AN ABSTRACT OF THE THESIS OF

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Title: THE EFFECT OF HYPOTHERMIA ON BIOCHEMICAL

AND MORPHOLOGICAL ASPECTS OF CARBON

TETRACHLORIDE HEPATOTOXICITY

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The temporal relationships among selected correlates of

hepatocellular damage were investigated in cordotomized, hypo-

thermic rats intoxicated with carbon tetrachloride (CCl_4). Rats

were spinally transected between C6 and C7 and allowed to become

hypothermic. CCl_4 (1.25 ml/kg ip) was administered as a 1:1 solu-

tion in corn oil. Plasma alanine aminotransferase (ALT) activity

and bilirubin concentrations, hepatic malondialdehyde (MDA) forma-

tion, glucose-6-phosphatase (G6Pase) activity, and microsomal diene

conjugations, as well as morphological changes were monitored over

a 48-hour time course.

Diene conjugation, ALT and morphologic changes were all

delayed and attenuated in CCl_4 treated transected rats. The depres-

sion of hepatic G6Pase after CCl_4 treatment was of the same magni-

tude in both transected and non-transected rats and was delayed only

slightly in the cordotomized animals. Elevation of plasma bilirubin

was delayed in transected rats, but the magnitude of the response was greater than that seen in nontransected rats. Parallel increases in MDA occurred in both CCl_4 and corn oil treated transected rats over the 48-hour period.

These results demonstrate that spinal cord transection has differential influences upon the developing hepatotoxic effects of CCl_4. Thus, the hypothermic rat may provide a model for a more detailed examination of the relationships among events associated with toxic hepatocellular degeneration.

The Effect of Hypothermia on Biochemical and
Morphological Aspects of Carbon
Tetrachloride Hepatotoxicity

by

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INTRODUCTION

Several studies have demonstrated that spinal cord transection

at vertebra C7 delays the morphological features of carbon tetra-

chloride (CCl_4) induced hepatocellular necrosis in rats (Brody et al.,

1961; Larson et al., 1964; Kurstak et al., 1973). Larson and Plaa

(1965) demonstrated that this delayed effect could be related to a

decreased metabolic rate that was associated with the hypothermia

generated by cordotomy. They established this by showing that

transected rats maintained at normal body temperature responded

to CCl_4 in a similar manner to that of non-transected rats. They

also demonstrated that non-transected rats, rendered hypothermic

by other means, responded to CCl_4 in a delayed manner similar to

that of transected, hypothermic animals. The attenuation of CCl_4-

induced hepatotoxicity, determined by morphologic changes up to

48 hours, has been described for hypothermic rats (Larson et al.,

1964).

Kurstak et al. (1973) have extended these findings by studying

the changes in hepatic ultrastructure that occurred through 24 hours

after CCl_4 administration in transected hypothermic rats. They also

observed a striking delay in the development of the lesion in transected

rats. Only mild degenerative changes, mostly associated with the mitochondria, occurred in these animals.

Some studies have employed other poikilothermic models to study aspects of CCl_4-induced hepatotoxicity. In rainbow trout, for example, spinal cord transection was found to have no effect on urine or bile flow or on sulfobromophthalein clearance from plasma relative to free swimming fish (Schmidt and Weber, 1973; Gingerich et al., 1977). Sulfobromophthalein was cleared from plasma of the cordotomized trout in a manner substantially similar to that of normothermic rats (Gingerich et al., 1977). Also, similar effects of CCl_4 upon sulfobromophthalein retention have been noted in cordotomized rainbow trout as are seen in normothermic rats (Gingerich et al., 1978 a, b). However, the toxicant seemed to primarily affect the uptake or storage mechanisms for the dye in this species rather than the secretory processes as in mammals (Gingerich et al., 1978b). Thus, this "hypothermic" model does not appear to differ greatly from normothermic rats in its response to CCl_4 at least in terms of sulfobromophthalein clearance.

To date, no studies have appeared that examine the biochemical correlates of CCl_4-induced hepatocellular degeneration in hypothermic rats. In view of the continuing and unresolved question of the molecular basis for the toxic degenerative effects of CCl_4 and other hepato-toxicants, the hypothermic rat might provide a useful model to study.

If the biochemical correlates of CCl_4-induced hepatotoxicity progress more slowly and at varying rates, more critical associations might possibly be made between initiating events and their consequences. In our study, we have compared some of the biochemical features of CCl_4 hepatotoxicity in cordotomized, hypothermic rats and in normo-thermic animals relating them to morphological features as well as to each other. In these studies, we have investigated the time course and magnitudes of change observed in plasma alanine aminotransfer-ase activity (ALT), plasma bilirubin concentration, hepatic malondi-aldehyde formation (MDA), hepatic microsomal diene conjugation, hepatic glucose-6-phosphatase activity (G6Pase), and histological changes seen by light microscopy through 48 hours following CCl_4 administration.

METHODOLOGY

Animal Treatments

All experiments were performed using male Sprague-Dawley
rats obtained from the colony at the School of Pharmacy at Oregon
State University. The animals weighed 275-375 g and were housed
under a 12 hour light/dark cycle at an ambient temperature of
$21 \pm 1°C$.

Two groups of animals were used, transected and non-transect-
ed. Transected animals had their spinal cords surgically severed
under ether anesthesia between the sixth and seventh cervical verte-
brae as described by Larson and Plaa (1964). All surgery was per-
formed between 7:30 and 9:30 a.m. After transection, the animals
were arranged in a prone position in wire bottomed cages to allow
free air circulation about them and to minimize respiratory em-
barrassment. Rectal temperatures were continuously monitored
using a TRI-R model TMB telethermometer and rectal probes. By
5 hours after surgery, the rectal temperatures of these transected
rats had stabilized at $27° \pm 1°C$. At this time, animals within each
group were treated with either 1.25 ml/kg (ip) reagent grade carbon
tetrachloride (CCl_4), as a 1:1 solution in corn oil, or an equivalent
volume of corn oil (controls).

Subgroups of transected rats were killed at 0, 2, 4, 6, 8, 16,

24, 36 and 48 hours after treatment. Non-transected rats were sacrificed at 0, 4, 8, 16 and 24 hours after treatment. Fewer time periods were studied in non-transected rats because preliminary studies had shown that the critical events had occurred by 24 hours after CCl_4 administration in these animals. All animals were allowed food and water ad libitum up to the time of surgery for transected rats. Transected rats in the 36 and 48 hour subgroups were given 8 cc of 5% dextrose in water sc every 12 hours to maintain hydration.

Blood samples were taken under light ether anesthesia by aortic puncture using heparinized syringes and the animals were killed by exsanguination. The livers were then rapidly removed for biochemical and histopathological analysis.

Histologic Techniques

After removal of the liver, small portions of the left lobe were fixed in a buffered formalin solution, embedded in paraffin and stained with hematoxylin and eosin. The specimens were coded and evaluated under single-blind conditions.

Enzymatic and Chemical Assays

Plasma alanine aminotransferase (ALT) activity was measured using the method of Reitman and Frankel (1963). The units defined

by Reitman and Frankel were converted to International Units/Liter

(Rosalki, 1969). Plasma bilirubin, total and direct, was determined

by the method of Van Den Bergh (Natelson, 1961) and expressed in

mg/100 ml. Hepatic malondialdehyde (MDA) was measured using

the thiobarbituric acid method of Bernheim et al. (1948) and expressed

in nmol MDA/g liver using an extinction coefficient of 1.56×10^5

(Sinnhuber and Yu, 1958). Microsomal conjugated diene formation

was measured by the method described by Klaassen and Plaa (1969)

and was expressed as the absorbance of the microsomal lipid extract

at 243 nm. Glucose-6-phosphatase (G6Pase) activity was assessed

using the method of Harper (1963). Activity was expressed as μmol

Pi/min/g liver.

RESULTS

Plasma Alanine Aminotransferase Activity

Transected rats treated with CCl_4 exhibited a temporal profile of ALT that was delayed and attenuated compared to that seen in the non-transected, normothermic rats (Fig. 1). Significant increases in ALT activity were first seen at 4 hr in non-transected rats but not until 16 hr in transected rats. A maximal response was seen by 16 hr in non-transected rats whereas the maximal response in the transected animals was delayed by an additional 8 hr. The magnitude of increase in GPT activity in transected rats was less than half that seen in the non-transected rats. The ALT activities in corn oil treated transected and non-transected rats were unchanged at 11.0 (± 3.0, S.E.; N = 39) and 9.0 (± 3.4, S.E.; N = 17) respectively over the time periods indicated.

Plasma Bilirubin

Total and direct plasma bilirubin concentrations in transected and non-transected rats subsequent to CCl_4 administration are presented in Table 1. Total plasma bilirubin in non-transected rats increased through 16 hr in contrast to transected rats in which total bilirubin continued to rise through 36 hr - a delay of 20 hr in achieving maximum. In addition, the magnitude of increase of total
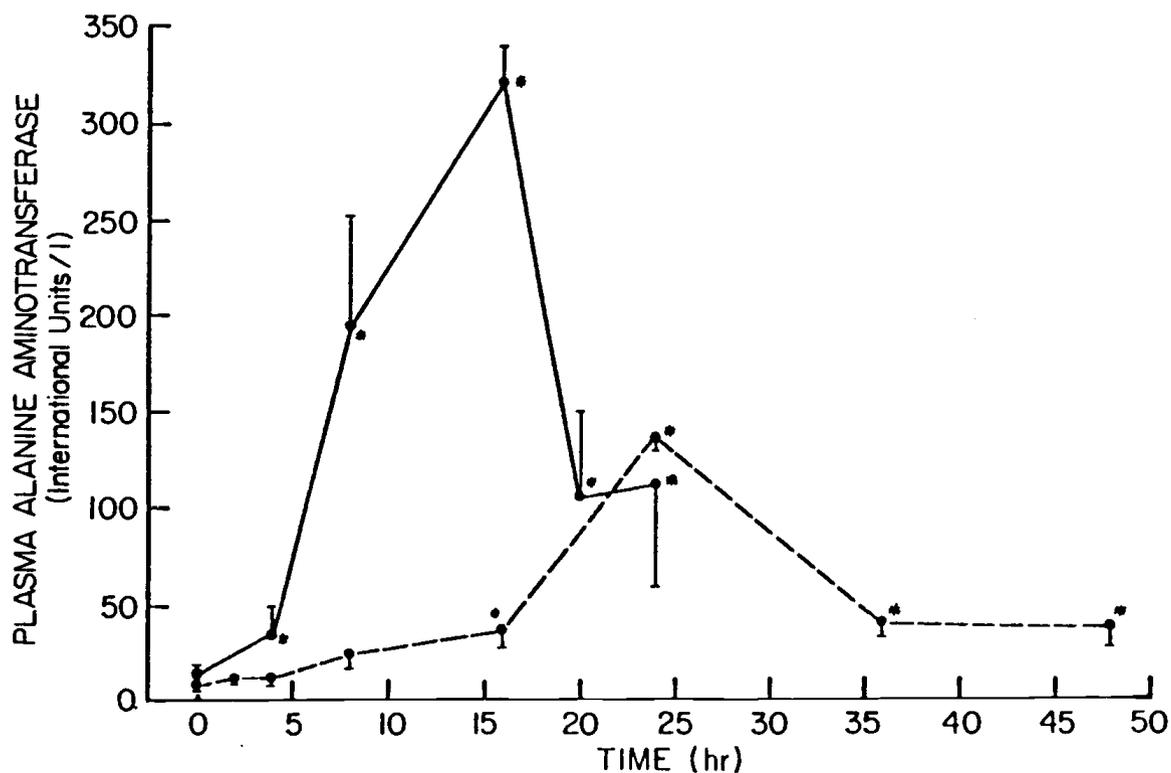
Figure 1. Temporal pattern of plasma alanine aminotransferase
(ALT) activity in transected (---) and non-transected
(___) rats after administration of 1.25 ml/kg CCl_4
given ip. Each point represents the ±S.E. of a mini-
mum of 4 animals. Asterisks denote significant differ-
ences ($p < 0.05$) from time 0 controls (Mann-Whitney
U-Test).

Table 1. PLASMA BILIRUBIN CONCENTRATIONS AFTER CARBON TETRACHLORIDE ADMINISTRA-TION IN NON-TRANSECTED AND TRANSECTED RATS[a]

Time (hr after CCl_4)	NON-TRANSECTED		TRANSECTED	
	Total	Direct	Total	Direct
0	.01 ± .01[b] (11)	0 (5)	.06 ± .02 (38)	0 (8)
4	.11 ± .03 (4)	0 (4)	.07 ± .01 (5)	0 (5)
8	.20 ± .06 (5)	.08 ± .04 (5)	.05 ± .05 (7)	0 (7)
16	.33 ± .22 (4)	.04 ± .04 (4)	.29 ± .10 (4)	0 (4)
24	.20 ± .09 (5)	.02 ± .03 (5)	.46 ± .14 (4)	.33 ± .14 (4)
36	---	---	.69 ± .22 (4)	.41 ± .20 (4)
48	---	---	.62 ± .55 (4)	.42 ± .42 (4)

[a] 1.25 ml/kg CCl_4 was given ip as a corn oil solution.

[b] Mean plasma bilirubin in mg/100 ml ± SE; number in parentheses is the number of animals in each group.

bilirubin in transected animals was twice that of non-transected animals. The contribution of direct-reacting bilirubin as a fraction of the total was approximately 15% in non-transected rats and 60% in transected animals at the times when total bilirubin concentrations were maximal. Total bilirubin levels in corn oil treated transected and non-transected rats remained between 0 and .15 mg/100 ml throughout the time course.

Hepatic Malondialdehyde Formation

Parallel increases in MDA formation occurred in both CCl_4 treated and corn oil treated transected rats over the 48 hr time course studied (Figure 2). Since MDA formation was not detected in either corn oil or CCl_4 treated non-transected rats, no comparison between transected and non-transected rats could be made. While the MDA content of the livers from CCl_4 treated rats was consistently higher than that of their corn oil controls, significant differences were noted only at 4 and 8 hr after treatment. In CCl_4 treated transected rats, significant increases ($p < 0.05$) compared to time 0, were observed by 16 hr and in the corn oil group, significant increases were seen at 24 hr. By 48 hr, there was about a 50% increase in both groups, when compared to time 0.
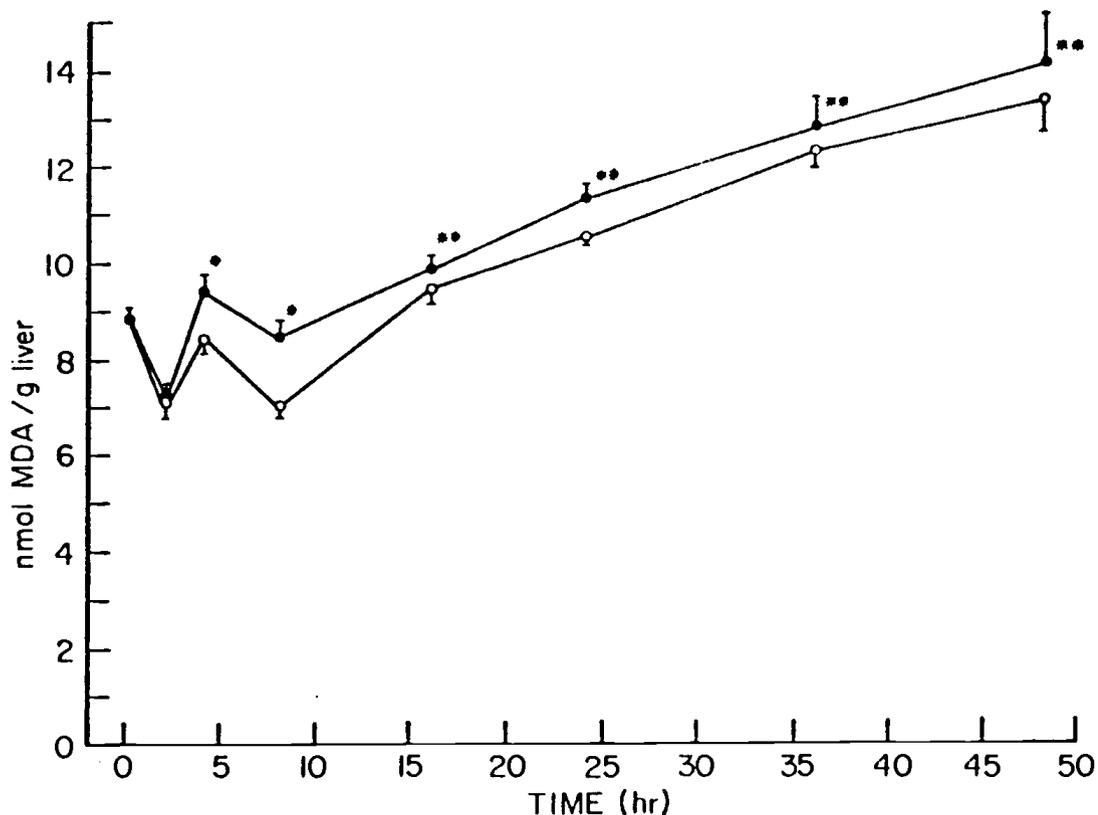
Figure 2. Temporal pattern of hepatic malondialdehyde (MDA) formation in corn oil (o) and CCl_4 (●) treated transected rats given either 1.25 mg/kg CCl_4 or an equivalent volume of corn oil ip. Each point represents the mean ± S. E. of a minimum of 3 animals. Single asterisks denote significant differences (p < 0.05) between CCl_4 and the respective corn oil treated transected rats. Double asterisks denote significant differences (p < 0.05) between sample time and time 0 (Student's t-Test).

Hepatic Microsomal Conjugated Dienes

Conjugated diene formation increased rapidly after CCl_4 admin-istration in non-transected rats as shown in Figure 3. A maximum occurred at 2 hr with levels approaching control values by 4 hr. In transected rats statistically significant increases in conjugated diene formation were not observed ($p > 0.05$). The absorbance of micro-somal lipid extracts from corn oil treated transected and non-transected rats remained constant over the entire time course ($A_{243} = 0.17$ to 0.32 OD units).

Hepatic Glucose-6-phosphatase Activity

The activity of hepatic G6Pase decreased significantly ($p < 0.05$) by 4 hr in non-transected rats which had received CCl_4 (Figure 4). In contrast, CCl_4 treated transected rats showed a slower onset of this effect with significantly lower values ($p < 0.05$) occurring at 8 hr. However, by 8 hr the magnitude of change in both transected and non-transected CCl_4 treated rats was similar. In corn oil treated tran-sected and non-transected rats, G6Pase activity increased, with a greater increase occurring in the non-transected rats.

Histologic Changes

Transected animals treated with CCl_4 showed an attenuated degree of degenerative liver changes when compared with CCl_4 treated
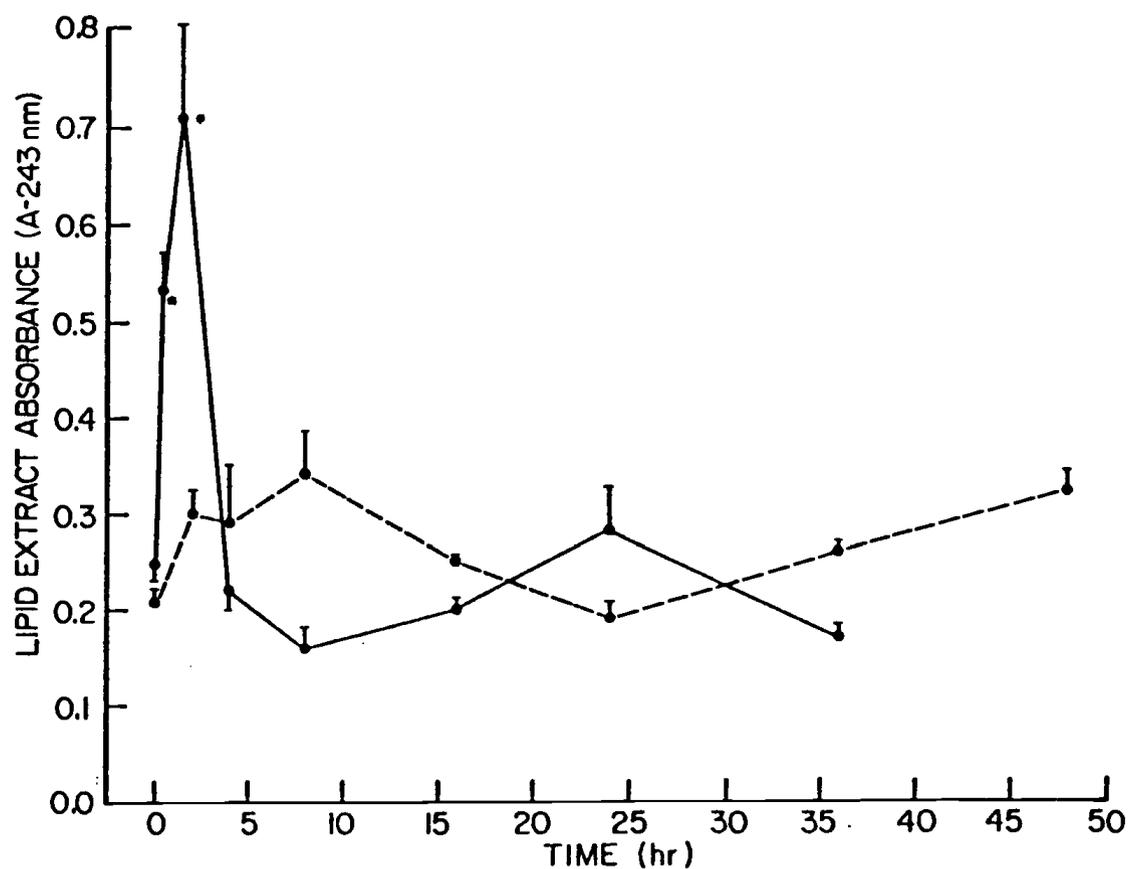
Figure 3.   Time course of microsomal conjugated diene formation
in transected (- - -) and non-transected (——) rats follow-
ing the administration of 1.25 ml/kg CCl_4 given ip.   Each
point represents the mean ± S. E. of a minimum of 3 ani-
mals.   Asterisks denote significant differences (p < 0.05)
from time 0 controls (Mann-Whitney U-test).
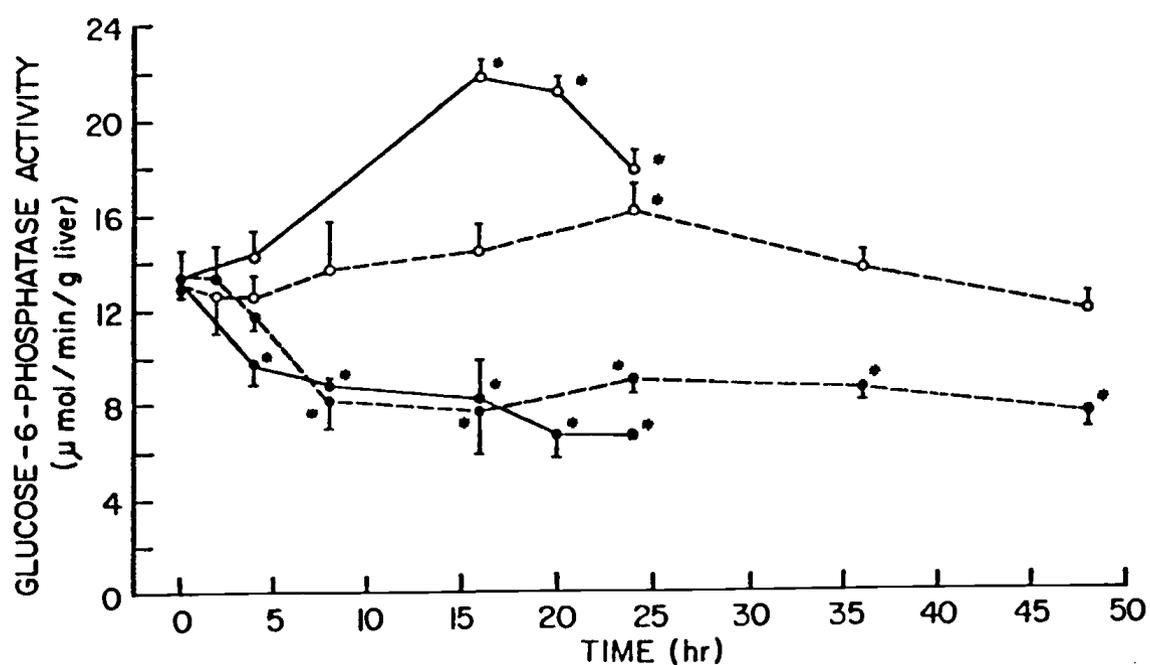
Figure 4.   Hepatic glucose-6-phosphatase (G6Pase) activity in
transected (- - -) and non-transected (———) rats follow-
ing ip administration of either corn oil (o) or CCl_4 (●)
1.25 ml/kg dissolved in corn oil.   Each point repre-
sents the mean ± S. E. of a minimum of 3 animals.
Asterisks denote significant differences ($p < 0.05$) be-
tween sample time and 0 controls (Mann-Whitney test).

non-transected rats. Through 48 hr, livers of transected rats

exhibited only a progressive mild degree of cellular swelling, cyto-

plasmic disruption and early fatty metamorphosis. In contrast,

livers taken from non-transected rats at 8 and 16 hr showed marked

cellular swelling and cytoplasmic vacuolization accompanied by

coagulative necrosis (i.e., the typical necrotic lesion associated

with CCl_4). The degenerative changes in livers from transected

rats 48 hr after treatment were always less severe than the changes

seen in livers from non-transected rats after only 8 hr. Photo-

micrographs of the developing lesion are not included herein because

they so closely paralleled previously published results (Larson et al.,

1964, Larson and Plaa, 1965). A more detailed evaluation of certain

aspects of the morphological changes is the subject of another study

the results of which will be published separately.

DISCUSSION

These results demonstrate that cervical cordotomy, presumably

through the resultant hypothermia, differentially affects some of the

biochemical and morphological correlates of the hepatotoxic response

to CCl_4. For example, an 8 hr delay in peak ALT activity was ob-

served in hypothermic rats when compared to normothermic rats.

Despite the fact that morphological disruption continued to progress

in a slow and mild fashion through 48 hr, ALT activity in the hypo-

thermic animals had maximized and returned to near baseline levels

by 36 hr after CCl_4 administration. This is in contrast to non-

transected rats in which elevations in ALT activity were essentially

coincident with the development of the lesion (Fig. 1). Others (e. g.,

Rouiller, 1964) have also noted that in normothermic rats the necrotic

lesion associated with CCl_4 intoxication is fully developed by about 16

hours and signs of regeneration are evident at that time. The attenu-

ated maximum in ALT activity exhibited by CCl_4 treated transected

rats may be indicative of the diminished hepatocellular degeneration

in this group when compared to non-transected rats. Thus, it appears

that only part of degenerative response is associated with ALT release

to the plasma in hypothermic rats.

In non-transected rats, plasma bilirubin levels followed a

temporal pattern similar to that of ALT activity. Most of the increase

in these rats was in the form of non-conjugated bilirubin. The appearance of hyperbilirubinemia was delayed 12 hours in CCl_4 treated transected rats when compared to non-transected rats; however, the peak level eventually attained was higher in the transected rats. Moreover, at its peak, most of the increase was in the form of conjugated bilirubin (Table 1). Thus, it appears that in transected rats treated with CCl_4, there is a relatively greater impairment of bilirubin secretion than of uptake and conjugation. Hence, even though the hyperbilirubinemia was delayed, protection against this effect was not provided by hypothermia. This observation can be accounted for from at least two standpoints. Firstly, bilirubin uptake, conjugation and secretion are enzyme or carrier mediated processes (Schmid, 1971) and are all probably temperature sensitive. Perhaps the secretory mechanism is more temperature sensitive resulting in intra-hepatic cholestasis in the hypothermic rats. Secondly, the post-surgical status of the transected rats was compromised in that they remained in a prone position. This, together with the developing hypothermia, may have had an effect on hepatic hemodynamics and bile flow. However, hyperbilirubinemia did not tend to develop in transected control rats which were not exposed to CCl_4 which argues against the latter explanation to some extent. The question may be resolved by studying the development of hyperbilirubinemia following CCl_4 intoxication of transected rats that are maintained at

normothermic body temperature.

No clear indication of accelerated lipid peroxidation was observed in CCl_4 treated transected rats. A parallel increase in MDA formation occurred in the livers of both the CCl_4 and corn oil treated transected rats beginning at 16 hr. This parallel increase may indicate a decreased rate of MDA catabolism by hepatic mitochondria (Recknagel and Ghoshal, 1966) in hypothermic rats. These data also suggest that perhaps a low level of lipid peroxidation is normally occurring as is suggested by the data of Dillard et al. (1978). Alternatively, the surgical procedure and its sequelae may have in some way promoted lipid peroxidation. Significantly higher levels of MDA formation occurred in CCl_4 treated transected rats only at 4 and 8 hr, when compared to corn oil treated transected rats. This suggests that a somewhat accelerated rate of lipid peroxidation was occurring in the presence of CCl_4 at these times. After 8 hr, MDA formation in CCl_4 treated, transected rats remained consistently, but not significantly, higher than control transected rats; nevertheless, this trend may be indicative of a low level of accelerated lipid peroxidation. MDA formation was not detected through 36 hr in CCl_4 treated nontransected rats when compared to control non-transected rats, which is in agreement with the observations of Recknagel and Ghoshal (1966).

Statistically significant conjugated diene formation was not observed ($p > 0.05$) in transected rats treated with CCl_4. However,

the data consistently showed a modest rise in the formation of these products with a tendency to maximize at around 8 hr after CCl_4 treatment suggesting that perhaps some low level of accelerated lipid peroxidation occurred. The fact that the rise coincided with significantly higher levels of MDA formation in the CCl_4 treated rats tends to support this idea. Perhaps a clearer indication as to whether lipid peroxidation is stimulated by CCl_4 in hypothermic rats could be gained by monitoring expired pentane and ethane patterns in these animals (Dumelin and Tappel, 1977; Köster et al., 1977; Dillard et al., 1978).

The relationship between G6Pase activity and lipid peroxidation is presently unresolved (Cignoli and Castro, 1971; Recknagel and Glende, 1973; Recknagel et al., 1976). We observed a 4 hour delay in the appearance of a significant ($p < 0.05$) depression of this enzyme activity in CCl_4 treated transected rats over that seen in non-transected rats treated with CCl_4. However, the extent to which G6Pase activity was depressed was the same for both groups. The probability of an association between the depression of this enzyme activity and lipid peroxidation as measured by diene conjugation is cast in doubt by these data since diene conjugation was markedly attenuated in hypothermic rats whereas the depression of G6Pase activity was not. Moreover, MDA accumulation (if taken as evidence of lipid peroxidation) was not accompanied by depressed G6Pase

activity in hypothermic rats not exposed to CCl_4 (Fig. 2 and 4). It is

therefore possible that the depression of G6Pase activity seen in CCl_4

intoxication is unrelated to lipid peroxidation. Alternatively, near

maximal depression of G6Pase may occur in the presence of low

levels of peroxidative activity. This view may be given credence by

the observations of Högberg et al. (1973) which demonstrated such

an effect in microsomal suspensions subjected to varying degrees of

peroxidation. In any event, the effect of CCl_4 on G6Pase seems to

be relatively refractory to the hypothermic state induced by spinal

cord transection.

In both transected and non-transected control rats, an increase

in G6Pase activity was observed. This phenomenon was probably due

to fasting which results in increased gluconeogenesis and glyco-

genolysis, both of which stimulate G6Pase activity (Scrutton and Utter,

1968).

Our histopathological evaluations are in agreement with those

of Larson et al. (1964). As an extreme comparison, livers of CCl_4

treated non-transected rats at 8 hr demonstrated more degenerative

changes than did livers of CCl_4 treated transected rats at 48 hr.

Nevertheless, the morphological changes became progressively more

marked through 48 hr in the hypothermic rats even though no clear

indication of accelerated lipid peroxidation was evident and other

biochemical markers had long since maximized or even normalized

(e.g., ALT, Fig. 1; G6Pase, Fig. 4).

From the data it appears that hypothermia induced by spinal cord transection differentially affects some of the parameters of CCl_4 hepatotoxicity in the rat. This study has demonstrated the potential utility of the hypothermic rat as a model in which to investigate the pathogenesis hepatotoxicity induced by CCl_4 and perhaps other toxicants. By slowing and separating the events that occur during in vivo intoxication, a better evaluation of their interrelationships may be possible.

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