROME P-450 AND DRUG METABOLISM

Introduction

The activities of a number of hepatic microsomal drug metabolizing enzymes are decreased by CCl_4 treatment. The hexobarbital sleeping time was prolonged in rats pretreated with ethionine or CCl_4, due to the depression of drug metabolizing enzyme activity (Neubert and Maibauer, 1959). Similar decreases in activity have been demonstrated for aminopyrine demethylase (Neubert et al., 1959), ring hydroxylation and azo dye cleavage (Smuckler, Arrhenius and Hultin, 1967). After gastric administration of CCl_4 in rats, Dingell and Heimberg (1968) confirmed the impairment of hexobarbital and aminopyrine metabolism. Sasame et al. (1968) made a similar observation for ethylmorphine metabolism. Lal, Puri and Fuller (1970) administered CCl_4 to rats by inhalation, and found that the hexobarbital sleeping time was prolonged, and the in vitro metabolism of hexobarbital was diminished. The hepatic damage resulting from CCl_4 inhalation was reversible within 48 hours, whereas after oral administration of CCl_4 the hepatic damage was considered irreversible (Sasame et al., 1968) or took at least several days to reverse (Dingell et al., 1968).

Fujimoto and Plaa (1961) utilized the hexobarbital sleeping time to study the interaction of a microsomal stimulating agent and a microsomal depressing agent on hepatic drug metabolism. Using mice, they demonstrated that if CCl_4 was administered simultaneously with phenobarbital, the normal decrease in sleeping time produced by phenobarbital was no longer observed. If CCl_4 was given 24 hours prior to phenobarbital, however, this sleeping time response to phenobarbital was not blocked. Recknagel and Lombardi (1961) reported a decreased glucose-6-phosphatase activity after CCl_4. The comparative action on certain microsomal phosphatases and drug metabolizing enzymes was studied for the hepatotoxic agent CCl_4 and enzyme-inducers such as hexobarbital and phenobarbital, by Feuer and Granda (1970). They concluded that the concurrent administration of CCl_4 and the enzyme-inducers led to a balance between the inhibitory and stimulatory phases, which resulted in an interaction or antagonism on the enzyme activity of the endoplasmic reticulum. That CCl_4 exerts its initial deleterious effects on the endoplasmic reticulum and causes a rapid decline in the concentration of microsomal cytochrome P-450 has been shown by Dingell et al. (1968). According to Marshall and McLean (1969), the acute pretreatment of rats with DDT or phenobarbital produced massive increases in the microsomal hydroxylating enzyme systems in the liver. They believed this induction was responsible for an increased susceptibility of rats to CCl_4

following acute DDT pretreatment. In this context, the potentiation of CCl_4 hepatotoxicity was related to its metabolism by the drug metabolizing enzyme system. Decreases in the activities of certain hepatic microsomal drug metabolizing enzymes correlate well with decreases in the amount of cytochrome P-450 in the liver microsomes (Smuckler et al., 1967; Castro et al., 1968; Sasame et al., 1968). Greene, Stripp and Gillette (1969) have recently confirmed that this correlation exists, utilizing the in vitro N-demethylation of ethylmorphine in comparison to the measured concentrations of cytochrome P-450.

In the present investigation, the effects of spinal cord transection and lowered incubation temperatures on the in vitro metabolism of hexobarbital and ethylmorphine were determined. These results were compared with the effect of cord-sectioning on the hepatic microsomal concentration of cytochrome P-450. The primary goal was to distinguish whether hypothermia or a decreased cytochrome P-450 concentration was responsible for the protective effect of cord-sectioning against the CCl_4-induced hepatic necrosis.

The present study was also designed to assess whether the potentiation of CCl_4 hepatotoxicity by dietary DDT could enhance the effect of CCl_4 of depressing the cytochrome P-450 concentration. On the other hand, the hepatotoxic interaction of dietary DDT and acute CCl_4 could produce a compromise between the inducing effect of

DDT on cytochrome P-450, versus the action of CCl_4.

Several investigators have dealt with the liver-enlarging effects of agents such as DDT and CCl_4, and have discussed the relationship of hepatic hypertrophy to hepatotoxicity (Meldolesi, 1967; Platt and Cockrill, 1967; Hoffman et al., 1970). In this study, changes in the liver weight and rectal temperature were monitored to assess the effect of the DDT-CCl_4 hepatotoxic interaction on these variables.

Methods

Hexobarbital Metabolism

In these studies, the 9000 x g supernatant fractions from liver homogenates were utilized, essentially according to the method of Cooper and Brodie (1955), who studied the enzymatic metabolism of hexobarbital in the rabbit. The animals were stunned, exsanguinated, and the livers were quickly removed and placed in an ice bath containing 1.15% KCl. The livers were then homogenized in ice cold 1.15% KCl solution in a ratio of 5 g of liver per 10 ml of KCl. The homogenate was initially centrifuged at 2000-2500 rpm for 20 minutes at 4°C in a refrigerated high speed (Lourdes Beta-Fuge) centrifuge. The supernatant was decanted into a second 10 ml centrifuge tube, and recentrifuged at 9000 x g for 60 minutes at 4°C. The resultant supernatant was free of unbroken cells, nuclei and mitochondria, but

contained essential cofactors and an NADPH-generating system for in vitro oxidative drug metabolism. Incubation mixtures were prepared by adding the following constituents to a 25 ml Erlenmeyer flask: Nicotinamide adenine dinucleotide phosphate (NADP) (2 μM), glucose 6-phosphate (G6-P) (20 μM), nicotinamide (40 μM), $MgCl_2$ (20 μM), hexobarbital substrate (0.5-1.5 μM), glucose 6-phosphate dehydrogenase (G 6-PD) (2 e.u.), 2 ml of 9000 x g supernatant, and 2.3 ml of pH 7.4 tris (2-amino-2(hydroxymethyl)-1,2-propanediol) buffer to bring the total incubation mixture volume to 5 ml. The reaction mixtures were incubated in open 25 ml Erlenmeyer flasks in a Dubnoff Metabolic Shaking Incubator for 45 minutes at 37°C under an oxygen atmosphere. Following incubation, the hexobarbital was extracted and assayed according to the method of Brodie et al. (1953). The extraction efficiency for hexobarbital was 95 ± 1. The results were expressed as μM hexobarbital metabolized per gram of liver per 45 minutes.

Ethylmorphine Metabolism

The liver 9000 x g supernatant fractions were prepared as described above, and subsequently re-centrifuged at 100,000 x g for 90 minutes at 4°C in a Beckman preparative ultracentrifuge. The resulting microsomal pellet was re-suspended in fresh ice cold 1.15% KCl, and re-centrifuged at 100,000 x g for 90 minutes at 4°C. This

final, washed microsomal pellet was resuspended in 5 ml of ice cold 1.15% KCl. The metabolism of ethylmorphine was accomplished according to the following procedure. Into a 25 ml Erlenmeyer flask the following constituents were added, making up the reaction mixture: $MgCl_2$ (5 μM), NADP (0.33 μM), G 6-P (20 μM), G6-PD (2 e.u.), ethylmorphine substrate (10 μM), 1 ml of microsomal suspension, and 2.4 ml (50 μM) of pH 7.4 tris buffer. The final volume of this reaction mixture was 4 ml. The incubation time for this reaction was 15 minutes. This procedure was essentially that of Cochin and Axelrod (1953). The extent of ethylmorphine metabolism in vitro by the incubation mixture was assessed by the colorimetric determination of formaldehyde produced, according to the method of Nash (1953).

Cytochrome P-450

The microsomal suspension from the 100,000 x g washed microsomal pellet was prepared as described above, and then diluted five-fold with ice-cold 0.1 M phosphate buffer, pH 7.0. Aliquots were taken for the determination of total microsomal protein (method of Lowry et al., 1951) and the cytochrome P-450 concentration (method of Omura and Sato, 1964). The cytochrome P-450 concentration was determined in the following manner. First, approximately 5 mg of sodium dithionite powder was added to the diluted microsomal suspension, in order to reduce the cytochrome P-450 and thus enable

it to bind carbon monoxide gas (CO). After mixing, 3 ml of the suspension was poured into each of two cuvettes. Carbon monoxide gas was bubbled into the microsomal suspension in one of the cuvettes, whereas the other cuvette served as a reference. Carbon monoxide gas was generated in a special apparatus. About 50-100 ml of concentrated H_2SO_4 was poured into a 500 ml rubber-stoppered Erlenmeyer flask. Concentrated formic acid was added dropwise from a buret mounted in the rubber stopper. Usually, the addition of 5-6 drops of formic acid at once was sufficient to generate enough CO gas for the preparation of several samples. A thin metal tube (approximately 1/16" O. D.) was also inserted through the rubber stopper, and to the outer end of this tube a two-foot length of polyethylene tubing was attached. The generated CO gas in the flask was bubbled through this tubing into the cuvette. The concentration of cytochrome P-450 was determined utilizing a Bausch and Lomb Spectronic 600 spectrophotometer, by taking the optical density difference of readings taken at 450 and 500 nm. The resulting optical density difference was divided by the extinction coefficient for cytochrome P-450 (91 mM^{-1} cm^{-1}) and by the concentration of total microsomal protein (mg/ml) in the microsomal suspension. Results were expressed as nanomoles of cytochrome P-450 per mg of microsomal protein.

Outline of the Experiment

In the first portion of this study, the effects of a lowered incubation temperature and hypothermia in rats were studied for their effects on hepatic microsomal drug metabolism in rats. The in vitro hexobarbital metabolism was determined simultaneously at 25° and 37°C in the 9000 x g supernatant fractions prepared from the livers of control and DDT-pretreated (75 mg/kg by gavage for 36 hours) animals. Thus, the effect of a lowered incubation temperature was studied utilizing the in vitro hexobarbital metabolism. Then, the microsomal preparations from the livers of spinal cord transected control and DDT-fed (16 ppm x 8 wks) rats were incubated simultaneously at 25° and 37°C with ethylmorphine, in order to assess the effect of cord-sectioning upon drug metabolism. In conjunction with these in vitro drug metabolism studies, the cytochrome P-450 concentrations were measured in the liver microsomal preparations from spinal cord transected control and DDT-fed rats. In the second part of the study, the effect of the DDT-CCl_4 hepatotoxic interaction upon the hepatic microsomal cytochrome P-450 concentration was determined. Control and DDT-fed (16 ppm x 8 wks) rats were dosed with CCl_4 at increasing dosages of 0.125-2.0 ml/kg. The cytochrome P-450 concentrations were determined 24 hours post-CCl_4 exposure. In addition, groups of control and DDT-fed rats were treated with 0.5

ml/kg CCl_4, and the cytochrome P-450 concentrations were determined in subgroups of these animals at intervals of 0, 6, 12, 18, 24 and 48 hours after CCl_4. The liver weights and rectal temperatures were also recorded at the same time liver samples were taken.

Results

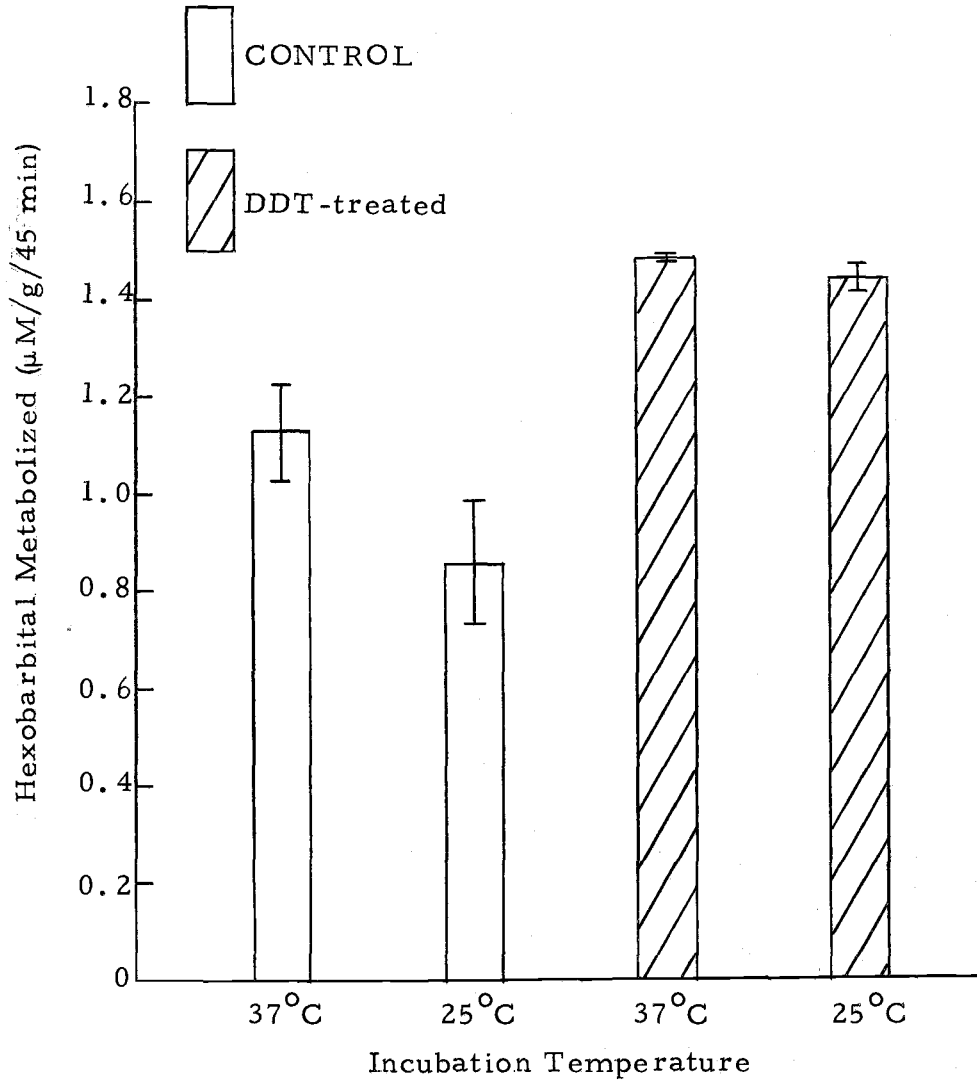
Effect of Incubation Temperature Upon *in vitro* Hexobarbital Metabolism

Lowering the incubation temperature from 37° to 25°C decreased the *in vitro* hexobarbital metabolism by 18% in the liver 9000 x g supernatant fractions from control rats (Figure 6-1). This 18% decrease was not significant ($P > 0.05$, Student's t-test). In similar liver preparations from the DDT-treated animals, the reduction in hexobarbital metabolism was only minimal ($P > 0.05$), since nearly 100% of the 1.5 μM hexobarbital substrate concentration was metabolized at both of the incubation temperatures employed. The amount of hexobarbital metabolized by the DDT-fed group at either 25° or 37° was significantly more ($P < 0.05$) than the control animals metabolized at either 25° or 37°C.

Effect of Spinal Cord Transection Upon *in vitro* Ethylmorphine Metabolism

The spinal cord transected control and DDT-fed rats were hypothermic, which resulted in a reduced rate of *in vitro*

Figure 6-1. Effect of incubation temperature on the in vitro metabolism of hexobarbital by liver 9000 x g supernatant fractions from control and DDT-treated rats. Acute DDT was dissolved in corn oil and administered by gavage at a dosage of 75 mg/kg, and livers were taken 36 hours later. Each bar represents the mean (± standard error) of 5 animals. Note the liver preparations from the DDT-treated rats metabolized significantly more hexobarbital ($P < 0.05$, Student's t-test) at both 37° and 25° C. than did the controls at 37°. The hexobarbital metabolism was not significantly decreased ($P > 0.05$) by lowering the incubation temperature from 37° to 25° in either control or DDT-treated groups.


CONTROL
DDT-treated
Hexobarbital Metabolized (μM/g/45 min)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
37°C
25°C
37°C
25°C
Incubation Temperature

ethylmorphine metabolism by liver microsomal preparations from both of these groups (Figure 6-2). The amount of formaldehyde (HCOH) produced (μg/g of liver) in the Nash reaction in the cord-sectioned control group was decreased by 60% when the incubation temperature was lowered from 37° to 25°C, however, this reduction was not significant ($P > 0.05$, Student's t-test). The liver microsomal preparations from the cord-sectioned DDT-fed rats metabolized significantly less ($P < 0.05$) ethylmorphine when the incubation temperature was lowered from 37° to 25°C. In this latter group, the production of HCOH declined from about 28.5 μg/g (37°) to 7.5 μg/g (25°), thus representing a 70% decrease in metabolism as a result of lowering the incubation temperature. In the DDT-fed group, the amount of ethylmorphine metabolism at 25° was significantly greater ($P < 0.05$) than that of controls at that temperature, but was not significantly different ($P > 0.05$) from the amount of ethylmorphine metabolized by controls at 37°.

Effect of Spinal Cord Transection Upon Cytochrome P-450 and Rectal Temperature

From the data presented in Figure 6-3, it can be seen that the rectal temperatures of control and DDT-fed rats declined about 10-12 degrees below normal control values (37-38°C) by five hours after spinal cord transection. The determination of the cytochrome P-450 concentrations in the hepatic microsomal preparations indicated that

Figure 6-2. Effect of spinal cord transection (C-7X) in control and DDT-fed (16 ppm X 8 wks) rats and a lowered incubation temperature on the in vitro metabolism of ethylmorphine. Each bar represents the mean (± standard error) of 5 animals. Note the liver microsomal (100,000 x g) preparations from DDT-fed rats metabolized significantly more ethylmorphine ($P < 0.05$, Student's t-test) at 37° and 25° than did their control counterparts. The rate of metabolism at 25° in the liver preparations from the DDT-fed group was comparable ($P > 0.05$) to that in the control group at 37°. Ethylmorphine metabolism by the microsomal preparations from the DDT-fed group was significantly reduced ($P < 0.05$) when the incubation temperature was lowered from 37° to 25°.

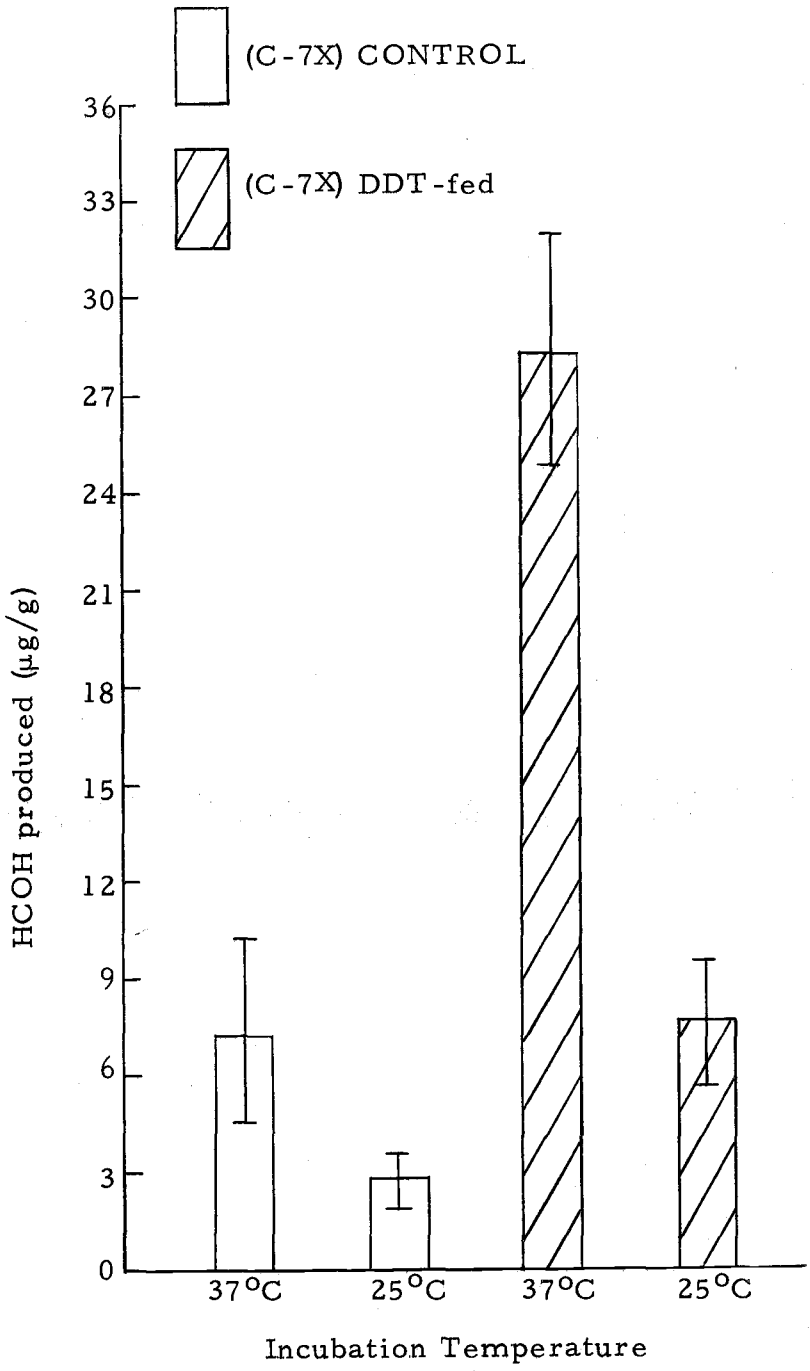
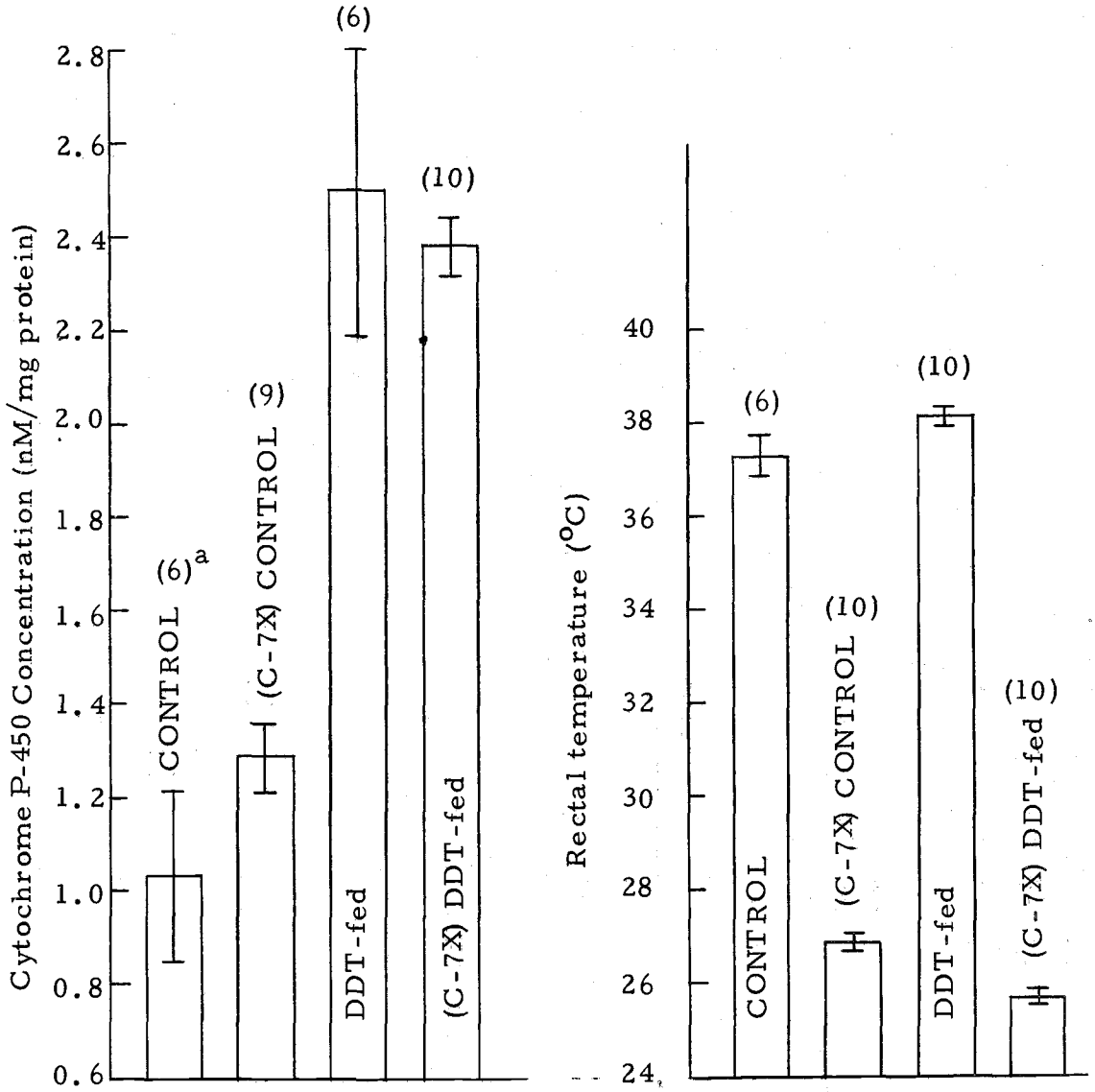

(C-7X) CONTROL
(C-7X) DDT-fed
HCOH produced (µg/g)
36
33
30
27
24
21
18
15
12
9
6
3
0
37°C
25°C
37°C
25°C
Incubation Temperature

Figure 6-3. Effect of spinal cord transection (C-7X) on the microsomal cytochrome P-450 concentration and the rectal temperature in control and DDT-fed (16 ppm X 8 wks) rats. Each bar represents the mean (± standard error) of from 6-10 animals. Note the cytochrome P-450 concentrations in control and DDT-fed rats were not significantly decreased ($P > 0.05$, Student's t-test) by spinal cord transection. The rectal temperatures were significantly decreased ($P < 0.05$) by this procedure in both groups of animals.

[a]The number in parentheses represents the number of animals per group.


Cytochrome P-450 Concentration (nM/mg protein)
2.8
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
(6)[a]
CONTROL
(9)
(C-7X) CONTROL
(6)
DDT-fed
(10)
(C-7X) DDT-fed
Rectal temperature (°C)
40
38
36
34
32
30
28
26
24
(6)
CONTROL
(10)
(C-7X) CONTROL
(10)
DDT-fed
(10)
(C-7X) DDT-fed

neither the surgical procedure nor the resultant hypothermia significantly lowered ($P > 0.05$) the cytochrome P-450 content in these cord-sectioned animals. The hepatic microsomal (100,000 x g) preparations from the transected control rats had a cytochrome P-450 concentration of 1.0 nM/mg, whereas the concentration of P-450 in the DDT-fed animals was induced to 2.5 nM/mg. These values were comparable to those generally measured in non-transected control and DDT-fed animals. Thus, the cord-sectioning probably did not result in protection against CCl_4-induced central necrosis by causing a prior reduction in the cytochrome P-450 content in the hepatic microsomes of transected animals.

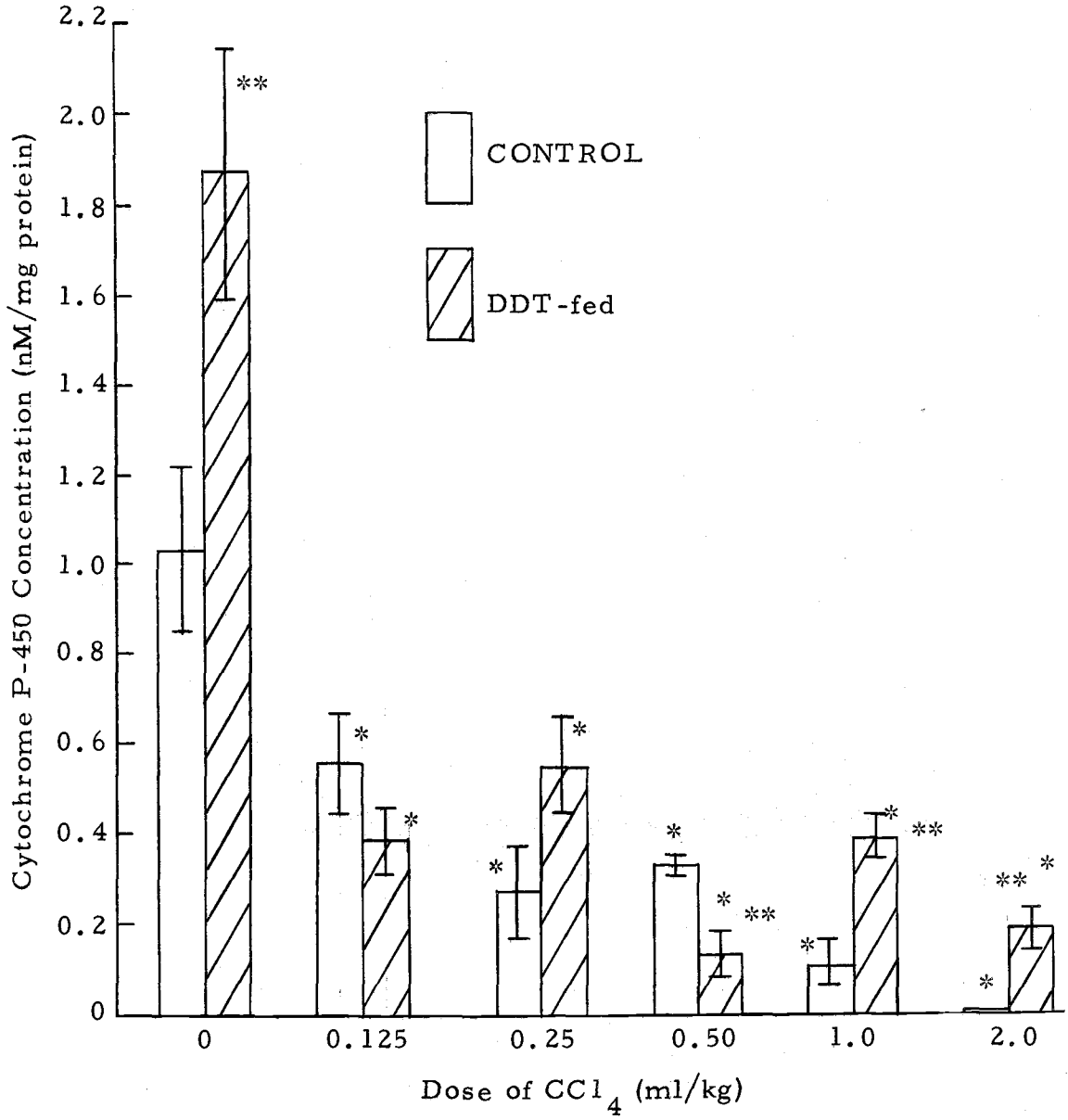
Effect of the DDT-CCl_4 Hepatotoxic Interaction Upon Cytochrome P-450

The effects of increasing doses of CCl_4 upon the hepatic microsomal cytochrome P-450 concentration in control and DDT-fed rats are illustrated in Figure 6-4. In the non-CCl_4-treated control and DDT-fed animals, the concentrations of cytochrome P-450 were 1.0 and 1.9 nM/mg, respectively. These mean values were significantly different ($P < 0.05$). After CCl_4 treatment in controls, there was a dose-related decline in the cytochrome P-450 concentrations, which decreased to 0 nM/mg after a dose of 2.0 ml/kg. In the DDT-fed animals, there was also a dose-related tendency for the hepatic microsomal cytochrome P-450 concentration to decrease after CCl_4.

Figure 6-4. Effect of dietary DDT (16 ppm X 8 wks) on the microsomal cytochrome P-450 response to increasing dosages of CCl_4. CCl_4 (0.125 - 2.0 ml/kg) was administered by gavage. Each bar represents the mean (± standard error) of at least 6 animals. Note the total decrease in P-450 concentration was greatest in the DDT-fed rats. However, the higher dosages of CCl_4, i.e. 1.0-2.0 ml/kg, produced the lowest absolute P-450 concentrations in the non-DDT-fed animals. The response of the cytochrome P-450 concentration was apparently dose-related to CCl_4 in both groups.

*Significantly different ($P < 0.05$, Student's t-test) from the mean of the respective control value.

**Significantly different ($P < 0.05$) from the mean of the respective non-DDT-fed group.


Cytochrome P-450 Concentration (nM/mg protein)
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
CONTROL
DDT-fed
0
0.125
0.25
0.50
1.0
2.0
Dose of CCl4 (ml/kg)

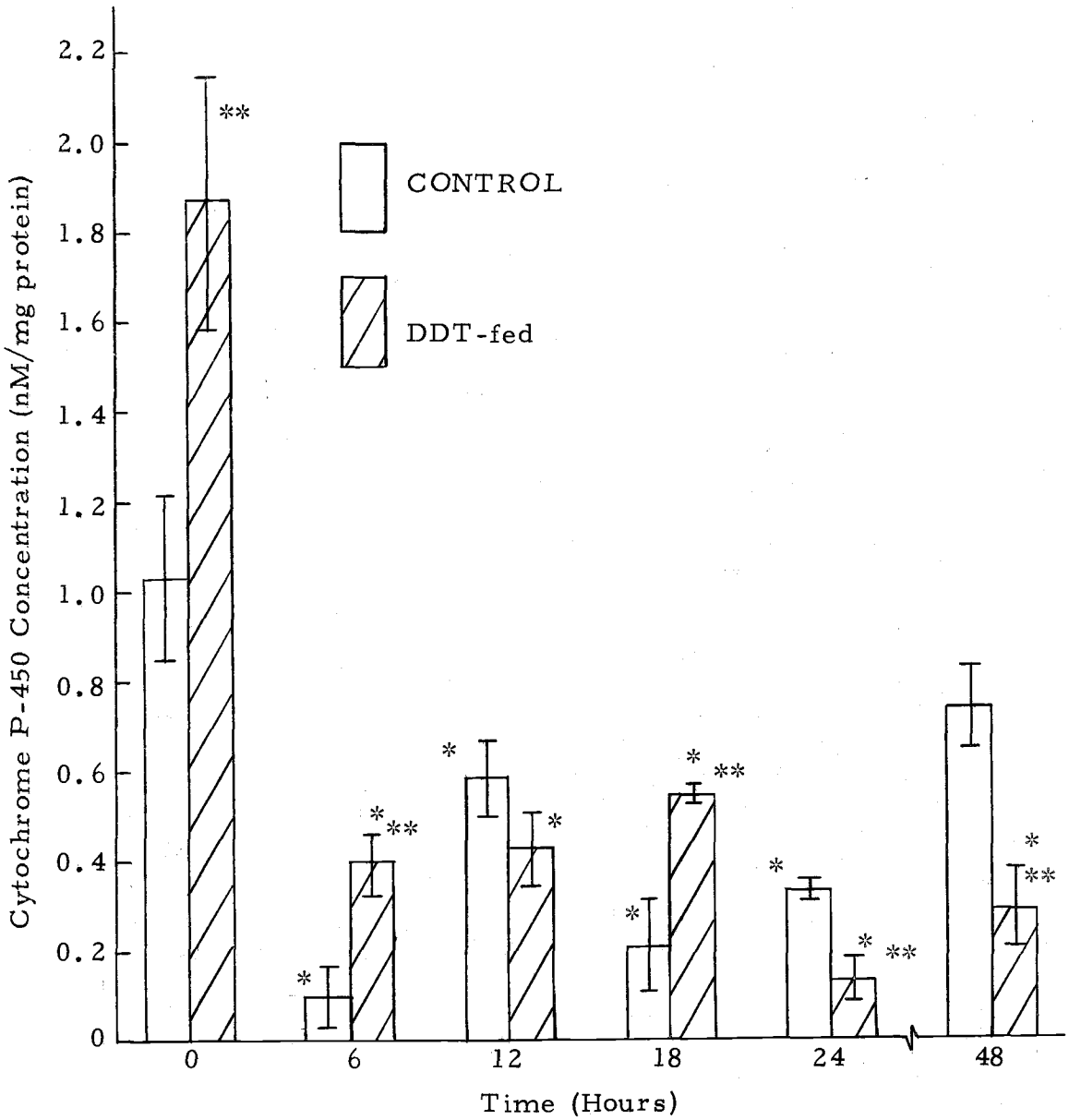
In fact, the lowest dose of CCl_4 employed, i.e., 0.125 ml/kg, produced a greater depression in the cytochrome P-450 concentration in the DDT-fed group (75%) than in the controls (45%). Moreover, in both the control and DDT-fed groups, the decreases in cytochrome P-450 were significant after all doses of CCl_4 employed. At doses of 0, 0.5, 1.0 and 2.0 ml/kg CCl_4, the mean cytochrome P-450 concentrations in the DDT-fed groups were significantly different ($P < 0.05$) from the respective control means.

The temporal patterns of cytochrome P-450 response to a challenging dose of 0.5 ml/kg CCl_4 in the control and DDT-fed rats are indicated in Figure 6-5. In the first six hours after CCl_4, there was a marked decline in the concentrations of cytochrome P-450 in both groups. The controls responded with a higher percent decrease (90%) in the cytochrome P-450 concentration six hours after CCl_4, whereas more cytochrome P-450 was destroyed in the DDT-fed animals (1.5 nM/mg). Following CCl_4 administration to the controls, the concentrations of cytochrome P-450 were significantly lower than the non-CCl_4-treated control mean at all times, with the exception of the 48 hour value. Thus, it was apparent that the cytochrome P-450 concentration had begun to regenerate between 24-48 hours after CCl_4 in the controls. In the DDT-fed animals, however, little change in the cytochrome P-450 concentration was noted between 6-18 hours, thereafter it declined to a low value at 48 hours. At all times after

Figure 6-5. Effect of dietary DDT (16 ppm X 8 wks) on the temporal response of the microsomal cytochrome P-450 concentration to 0.5 ml/kg CCl_4. CCl_4 was administered by gavage. Each bar represents the mean (± standard error) of at least 6 animals. Note the greatest decline in P-450 concentration occurred within the first 6 hr after CCl_4 administration in both non-DDT-fed and DDT-fed animals. In the non-DDT-fed rats, the P-450 concentration attained a low value at 18 hr, but at 24 and 48 hr it appeared to be returning toward the control value. In contrast, the P-450 concentration in the DDT-fed rats remained significantly decreased ($P < 0.05$) at 48 hr with respect to the control value and to the 48 hr value in the non-DDT-fed group.

*Significantly different ($P < 0.05$, Student's t-test) from the respective control mean.

**Significantly different ($P < 0.05$) from the mean of the respective non-DDT-fed group.


Cytochrome P-450 Concentration (nM/mg protein)
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
CONTROL
DDT-fed
0
6
12
18
24
48
Time (Hours)

CCl_4 in this group, the decrease in the cytochrome P-450 concentration was significant ($P < 0.05$), with respect to the non-CCl_4-treated DDT-fed group. In the DDT-fed groups, the mean cytochrome P-450 concentrations were significantly different ($P < 0.05$) from the means of the respective CCl_4-treated control groups at intervals of 0, 6, 18, 24 and 48 hours after CCl_4. Therefore, the effect of the DDT-CCl_4 hepatotoxic interaction upon the cytochrome P-450 concentration was an apparent compromise between the effects of induction and destruction, at least in the dose-response study. However, a larger amount of cytochrome P-450 was destroyed by CCl_4 in the DDT-fed animals, by virtue of the higher (induced) concentration of cytochrome P-450 present in this group prior to the CCl_4 challenge. Insofar as the concentration of cytochrome P-450 can be used as an indicator of hepatic damage, this damage seemed to worsen between 18-48 hours in the DDT-fed rats. This finding was in contrast to the situation observed in controls, which appeared to be rebounding from the hepatic damage between 18-48 hours. Therefore, it would seem that the process of hepatic regeneration was somewhat impaired in the DDT-fed animals, with respect to the controls. Thus, these differences in hepatic regenerative capacity could probably serve as an indication of the relative prognoses for recovery following a CCl_4 challenge in these two groups.

Effect of the DDT-CCl_4 Hepatotoxic Interaction Upon Liver Weight

The changes in liver weight were recorded and expressed as a percentage or ratio of the total body weight in Figure 6-6. In the controls, the liver weight responded in an apparent dose-related manner to CCl_4. The mean liver weights for the non-CCl_4-treated control and DDT-fed groups were 3.38 and 3.34%, respectively. After challenging controls with 0.125 or 0.25 ml /kg, but not after 0.5 ml/kg, the increases in liver weight were significant ($P < 0.05$). A sharp increase in the liver weight was noted in response to the next two higher doses, i.e., 1.0 and 2.0 ml/kg CCl_4. In the DDT-fed rats, the liver weight response to increasing doses of CCl_4 was similar, although there was a greater gain after 0.125 ml/kg in the latter group. The mean liver weights in the respective control and DDT-fed groups were not significantly different ($P > 0.05$) at any of the doses of CCl_4 employed.

The temporal patterns of liver weight response to 0.5 ml/kg CCl_4 were also studied in the control and DDT-fed animals. The results of these studies are illustrated in Figure 6-7. In the controls, the liver weight increased rapidly initially, and by 18 hours after CCl_4 a maximal value of 4.23% was attained. Thereafter, the control liver weights decreased to progressively lower mean values at 24 hours (3.85%) and 48 hours (3.5%). At 48 hours, however, the control

Figure 6-6. Effect of dietary DDT (16 ppm X 8 wks) on the liver weight response in rats challenged with increasing dosages of CCl_4. CCl_4 (0.125-2.0 ml/kg) was administered by gavage, and the liver weights were recorded 24 hr later. Each bar represents the mean (± standard error) of at least 6 animals. Note the liver weight increased markedly, and in a dose-related manner in both control and DDT-fed animals.

*Significantly different ($P < 0.05$, Student's t-test) from the respective control mean.

**Significantly different ($P < 0.05$) from the mean of the respective group receiving the CCl_4 treatment only.

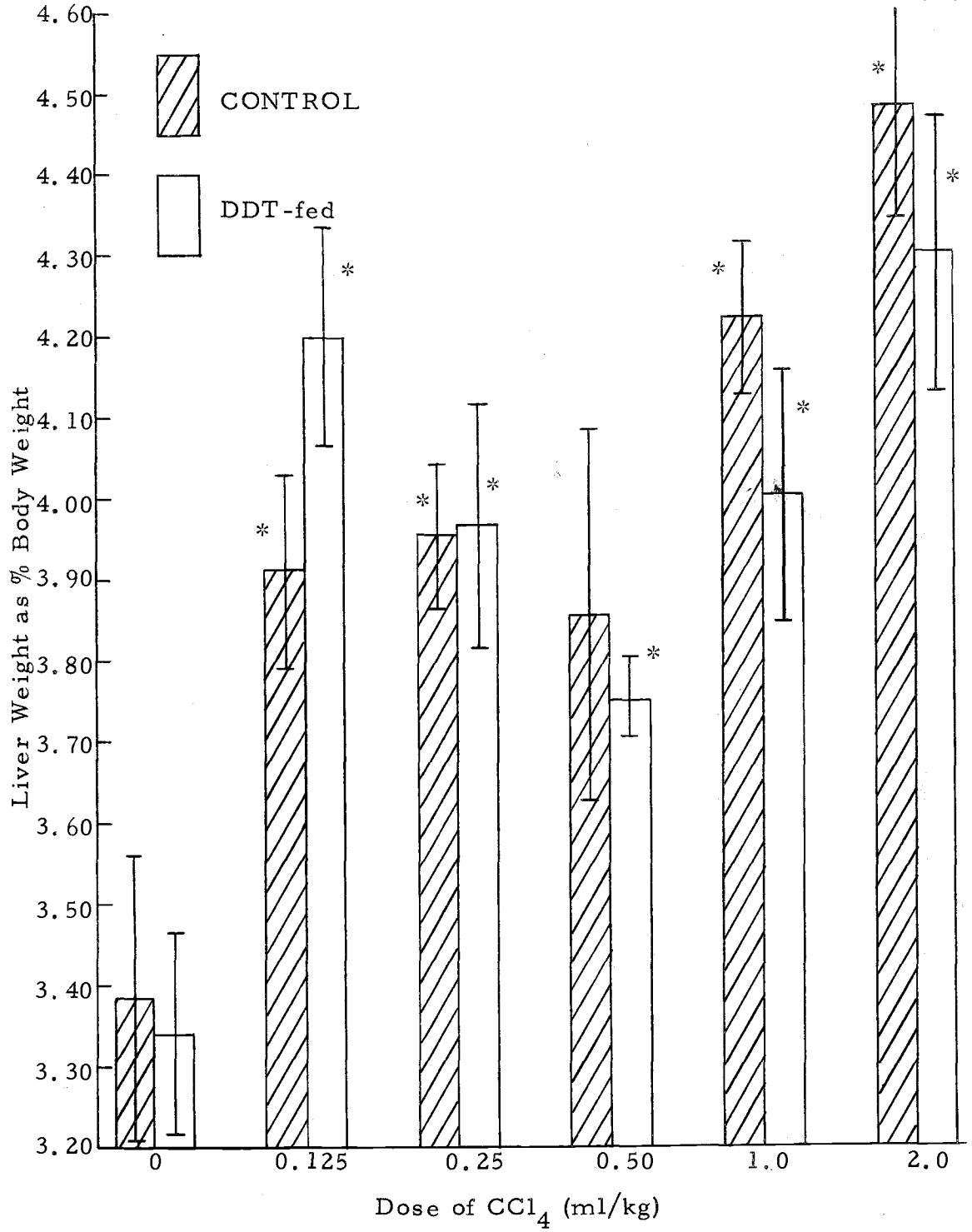
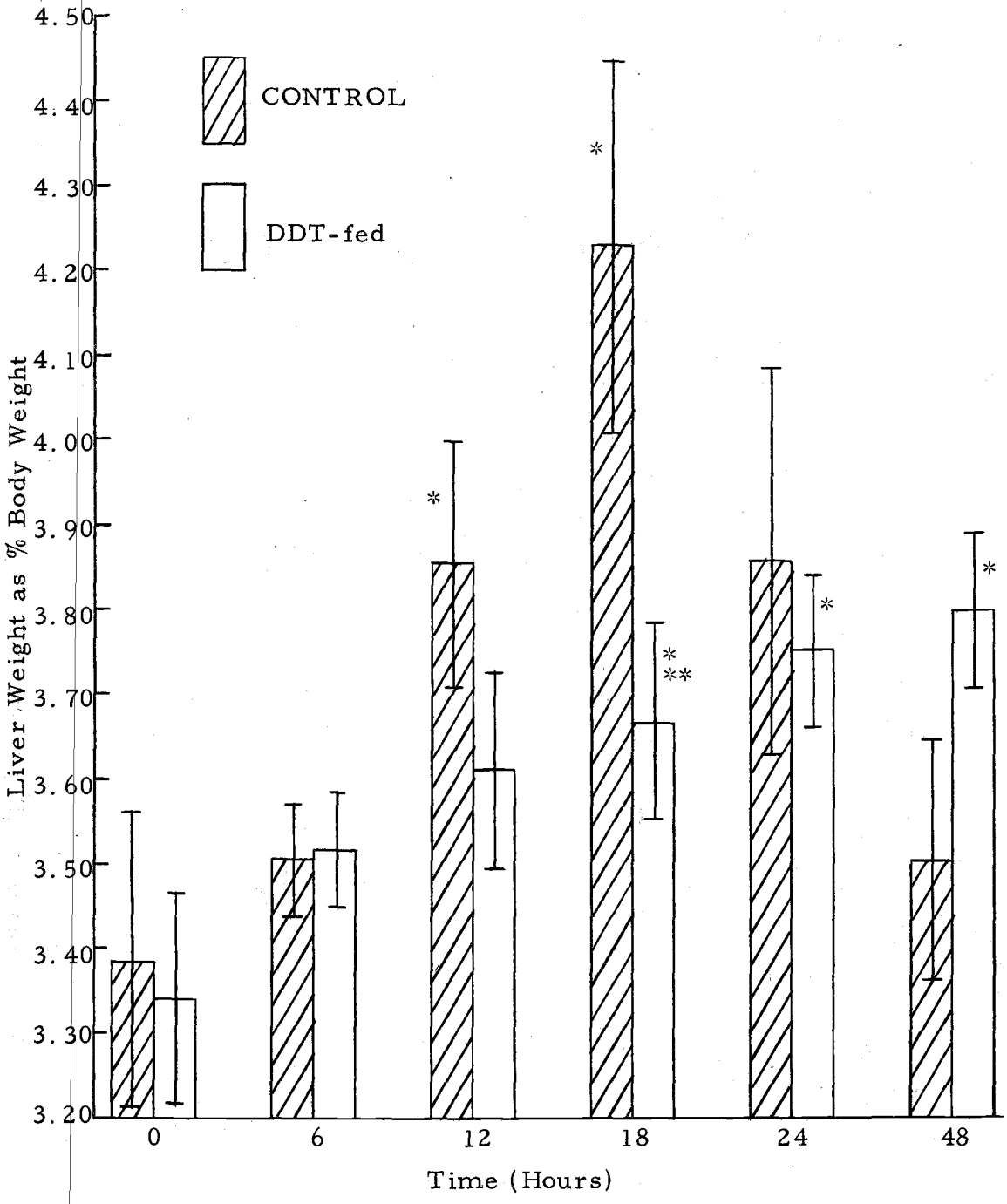

CONTROL
DDT-fed
Liver Weight as % Body Weight
4.60
4.50
4.40
4.30
4.20
4.10
4.00
3.90
3.80
3.70
3.60
3.50
3.40
3.30
3.20
0
0.125
0.25
0.50
1.0
2.0
Dose of CCl4 (ml/kg)

Figure 6-7. Effect of dietary DDT (16 ppm X 8 wks) on the temporal response of liver weight to 0.5 ml/kg CCl_4. CCl_4 was administered by gavage. Each bar represents the mean (± standard error) of at least 6 animals. Note the liver weight increased initially in the non-DDT-fed group to a maximal value of 4.2% at 18 hr after CCl_4. After 18 hr the liver weight decreased, and approached the control value of 3.4% by 48 hr. In the DDT-fed animals, however, the liver weight continued to increase at 48 hr following CCl_4 administration.

*Significantly different ($P<0.05$, Student's t-test) from the respective control mean.

**Significantly different ($P < 0.05$) from the mean of the respective group receiving the CCl_4 treatment only.


4.50
4.40
4.30
4.20
4.10
4.00
3.90
3.80
3.70
3.60
3.50
3.40
3.30
3.20
CONTROL
DDT-fed
Liver Weight as % Body Weight
0
6
12
18
24
48
Time (Hours)
*
*
*
**
*
*

liver weight was not significantly different ($P > 0.05$) from the respective non-CCl_4-treated control mean (3.38%). These results indicated that the control liver weight had returned essentially to a normal size by 48 hours after CCl_4. In contrast, in the DDT-fed animals the liver weights responded differently over a 48 hour period to the same dose of CCl_4 (0.5 ml/kg). In this latter group, the liver weight increase was more gradual, but continued to progress throughout the entire 48 hour temporal study. In fact, the maximal liver weight of 3.80% in the DDT-fed animals was recorded at 48 hours. This latter mean value was significantly different ($P < 0.05$) from the mean of the respective non-CCl_4-treated control group. Thus, unlike the situation in controls, in the DDT-fed group the liver weight continued to increase 48 hours after CCl_4. These results indicated that the hepatic response to CCl_4, in terms of an increase in liver weight, was of longer duration in the DDT-fed animals in comparison to the controls.

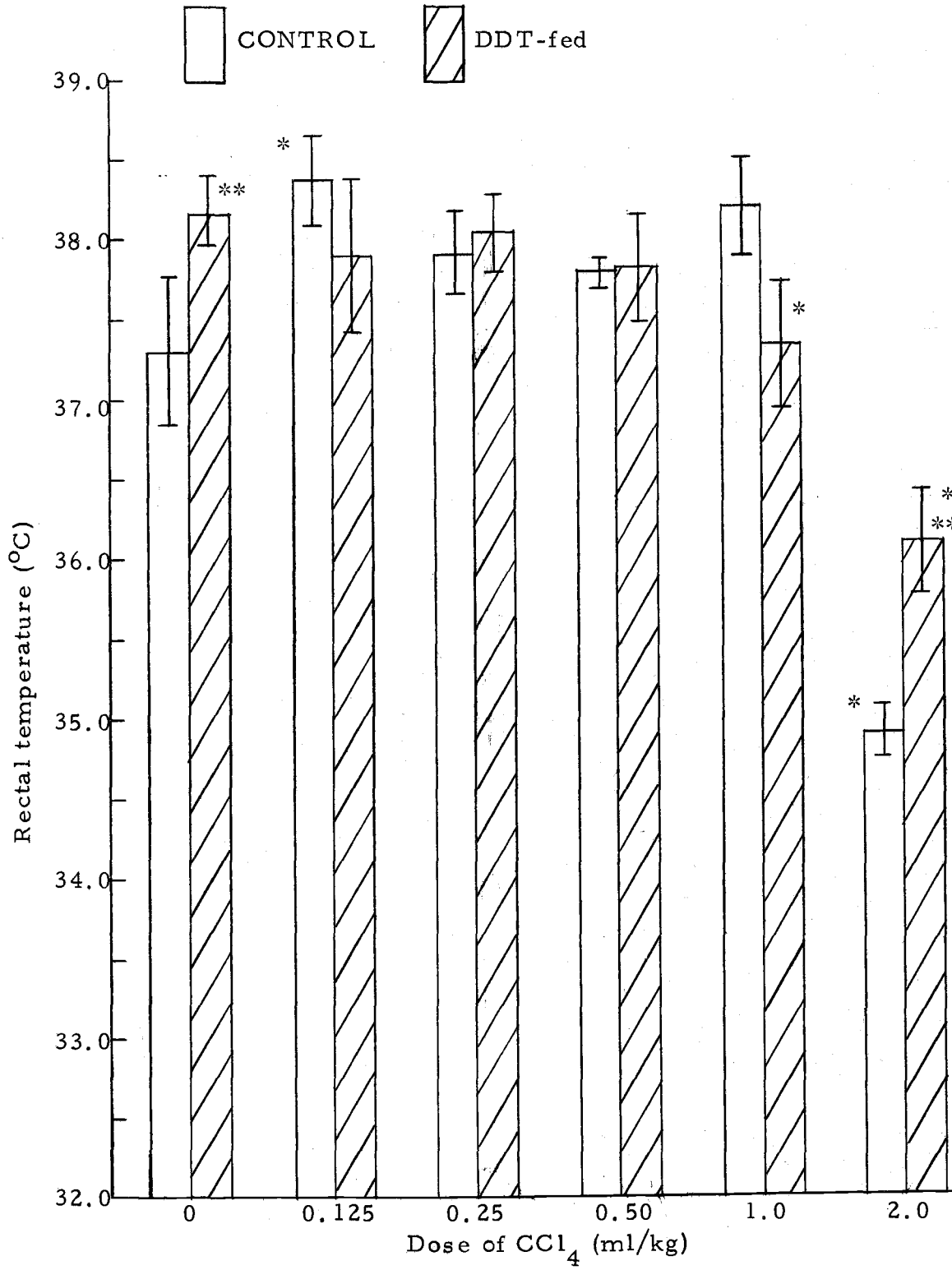
Effect of the DDT-CCl_4 Hepatotoxic Interaction Upon Rectal Temperature

The rectal temperature responses to increasing doses of CCl_4 in the control and DDT-fed groups are indicated in Figure 6-8. The mean rectal temperatures in the non-CCl_4-treated control (37.3°C) and DDT-fed (38.4°C) groups were significantly different ($P < 0.05$). In the controls, there was no apparent dose-response relationship

Figure 6-8. Effect of dietary DDT (16 ppm X 8 wks) on the rectal temperature response to increasing dosages of CCl_4. CCl_4 (0.125 - 2.0 ml/kg) was administered by gavage, and the rectal temperatures were recorded 24 hr later. Each bar represents the mean (± standard error) of at least 6 animals. Note the rectal temperature was significantly decreased ($P < 0.05$) at 1.0 and 2.0 ml/kg in the DDT-fed group, and at 2.0 ml/kg in the control group.

*Significantly different ($P < 0.05$, Student's t-test) from the respective control mean.

**Significantly different ($P < 0.05$) from the mean of the respective group receiving the CCl_4 treatment only.


CONTROL
DDT-fed
39.0
38.0
37.0
36.0
35.0
34.0
33.0
32.0
Rectal temperature (°C)
0
0.125
0.25
0.50
1.0
2.0
Dose of CCl4 (ml/kg)

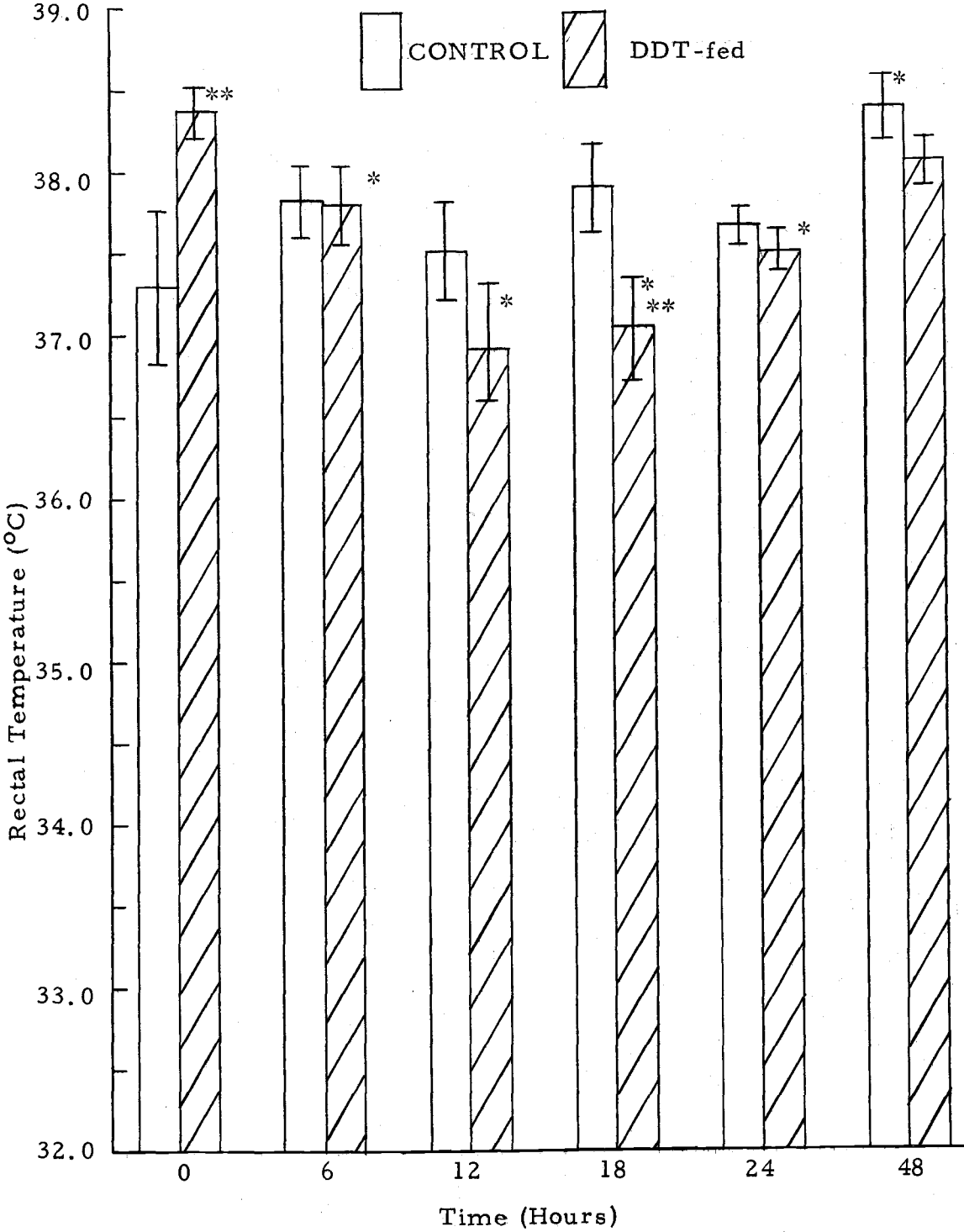
between the dose of CCl_4 employed and the rectal temperature response. After treating controls with 0.125 ml/kg CCl_4, a significant increase ($P < 0.05$) in rectal temperature was observed, however, significant differences were not observed after dosing with 0.25, 0.5, or 1.0 ml/kg. In the controls, 24 hours after a dose of 2.0 ml/kg CCl_4, the mean rectal temperature declined to 34.7°C. This latter value was significantly different ($P < 0.05$) from the mean rectal temperatures in the respective non-CCl_4-treated control group (37.3° C) and the respective DDT-fed group (36.1°C). In the DDT-fed animals, there was an apparent dose-related decline in rectal temperatures as the dose of CCl_4 was increased from 0.125 to 2.0 ml/kg. This response was maximal at 2.0 ml/kg, where a decrease of more than two degrees to 36.1°C was noted. In the DDT-fed animals, the decreases in rectal temperature were significantly different ($P < 0.05$) from the mean of the respective non-CCl_4-treated control group at doses of 1.0 and 2.0 ml/kg.

The results of the temporal studies of rectal temperature response to 0.5 ml/kg CCl_4 are shown in Figure 6-9. The mean rectal temperatures prior to CCl_4 treatment in control and DDT-fed rats were 37.3° and 38.38°C, respectively, and these values were significantly different ($P < 0.05$). After CCl_4 treatment in controls, only the 48 hour mean rectal temperature (38.4°C) was significantly ($P < 0.05$) increased with respect to the non-CCl_4-treated control

Figure 6-9. Effect of dietary DDT (16 ppm X 8 wks) on the temporal pattern of rectal temperature response to 0.5 ml/kg CCl_4. CCl_4 was administered by gavage. Each bar represents the mean (± standard error) of at least 6 animals. Note the rectal temperatures were significantly decreased ($P < 0.05$) at 6, 12, 18, and 24 hr after CCl_4 administration to the DDT-fed animals.

*Significantly different ($P < 0.05$, Student's t-test) from the respective control mean.

**Significantly different ($P < 0.05$) from the mean of the respective group receiving the CCl_4 treatment only.


CONTROL
DDT-fed
Rectal Temperature (°C)
39.0
38.0
37.0
36.0
35.0
34.0
33.0
32.0
0
6
12
18
24
48
Time (Hours)
**
*
*
*
**
*
*

mean. In the DDT-fed group, on the other hand, the mean rectal temperatures were significantly decreased ($P < 0.05$) from the respective non-CCl_4-treated control mean at 6, 12, 18 and 24 hours after CCl_4, but not at 48 hours.

Discussion

The effect of hypothermia in rats (produced by spinal cord transection) and/or lowering the incubation temperature in the *in vitro* drug metabolism studies from 37° to 25°C, was to decrease the rate of drug metabolism. The optimal temperature for the liver microsomal drug-metabolizing enzyme system, at least for those enzymes concerned with the oxidation of hexobarbital and ethylmorphine, was apparently nearer 37° than 25°C. The normal *in vivo* temperature for hepatic drug metabolism in rats is about 37°. In the cord-sectioned DDT-fed rats, the *in vitro* metabolism of ethylmorphine was significantly reduced when the incubation temperature was maintained at 25°, but was still equivalent at 25° to that of the control group at 37°. Thus, the cord-sectioned DDT-fed animals apparently had an ability to metabolize these substrates which was at least comparable to that of the intact control animals.

Some investigators believe that the activation (Slater, 1966) or metabolism (McLean *et al.*, 1966; Seawright *et al.*, 1967) of CCl_4 to a toxic metabolite is essential to the toxic mechanism of this agent.

McLean postulated that the potentiation of CCl_4 hepatotoxicity by acute DDT pretreatment of rats was based on the ability of DDT to stimulate the hepatic microsomal oxidative drug-metabolizing enzyme systems. In the present investigation, the SGOT activity measured in the spinal cord transected DDT-fed rats was nearly identical to that of the intact control group 24 hours after CCl_4 (Chapter V). Although both of these groups were treated with the same dose of CCl_4, only the cord-sectioned DDT-fed animals were protected histologically against the effects of CCl_4. At least two interpretations could be made to explain these observations. First, it was previously observed the cordotomized DDT-fed rats metabolized as much ethylmorphine in vitro at 25° as did the controls at 37°. According to McLean's hypothesis, if the CCl_4 had been metabolized to a similar extent in both of these groups, then the damage attained histologically should also have been comparable. Therefore, it could be argued that the in vivo metabolism of CCl_4 to a toxic intermediate metabolite did not occur in the cord-sectioned animals even though the potential for drug metabolism was present. The high concentration of SGOT measured in this latter group could have resulted from an increased hepatic synthesis of GOT, with subsequent leakage of small amounts of fluid having higher GOT concentration into the plasma. In the second interpretation of these data, the SGOT activities correlated better with the rate of in vitro ethylmorphine metabolism than did the histologic findings. Thus,

the intact control and transected DDT-fed groups both had SGOT activities of about 700 units 24 hours after 1.0 ml/kg CCl_4. It is difficult to explain the differences in hepatic histologic appearance in these two groups unless more than one mechanism is operative. Nevertheless, it is conceivable that the degree of membrane damage in the central parenchymal cells was comparable in both groups. If this were true, however, the histopathologic evidence was not a direct reflection of the amount of CCl_4 metabolized *in vivo* in both groups. Interpreted in this way, the results corroborate the hypothesis of McLean, since the capacity for CCl_4 metabolism in terms of drug metabolizing enzyme activity correlated with the degree of hepatotoxicity observed.

In this study, it was demonstrated that the operative mechanism of the protection afforded by spinal cord transection against CCl_4-induced hepatic necrosis was not related to a reduction in the cytochrome P-450 content in the hepatic microsomes. The concentrations of cytochrome P-450 in either the control or DDT-fed rats were unaltered by surgical transection of the spinal cord or the resulting hypothermia. The rate of ethylmorphine metabolism *in vitro* usually correlates with the cytochrome P-450 concentration in the microsomes (Sasame *et al.*, 1968). Therefore, the protection observed in these cord-sectioned groups must have been related to the reduced hepatic metabolism resulting from hypothermia, as Larson *et al.* (1965) have proposed.

A greater destruction of cytochrome P-450 was noted in the DDT-fed rats in comparison to the controls. The chronic pretreatment of rats with DDT via the diet produced an induction of the microsomal cytochrome P-450 concentration. Approximately 75% of this induced cytochrome P-450 concentration, or about 1.5 nM/mg, was destroyed within the first six hours after CCl_4. These results corroborated the observations of Sasame et al. (1968), who demonstrated a greater destruction of cytochrome P-450 within three hours after CCl_4 in rats pretreated with phenobarbital (70%), with respect to the non-induced animals. The results of the present study and those of Sasame indicate that CCl_4 is capable of rapidly binding the available concentration of cytochrome P-450, and producing a nearly complete destruction of this cytochrome within 3-6 hours after CCl_4. Thus, after six hours, the concentrations of cytochrome P-450 remaining in the microsomes in the control and DDT-fed groups were similar. In essence, however, the DDT-fed animals had lost a greater amount of P-450 at this time, due to the initial induced concentrations of this cytochrome. Therefore, at least in the dose-response study, the resultant concentration of cytochrome P-450 in the DDT-fed rats was an apparent compromise between the effects of induction and destruction of cytochrome P-450. Recently Sasame et al. (1968) demonstrated that the irreversible damage to cytochrome P-450 subsequent to CCl_4 treatment of rats was the probable cause of

the impairment of the NADPH-dependent oxidative drug metabolizing enzymes in the hepatic microsomes. The concentration of cytochrome P-450 was observed to increase and decrease in parallel with the drug metabolizing enzyme activity.

The temporal alterations in the cytochrome P-450 concentration correlated with the temporal development of CCl_4-induced hepatotoxicity in the control and DDT-fed rats. The relationship between the hepatic damage (serum transaminase activities, Chapters II and III) and functional impairment (BSP test, Chapter IV) and the temporal uptake of CCl_4 into the blood and livers has been extensively discussed above (Chapter V). Much of the cytochrome P-450 concentration (45%) was destroyed within six hours after CCl_4 administration in the control group, which closely paralleled the temporal attainment of maximal CCl_4 concentrations in the livers of these animals. Thus, the initial massive destruction of P-450 preceded the onset of detectable hepatic damage and functional impairment which began about six hours after CCl_4. In the control group the nadir of the cytochrome P-450 concentration occurred about 18 hours after CCl_4, whereas the greatest elevation in SGOT activity was measured at 36 hours, and a maximal BSP retention was noted at 24 hours. The hepatic microsomal content of cytochrome P-450 was essentially entirely replenished by 48 hours in the control group, and at this same time a distinct reduction in hepatic functional impairment and a decreased

SGOT activity were also measured. Thus, it appeared that the maximal control liver damage occurred about 18-24 hours after CCl_4, but that extensive repair and regeneration had taken place between 24-48 hours. In the DDT-fed animals, the massive destruction of cytochrome P-450 within the first six hours after CCl_4 also preceded the onset of potentiated hepatic damage and dysfunction, which was detectable about six hours after CCl_4. It was previously observed (Chapter V) that the maximal concentrations of CCl_4 attained in the blood and livers of the control and DDT-fed groups were comparable, and were measured about four hours after CCl_4. Apparently the concentration of CCl_4 in the blood declined more rapidly between 4-12 hours in the DDT-fed group, which could have indicated a more rapid metabolism of CCl_4 was taking place in vivo during this time interval. From six hours, the microsomal content of cytochrome P-450 continued to decline, and the potentiated hepatic damage and functional impairment continued to increase steadily throughout the 48 hour temporal study. Thus, the onset, development and prolongation of the potentiated CCl_4-induced hepatotoxicity was influenced by the concentrations of CCl_4 attained in the blood and livers, and paralleled the decreases in the cytochrome P-450 concentration. The ability of the livers to regenerate in response to the toxic agent was greatly retarded in the DDT-fed group, in comparison to the controls.

Following the administration of increasing doses of CCl_4 to

control or DDT-fed rats, the concentration of cytochrome P-450 decreased as the degree of hepatotoxicity increased. In the controls, a progressive decline in the cytochrome P-450 concentration was observed as the dose of CCl_4 was increased from 0.125 to 1.0 ml/kg. These cytochrome P-450 changes apparently correlated with a steadily increasing serum transaminase activity and BSP retention. When the dose was increased from 1.0 to 2.0 ml/kg, the cytochrome P-450 content in the hepatic microsomes declined to 0 nM/mg. Previously it was shown that this dose of CCl_4 (2.0 ml/kg) produced a considerable elevation of the serum enzyme activities and a tremendous impairment of hepatic function in the control animals. In the DDT-fed group, the degree of potentiation of CCl_4-induced hepatotoxicity also increased in a dose-related manner as the dose of CCl_4 was increased from 0.125 to 1.0 ml/kg. The severity of the potentiated hepatotoxic response correlated closely with the amount of cytochrome P-450 destroyed at these doses. In the DDT-fed animals, the cytochrome P-450 loss at a dose of 2.0 ml/kg was comparable to the amount of cytochrome P-450 destroyed in the control group. The potentiating effect of dietary DDT could not be demonstrated at 2.0 ml/kg CCl_4, due to the severity of the lesion resulting from that dose of CCl_4 given alone in the control animals. Undoubtedly, the temporal differences in regeneration observed in the control and DDT-fed rats in response to CCl_4 would affect the dose-response relationship at all doses of CCl_4 employed.

There are at least two alternative explanations for the correlation of a decreasing P-450 concentration in the dose-response and temporal studies with the concurrent increases in hepatotoxicity. Obviously, the measurement of cytochrome P-450 destruction could be considered as merely another index of hepatotoxicity. In this context, the cytochrome P-450 concentration at any time interval after CCl_4, or after any dose of CCl_4, should decrease in parallel with an increase in hepatic damage. The amount of destruction of cytochrome P-450 correlated with the degree of hepatotoxocity observed, and the massive cytochrome P-450 destruction preceded the onset of the potentiated CCl_4-induced hepatic damage in the DDT-fed group. Therefore, it is possible that the concentration of cytochrome P-450 and its associated NADPH-linked oxidative drug-metabolizing enzyme system plays a more integral and causative role in the mechanism of CCl_4-induced hepatic necrosis. Phenobarbital, another enzyme inducer, also potentiated the CCl_4 hepatotoxicity, and this agent is known to increase the microsomal content of cytochrome P-450 (Garner et al., 1969). It is now believed that an interaction of CCl_4 with cytochrome P-450 in vivo could effect the metabolism (McLean et al., 1969) or activation (Slater, 1966) of CCl_4 to a toxic metabolite (CCl_3?) as the cytochrome P-450 is destroyed. Or the binding of CCl_4 with cytochrome P-450 may prevent oxygen utilization by the NADPH-dependent mixed function oxidase system and shift it

into NADPH-dependent lipoperoxidation. Either the increased concentration of a free-radical metabolite of CCl_4 or oxygen could promote endogenous free radical formation, with the resulting lipoperoxidative attack on the membranes of the endoplasmic reticulum. Recknagel (1967) postulated that the toxicity of CCl_4 is mediated via a lipoperoxidative destruction of the unsaturated fatty acid side chains of the lipid portions of the lipoprotein membranes by free radicals. The two mechanisms proposed above, which are based on the binding of CCl_4 with cytochrome P-450, could both account for the protective effect of cord-sectioning against CCl_4-induced hepatic necrosis. The hypothermia produced by spinal cord transection was shown to decrease the rate of oxidative N-demethylation of ethylmorphine *in vitro*, and a similar decrease in CCl_4 metabolism could also be operative *in vivo*. The protection could also be mediated by hypoxia in the hepatic parenchymal cells of the centrilobular zone. Larson *et al.* (1965) have demonstrated a dramatic decline in oxygen consumption by rats having undergone cord-sectioning or cold-water immersion.

DDT and CCl_4 produce the common effect of liver hypertrophy, but have very dissimilar patterns of enzyme activity changes (Platt and Cockrill, 1967, 1969). CCl_4 inhibited microsomal protein synthesis, decreased microsomal drug metabolizing enzyme activity, decreased glucose 6-phosphatase and raised glucose 6-phosphate

dehydrogenase activities. DDT produced exactly the opposite changes in enzyme activity in all cases, except that it also produced a decreased glucose 6-phosphatase activity. Meldolesi (1967) believed that agents with diverse biochemical spectra of actions could produce the common effect of liver enlargement by acting as inducers to stimulate the proliferation of the smooth endoplasmic reticulum (SER). He suggested that such an adaptive response would be effective if protein synthesis was also increased, but ineffective in cases where protein synthesis was inhibited.

In the present study, a dietary concentration of 16 ppm DDT fed to rats for eight weeks did not produce an increase in liver weight. Hoffman et al. (1970) noted that liver weight in rats did not increase after DDT feeding until a concentration of DDT was attained which could not be metabolized by the existing levels of drug metabolizing enzymes. They found that the liver weight increased in proportion to the dose at dietary concentrations of 128-512 ppm DDT administered for two weeks, but observed no effect below 128 ppm. In view of their results, the liver weight response in DDT-fed rats in the present investigation was not surprising.

The CCl_4-induced hepatic hypertrophic response in control rats could have been influenced by at least three contributing factors: 1) water imbibition, 2) triglyceride accumulation, and 3) an adaptive proliferation of the SER. The histologic evidence of extensive central

cloudy swelling and midzonal hydropic degeneration indicated that the uptake of water, particularly in the centrilobular parenchymal cells, was marked after CCl_4. The increase in the hepatic triglyceride concentration after CCl_4 intoxication in rats has been well-documented, both in vitro (Heimberg et al., 1962) and in vivo (Poggi and Paoletti, 1967; Klaassen and Plaa, 1969). Lombardi (1965) reported finding concommitant decreases in serum triglycerides along with increases in hepatic triglycerides. He concluded that the inhibition of hepatic protein synthesis, resulting from the CCl_4-induced swelling and disruption of the SER, prevented the formation and release of triglycerides as lipoproteins. Since SER destruction occurs after CCl_4, it is doubtful that the adaptive response played a significant role in the control liver weight response. Of the three factors considered above, the uptake of water probably accounted for the highest percentage of the weight gain.

The temporal response of the liver weight to CCl_4 in the DDT-fed rats correlated mainly in terms of its prolongation with the temporal changes measured using the other indices of hepatic damage. It was evident that prior DDT feeding altered the liver weight response to CCl_4. Since CCl_4 hepatotoxicity was potentiated by dietary DDT pretreatment, a larger weight gain response than that observed in controls was expected. Instead the increase in liver weight was more gradual and less severe, but prolonged. In the DDT-fed group, the

extensive hepatocellular destruction by CCl_4 could have resulted in losses of water, proteins and lipids from the liver. The loss of these materials through leakage would tend to decrease the liver weight, and thus could offset the increase in liver weight resulting from water and triglyceride accumulation. It is also possible that the adaptive response played a more important role in the liver weight response of the DDT-fed animals. The initial drug metabolizing enzyme activity was higher in these animals than in controls. Hence, the adaptive response could have been slower, resulting in a more gradual increase in liver weight. In the context of this hypothesis, as more cytochrome P-450 (and its associated NADPH-linked drug metabolizing enzyme system) was destroyed, the liver weight would continue to increase in an effort to re-instate the initial drug metabolizing enzyme activity. Such a proliferative response in the DDT-fed animals would have to occur in hepatocytes involved in regeneration, since SER membranes in the injured cells would have been destroyed by CCl_4.

General biochemical mechanisms involved in hepatic regeneration after injury mainly by partial hepatectomy, but which are also involved to a large extent in regeneration after toxic injuries, have been reviewed by Stenger and Confer (1966) and by Bucher (1967). The effects on hepatic regeneration after hepatectomy in rats induced with phenobarbital (Chiesara, Conti and Meldolesi, 1970) and DDT (Ortega, 1969) have also been described. Ortega demonstrated that

there was a larger proliferative response in the SER in DDT-fed rats than in controls during the regenerative response. Chiesara and co-investigators believed that the prolonged microsomal induction occurring in induced animals (phenobarbital or DDT-pretreated) was due to a decreased microsomal breakdown. Barker, Arcasoy and Smuckler (1969) compared the regeneration of cytochrome P-450 and microsomal oxidative N-demethylation in rats receiving partial surgical or chemical (2.5 ml/kg CCl_4) hepatectomy. Regeneration was more severely impaired in the CCl_4-treated animals, which had an initial 70% reduction in cytochrome P-450, compared to a 30% decline in the surgically hepatectomized animals. In the former group, the cytochrome P-450 concentration was still significantly depressed by 120 hours after CCl_4.

In the present study, the cytochrome P-450 concentration continued to decline at 48 hours in the DDT-fed group, and thus was similar to the response observed in controls treated with 2.5 ml/kg CCl_4 by Barker et al. (1969). In Chapter IV, it was observed that the potentiation in DDT-fed rats was not demonstrable at doses of CCl_4 approaching 2.0 ml/kg, due to the severe degree of hepatic damage resulting from such doses of CCl_4 given alone. Thus, if the present studies had been extended to 120 hours post-CCl_4 exposure, the regeneration of cytochrome P-450 in the DDT-fed animals probably would have followed a temporal pattern similar to that observed by Barker et al.

A high dose of 2.0 ml/kg CCl_4 significantly lowered the 24 hour rectal temperatures in control and DDT-fed animals. This high dose probably depressed the central nervous system centers responsible for temperature regulation. Also, peripheral cardiovascular changes could have resulted in shock. Prior to treatment with CCl_4, the mean rectal temperature in the DDT-fed rats was significantly higher than that in controls. This elevation was probably too small, however, to effect a potentiation of CCl_4-induced hepatotoxicity through a mechanism opposite to the protective action of hypothermia. In the temporal study, core temperatures were significantly depressed between 6-24 hours after CCl_4 only in the DDT-fed group. It is unknown whether this hypothermic effect was caused by the hepatic or extrahepatic action of CCl_4 in these animals.

VII. THE USE OF VARIOUS PHARMACOLOGIC AGENTS TO INHIBIT THE DDT-CCl_4 HEPATOTOXIC INTERACTION

Introduction

These studies were designed to provide some insight into the mechanism of the potentiation of CCl_4-induced hepatotoxicity by dietary DDT pretreatment in rats. It was previously shown (Chapter VI) that the interaction of CCl_4 with cytochrome P-450, which resulted in the destruction of this cytochrome, could also have resulted in the metabolism (McLean et al., 1969) or activation (Slater, 1966) of CCl_4 to a toxic form. It was believed that the judicious use of β-ethyl-aminoethyl diphenylpropylacetate (SKF 525-A), a pharmacologic inhibitor of microsomal drug metabolism that binds with cytochrome P-450, could help to clarify the role of cytochrome P-450 in the potentiation effect. High doses of this inhibitor could conceivably bind most of the cytochrome P-450 synthesized during the process of enzyme induction by dietary DDT, thus making the cytochrome P-450 unavailable for binding with CCl_4. In this way, the metabolism of CCl_4 to a toxic intermediate, which was supposedly enhanced after dietary DDT pretreatment, could be inhibited. Further, if this inhibition did not occur as predicted, then this finding would give indirect evidence that the potentiated CCl_4-induced hepatotoxicity was independent of the cytochrome P-450 concentration in the hepatic

microsomes. Several groups of investigators have used a similar reasoning process in their experiments with non-induced rats. Slater (1966) reported that pretreatment of rats with SKF 525-A protected the animals from the CCl_4-induced hepatic necrosis. Castro et al. (1968) corroborated that this protection existed at three hours, but not at 24 hours after CCl_4. In their study, SKF 525-A also prevented the massive destruction of cytochrome P-450 and the decrease in oxidative N-demethylation of ethylmorphine usually detected within three hours after CCl_4. Seawright et al. (1967) demonstrated that SKF 525-A reduced the effect of CCl_4 of decreasing the incorporation of ^{14}C-leucine into protein by liver slices in vitro. Thus, the present utilization of SKF 525-A to block the potentiation of CCl_4-induced hepatotoxicity by dietary DDT seemed to be a rational approach in assessing the role of cytochrome P-450 in potentiation.

Wei et al. (1971) have recently indicated that the potentiating effect of ethanol upon the CCl_4-induced hepatotoxicity was mediated by the sympathetic nervous system and the release of norepinephrine. They found that pentolinium, a ganglionic blocking agent, protected against the ethanol-induced potentiation. They surmized that pentolinium inhibited the sympathomimetic action of ethanol. Stavinoha and Rieger (1966) reported that an oral dose of 100 mg/kg DDT increased the urinary excretion of norepinephrine, and they speculated that this norepinephrine was probably released from the sympathetic

nerve endings. At a higher dosage of DDT, i.e., 150 mg/kg, Stavinoha et al. showed that the urinary excretion of epinephrine was also increased, which was probably due to a release of this hormone from the adrenal medulla. It is apparent that DDT, acting as a stressor in the rat, can stimulate the sympathetic nervous system and the ensuing release of epinephrine and norepinephrine. Conceivably, the potentiation of CCl_4-induced hepatotoxicity by dietary DDT could be mediated by a centrally-directed release of norepinephrine. If this were true, then the ganglionic blocking agent mecamylamine should inhibit the potentiation by preventing norepinephrine release. If potentiation was not altered by mecamylamine pretreatment, then this would provide indirect evidence that the sympathomimetic properties of DDT were not involved in the potentiation effect. In order to obtain a pronounced effect on norepinephrine and epinephrine release, a high oral dose of DDT, i.e., 150 mg/kg, was utilized instead of chronic dietary pretreatment with DDT. This dose of DDT was chosen from the study of Stavinoha et al.

Certain barbiturates and other drugs are known to induce the synthesis of porphyrins by stimulating the formation of the enzyme delta-aminolevulinic acid synthetase in the liver mitochondria. Conceivably, this mitochondrial induction of porphyrins could be related to the drug-induced synthesis of cytochrome P-450 observed with these agents (Conney, 1967). Therefore, the induction of hepatic

microsomal cytochrome P-450 and drug metabolizing enzymes occurring after dietary DDT pretreatment could be related to the induction of porphyrin biosynthesis. Lead is known to block the porphyrin biosynthetic pathway in at least two places (Kreimer-Birnbaum and Grinstein, 1965). Cress (1970) demonstrated a dose-related decrease in the cytochrome P-450 concentration after chronic lead pretreatment in mice. Therefore, the chronic pretreatment of rats with lead should block the induction of cytochrome P-450 in a similar fashion. If the induction of cytochrome P-450 and drug metabolism in DDT-fed rats could be inhibited by lead, then the role of cytochrome P-450 in potentiation could be further clarified. The exact mechanisms operative in the process of microsomal enzyme induction by various agents have not been completely elucidated.

In the present study, the pharmacologic agents SKF 525-A (drug metabolism inhibitor), mecamylamine (ganglionic blocking agent) and chronically-administered lead (porphyrin biosynthesis inhibitor) were utilized to inhibit the potentiating effect of DDT on the CCl_4-induced hepatotoxicity. The primary objective was to clarify the mechanism involved in potentiation.

Methods

In the present study, all of the following methods were employed: SGOT activity (Reitman et al., 1957); BSP test (Kutob et al., 1962a);

cytochrome P-450 (Omura et al., 1964); in vitro hexobarbital metabolism (Cooper et al., 1955); and histopathology. All of these methods have been previously described in detail in this dissertation.

Outline of the Experiment

SKF 525-A. SKF 525-A was dissolved in 0.9% saline, and administered by i.p. injection. DDT was dissolved in corn oil. In preliminary studies, pretreatment with 50 mg/kg SKF 525-A for two hours significantly ($P < 0.05$) increased the hexobarbital sleeping time in rats. The same pretreatment schedule used in DDT-fed animals demonstrated the ability of SKF 525-A to inhibit the in vivo hexobarbital metabolism in animals having induced drug metabolizing enzyme activities. In this latter group, the sleeping times were increased about nine-fold with respect to non-SKF 525-A-pretreated animals (unpublished data). The pretreatment schedule with SKF 525-A that was used in the present study was modified slightly according to the procedure employed successfully by Castro et al. (1968). Fifteen minutes after the administration of SKF 525-A (50 mg/kg, i.p.) to control or DDT-fed (16 ppm x 8 wks) rats, CCl_4 (1.0 ml/kg) was administered by gavage. In the acute study, DDT (100 mg/kg) was administered by gavage 24 hours prior to CCl_4 (1.0 ml/kg). Liver sections were taken at intervals of 6-24 hours after CCl_4, and prepared for histopathology.

Mecamylamine. Mecamylamine was dissolved in 0.9% saline. DDT was dissolved in corn oil. Rats were pretreated with a single i.p injection of mecamylamine (10 mg/kg) 30 minutes prior to acute DDT (150 mg/kg) administration by gavage. Twenty-four hours after DDT was given, CCl_4 (0.5 ml/kg) was administered by gavage. This pretreatment schedule was similar to that employed by Wei et al. (1971). The dose of mecamylamine employed was sufficient to produce a strong ganglionic blockade (L. J. Weber, personal communication, 1971). Blood and liver samples were taken 24 hours after CCl_4 for subsequent analyses of SGOT activity, BSP retention, and histopathology. The treatment groups studied were: no treatment, mecamylamine only, CCl_4 only, and DDT + CCl_4. An adequate amount of information for DDT-treated controls was available from previous experiments.

Lead. Lead was administered chronically via the drinking water, which consisted of lead acetate dissolved in laboratory tap water. Glacial acetic acid (0.1 ml/l) was added to aid in the dissolution of the lead acetate. The calculated concentration of the lead ion (Pb^{++}) in the drinking water was 300 ppm. Lead-containing drinking water was administered concurrently with feed containing 16 ppm DDT for eight weeks. The animals were allowed free access to food and water during the exposure period. At the end of the exposure period, subgroups of 5-8 animals were given CCl_4 (0.25 ml/kg) by

gavage. Twenty-four hours after CCl_4, blood and liver samples were taken for analyses of plasma BSP retention, cytochrome P-450, in vitro hexobarbital metabolism and histopathology. The treatment groups studied were: no treatment, lead only, DDT only, CCl_4 only, lead + DDT, lead + CCl_4, DDT + CCl_4 and lead + DDT + CCl_4

Results

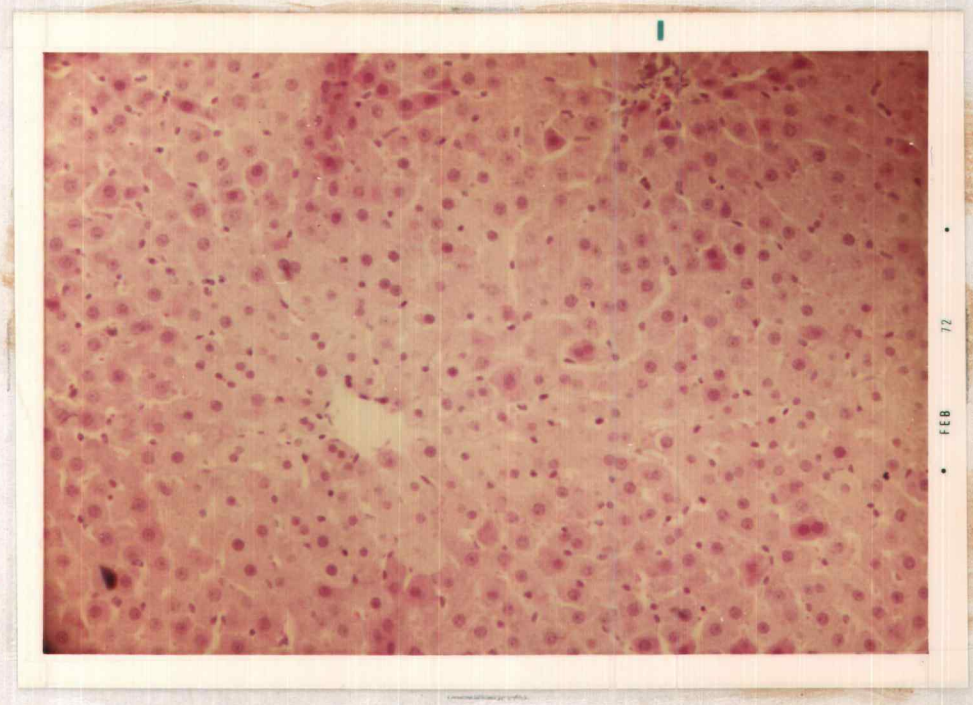
SKF 525-A

The histologic evidence of hepatic damage 24 hours after CCl_4 in control and DDT-fed rats was previously described in Chapter III. Pretreating controls with SKF 525-A provided some degree of protection against CCl_4-induced hepatic necrosis. This liver section is shown in Figure 7-1. The most obvious histologic change was the loss of the characteristic inter- and intralobular spatial relationships and general architectural pattern of the liver, resulting from SKF 525-A treatment. The appearance of midzonal balloon cells gave evidence of hydropic degeneration. The parenchymal cells generally appeared healthy, with well-defined cellular boundaries. The overall effect of SKF 525-A pretreatment in controls was a marked degree of protection against CCl_4-induced hepatic necrosis.

It was previously observed in Chapter IV that the onset of the potentiated hepatic damage and functional impairment occurred about six hours after CCl_4 in the DDT-fed animals. Therefore, it was

Figure 7-1. Liver section taken from an SKF 525-A (50 mg/kg) - pretreated rat 24 hours after the administration of 1.0 ml/kg CCl_4 by gavage. SKF 525-A was dissolved in 0.9% saline solution and given intraperitoneally 15 min prior to the CCl_4 challenge. Note the architectural pattern of the central zone was disrupted, which was accompanied by cloudy swelling and a loss of the cytoplasmic staining properties of the hepatocytes in that area. Note the absence of midzonal balloon cells and particularly the reduction in centrilobularly-oriented coagulative necrosis usually observed in CCl_4-treated rats. H and E stain (400X)

Figure 7-2. Liver section taken from a DDT (100 mg/kg) -pretreated rat 11 hours after the administration of 1.0 ml/kg CCl_4 by gavage. DDT was dissolved in corn oil and administered by gavage 24 hours prior to the CCl_4 challenge. Note the extensive central cloudy swelling and necrosis along with midzonal ballooning, which is characteristic of the potentiated CCl_4-induced lesion. H and E stain (400X)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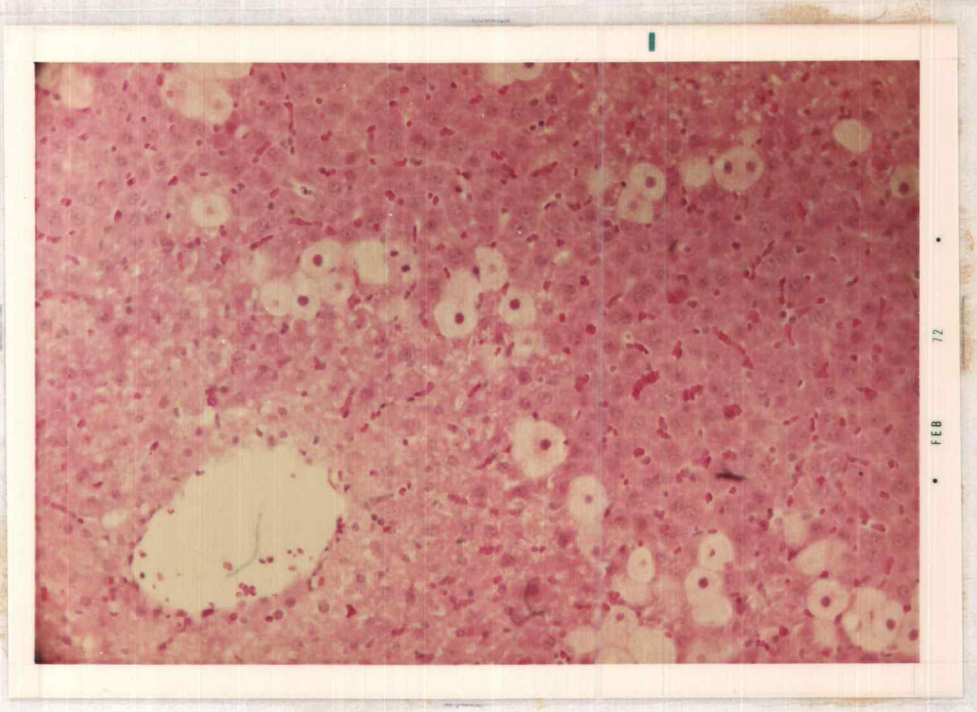
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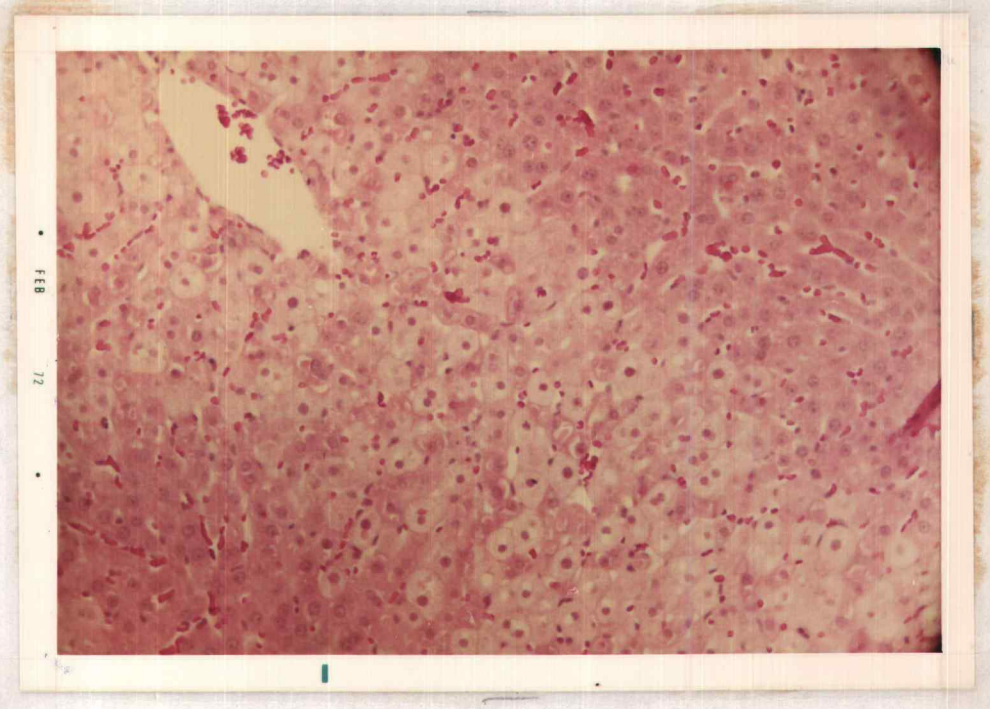
difficult to detect gross hepatic damage at six hours in those rats. In the present study, the histological assessment of hepatic damage six hours after CCl_4 was also difficult in the DDT-fed animals, regardless of whether they had been pretreated with SKF 525-A. Thus, it was unknown whether the lack of evident damage in DDT-fed rats receiving the SKF 525-A pretreatment actually represented protection. Therefore, these liver sections are not shown. When acute pretreatment with DDT was employed instead of chronic dietary exposure, SKF 525-A protected against the CCl_4-induced hepatic necrosis 11 hours after CCl_4. The liver section taken from an animal not pretreated with SKF 525-A 11 hours after CCl_4 is shown in Figure 7-2. The potentiated CCl_4-induced hepatic lesion was manifested by a severe centrilobularly-oriented submassive coagulative necrosis. There was also evidence of hemorrhagic changes. The liver illustrated in Figure 7-3 was taken from an SKF 525-A-pretreated rat. There was considerable central cloudy swelling, midzonal hydropic degeneration and ballooning. Nevertheless, the degree of centrilobular necrosis was greatly reduced in comparison to the situation in non-SKF 525-A-pretreated animals. The overall result of pretreating induced rats with SKF 525-A was a marked histologic evidence of protection against the potentiated CCl_4-induced hepatic necrosis.

Figure 7-3. Liver section taken from a rat pretreated with DDT (100 mg/kg) and SKF 525-A (50 mg/kg), 11 hours after the administration of 1.0 ml/kg CCl_4 by gavage. DDT was dissolved in corn oil and administered by gavage 24 hours before CCl_4. SKF 525-A was dissolved in 0.9% saline and injected i.p. 15 minutes prior to the CCl_4 challenge. There was a loss of the characteristic basophilic stipling properties in the cytoplasm in the centrilobular parenchymal cells as well as a loss of the normal hepatic cellular arrangement. Note the reduction in central necrosis afforded by SKF 525-A pretreatment, in comparison to the degree of hepatic damage noted in Figure 7-2. H and E stain (400X)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Mecamylamine

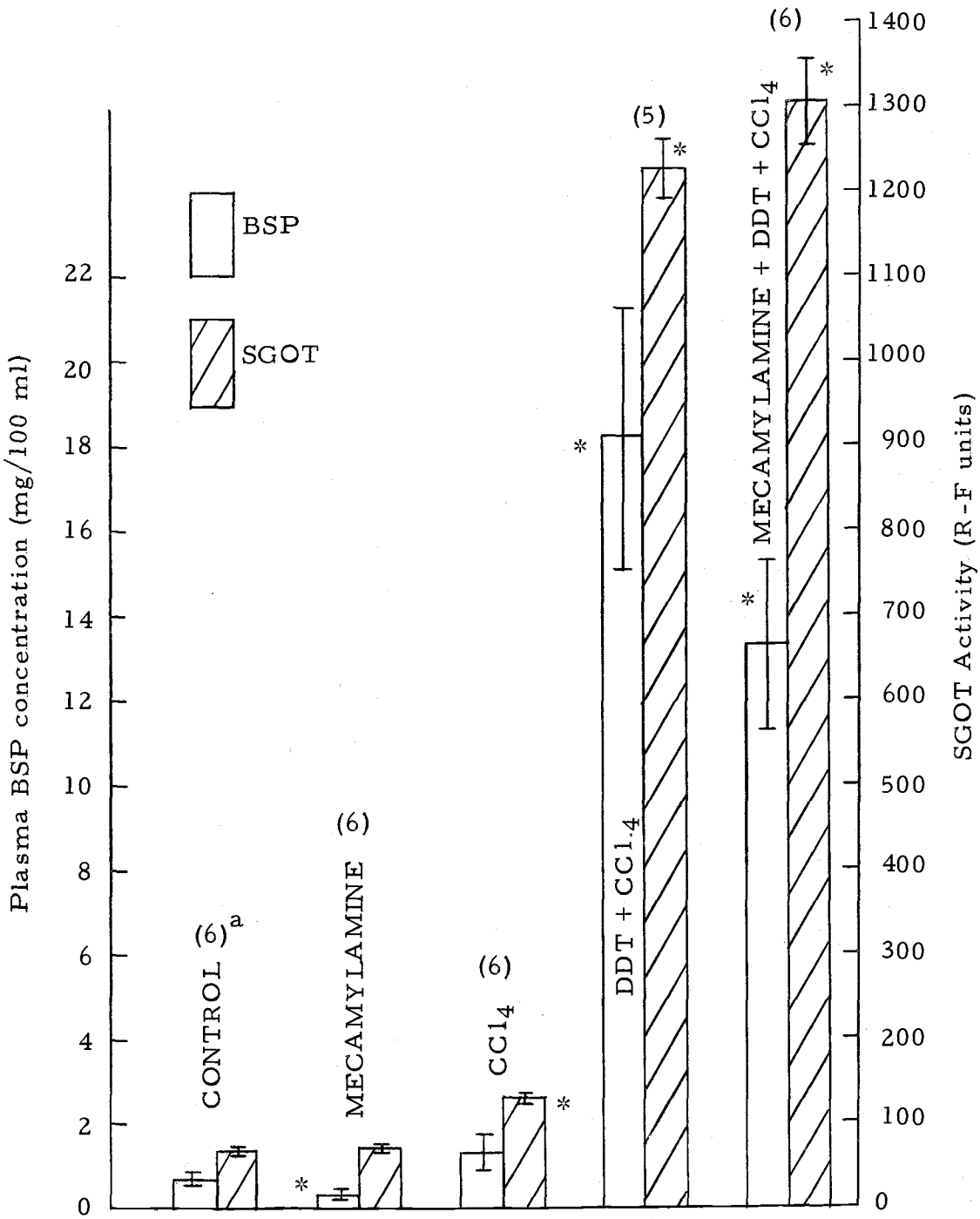
SGOT Activity. As shown in Figure 7-4, the SGOT activity in controls approximated 60 units. Mecamylamine pretreatment did not significantly ($P > 0.05$) alter this activity. CCl_4-treated controls had a significantly ($P < 0.05$) elevated serum enzyme activity to about 130 units. The acute pretreatment with DDT prior to the CCl_4 challenge potentiated the hepatic damage to 1230 units. Mecamylamine pretreatment did not protect against this potentiated response, since the SGOT activity in this latter group was about 1300 units.

BSP Retention. Rats receiving only the mecamylamine pretreatment had a plasma retention of BSP similar to controls (Figure 7-4). CCl_4-treated controls were slightly impaired in their ability to clear BSP. Acute DDT pretreatment potentiated the CCl_4-induced BSP retention to 18.2 mg/100 ml. By decreasing the amount of BSP retained in the latter group to 13.3 mg/100 ml, mecamylamine produced an insignificant ($P > 0.05$) tendency to protect against the potentiated hepatic dysfunction.

Histopathology. Submassive centrilobularly-oriented coagulative necrosis was noted in the livers of rats given the DDT and CCl_4 treatments, regardless of whether they had been pretreated with mecamylamine. Thus, the histologic evidence of protection by mecamylamine against the potentiated CCl_4-induced hepatic lesion was

Figure 7-4. Influence of mecamylamine pretreatment on the potentiated plasma BSP retention and SGOT activity resulting from the DDT-CCl_4 hepatotoxic interaction. (Mecamylamine (10 mg/kg) was dissolved in 0.9% saline and injected i.p. 30 minutes prior to the DDT challenge. DDT (150 mg/kg) was dissolved in corn oil and administered by gavage 24 hours before CCl_4 was given. CCl_4 (0.25 ml/kg) was administered by gavage. Each bar represents the mean (± standard error of at least 5 animals. Note the mecamylamine pretreatment produced an insignificant tendency ($P > 0.05$, Student's t-test) to reduce the potentiated BSP retention, but not the SGOT activity, in the rats receiving both DDT and CCl_4.

[a]The number in parentheses represents the number of animals per group.


Plasma BSP concentration (mg/100 ml)
SGOT Activity (R-F units)
BSP
SGOT
0
2
4
6
8
10
12
14
16
18
20
22
100
200
300
400
500
600
700
800
900
1000
1100
1200
1300
1400
(6)a
CONTROL
(6)
MECAMYLAMINE
(6)
CCl4
(5)
DDT + CCl4
(6)
MECAMYLAMINE + DDT + CCl4
*

negligible. Therefore, these livers are not shown.

Lead

BSP Retention. The results of this study are illustrated in Figure 7-5. CCl_4 increased BSP retention about five-fold to 2.6 mg/100 ml with respect to the control mean of 0.54 mg/100 ml. Pretreatment with lead or DDT did not significantly ($P > 0.05$) alter the amount of BSP retained. The combination of chronic lead pretreatment and acute CCl_4 resulted in a slightly enhanced effect of CCl_4 to 3.1 mg/100 ml. In the DDT-fed rats, the CCl_4-induced BSP retention was potentiated to 14.4 mg/100 ml, but this effect was significantly ($P < 0.05$) reduced to 8.2 mg/100 ml by chronic lead pretreatment. These results indicated that chronic lead exerted a significant degree of protection against the potentiated CCl_4-induced hepatic functional impairment in DDT-fed rats.

Cytochrome P-450. It can be seen in Figure 7-6 that the control microsomal concentration of cytochrome P-450 approximated 0.84 nM/mg protein. The cytochrome P-450 content was significantly ($P < 0.05$) induced to 2.5 nM/mg by DDT feeding. Chronic lead administration concurrent with DDT feeding tended ($P > 0.05$) to enhance the induction effect of DDT. Lead given alone produced an insignificant ($P > 0.05$) elevation of the cytochrome P-450 concentration to about 1.16 nM/mg. CCl_4 given alone and the DDT-CCl_4

Figure 7-5. Effect of chronic lead pretreatment on the potentiated BSP retention in DDT-fed (16 ppm X 8 wks) rats treated with 0.25 ml/kg CCl_4. CCl_4 was administered by gavage. Chronic lead was given in the drinking water for 8 wks as 300 ppm of the Pb^{++} ion. Each bar represents the mean (± standard error) of at least 4 animals. Note the chronic lead pretreatment produced a significant reduction ($P < 0.05$, Student's t-test) of the potentiated plasma BSP retention resulting from the administration of CCl_4 to DDT-fed animals.

[a]The number in parentheses represents the number of animals per group.

*Significantly different ($P < 0.05$, Student's t-test) from the control mean.

**Significantly different ($P < 0.05$) from the mean of the DDT + CCl_4 group.

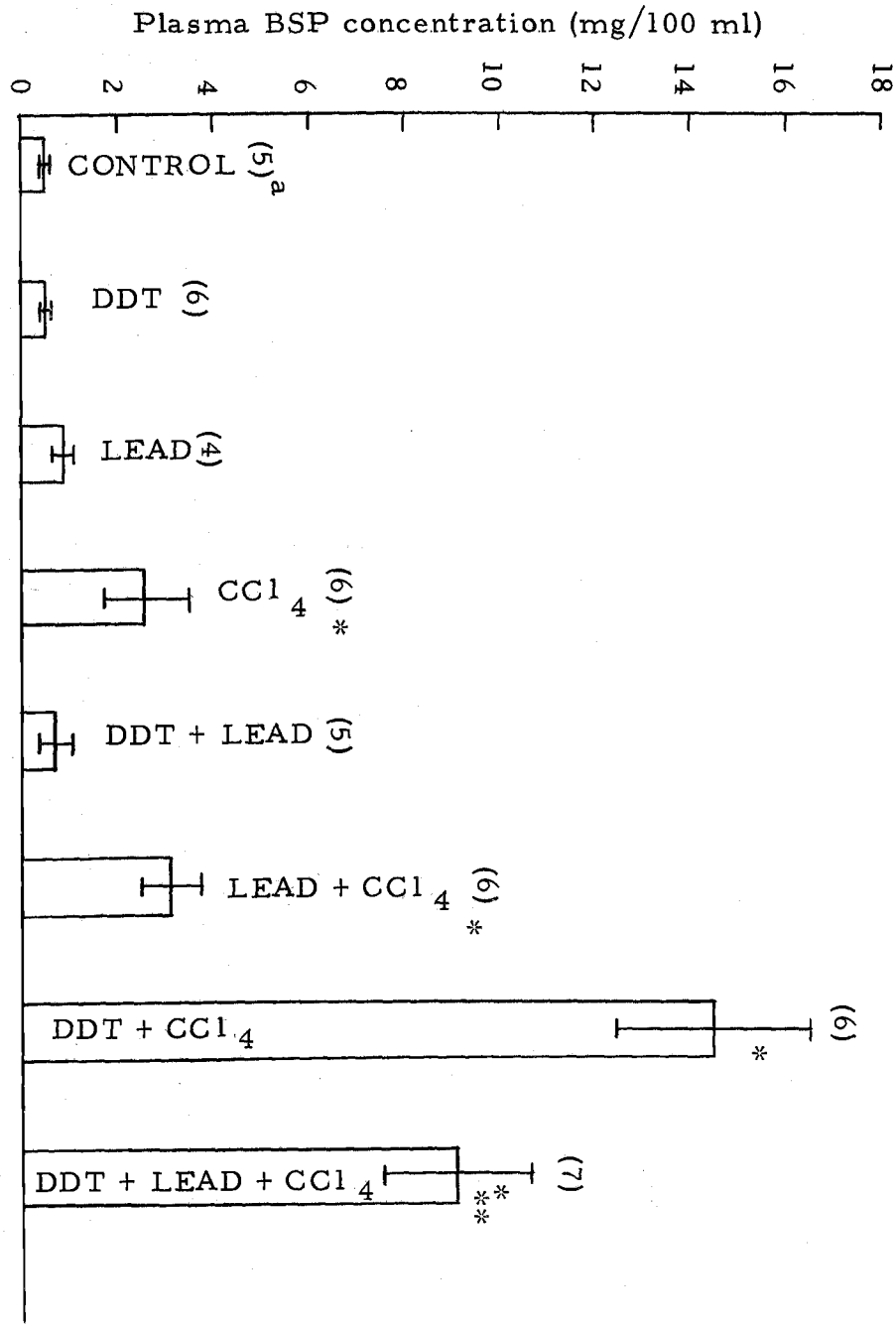
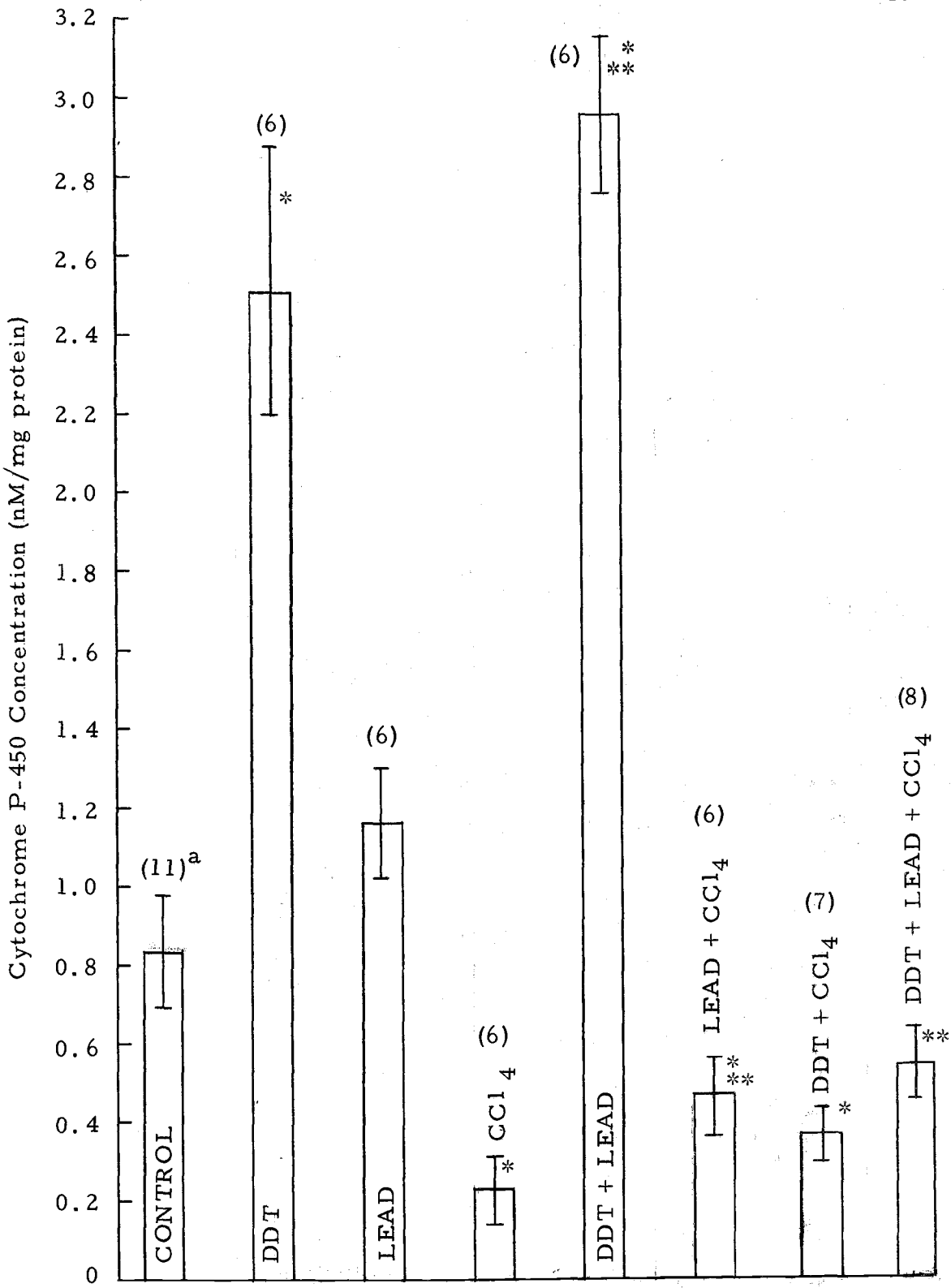

Plasma BSP concentration (mg/100 ml)
0
2
4
6
8
10
12
14
16
18
CONTROL (5)a
DDT (6)
LEAD (4)
CCl4 (6) *
DDT + LEAD (5)
LEAD + CCl4 (6) *
DDT + CCl4 (6) *
DDT + LEAD + CCl4 (7) **

Figure 7-6. Effect of chronic lead pretreatment on the cytochrome P-450 concentration in DDT-fed (16 ppm X 8 wks) rats treated with 0.25 ml/kg CCl_4. CCl_4 was administered by gavage. Chronic lead was given in the drinking water for 8 wks as 300 ppm of the Pb^{++} ion. Each bar represents the mean (± standard error) of at least 6 animals. Note the chronic lead pretreatment reduced the effects ($P > 0.05$, Student's t-test) of CCl_4 and the DDT-CCl_4 interaction of lowering the cytochrome P-450 concentration.

[a] The number in parentheses represents the number of animals per group.

* Significantly different ($P < 0.05$, Student's t-test) from the control mean.

** Not significantly different ($P > 0.05$) from the mean of the respective non-lead treated group.


Cytochrome P-450 Concentration (nM/mg protein)
3.2
3.0
2.8
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
(11)a
CONTROL
(6)
*
DDT
(6)
LEAD
(6)
*
CCl4
(6)
*
**
DDT + LEAD
(6)
**
LEAD + CCl4
(7)
*
DDT + CCl4
(8)
**
DDT + LEAD + CCl4

hepatotoxic interaction significantly ($P < 0.05$) reduced the cytochrome P-450 concentration, but chronic lead pretreatment decreased those effects.

Hexobarbital Metabolism. From the results shown in Figure 7-7, it is apparent that the DDT-fed rats metabolized significantly more ($P < 0.05$) hexobarbital in vitro than did the controls. The mean concentrations of substrate metabolized were 0.88 and 0.54 μM/g/45 minutes, respectively. Chronic lead pretreatment did not significantly ($P > 0.05$) alter the rate of hexobarbital metabolism with respect to controls. CCl_4 given alone decreased the rate of metabolism to 0.34 μM/g/45 minutes, and this effect was unaltered by lead. Lead slightly reduced the stimulatory effect of DDT. The effect of the DDT-CCl_4 hepatotoxic interaction of decreasing the rate of hexobarbital metabolism was significantly ($P < 0.05$) greater than the effect of CCl_4 given alone. The reduction of the former effect by lead was not significant ($P > 0.05$).

Histopathology. These results were similar to those observed after mecamylamine pretreatment, and therefore will not be discussed in detail. Chronic lead pretreatment did not give histologic evidence of protection from the potentiated CCl_4-induced hepatic necrosis in the DDT-fed animals. These livers are not shown.

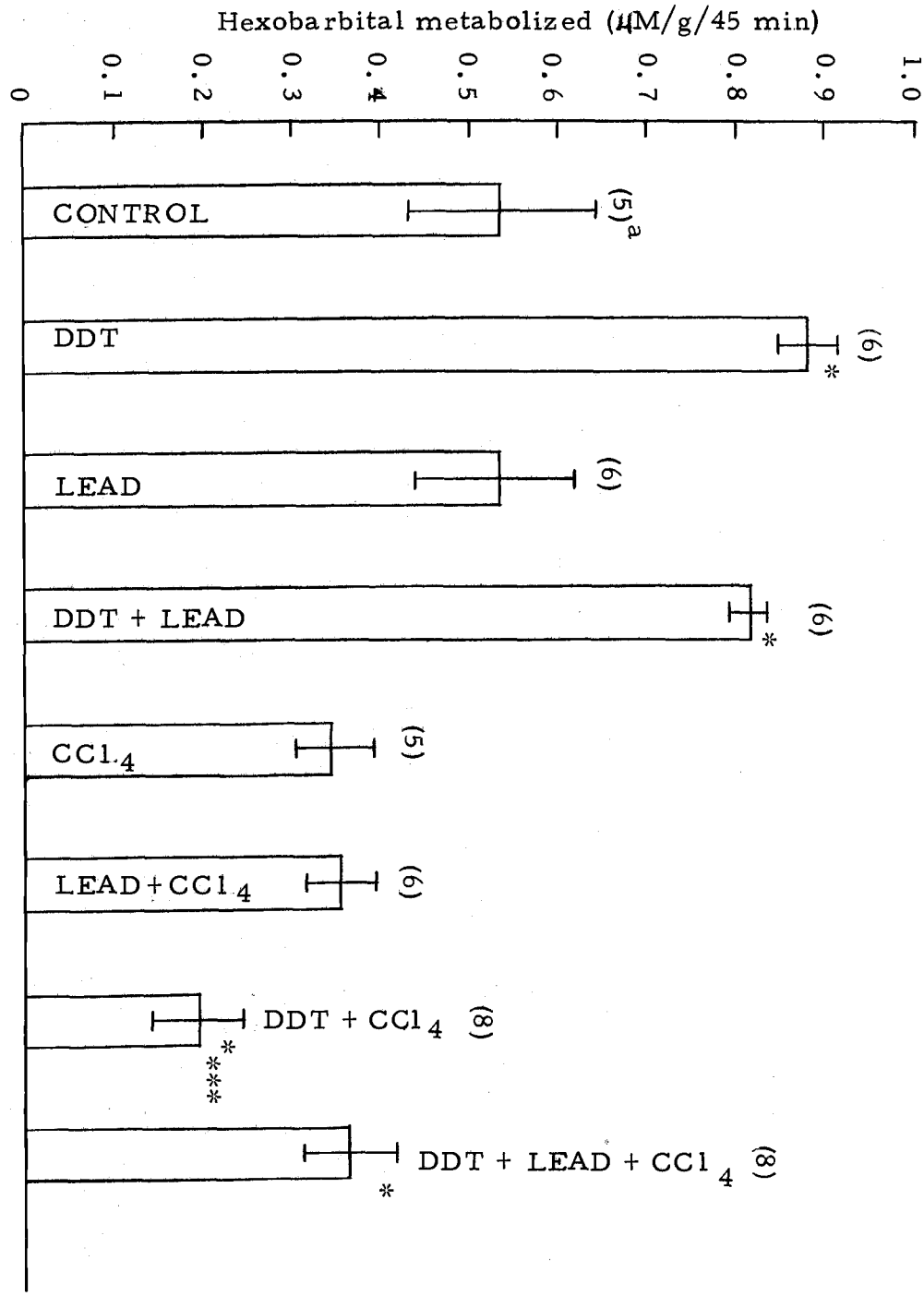
Figure 7-7. Effect of chronic lead pretreatment on the in vitro metabolism of hexobarbital by hepatic microsomal preparations from DDT-fed (16 ppm X 8 wks) rats treated with 0.25 ml/kg CCl_4. CCl_4 was administered by gavage. Chronic lead was given in the drinking water as 300 ppm of the Pb^{++} ion. Each bar represents the mean (± standard error) of at least 6 animals. Note the chronic lead pretreatment reduced the effect ($P > 0.05$, Student's t-test) of the DDT-CCl_4 interaction of decreasing hexobarbital metabolism.

[a] The number in parentheses represents the number of animals per group.

* Significantly different ($P < 0.05$, Student's t-test) from the control mean.

** Significantly different ($P < 0.05$) from the mean of the group receiving DDT+CCl_4 treatments.

*** Significantly different ($P < 0.05$) from the mean of the group receiving the CCl_4 treatment only.


Hexobarbital metabolized (μM/g/45 min)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
CONTROL
(5)a
DDT
(6)
*
LEAD
(6)
DDT + LEAD
(6)
*
CCl4
(5)
LEAD+CCl4
(6)
DDT + CCl4
(8)
*
***
DDT + LEAD + CCl4
(8)
*

Discussion

The mechanism of action of SKF 525-A as an inhibitor of drug metabolism remains to be elucidated. Rogers and Fouts (1964) described its biphasic action on drug metabolism, which is the principal pharmacologic effect of SKF 525-A. This drug inhibited the metabolism in a variety of hepatic microsomal drug metabolism pathways concerned with oxidation, reduction and conjugation reactions, for up to 12 hours after its administration to rats. Thereafter SKF 525-A stimulated drug metabolism, which was attributed to the adaptation or recovery of the drug metabolizing enzyme systems. These investigators suspected that there was a correlation between the rate of drug metabolism and the structure of the endoplasmic reticulum. Early studies by Brodie (1962) led him to conclude that SKF 525-A could have a physico-chemical effect on the microsomal membrane, resulting in an altered permeability of the membrane to drugs.

In the present study, pretreatment of rats with SKF 525-A provided histologic evidence of protection from the central necrosis usually observed 24 hours after treatment with CCl_4. In DDT-treated rats, SKF 525-A protected against the potentiated CCl_4-induced hepatic lesion at 11 hours. These results were in accord with the observations of Slater (1966). Castro *et al.* (1968) demonstrated that

SKF 525-A pretreatment afforded protection against the impairment of ethylmorphine metabolism, cytochrome P-450 destruction and central necrosis three hours after 2.5 ml/kg CCl_4. No protection was observed with respect to cytochrome P-450 or histopathologically, however, when the determinations were made 24 hours after CCl_4. This latter finding of Castro et al. apparently conflicted with the histologic evidence of protection observed at 24 hours in this study; however, they used a much higher dose of CCl_4 (2.5 ml/kg) than the one used here (1.0 ml/kg). A look in retrospect at the data in Chapter II, Figure 2-2 and Chapter IV, Figure 4-2 revealed that the hepatic damage and dysfunction produced in controls by 2.0 ml/kg CCl_4 was considerably greater than that resulting from 1.0 ml/kg. Therefore, protection against hepatic necrosis was observed at 24 hours in the present study because the dose of CCl_4 employed produced considerably less hepatic damage. According to Castro et al., their failure to demonstrate protection at 24 hours was explained by the biphasic action of SKF 525-A on hepatic microsomal drug metabolism. Thus, the biphasic nature of the protective effect was supposedly due to the inhibitory-stimulatory effect of SKF 525-A upon the metabolism of CCl_4 to a toxic form. Slater (1966) postulated a similar mechanism for the protective action of SKF 525-A.

Slater, Sawyer and Strauli (1966) attempted to correlate the protective action of SKF 525-A against CCl_4-induced hepatic necrosis

at 24 hours with the prevention of alterations in the pyridine nucleotide ($NADP+NADPH_2$) content one hour after CCl_4. Smuckler and Hultin (1966) showed that SKF 525-A had a biphasic inhibitory-stimulatory effect upon the CCl_4-induced inhibition of microsomal amino acid incorporation in rats. Slater (1966) classified five groups of drugs according to their proposed mechanisms of protection in CCl_4 hepatotoxicity. According to Slater, these protective agents should be examined for possible hypothermogenic properties, in view of the finding by Larson et al. (1963) that hypothermia resulted in protection against CCl_4-induced hepatic necrosis in rats. In the present study, SKF 525-A did have a transient effect of significantly ($P < 0.05$) lowering the rat rectal temperatures from 38° to 36°C for four hours after CCl_4. However, Larson et al. (1965) observed that protection did not occur unless the rectal temperatures declined below 32°C. Thus, it is doubtful that the hypothermic effect of SKF 525-A contributed significantly to its protective action.

As in the present investigation, most studies relating to the protective effect of SKF 525-A have been based on the ability of this agent to inhibit hepatic microsomal drug metabolism. Marchand, McLean and Plaa (1970) recently provided another plausible explanation for this observed phenomenon. Utilizing labeled $^{14}CCl_4$, they demonstrated that SKF 525-A had a biphasic action of altering the gastrointestinal absorption of CCl_4. Also, according to Cignoli and

Castro (1971), the preventive action of inhibitors of drug metabolizing enzymes against CCl_4-induced hepatic necrosis cannot be simply ascribed to their effects on drug metabolism. Therefore, it is quite possible that the protective effect of SKF 525-A observed in the present study was not directly related to its ability to inhibit drug metabolism. In most investigations of this nature, a drug is employed as a "tool" for its principal pharmacologic action. It is apparent that the secondary properties of the drug should not be forgotten, as they could exert a significant influence upon the outcome of the experiment.

Mecamylamine pretreatment did not provide histologic evidence of protection from the potentiated CCl_4-induced hepatic necrosis. Moreover, mecamylamine did not reduce the potentiated SGOT activity in the group receiving both the DDT and CCl_4 treatments. There was a strong, but statistically insignificant ($P > 0.05$), tendency for mecamylamine to protect against the potentiated CCl_4-induced hepatic functional impairment (BSP retention). Also, mecamylamine produced a transient decline in rat rectal temperatures to about 36°C for the first four hours after its administration; however, by 6 hours the mean rectal temperature again approached the normal control value of 38° (unpublished observation). In view of the results of Larson et al. (1965) discussed above, it is doubtful that the mecamylamine-induced decrease in rectal temperature could have contributed to any protective effect. The blood and liver uptakes of

CCl_4 were not monitored in this study. If mecamylamine pretreatment had given protection, then the contributions of hypothermia and an altered gastrointestinal absorption of CCl_4 should have been thoroughly examined as potential mechanisms. All of these possibilities having been eliminated, a protective action of mecamylamine could then be ascribed to its ganglionic blocking properties. From these results, it is doubtful that a direct central stimulatory effect on the sympathetic nervous system is related to the mechanism of potentiation of CCl_4-induced hepatotoxicity by dietary DDT. No conclusive statements are possible at this time.

Chronic lead pretreatment was utilized in an attempt to inhibit the potentiation of the CCl_4-induced hepatic lesion in DDT-fed rats, by inhibiting the porphyrin biosynthetic pathway. This study was suggested by the results of Cress (1970), who demonstrated that chronic lead pretreatment resulted in a dose-related decline in the cytochrome P-450 concentration in mice. In the present study, histologic evidence of protection was not apparent after chronic lead pretreatment. However, lead did significantly ($P < 0.05$) reduce the potentiated CCl_4-induced hepatic functional impairment, as measured by the amount of BSP retention. A slight elevation in the microsomal cytochrome P-450 concentration was noted, rather than a decline, after the chronic lead administration to rats. In addition, the chronic lead pretreatment concurrent with DDT feeding apparently slightly

enhanced the induction of cytochrome P-450 by dietary DDT. These effects of lead on the cytochrome P-450 concentration were completely unanticipated. The present study was conducted on the assumption that chronic lead pretreatment would lower the cytochrome P-450 concentration by exerting an inhibitory effect on porphyrin biosynthesis. The hematocrit was apparently decreased by lead in these animals (unpublished observation). Therefore, it is conceivable that lead had a more marked effect of inhibiting the biosynthesis of porphyrin moieties for incorporation into hemoglobin, and that the production of cytochrome P-450 was relatively unimpaired. The underlying basis for the apparent protection from the potentiated hepatic functional impairment by lead is unknown, due to the unexpected increase in the cytochrome P-450 concentration following pretreatment with lead.

VIII. SUMMARY AND CONCLUSIONS

The results of the present study give strong evidence that chronic low-level exposure to DDT via the diet, and the resultant total DDT body burden, renders animals highly susceptible to the hepatotoxic effects of CCl_4. The most important findings were: 1) Chronic dietary pretreatment of rats with DDT greatly potentiated (ten-fold or more) CCl_4-induced hepatotoxicity as measured by serum transaminase (SGPT and SGOT) activities, BSP retention and histopathologic evaluation. 2) This potentiation was dose-related to CCl_4 and was demonstrable at doses below 2.0 ml/kg. 3) The potentiation was dose-related to the total DDT body burden in the range of 6-30 ppm DDT (including its metabolites). 4) The response to CCl_4 in DDT-fed rats was not only potentiated in magnitude, but it was also greatly prolonged; or, the regenerative process was considerably impaired with respect to the controls. This finding was also verified with regard to the hepatic microsomal cytochrome P-450 content and liver weight responses following CCl_4 challenge.

The total body burden of DDT attained in these rats was less than 11 ppm in three of the four groups studied initially. These body burdens are comparable to the human body burden, which was estimated at 10 ppm of DDT and DDE combined by Durham (1965). Since man responds very similarly to the hepatotoxic effects of CCl_4,

it is not unlikely that these findings are directly applicable to human exposures to these two agents.

Potentiated hepatotoxic responses occurred at doses of DDT or CCl_4 which were not in themselves damaging. For example, a dietary exposure of rats to 6 ppm DDT for three weeks resulted in a potentiated response to a low challenging dose of 0.125 ml/kg CCl_4. The histologic evidence of hepatic damage or elevation in serum enzyme activities resulting from this DDT pretreatment given alone were not detectable. This dose of CCl_4 produced only mild hepatic damage and rises in serum transaminase activities. When CCl_4 treatment was superimposed on DDT feeding, however, a potentiated response ensued.

Clinical tests were utilized for the assessment of hepatic damage in rats. Measurements of serum transaminase activities and BSP retention are widely-used in routine clinical screening for hepatic damage in man. Since these tests provided sensitive indices for detecting potentiated hepatotoxic responses in rats, they are probably sufficient to detect such responses in man. Clinicians should be made aware that elevations in serum transaminase activity and BSP retention may be inordinately severe in relation to the dosage of CCl_4 in the presence of commonly acquired body burdens of DDT. Drill (1965) reported that man's hepatotoxic response to CCl_4 frequently did not correlate with the degree (incidence) of CCl_4 exposure. The lack

of correlation between dose and response could be explained by potentiated responses to low doses of CCl_4, which are a function of the total DDT body burden.

The maximal allowable concentration (m. a. c.) of 10 ppm CCl_4, which is the current standard of the American conference of Governmental and Industrial Hygienists (A. C. G. I. H.), probably should be re-evaluated in view of the present findings. The severe hepatotoxic potential of CCl_4 has long been recognized by the A. C. G. I. H., and as a result the m. a. c. has been lowered twice in the past 13 years in an effort to establish a safe limit of exposure. The A. C. G. I.H. should also be made aware of the phenomenon of potentiated hepatotoxic responses to CCl_4 in induced organisms. The m. a. c. for CCl_4 may have to be lowered again in an attempt to avoid such potentiated responses. In addition, the A. C. G. I. H. could recommend that epidemiologic studies be made to determine the incidence of potentiated hepatotoxic responses to CCl_4.

Attempts have been made to set "no-effect levels" for DDT and other persistent organochlorine insecticides. Hodge et al. (1966) discussed this possibility for aldrin and dieldrin. The concept of a "no-effect level" is based on the assumption that an exposure concentration of the pesticide can be attained which should produce no long-term toxicity. According to Eisler (1969, p. 729):

> An acceptable daily intake (ADI) of pesticide chemicals is determined for specific pesticides by an examination of the toxicologic data by WHO (World Health Organization). This is the amount of the chemical ingested throughout the lifetime of the individual without any effect toxicologically. It should also be kept in mind that built into toxicologic safety standards are many factors. Among them, after a "no-effect level" is determined in animals, is a safety factor of at least 1:100.

Despite the attainment of a "no-effect level" and the safety factors introduced, this toxicologic concept of protection is based upon the achievement of pesticide residue concentrations in man which are "not in themselves damaging." The results of the present investigation indicated that these measures may not be ultimately sufficient. The general human body burden of DDT, though it is not toxic to man directly, may produce an increased susceptibility to disease or toxicity from subsequent chemical exposures.

The demonstration of such potentiations probably occurs outside the laboratory as well as in it. Brodie et al. (1971) reported that Australian sheepherders lost entire groups of sheep after treating them with low therapeutic doses of CCl_4 for removal of worms. This potentiated response to CCl_4 could have resulted from prior ingestion of high pesticide residue concentrations in the food. Therefore, doses of CCl_4 normally considered harmless when given to non-induced animals, could result in lethality in the induced animals. Conceivably, such responses could also occur in certain environmentally or occupationally exposed subgroups of the general human population, which have high body burdens of DDT.

The observed differences in hepatic regenerative capacities in control and DDT-fed rats undoubtedly gave an indication of their relative prognoses for recovery after a challenging dose of CCl_4. The degree of potentiated hepatotoxic response in DDT-fed rats was equivalent to, or greater than, the degree of responses observed after acute pretreatment with 35, 75 or 150 mg/kg DDT. McLean et al. (1966) reported that the one-week oral LD50 of CCl_4 decreased from 6.4 to 4.2 ml/kg as a result of pretreating rats acutely with 75 mg/kg DDT, s.c. Similarly, Garner et al. (1969) demonstrated that phenobarbital pretreatment decreased the oral LD50 of CCl_4 in rats from 3.9 to 0.5 ml/kg. Thus, it could be assumed that a reduction in the oral LD50 of CCl_4 would also occur after chronic dietary pretreatment with DDT.

Additional studies are needed to assess whether the toxicities of other aliphatic (or aromatic) hydrocarbons are potentiated by prior exposure to enzyme inducers like DDT or phenobarbital. Many of these agents are used in industry as solvents and in the clinic as anesthetics, so perhaps these agents should be examined first for possible potentiated hepatotoxic responses. The anesthetic halothane is known to produce hepatic necrosis upon repeated exposure in some patients (Lindenbaum and Liefer, 1963). Preliminary studies in this laboratory indicated that prior dietary exposure of rats to DDT did not potentiate halothane toxicity as measured by serum transaminase

activities and histopathology. Brodie et al. (1971) demonstrated that the hepatotoxic response to bromobenzene was potentiated by phenobarbital pretreatment in rats. He suggested that the study of certain drugs and other chemical agents, which evidently depend upon metabolism in specific organs for their toxicities, e.g., bromobenzene, CCl_4, will be an important area of research in the future of clinical toxicology.

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