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The Effects of Carbon Tetrachloride on Rat Liver Microsomes during the First Hour of Poisoning *in vivo*, and the Modifying Actions of Promethazine

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The effects of an oral administration of carbon tetrachloride on various liver microsomal and supernatant components were studied 1hr. and 2hr. after dosing. The modifications of such early changes resulting from a concomitant administration of promethazine together with the carbon tetrachloride were also investigated. The microsomal components studied were: cytochromes *P*-450 and b_5; inorganic pyrophosphatase; NADH- and NADPH-cytochrome *c* reductases; NADH- and NADPH-neotetrazolium reductases; a lipid-peroxidation system associated with the oxidation of NADPH and stimulated by ADP and Fe^{2+}. NAD- and NADP-DT-diaphorases were measured in the supernatant solution remaining after isolation of liver microsomes, and the distribution of RNA phosphorus between the microsomes and supernatant solution was also determined. Carbon tetrachloride produced a rapid fall in inorganic pyrophosphatase activity, a rather slower decrease in cytochrome *P*-450 content of the microsomes and small increases in the activities of NADH-cytochrome *c* reductase and neotetrazolium reductases. The activities of NADPH-cytochrome *c* reductase, the NADPH-ADP/Fe^{2+}-linked lipid-peroxidation system, DT-diaphorases and the content of cytochrome b_5 in the microsomes were unchanged. There was also a loss of RNA phosphorus from the microsomes into the supernatant solution. The RNA phosphorus redistribution, the decrease in inorganic pyrophosphatase and the increases in neotetrazolium reductase activities were at least partially prevented by a concomitant dosing with promethazine. However, the decrease in cytochrome *P*-450 was not affected by promethazine treatment. These early changes are discussed in terms of the liver necrosis produced by carbon tetrachloride and which is greatly retarded in its onset by the administration of promethazine.

The massive accumulation of fat and the development of centrilobular necrosis are well-documented hepatotoxic effects known to result from the administration of carbon tetrachloride to a variety of species (Cameron & Karunaratne, 1936; Van Oettingen, 1955). It has been shown that the metabolic disturbances producing these two gross effects can be separated from each other by a variety of procedures; for example, the administration of promethazine together with carbon tetrachloride will greatly retard the onset of necrosis while having little effect on the accumulation of fat (Rees, Sinha & Spector, 1961).

The accumulation of fat and the occurrence of centrilobular necrosis are not apparent histologically until several hours after the oral administration of the toxic agent, whereas many biochemical changes are known to occur during the first hour of poisoning. These changes are particularly evident in the endoplasmic reticulum (Recknagel & Lombardi, 1961; Ghoshal & Recknagel, 1965*a*; Smuckler & Benditt, 1965; Slater, 1965). From the viewpoint of elucidating the mechanism of the necrogenic action of carbon tetrachloride in chemical terms it is of obvious importance to know which, if any, of these early disturbances in the endoplasmic reticulum are related in a primary manner to the resultant necrosis. An attempt to obtain such information was made in this study by evaluating the modifying action of promethazine on the early changes that are normally produced by carbon tetrachloride in microsomal components. A preliminary account of some of this work has been published (Slater, 1965).

METHODS

The rats used were albino females, body weight approx. 130g. Rats were allowed free access to food (modified diet 41B; Oxo Ltd., London, E.C. 4) and water before being killed by cervical dislocation 1hr. after being dosed with

either liquid paraffin or CCl_4–liquid paraffin (1:3, v/v) mixture; 0·5ml. of the mixture or liquid paraffin was given/100g. body wt. by stomach tube with the rat under light ether anaesthesia. Immediately after oral dosing the rats were injected intraperitoneally with promethazine (2·5mg./100g. body wt.) or an equivalent volume of water (0·1ml./100g. body wt.). Rats dosed with CCl_4 were kept isolated from control animals dosed with liquid paraffin. Rats were thus treated in four different ways: (1) control group (liquid paraffin by stomach tube; water by intraperitoneal injection); (2) CCl_4-treated group (CCl_4; water); (3) promethazine-treated group (liquid paraffin; promethazine); (4) CCl_4+promethazine-treated group (CCl_4; promethazine).

During the early part of this study rats from two different colonies were used and it was observed that there were colony differences in the amounts of certain liver components. The study was subsequently extended to include rats from a third source. The three sources of the rats used were as follows, with the numbers of rats used in each category given in parentheses: colony A, University College London (30); colony B, Twyford Laboratories (34); colony C, Carworth Ltd. (55). In general, except where it was required to investigate specifically differences between colonies A, B or C, rats of only one source were used in each experiment. Four rats were used in each experiment, two of which received one of the treatments given above and the other two rats received a different treatment. The numbers of pairs of rats compared in this direct manner were: treatment (1) versus treatment (2), 24 pairs; treatment (1) versus treatment (3), eight pairs; treatment (2) versus treatment (4), 11 pairs; treatment (3) versus treatment (4), six pairs. Comparisons between treatments, by using only data obtained from such paired groups of rats, are termed 'corresponding-group values' in this paper. Since the effects of CCl_4 or promethazine or both that were observed on components of rat liver microsomes were in fact independent of the source of rat, the corresponding group values for the three colonies of rats are grouped together in the results.

The liver was removed immediately after the rat was killed, weighed and homogenized in ice-cold 0·25M-sucrose and made up to a final volume of 35ml. with ice-cold sucrose. Then 30ml. of this suspension was centrifuged at 12000*g* for 10min. (MSE Angle 13 centrifuge) at 2°. The supernatant mixture was sucked off by using a pipette with a bent tip and the pellet of material at the bottom of the tube was rejected. This procedure was repeated. The supernatant mixture from the second centrifuging was divided into two equal parts and centrifuged at $157000g_{av.}$ for 40min. (MSE Superspeed 50 centrifuge) at 2°. The supernatant solutions from the two tubes were decanted, pooled and stored at 0°. The microsomal pellets were gently rinsed free of supernatant with either cold 0·15M-KCl or cold 0·25M-sucrose and then one pellet was resuspended in cold 0·15M-KCl and the other pellet in cold 0·25M-sucrose, such that the microsomes from 1g. wet wt. of liver were present in a final volume of 1ml. Microsomes prepared in the above fashion are termed high-*g* microsomes in the Results and Discussion sections. In other experiments the final centrifuging was carried out at $105000g_{av.}$ for 30min. at 2°; these microsomal fractions are termed low-*g* fractions.

Chemical assays. Protein in the homogenate, microsomal suspensions and supernatant solution was estimated by the procedure of Lowry, Rosebrough, Farr & Randall (1951) with crystallized bovine plasma albumin [Sigma (London) Chemical Co. Ltd., London, S.W. 6] as a standard.

RNA was extracted from the tissue suspensions by the method of Greenbaum & Slater (1957) and was estimated by the orcinol procedure with precaution against interference by sucrose (Slater, 1958); values were calculated in terms of the phosphorus content of RNA (RNA P).

Cytochrome b_5 was estimated spectrophotometrically in the microsomal–KCl suspension by the method of Ernster, Siekevitz & Palade (1962*b*). Cytochrome *P*-450 was measured in the same suspension by the method of Omura & Sato (1962) by using either an Optica CF4 or a Unicam SP.800 recording spectrophotometer. The final concentration of microsomal protein in the cuvettes in both estimations was approx. 1mg./ml. in 0·1M-NaH_2PO_4–Na_2HPO_4 buffer, pH7·4. The results were converted into mμmoles/mg. of protein by using the molar extinction values: (i) cytochrome b_5, 112·1mM^{-1}cm.$^{-1}$ at 423mμ (Raw & Mahler, 1959); (ii) cytochrome *P*-450, 91mM^{-1}cm.$^{-1}$ at 450mμ (Omura & Sato, 1964).

Enzyme assays. NADH– and NADPH–neotetrazolium reductases were measured in microsomal–sucrose suspensions by a modification of the method of Slater (1959): 1·5ml. of 0·1M-tris–HCl buffer, pH7·4, 0·2ml. of 2·8mM-NADH or 2·4mM-NADPH (in 0·1M-NaH_2PO_4–Na_2HPO_4 buffer, pH7·4), 0·05ml. of microsomal suspension and 0·05ml. of neotetrazolium chloride (1%, w/v, in water) were incubated for 5min. at 37° for NADH–neotetrazolium reductase and for 10min. at 37° for NADPH–neotetrazolium reductase. The reaction was stopped by the addition of 1ml. of 10% (w/v) trichloroacetic acid; the formazan was extracted into 4ml. of ethyl acetate (peroxide-free) and the E_{510} was determined. The neotetrazolium chloride used was recrystallized twice from hot water and known amounts were chemically reduced with $Na_2S_2O_4$ to allow the construction of a calibration curve relating the E_{510} in ethyl acetate with the amount of formazan present.

Inorganic pyrophosphatase was measured by a modification of the method of Nordlie & Gehring (1963); 1·0ml. of 0·1M-sodium acetate buffer, pH5·0, 0·4ml. of 0·25M-sucrose, 1·2ml. of water, 0·3ml. of 0·05M-$Na_4P_2O_7$ and 0·1ml. of a microsomal–sucrose suspension were incubated for 15min. at 37°. The reaction was stopped by addition of 3ml. of 10% (w/v) trichloroacetic acid. The protein was sedimented by a short period of centrifuging and orthophosphate was estimated in the supernatant solution by the method of Flynn, Jones & Lipmann (1954).

NADH– and NADPH–diaphorases ('DT-diaphorase') were estimated in the supernatant solution obtained by high-speed centrifuging by a modification of the procedure described by Ernster, Danielson & Ljunggren (1962*a*): 1·5ml. of 0·1M-Na_2HPO_4–NaH_2PO_4 buffer, pH7·4, 0·2ml. of 0·5mM-2,6-dichlorophenol-indophenol, 0·6ml. of water, 0·3ml. of 0·7% (w/v) crystallized bovine plasma albumin [Sigma (London) Chemical Co. Ltd.], 0·1ml. of 10mM-KCN, 0·1ml. of 0·09mM-NADH or -NADPH [Sigma (London) Chemical Co. Ltd.] and 0·10ml. of 0·25M-sucrose or diluted supernatant (1:100 in 0·25M-sucrose) were mixed in a cuvette, path length 1cm. The change in E_{600} was recorded by using an Optica CF4 recording spectrophotometer; the temperature was approx. 19°. For calculating the results in terms of μmoles of 2,6-dichlorophenol-indophenol reduced/min./g. of protein a molar extinction value of

21 mM^{-1}cm.$^{-1}$ was used (Savage, 1957). The supernatant solution was diluted 1:100 with 0·25 M-sucrose immediately before assay.

NADH- and NADPH–cytochrome *c* reductases were measured by a modification of the method of Ernster *et al.* (1962*b*): 2·5 ml. of 0·1 M-tris–HCl buffer, pH 7·4, 0·5 ml. of crystallized cytochrome *c* (0·18%, w/v, in water) [Sigma (London) Chemical Co. Ltd.], 0·1 ml. of 10 mM-KCN, 0·1 ml. of diluted microsomes in 0·25 M-sucrose and 0·1 ml. of either 1·0 mM-NADH or -NADPH were mixed in a cuvette, 1 cm. path length, and E_{550} was measured by using an Optica CF4 recording spectrophotometer; the temperature was 16°. The reference cuvette contained all components with the exception of NADH or NADPH. The microsomes were diluted immediately before use to prevent the changes in activity that occur on aging in dilute suspensions (Ernster *et al.* 1962*b*). The molar extinction value for reduced minus oxidized cytochrome *c* used to calculate the results was 18·5 mM^{-1}cm.$^{-1}$ (Margoliash, 1954).

The uptake of O_2 coupled to the process of lipid peroxidation in microsomes in the presence of NADPH, ADP and Fe^{2+} was measured as described by Slater (1968).

The significance of differences between mean values was tested by using Student's *t* test.

Table 1. *Distribution of RNA phosphorus between the microsomal and supernatant fractions of rat liver after treatment with carbon tetrachloride or promethazine or both*

Values are given as the ratio of RNA P (μg. of P/mg. of protein) in the microsomal fraction to that in the supernatant fraction. Mean values are given ± S.E.M. The numbers of rats used are in parentheses. Results are shown for two different centrifuging procedures (low-*g* and high-*g*); for experimental details see the Methods section.

Treatment	RNA P ratio	
	Low-*g*	High-*g*
Control	5·8 ± 0·73 (8)	9·4 ± 0·44 (14)
CCl_4	4·2 ± 0·37 (10)*	8·5 ± 0·61 (14)†
Promethazine	5·9 ± 0·47 (6)	—
CCl_4 + promethazine	6·7 ± 0·80 (8)‡	—

* $P = 0·05$ (for difference from control).

† Not significant (for difference from control).

‡ Not significant (for difference from promethazine-treated group).

RESULTS

Source of rat. The effects of the four treatments described in the Methods section on the various microsomal components were studied in most cases with rats from each colony and no significant differences were found in the response of the different colonies to the various treatments applied. In general, therefore, the results obtained for the three colonies are grouped together for each treatment; the control activities of microsomal inorganic pyrophosphatase and NADH–cytochrome *c* reductase, however, showed variations between colonies (the range of variation observed between colonies was approximately twofold) that made such a grouping of results unfeasible. Colony variations in the activities of these two enzymes are not dealt with in detail in this paper. With the other microsomal and supernatant components studied no significant variation in the control values occurred between the three colonies of rats studied here.

RNA phosphorus. The results obtained for the distribution of RNA phosphorus between the microsomal and supernatant fractions are grouped in Table 1 according to treatment and to the centrifuging conditions used during isolation.

In the early phase of this investigation it was observed that treatment with carbon tetrachloride had apparently little effect on the RNA phosphorus ratio when the microsomal fractions were isolated by the high-*g* procedure (Table 1). Since previously published data have shown that RNA-phosphorus-rich particles dissociate from the endoplasmic reticulum early during the liver damage induced by carbon tetrachloride (for references see the Discussion section) it was decided to isolate the microsomes by an alternative (low-*g*) procedure. With the latter procedure there is a pronounced effect on the RNA phosphorus ratio 1 hr. after treatment with carbon tetrachloride (Table 1). The altered ratio results from a redistribution of RNA phosphorus between the microsomal and supernatant fractions; the sum of the RNA phosphorus contents in these two fractions was constant among the four groups of rats studied. Treatment with promethazine prevented the decrease in the RNA phosphorus ratio produced by carbon tetrachloride treatment. It is shown in Table 1 that the control value for the RNA phosphorus ratio was considerably changed by using the low-*g* in place of the high-*g* isolation procedure.

In view of the above-mentioned findings all of the other components studied in this investigation were estimated on microsomal suspensions isolated by both the high-*g* and the low-*g* procedures. No significant differences in either the endogenous enzyme activities or the effects produced by any of the treatments were observed between the results found by the two methods of isolation except for microsomal protein and inorganic pyrophosphatase. Microsomal protein, for example, averaged 14·2 mg./ml. of suspension after low-*g* isolation in colony C and 19·5 mg./ml. of suspension after high-*g* isolation. The results observed with inorganic pyrophosphatase are described below.

Cytochromes P-450 *and* b_5. The values obtained for cytochrome *P*-450 are given in Table 2. Although treatment with carbon tetrachloride decreased the mean value by approx. 22% this difference was not

Table 2. *Effect of treatment with carbon tetrachloride or promethazine or both on the concentration of cytochrome P-450 in rat liver microsomes*

Mean values are given ± S.E.M. The numbers of rats used are in parentheses. For the calculation of corresponding-group values and for other experimental details see the Methods section.

Treatment		Cytochrome *P*-450 (mμmole/mg. of protein)			
		All values	Corresponding groups		
Control	1 hr.	0·447 ± 0·029 (19)	0·436 ± 0·038 (9) †	0·479 ± 0·054 (7) ‡	
	2 hr.	0·589 ± 0·044 (9)†			
CCl_4	1 hr.	0·349 ± 0·063 (14)	0·305 ± 0·034		0·425 ± 0·017 (5) *
	2 hr.	0·425 ± 0·022 (9)†			
Promethazine	1 hr.	0·327 ± 0·039 (12)		0·371 ± 0·044	
CCl_4 + promethazine	1 hr.	0·305 ± 0·032 (9)			0·359 ± 0·013

* $P = 0·02$.
† $P < 0·05$.
‡ Not significant.

significant. However, by using the data obtained from corresponding groups it does appear that there was a significant decrease in cytochrome *P*-450 1 hr. after dosing with carbon tetrachloride. A similar decrease ($P < 0·05$; Table 2) was found 2 hr. after dosing with a carbon tetrachloride–liquid paraffin mixture (1:1, v/v). It may be noticed from Table 2 that the mean values obtained for the 1 hr. and 2 hr. control groups differ significantly. This may result from the estimations of the 2 hr. values for both the control and carbon tetrachloride-treated groups being carried out on rats of colony C over a short period of time (approx. 2 weeks). The values for the 1 hr. groups were obtained from colonies B and C and analyses were carried out over a period of approx. 3 years. It is known that the cytochrome *P*-450 content is rapidly responsive to changes in diet and particularly to certain additives such as DDT (McLean & McLean, 1966). Treatment with promethazine also decreased the mean value obtained, but the decrease was not significant. Treatment with carbon tetrachloride + promethazine decreased the content of cytochrome *P*-450 even more than treatment with carbon tetrachloride alone.

No significant differences were observed in the concentration of cytochrome b_5 after any of the treatments studied. The control concentration in liquid-paraffin-treated rats was 1·45 ± 0·08 mμmoles/mg. of protein (mean of ten rats).

Neotetrazolium reductases. The results obtained with these enzymes were not sufficient to decide whether significant differences existed in the activities of NADH- or NADPH–neotetrazolium reductases between colonies; most of the values were obtained on rats of colonies A and B, and with the low-*g* isolation procedure.

It is shown in Table 3 that treatment with carbon tetrachloride increased the activities of both

Table 3. *Effect of treatment with carbon tetrachloride or promethazine or both on the activities of NADH- and NADPH–neotetrazolium reductases in rat liver microsomes*

The values are given as mμmoles of neotetrazolium reduced/min./mg. of protein. Mean values are given ± S.E.M. The numbers of rats used are in parentheses. For other experimental details see the Methods section.

Treatment	NADH–neotetrazolium reductase	NADPH–neotetrazolium reductase
Control	36·8 ± 1·9 (8)	9·3 ± 0·8 (8)
CCl_4	51·1 ± 4·1 (11)*	11·2 ± 0·8 (7)†
Promethazine	34·6 ± 2·9 (6)	7·6 ± 0·5 (6)
CCl_4 + promethazine	40·2 ± 3·5 (9)§	8·5 ± 0·4 (4)‡

* $P < 0·05$ (for difference from control).
† $0·15 > P > 0·10$ (for difference from control).
‡ $P < 0·05$ (for difference from CCl_4-treated group).
§ $P < 0·10$ (for difference from CCl_4-treated group).

enzymes by a similar amount. Treatment with promethazine had no effect on NADH–neotetrazolium reductase, but appeared to inhibit the NADPH-linked enzyme to some extent ($0·15 > P > 0·10$). The carbon tetrachloride + promethazine-treated-group values are indistinguishable both from those of the control groups and from those of the promethazine-treated groups, but are lower than the values obtained after carbon tetrachloride treatment.

NADPH–cytochrome c reductase. None of the treatments had any significant effect on the activity of this enzyme. The control activity of this enzyme in liquid-paraffin-treated rats was 0·32 ± 0·02 μmole of cytochrome *c* reduced/min./mg. of protein (mean of six rats). Analysis of corresponding groups showed that the activity found after dosing with

Table 4. *Effect of treatment with carbon tetrachloride or promethazine or both on the activity of NADH–cytochrome c reductase in rat liver microsomes*

The values are given as μmoles of cytochrome *c* reduced/min./mg. of protein; mean values are given ± S.E.M. The numbers of rats used are in parentheses. For the calculation of corresponding-group values and for other experimental details see the Methods section.

Treatment	NADH–cytochrome *c* reductase			
	Colony B	Corresponding groups		
Control	13·9 ± 1·05 (8)	100%	100%	
CCl_4	16·1 (2)	110 ± 9% (6)	–	
Promethazine	12·4 ± 0·40 (8)	–	92 ± 6% (4)	100%
CCl_4 + promethazine	11·3 ± 0·29 (4)*	–	–	98 ± 5% (6)

* Not significant (for difference from control).

carbon tetrachloride was 109% compared with the control value of 100%. In a pooled sample of microsomes from four rats killed 2hr. after treatment the corresponding value was 117% of the value obtained with a pooled sample from control rats. In agreement with the work of Ernster *et al.* (1962*b*), the activity of NADPH–cytochrome *c* reductase did not show appreciable variations over a period of 1–2hr. after preparation of the microsomal suspensions.

NADH–cytochrome c reductase. No significant difference was observed between colonies of rats in the response of NADH–cytochrome *c* reductase to any of the four treatments used. Table 4 gives the results obtained with microsomes isolated from rats of colony B; the corresponding-group values were calculated by using data from experiments involving rats of all three colonies. The control activity in microsomes from colony C was 8·30 ± 1·24 μmoles of cytochrome *c* reduced/min./mg. of microsomal protein (mean of seven rats), which is significantly different from the activity found in microsomal pellets isolated from rats of colony B. Treatment with carbon tetrachloride tended to increase the activity of NADH–cytochrome *c* reductase, whereas treatment with promethazine tended to decrease the activity. It may be noted that the control activity of this enzyme was considerably higher than that found for NADPH–cytochrome *c* reductase, as reported by Ernster *et al.* (1962*b*).

NADPH–ADP/Fe^{2+}-linked lipid peroxidation. Treatment with carbon tetrachloride had no significant effect on the activity of this complex enzyme system (Table 5), whereas promethazine treatment decreased the activity by a substantial amount. This effect of promethazine was prevented by concomitant dosing with carbon tetrachloride. These experiments were done by using the low-*g* isolation procedure and with colonies A and B; no colony difference was apparent. The activity of untreated rats of colony C was 62 ± 2·5 mμmoles/min./mg. of microsomal protein, which is indistinguishable from the control-group values obtained for colonies A and B.

Table 5. *Effect of treatment with carbon tetrachloride or promethazine or both on the NADPH–ADP/Fe^{2+} lipid-peroxidation system in rat liver microsomes*

The results are expressed as mμmoles of O_2 utilized/min./mg. of protein; mean values are given ± S.E.M. The numbers of rats used are in parentheses.

Treatment	O_2 utilized (mμmoles/min./mg. of protein)
Control	62 ± 9 (11)
CCl_4	65 ± 7 (8)
Promethazine	31 ± 9 (7)
CCl_4 + promethazine	64 ± 12 (6)

NAD- and NADP-DT-diaphorases. No significant alterations in the activities of NAD- or NADP-DT-diaphorase in the supernatant fraction were observed after any of the treatments studied; the activity found with NADH was the same as that found with NADPH in all cases. These experiments were performed on supernatant solutions remaining after a low-*g* isolation of microsomes; rats of colonies A and B were used and no colony difference was apparent. The activity of the enzyme in liquid-paraffin-treated rats was 361 ± 41 μmoles of 2,6-dichlorophenol-indophenol reduced/min./g. of protein (mean of five rats) with NADH and 387 ± 45 μmoles/min./g. of protein (mean of five rats) with NADPH.

Inorganic pyrophosphatase. The results found for this enzyme are arranged in Table 6; considerable variations were observed in the activities of this enzyme between different colonies of rats. For example, in the control (liquid-paraffin-treated) group the activities found in microsomal fractions obtained by the low-*g* isolation procedure were (in μg. of phosphorus liberated/min./g. of microsomal protein): colony A, 157 ± 7 (six rats); colony B, 71 ± 5 (eight rats); colony C, 127 ± 19 (six rats).

It is shown in Table 6 that treatment with carbon

Table 6. *Effect of treatment with carbon tetrachloride or promethazine or both on the activity of inorganic pyrophosphatase in rat liver microsomes*

For the calculation of corresponding-group values and other experimental details see the Methods section. The number of rats in each pair of comparisons is shown in parenthesis.

	Inorganic pyrophosphatase					
	Corresponding groups					
Treatment	Low-*g*			High-*g*		
Control	100%	100%	–	100%	100%	–
CCl_4	76±2% (8)	–	–	90±2% (6)	–	100%
Promethazine	–	107±9% (4)	100%	–	107±1% (4)	–
CCl_4+promethazine	–	–	110±1% (4)	–	–	106±6% (8)

tetrachloride decreased the activity of this enzyme and that the decrease was more marked in microsomal fractions isolated by the low-*g* procedure. Concomitant treatment with promethazine only partially reversed the decrease in enzyme activity produced by carbon tetrachloride alone.

Treatment with promethazine alone produced a small increase in the activity of inorganic pyrophosphatase, although the increase was not significant when analysed in corresponding groups. An activating effect of promethazine on inorganic pyrophosphatase *in vitro* was also noted. With promethazine concentrations in the range 0·1–10 μM and an incubation time of 30min. at 37° a mean increase of 54% was observed (mean of ten experiments).

DISCUSSION

The results of this investigation obtained 1hr. after oral dosing of rats with 0·125ml. of carbon tetrachloride/100g. body wt. are in accord with many previous studies showing that the administration of carbon tetrachloride to rats produces very early changes in several components of the endoplasmic reticulum (for references see Recknagel, 1967).

It is important to note that the results given here are mostly concerned with the first hour of carbon tetrachloride poisoning and, as such, involve very early disturbances in the injury process that is manifest later by a variety of gross effects such as necrosis and fat accumulation. It is possible that the small colony differences found here in response to carbon tetrachloride or promethazine or both are a consequence of studying the very early phase of the injury, for the carbon tetrachloride was administered orally and differences in absorption rates between and within different colonies not only could affect the rate of development of the lesions but also would tend to increase variability within each single group.

The decrease in the ratio of RNA phosphorus in the microsomal fraction to that in the supernatant fraction (Table 1) is consistent with an earlier report on the mouse (Börnig, Richter & Frunder, 1960) and with electron-microscopic observations on rat liver that membrane-bound ribosomes dissociate from the membrane early during carbon tetrachloride poisoning (Smuckler, Iseri & Benditt, 1962) in the rat.

When the microsomal fractions were isolated by the high-*g* procedure the RNA phosphorus ratios obtained for the control and carbon tetrachloride-treated groups were not significantly different (Table 1). This suggests that the effects observed with the low-*g* experiments after dosing with carbon tetrachloride are the result of the displacement of polysomes from the membranes of the endoplasmic reticulum, and that these relatively large aggregates are sedimented with the microsomal fraction in the high-*g* experiments. Another possibility is that polysome dissociation has occurred to a limited extent by 1hr. after dosing with carbon tetrachloride and yet the dissociated fragments sediment under the high-*g* conditions. It is well established that both effects, displacement and dissociation, occur early during carbon tetrachloride poisoning, although most data bearing on this point have concerned rats dosed with a larger amount of carbon tetrachloride than used here (Smuckler & Benditt, 1965). Whatever the reason is for the differences reported in Table 1 for the effects of carbon tetrachloride, it is obvious that the centrifuging stage is a critical one for the biochemical demonstration of this early and important effect.

The decrease in the concentration of cytochrome *P*-450 in microsomes 1hr. and 2hr. after dosing with carbon tetrachloride (Table 2) is consistent with previous reports showing a decreased capacity for drug metabolism after dosing with carbon tetrachloride (Neubert & Maibauer, 1959; Clifford & Rees, 1966; Smuckler, Arrhenius & Hultin, 1967)

and a measured decrease in cytochrome *P*-450 2hr. after dosing of rats with 0·25ml. of carbon tetrachloride/100g. body wt. (Smuckler *et al.* 1967).

It is important to point out that promethazine does not overcome the decrease in cytochrome *P*-450 produced by carbon tetrachloride (Table 2), but, on the contrary, accentuates the tendency. Promethazine, however, retards the appearance of necrosis *in vivo*, although having little effect on the accumulation of fat (Rees *et al.* 1961).

It is significant that dosing with carbon tetrachloride has no effect on the concentration of cytochrome b_5 in rat liver microsomes. In rat liver cytochrome b_5 is reduced by NADH whereas the reduction of cytochrome *P*-450 involves NADPH; the concentration of $NADP^+ + NADPH$ in liver is rapidly decreased after dosing with carbon tetrachloride, whereas the concentration of $NAD^+ + NADH$ is unaltered (Slater, Sträuli & Sawyer, 1964). Thus two components (NADPH and cytochrome *P*-450) of a microsomal electron-transport chain are decreased early during carbon tetrachloride poisoning, whereas the corresponding members of a similar chain (NADH and cytochrome b_5) are not affected. This suggests that the initial lesion to the microsomes resulting in the decreased contents of NADPH and cytochrome *P*-450 must be confined to a relatively small sphere of influence, at least in the very early stages of the development of the injury studied here.

The tendency towards an increased activity of NADH–cytochrome *c* reductase after treatment with carbon tetrachloride (Table 4) is consistent with previous studies made at later stages of the poisoning. Increases in the activity of NADH–cytochrome *c* reductase have been reported by 3hr. after poisoning (Rees & Shotlander, 1963) and by 2hr. after poisoning with an increased dose (Recknagel & Lombardi, 1961). It is worth noting that the activity of NADH–cytochrome *c* reductase increased transiently in microsomal fractions undergoing lipid peroxidation *in vitro* (Nordenbrand, Hochstein & Ernster, 1964), whereas NADPH–cytochrome *c* reductase was unaffected.

No changes in the activity of NADPH–cytochrome *c* reductase were found 1hr. and 2hr. after poisoning, suggesting that this enzyme is relatively resistant to the effects of the hepatotoxin, despite an interaction between carbon tetrachloride and the NADPH electron-transport chain that is related to the development of necrosis (McLean & McLean, 1966; Slater, 1966*a*).

Table 5 shows that treatment with carbon tetrachloride has no significant effect on the activity of the NADPH–ADP/Fe^{2+} system linked to lipid peroxidation and associated with oxygen uptake. In a preliminary communication it was reported that carbon tetrachloride increased the activity of this system by 18% (Slater, 1965). However, further studies of the system indicate that the increase produced by carbon tetrachloride is not so extensive as first thought, and in fact is not statistically significant. This system shows a behaviour pattern after dosing with carbon tetrachloride that is similar to that found with the neotetrazolium and cytochrome *c* reductases: the activities are all raised or at least unchanged compared with the control situation. Other microsomal components, like the RNA phosphorus ratio, inorganic pyrophosphatase and the cytochrome *P*-450 content, are, on the contrary, decreased, as also is the sum of $NADP^+ + NADPH$ in the whole liver (Slater *et al.* 1964).

Promethazine treatment significantly decreases the activity of the NADPH–ADP/Fe^{2+} system *in vitro*, but concomitant dosing with carbon tetrachloride raises the activity to the control value. The action of promethazine *in vitro* in depressing the activity of the NADPH–ADP/Fe^{2+} system has been discussed by Slater (1968).

The decrease in inorganic pyrophosphatase 1hr. after dosing with carbon tetrachloride is a finding that has been reported previously with higher doses of carbon tetrachloride, with longer times of poisoning or by using glucose 6-phosphate as substrate. There is considerable evidence that the microsomal enzymes hydrolysing inorganic pyrophosphate and glucose 6-phosphate are one and the same (Stetten, 1964; Nordlie & Arion, 1964). With microsomal fractions isolated by both low-***g*** and high-***g*** conditions the activity of inorganic pyrophosphatase after dosing with carbon tetrachloride + promethazine is at least as high as the control value, in marked contrast with the decreased value observed after carbon tetrachloride alone. Analysis of the corresponding-group values showed that dosing with carbon tetrachloride alone decreased the mean activity of inorganic pyrophosphatase by 24% in the low-***g*** experiments and by 10% in the high-***g*** experiments. In comparison the mean value in the low-***g*** experiments for the carbon tetrachloride + promethazine-treated group was 10% higher than obtained for the promethazine-treated group; in the high-***g*** experiments, although the mean value for the carbon tetrachloride + promethazine-treated group was significantly lower than that for the promethazine-treated group, it was still higher than the control value. It may be concluded that promethazine treatment affords partial protection to microsomal inorganic pyrophosphatase during carbon tetrachloride poisoning. This protection seems due in part to the activating action of promethazine on this enzyme *in vivo* and *in vitro*. It is noteworthy that Hele (1963) has shown an interaction between phenothiazines and biologically occurring polyphosphates *in vitro*.

In connexion with the partially protective action of promethazine on inorganic pyrophosphatase *in vivo* discussed above, it has also been found (Ghoshal & Recknagel, 1965*b*; Slater, 1966*b*) that carbon tetrachloride decreases the activity of this enzyme when allowed to diffuse into microsomal suspensions *in vitro* and that this effect *in vitro* is prevented by 10 μM-promethazine. This result is, however, in marked contrast with another effect of promethazine on a microsomal change *in vitro* mediated by low concentrations of carbon tetrachloride: the increase in lipid peroxidation. Promethazine prevents the stimulation in lipid peroxidation produced by carbon tetrachloride at concentrations as low as 10mμM (Slater, 1968).

In summary, the effects of promethazine in retarding the appearance of necrosis after dosing with carbon tetrachloride (Rees *et al.* 1961) formerly studied by gross histological observation have now been studied in some detail at a biochemical level during the first hour of poisoning. At this time promethazine either totally or partially prevents several of the disturbances to the endoplasmic reticulum produced by carbon tetrachloride: the decreased inorganic pyrophosphatase activity, the displacement of RNA from the endoplasmic reticulum to the cell sap and the increases in NADH– and NADPH–neotetrazolium reductases. To these must be added previous findings on the inhibition by promethazine both of the decreased liver content of $NADP^+ + NADPH$ resulting from the administration of carbon tetrachloride and of leakage of liver enzymes into the plasma (Rees *et al.* 1961; Rees & Shotlander, 1963; Slater *et al.* 1964; Slater & Greenbaum, 1966). In contrast, promethazine has little effect on the decreased protein synthesis that occurs during the first hours of carbon tetrachloride poisoning (Rees & Shotlander, 1963) or on the decrease in cytochrome *P*-450 concentration (Table 2). It seems reasonable to assume that some or all of the early microsomal changes that are largely prevented by promethazine are involved in the spreading perturbations of metabolism that progress to necrosis. Other changes not so affected by promethazine are possibly of more relevance to the development of the fatty liver or other facets of the overall injury process or both.

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REFERENCES

Börnig, H., Richter, G. & Frunder, H. (1960). *Hoppe-Seyl. Z.* **322**, 213.
Cameron, G. R. & Karunaratne, W. A. E. (1936). *J. Path. Bact.* **42**, 1.
Clifford, J. & Rees, K. R. (1966). *J. Path. Bact.* **91**, 215.
Ernster, L., Danielson, L. & Ljunggren, M. (1962*a*). *Biochim. biophys. Acta*, **58**, 171.
Ernster, L., Siekevitz, P. & Palade, G. E. (1962*b*). *J. Cell Biol.* **15**, 541.
Flynn, R. M., Jones, M. E. & Lipmann, F. (1954). *J. biol. Chem.* **211**, 701.
Ghoshal, A. K. & Recknagel, R. O. (1965*a*). *Life Sci.* **4**, 1521.
Ghoshal, A. K. & Recknagel, R. O. (1965*b*). *Life Sci.* **4**, 2195.
Greenbaum, A. L. & Slater, T. F. (1957). *Biochem. J.* **66**, 155.
Hele, P. (1963). *Biochim. biophys. Acta*, **76**, 647.
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
McLean, A. E. M. & McLean, E. K. (1966). *Biochem. J.* **100**, 564.
Margoliash, E. (1954). *Biochem. J.* **56**, 535.
Neubert, D. & Maibauer, D. (1959). *Arch. exp. Path. Pharmak.* **235**, 291.
Nordenbrand, K., Hochstein, P. & Ernster, L. (1964). *Abstr. 6th int. Congr. Biochem., New York*, vol. 8, p. 76.
Nordlie, R. C. & Arion, W. J. (1964). *J. biol. Chem.* **239**, 1680.
Nordlie, R. C. & Gehring, A. W. (1963). *Biochim. biophys. Acta*, **77**, 100.
Omura, T. & Sato, R. (1962). *J. biol. Chem.* **237**, PC1375.
Omura, T. & Sato, R. (1964). *J. biol. Chem.* **239**, 2379.
Raw, I. & Mahler, H. (1959). *J. biol. Chem.* **234**, 1867.
Recknagel, R. O. (1967). *Pharmacol. Rev.* **19**, 145.
Recknagel, R. O. & Lombardi, B. (1961). *J. biol. Chem.* **236**, 564.
Rees, K. R. & Shotlander, V. L. (1963). *Proc. Roy. Soc.* B, **157**, 517.
Rees, K. R., Sinha, K. P. & Spector, W. G. (1961). *J. Path. Bact.* **81**, 107.
Savage, N. (1957). *Biochem. J.* **67**, 146.
Slater, T. F. (1958). *Biochim. biophys. Acta*, **27**, 201.
Slater, T. F. (1959). *Nature, Lond.*, **183**, 1679.
Slater, T. F. (1965). *Biochem. J.* **97**, 22C.
Slater, T. F. (1966*a*). *Nature, Lond.*, **209**, 36.
Slater, T. F. (1966*b*). *Biochem. J.* **101**, 16P
Slater, T. F. (1968). *Biochem. J.* **106**, 155.
Slater, T. F. & Greenbaum, A. L. (1966). *Biochem. J.* **96**, 484.
Slater, T. F., Sträuli, U. D. & Sawyer, B. C. (1964). *Biochem. J.* **93**, 260.
Smuckler, E. A., Arrhenius, E. & Hultin, T. (1967). *Biochem. J.* **103**, 55.
Smuckler, E. A. & Benditt, E. P. (1965). *Biochemistry*, **4**, 671.
Smuckler, E. A., Iseri, O. A. & Benditt, E. P. (1962). *J. exp. Med.* **116**, 55.
Stetten, M. R. (1964). *J. biol. Chem.* **239**, 3576.
Van Oettingen, W. F. (1955). *The Halogenated Aliphatic, Olefinic, Cyclic, Aromatic, Aliphatic-Aromatic Hydrocarbons, including the Halogenated Insecticides, their Toxicity and Potential Dangers.* Washington: U.S. Government Printing Office.