Correlations between common tests for assessment of liver damage: indices of the hepatoprotective activity of promethazine in carbon tetrachloride hepatotoxicity

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The effects of promethazine (PM) on different aspects of the hepatotoxic action of CCl_4 in the rat were investigated with the objective of finding rapid and reliable indicators of hepatoprotective effects. The study was based on definitive histological assessment of liver damage caused by CCl_4 in the presence and absence of PM: PM (78 μmol kg^{-1}, i.p.) protected against CCl_4-induced hepatic necrosis 24 h after a low dose of CCl_4 (1.3 mmol kg^{-1}) but not against a higher dose (13.0 mmol kg^{-1}). The large increases in plasma activities of GOT, GPT and LDH produced by dosing with CCl_4 were partially inhibited by the administration of PM. PM and CCl_4 caused a synergistic and long-lasting decrease in body temperature (2–3 °C for 8–10 h). Modifying the toxicity with PM, together with a low dose of CCl_4, helped to minimize secondary effects of CCl_4, to clarify the sequence of toxic events, and to assess the sensitivity of some standard tests of hepatotoxicity. Simultaneous measurement of over 20 commonly used biochemical screening tests in individual animals 3 or 6 h after treatment permitted direct correlation of a wide variety of concentrations, activities and effects. For example, liver $CHCl_3$ concentrations (as a measure of CCl_4 metabolism) correlate strongly with increases in diene conjugation of microsomal lipids (as a measure of CCl_4-induced lipid peroxidation); malonaldehyde production appears to be less sensitive as a measure of lipid peroxidation in vivo *than diene conjugation. The changes induced in each parameter and the correlations between them are discussed with reference to the overall nature of the hepatotoxic reaction and its modification by PM.*

Keywords: Carbon tetrachloride, liver damage, promethazine, free radicals, lipid peroxidation, hepatoprotective agents

Introduction

A frequently used model for studying mechanisms of hepatocellular injury is that based on exposure to carbon tetrachloride. Numerous approaches have been used in such studies in order to assess the extent of liver cell damage, and the attenuating influences of hepatoprotective agents[1, 2].

Histological assessments of necrosis and fatty degeneration of the liver following CCl_4 poisoning are still the most important means of determining the overall extent of the toxicity, as well as the success or failure of any hepatoprotective treatment. However, although histological procedures remain the ultimate criteria for assessing tissue damage, they are extremely time consuming and difficult to quantitate. It is inconvenient therefore to base the routine screening of large numbers of compounds, or studies of the time course of hepatotoxic events *in vivo*, primarily on histological procedures.

Evidence of liver cell damage may be detected at the biochemical level well before morphological changes in cellular structure can be observed. A problem to decide, however, is which of the multitude of biochemical tests for liver damage most accurately reflect the specific disturbances taking place, and which predict satisfactorily the ultimate extent and clinical importance of the resultant liver damage.

The choice of tests is inevitably biased in some way, not least by inadequate consideration of the importance of the time elapsing between dosing and sampling. The use of the most sensitive methods will be of no avail if the time of observation is inappropriate such that measurements are made too early or too late in the sequence of

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damaging reactions. Such considerations apply particularly to the screening of hepatoprotective agents. With toxic agents that are metabolically activated to highly reactive intermediates, the importance of having the protective agent in the right intracellular site, at the right time and in the right concentration for effective protection has been emphasized[2].

It is obvious, then, that no simple solution is available to the investigator who wishes to know what test or tests will provide all the immediate or predictive information he needs in studies on hepatotoxicity. In this study, samples from individual rats were subjected simultaneously to more than 20 frequently used screening tests in order to assess whether any important and meaningful correlations exist between the various changes commonly observed. The specific effects of promethazine (PM) on these changes were also investigated as part of a general assessment of the sensitivity and specificity of some procedures commonly used in screening for hepatoprotective activity. Additional information on potentially useful indicators of hepatic and metabolic function has been obtained using the model based on PM administration to rats intoxicated with CCl_4, and by taking advantage of gas–liquid chromatographic (g.l.c.) methods developed for determination of CCl_4, PM and their respective metabolites chloroform and desmethylpromethazine (DPM) *in vivo*[3–6].

An important aspect of the model chosen for this study is that hepatotoxicity was produced using a very small dose of CCl_4, according to the 'principle of the least effective dose'[2]. The experimental advantages of employing the smallest dose of a toxic agent capable of producing any desired toxic effect are that the physiological and, especially, the metabolizing systems of the organism are not overwhelmed, and that secondary effects of the toxic agent (e.g. lipophilic solvent effects, and the disturbing influence of metabolites of the primary toxic agent) are minimized. In consequence, the specific metabolic and damaging events produced as a direct result of the primary toxic substance are less likely to be masked by non-specific effects, and the relationships between primary events, later functional and structural perturbations and, ultimately, cell death should be more amenable to evaluation.

In previous concentration studies[5] an approximately constant production of $CHCl_3$ was observed over a ten-fold dose range of CCl_4: $CHCl_3$ production is generally believed to result from the activation of CCl_4 to a more toxic free-radical species that is essential for the production of liver injury. It was thus considered appropriate to establish which differences in the overall hepatotoxicity of CCl_4 and the protective effects of PM exist between the lower and upper end of this CCl_4 dose-range. The results of such studies, which deal with histological examinations, measurements of changes in body temperature and breathing rate, are reported under 'preliminary studies'. In our 'correlation studies' which deal with a variety of biochemical measurements, only one level of CCl_4 (1.3 mmol kg^{-1}) was used.

Materials and methods

Chemicals

CCl_4 (spectroscopic grade) from E. Merck, Darmstadt was used throughout the experiments. Promethazine hydrochloride was a gift of Specia, Paris, France. All other chemicals and reagents described in the Methods were of AR grade.

Treatment of animals

Young, adult, male Tiff (SPF) rats (Ciba-Geigy, Basle, Switzerland) fed on a standard diet (NAFAG 890 pellets), maintained in a climate-controlled room with a 12 h light/dark cycle, and weighing about 200 g were fasted for 15 h in cages with stainless steel grids above the cage floor. Tap water was available *ad libitum* throughout. At mid-morning (0830–1130) the rats were administered a dose of CCl_4 (0.26–13.0 mmol kg^{-1}) as a solution in liquid paraffin, or an equivalent volume of liquid paraffin alone, by intra-gastric cannula (0.5 ml/200 g body wt) under light ether anaesthesia. Promethazine (78 μmol kg^{-1}), as a freshly prepared 1% (w/v) aqueous solution, or an equivalent volume of physiological saline was administered simultaneously by intraperitoneal (i.p.) injection.

In preliminary studies rats were given at 6 h a further dose of PM (39 μmol kg^{-1}) i.p., or an equivalent volume of physiological saline.

Sample collection

At the time of sampling or before killing, the rats were anaesthetized with ether. When immediate g.l.c. determinations of PM, DPM, CCl_4 and $CHCl_3$ in whole blood were required, a heparinized haematocrit tube was inserted into the retro-orbital capillary net of the rat and about 4–6 drops of blood collected into tared extraction tubes containing the prescribed extractant/borax buffer pH 10.0 mixture (see g.l.c. analysis, below).

The peritoneal and thoracic cavities of anaesthetized rats were opened quickly; the inferior vena cava was severed cranially to the diaphragm and blood collected from the thoracic cavity in a heparinized syringe. When plasma enzyme activities were required, the blood was placed in heparinized 5 ml tubes and centrifuged at 3500 rev per min for 10 min. The plasma was removed by Pasteur pipette to duplicate sample tubes and stored at −20°C until analysis (see plasma enzyme determinations, below). The liver was excised, weighed and chilled by placing on ice. For histological assessment, thin slices were placed in buffered 4% (v/v) formalin solution and stored for 1 week. Sections were washed and stained for assessment of the degree of necrosis and extent of fat accumulation with haematoxylin–eosin and Oil red-O, respectively. For malonaldehyde determination, a small lobe (~2 g) was isolated, clamped firmly between aluminium freezing-blocks and plunged into liquid nitrogen. The frozen samples were stored at −20°C until analysis

(see liver determinations). For glutathione determination, exactly 1 g of liver was homogenized with 9 g ice-cold water in an ice-cooled Potter-Elvehjem homogenizer and the homogenate analysed immediately (see liver determinations). A sample of this homogenate was stored at −20°C for later determination of DNA, RNA and total protein.

Exactly 4 g of the liver remaining after the above sampling procedures was homogenized with ice-cold 1.15% (w/v) KCl in an ice-cooled Potter-Elvehjem homogenizer and the volume made up to 20 ml. For immediate g.l.c. determinations of the liver concentrations of PM, DPM, CCl_4 and $CHCl_3$ small (200–500 μl) samples of the liver homogenate were taken and pipetted directly into the prescribed extraction mixtures in tared tubes (see g.l.c. analysis). For the preparation of microsomes, the homogenate was then centrifuged at 10 000*g* for 20 min, and then at 100 000*g* for 20 min at 4°C. Microsomal pellets were stored at −20°C until analysis (see liver determinations).

G.l.c. analysis

PM and DPM were determined by g.l.c. using a nitrogen-selective flame ionization detector[3]. CCl_4 and $CHCl_3$ were determined by g.l.c. electron capture detector analysis of toluene extracts of micro-samples of blood and homogenate[4].

Plasma enzyme determinations

Following thawing and centrifuging at 3000 rev per min for 5 min, the plasma samples were analysed for glutamate-oxaloacetate transaminase (GOT), glutamate-pyruvate transaminase (GPT), lactate dehydrogenase (LDH), alkaline phosphatase (AP) and sorbitol dehydrogenase (SDH). GOT, GPT, LDH and AP activities were determined using Roche diagnostic kits and a 20-channel Union-Carbide Centrifichem system. SDH activity was determined manually using a Boehringer diagnostic kit.

Liver determinations

Glutathione was determined in a fresh 20% homogenate with DTNB reagent according to the method of Beutler *et al.*[7]. The microsomal pellets were resuspended in 1.15% KCl for the determinations of microsomal enzymes and protein.

Aminopyrene demethylase was assayed by estimation of formaldehyde production[8]. The incubation mixture contained 140 μmol phosphate buffer pH 7.4, 10 μmol glucose 6-phosphate, 2.8 U glucose 6-phosphate dehydrogenase, 0.75 μmol $NADP^+$, 25 μmol nicotinamide, 12.5 μmol $MgCl_2$, 2.5 μmol aminopyrene and ~2 mg microsomal protein in a total volume of 2.5 ml. The incubation (10 min at 37°C) was terminated with $ZnSO_4$ and $Ba(OH)_2$.

Cytochrome P_{450} was determined using the method of Omura and Sato[9]. Glucose 6-phosphatase was determined by the method of Poli *et al.*[10]. Microsomal lipid was extracted and the extent of diene conjugation was assessed using u.v. absorption spectroscopy[11]. Malonaldehyde was determined as thiobarbituric acid-reacting material by measurement of the E_{535} in butanol according to the method of Jose and Slater[12], using free-clamped liver samples. The content of liver neutral lipids was determined by the method of Chiang *et al.*[13] and liver triglycerides by the method of van Handel and Zilversmit[14] as modified by van Handel[15].

Finally, a deep-frozen sample of 10% homogenate was thawed, homogenized and used for the determination of liver DNA and RNA according to the method of Fleck and Munro[16]. The alcoholic hydrolysis of RNA was carried out in 0.3 N NaOH at 37°C for 1 h. DNA was precipitated with 1 N perchloric acid, hydrolysed with 0.75 N perchloric acid at 80°C for 35 min and determined by the method of Burton[17]. Total liver protein was assayed with this homogenate sample according to the method of Lowry[18], as modified by Miller[19]; microsomal protein was determined by the same method.

Body temperature and breathing rate

After dosing, the rats were placed in restraining cages with tail-holes and a rectal thermocouple was inserted and taped in place; the restrained rats were then put inside cabinets maintained at 28°C. Body temperature was recorded automatically to the nearest 0.1°C at 15 min intervals for up to 10 h. The breathing rate was determined at 30 min intervals by observing the rise and fall of the abdomen of the rat; the time taken for 50 cycles was used as the standard measurement.

Data analysis

The significance of the difference between two mean values was assessed by Student's t-test. Differences were considered significant when $p < 0.05$. The correlation between two variables was assessed by calculation of the correlation coefficient r by least-squares linear regression analysis or by Spearman's rank correlation method.

Results

Preliminary studies

Histology. We have repeated the original experiment of Rees *et al.*[20] and have assessed the sections of liver so obtained for necrosis and fatty degeneration. Following a dose of CCl_4 of 13.0 mmol kg^{-1} i.g., both centrilobular necrosis and fatty degeneration were clearly evident in all treatment-groups, and no more than slight protection was evident at + 24 h in either those rats treated with a single dose of PM (78 μmol kg^{-1}, i.p.) or those treated with a supplementary dose of PM (39 μmol kg^{-1}, i.p.) at + 6 h.

A significant reduction in the concentrations in blood of $CHCl_3$ has been observed previously in PM-treated rats during the first 3 h following CCl_4 dosing at 1.3 mmol kg^{-1} (Ref. 5). The histology experiment was therefore repeated at this lower CCl_4 dose, with histological assessment at 24 h, and a protective effect of PM on the extent of necrosis was observed. There was no significant difference

in this latter respect, however, between the groups treated with either the single or the double dose of PM. Fatty degeneration was again similar in extent in all treatment groups; additionally, however, a strong band of fat was present in the intermediate zone of the liver lobules of the group treated with CCl_4 in the absence of promethazine.

Plasma enzymes

The release of several enzymes into the plasma of rats treated with a low dose of CCl_4 (1.3 mmol kg^{-1}, i.g.) was measured over 24 h. This study revealed that GOT, GPT and LDH levels were significantly elevated by 6 h of intoxication. Several studies have demonstrated beneficial effects of PM on the release of enzymes but, even with this low dose of CCl_4, we were unable to detect significant protection by PM on GOT, GPT and LDH release in the first 18 h of intoxication, though some protection became apparent at later times (these data not shown).

Body temperature

At CCl_4 dose levels of 1.3 and 13.0 mmol kg^{-1} i.g., a synergistic effect of CCl_4 and PM on decreases in body temperature and a strong influence of the second 'protective' dose of PM[20] on body temperature were clearly demonstrated (*Figure 1*).

Breathing rate

PM was observed to cause a large increase in breathing rate in the first 4–6 h after administration. This effect of PM was even more marked in the presence of CCl_4[5].

Correlation studies

On the basis of the above-mentioned results it is apparent that, at least with this strain of rat, the 'standard' 13.0 mmol kg^{-1} oral dose of CCl_4 used in many early studies is too large for the investigation of primary hepatotoxic events and the mode of hepatoprotection by PM. The lower dose of 1.3 mmol kg^{-1} was therefore employed in a detailed study of the effects of PM (78 μmol kg^{-1}, i.p.) on 22 diverse parameters measured in individual rats killed 3 h or 6 h after treatment. These particular time points were chosen as it has been shown elsewhere[6] that PM delays the onset of absorption of CCl_4 administered orally by about 2 h, resulting in peak concentrations of CCl_4 in the liver at + 3–4 h instead of at + 2 h after dosing.

Since this experiment was performed principally to permit direct correlations of a wide range of measurements obtained from the same animals, a detailed examination of each of the parameters measured will not be made. However, from examination of the mean values shown in *Table 1* the following effects are readily apparent:

Lipid peroxidation. A clear increase in diene conjugation was measured at both time points (*Figure 2*), though there was no significant increase in malonaldehyde levels in freeze-clamped samples.

Microsomal enzymes. Destruction of glucose 6-phosphatase and cytochrome P_{450} was substantial at both time points but the decrease in aminopyrine *N*-demethylase activity was less substantial, being insignificant at + 3 h and only barely significant at the 10% level at + 6 h.

Relative liver weight. A significant increase was observed in the CCl_4 treated rats.

Liver triglyceride content. The increase in treated rats was not significant at + 3 h but was significant at + 6 h.

Plasma enzymes. The recorded increases were either barely significant or not significant at + 3 h but were all significant by + 6 h.

CCl_4 and PM concentrations. The concentrations of CCl_4 and $CHCl_3$ in the liver at + 3 h were significantly reduced in the presence of PM, but similar levels existed at + 6 h in both treatment groups. Most concentrations fell within expected ranges (based on detailed kinetic studies[5]). However, an irregular relationship appears to exist between CCl_4 concentrations in liver and blood with the dose of CCl_4 administered. High concentrations of PM were recorded in the liver at both time points.

Glutathione content. Small but statistically significant increases *versus* controls were recorded at both time points in treated rats.

Liver DNA, RNA and total protein. Determinations were conducted at + 6 h only (data not included), but no significant changes *versus* controls were observed.

Microsomal lipid and protein. No significant changes were seen at 3 h in these components; at 6 h there was an ~20% decrease in microsomal lipid and an ~10% decrease in microsomal protein

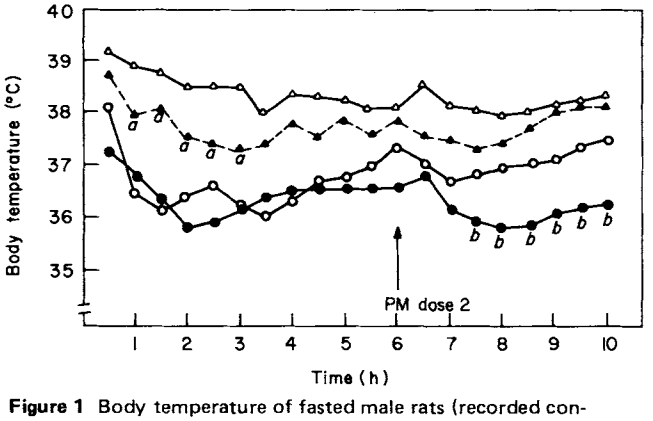

Figure 1 Body temperature of fasted male rats (recorded continuously by rectal thermocouple) following repeated-dose promethazine treatment (78 μmol kg^{-1}, i.p. at 0 h; 39 μmol kg^{-1}, i.p., at 6 h) and concomitant (0 h) administration of CCl_4 13 mmol kg^{-1}, i.g., (●), CCl_4 1.3 mmol kg^{-1}, i.g. (○) or liquid paraffin, i.g., (▲). Vehicle-treated rats (△) served as controls. Data are means (n = 4–5). All CCl_4 + PM means (○, ●) are significantly lower than others. Points marked a are significantly lower than controls ($p < 0.05$); points marked b are significantly lower than CCl_4 (low dose) + PM ($p < 0.05$). △, Liquid paraffin alone; ▲, liquid paraffin + PM; ○, low dose CCl_4 + PM; ●, high dose CCl_4 + PM

Table 1 Simultaneous measurements on individual rats after concomitant administration of CCl_4 (1.3 mmol kg^{-1}, i.g.), and promethazine (78 μmol kg^{-1}, i.p.) or saline. Controls received liquid paraffin i.g. and saline i.p. Data presented as mean ± s.d. ($n = 6$, except * —$n = 5$). Significance level (Student's t-test) indicated by p value: (1) control *versus* CCl_4; (2) control *versus* CCl_4 + PM; (3) CCl_4 *versus* CCl_4 + PM. p value omitted if N.S. = not significant

	After 3 h				After 6 h			
	Treatment group			Significance	Treatment group			Significance
Measurement	Control	CCl_4	CCl_4 + PM	$p <$	Control	CCl_4	CCl_4 + PM	$p <$
Body weight (g)	220 ± 4.7	222 ± 3.4	220 ± 6.8	N.S.	208 ± 9	209 ± 5	207 ± 7	N.S.
Liver weight (g/100 g body weight)	3.61 ± 0.15	3.78 ± 0.10	3.63 ± 0.15	(1) 0.05 (3) 0.1	3.3 ± 0.19	3.81 ± 0.18	3.67 ± 0.14	(1) 0.005 (2) 0.01
CCl_4 conc.								
Liver (nl g^{-1})		0.90 ± 1.03	<0.01*	(3) 0.001		0.24 ± 0.19	0.40 ± 0.63	N.S.
Blood (nl g^{-1})		0.78 ± 0.29	0.15 ± 0.12*	(3) 0.005		0.89 ± 0.19	0.83 ± 0.45	N.S.
$CHCl_3$ conc.								
Liver (nl g^{-1})		2.55 ± 0.53	0.64 ± 0.17*	(3) 0.001		1.58 ± 0.75	1.59 ± 1.15	N.S.
Blood (nl g^{-1})		0.57 ± 0.08	0.16 ± 0.11*	(3) 0.001		0.14 ± 0.07	0.11 ± 0.19	N.S.
PM conc.								
Liver (μg g^{-1})			6.02 ± 0.47				3.99 ± 2.13	
Blood (ng g^{-1})			372 ± 72				293 ± 178	
DPM conc.								
Liver (μg g^{-1})			2.04 ± 0.62				2.28 ± 0.39	
Blood (ng g^{-1})			83 ± 43				82 ± 178	
Diene conjugation E_{235} (0.1% extract)	0.203 ± 0.034	0.292 ± 0.019	0.259 ± 0.035	(1) 0.001 (2) 0.025	0.211 ± 0.019	0.308 ± 0.028	0.325 ± 0.049	(1) 0.001 (2) 0.001
Malonaldehyde ($E_{535-580} \times 10^3$ × liver wt^{-1} g^{-1} 100^{-1} body wt)	5.13 ± 1.07	5.50 ± 0.98	4.69 ± 1.16	N.S.	5.41 ± 0.96	5.18 ± 0.63	4.75 ± 0.77	N.S.
Triglycerides (mg liver wt^{-1} body wt $100\,g^{-1}$)	116 ± 28	122 ± 29	124 ± 27	N.S.	64.2 ± 12.7	121.6 ± 22.5	110.8 ± 31.4	(1) 0.001 (2) 0.02
Glutathione (μmol liver wt^{-1} body wt. $100\,g^{-1}$)	19.3 ± 2.6	22.6 ± 1.5	19.4 ± 3.0	(1) 0.05 (3) 0.1	16.1 ± 1.6	22.8 ± 2.3	19.6 ± 2.7	(1) 0.001 (2) 0.05
Cytochrome P_{450} (nmol mg^{-1} protein)	1.07 ± 0.01	0.83 ± 0.04	1.09 ± 0.06	(1) 0.001 (3) 0.001	1.07 ± 0.08	0.77 ± 0.07	0.88 ± 0.26	(1) 0.001 (2) 0.05 (3) 0.2
Aminopyrine *N*-demethylase (nmol/mg prot./min)	3.45 ± 0.93	3.00 ± 1.07	3.87 ± 1.14	N.S.	2.28 ± 0.60	1.76 ± 0.25	2.63 ± 0.93	(1) 0.1 (3) 0.1
Glucose 6-phosphatase (nmol/mg prot./min)					1.75 ± 0.62	0.95 ± 0.13	1.07 ± 0.18	(1) 0.02 (2) 0.05
Plasma enzymes								
GOT ($U\,l^{-1}$)	118 ± 87	95 ± 10	158 ± 82	(3) 0.1	88 ± 8	190 ± 56	271 ± 107	(1) 0.005 (2) 0.005
GPT ($U\,l^{-1}$)	28 ± 17	28 ± 9	36 ± 15	N.S.	24 ± 5	54 ± 33	55 ± 20	(1) 0.1 (2) 0.005
LDH ($U\,l^{-1}$)	669 ± 911	417 ± 102	635 ± 450	N.S.	325 ± 91	723 ± 143	1116 ± 436	(1) 0.001 (2) 0.005 (3) 0.005
SDH ($U\,l^{-1}$)	3.30 ± 1.5	6.30 ± 1.8	3.27 ± 1.1	(1) 0.02 (3) 0.01	1.7 ± 0.8	15.7 ± 3.8	5.3 ± 4.8	(1) 0.001 (2) 0.1 (3) 0.005
AP ($U\,l^{-1}$)	537 ± 72	541 ± 62	517 ± 51	N.S.	553 ± 75	586 ± 45	552 ± 73	N.S.

in the CCl_4-treated group ($p < 0.02$ and $p < 0.05$, respectively). Promethazine had no significant effects. These data are not included in *Table 1*; control values for microsomal lipid and protein were 11.7 ± 1.3 and 22.0 ± 2.3 mg/g liver (3 h) and 9.8 ± 1.0 and 23.0 ± 1.5 mg/g liver (6 h), respectively.

Within treated groups, statistically significant 'protective' effects of PM were observed at 3 h in relation to the concentrations of CCl_4 and $CHCl_3$ in the liver and in relation to cytochrome P_{450} content. Strong 'protective' trends ($p < 0.1$) were observed in relation to the changes produced by CCl_4 in glucose 6-phosphatase, diene conjugation and relative liver weight values. PM also caused a large but not significant ($p < 0.1$) reduction in the CCl_4-induced increase in hepatic GSH; and, confirming earlier results[5], produced a pronounced increase in breathing rate which lasted about 4 h (breathing rate data not shown).

As the complete range of measurements described above was performed on each individual rat, it was possible to conduct correlation analyses on those parameters that were expected to correlate, or which appeared to follow similar or opposite trends as judged by data ranking: the significant correlations we found are presented in *Table 2*.

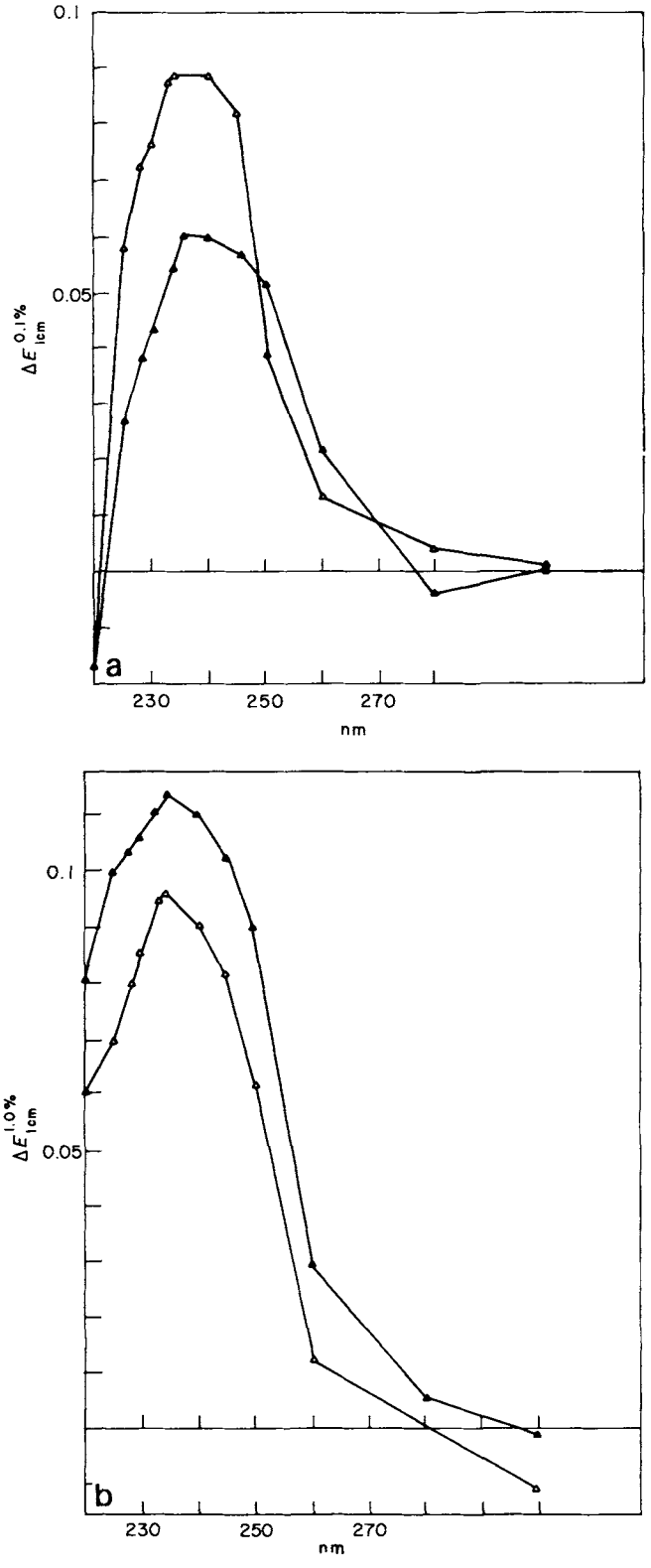

Figure 2 Diene conjugation difference spectra of microsomal lipids (1 mg ml^{-1} in cyclohexane) from male, fasted rats administered CCl_4, 1.3 mmol kg^{-1}, i.g., either with promethazine 78 μmol kg^{-1}, i.p. (▲) or with physiological saline i.p. (△), in each case relative to microsomal lipids of untreated controls. Standard deviation and significance are indicated in Tables 1 and 2. a, 3 h; b, 6 h

Discussion

Using a wide range of biochemical indices of hepatotoxicity we have attempted to elucidate the early sequential processes involved in the CCl_4 model of hepatotoxicity, and the mechanisms by which PM might act as an hepatoprotective agent. This study was placed on a firm base by preliminary studies that established, (i) the minimum effective dose of CCl_4, and (ii) the dose of PM required for the prevention of CCl_4-induced necrosis.

Rees *et al.*[20] demonstrated that the use of two doses of PM, at time 0 and + 6 h, caused a significant reduction in the extent of CCl_4-induced hepatic necrosis as assessed at + 24 h. This work was repeated using the same dose regimens of CCl_4 and PM, but a protective effect of PM was only observed with a dose of CCl_4 ten-times lower than that used originally. This result strongly suggests that the strain of rats used in the present studies is particularly sensitive to CCl_4, and points to the need for special consideration of dose-response relationships when applying previously reported treatment regimens to rats of different origin.

The importance of using a correct *time* of sampling and measurement was effectively demonstrated in screening for lipid peroxidation by monitoring the extent of diene conjugation of microsomal lipid, a method frequently employed in the CCl_4 hepatotoxicity model. In the present study, a clear protective effect of PM *versus* diene conjugation was apparent at + 3 h, but the relative extent of conjugation in the two treatment groups was reversed at + 6 h (*Figure 2*). Bearing in mind

Table 2 Significant correlations found between parameters measured simultaneously in individual rats 3 or 6 h after concomitant administration of CCl_4 (1.3 mmol kg^{-1}, i.g.) and either promethazine (78 μmol kg^{-1}, i.p.) or an equivalent volume of physiological saline

Correlations observed at significance level $p < 0.001$		
$CHCl_3$ concentrations in liver	*and*	$CHCl_3$ concentration in blood, square-root of CCl_4 conc. in liver, microsomal lipid diene conjugation (OD_{235}), cytochrome P_{450} (3 h only)[a]
$CHCl_3$ concentration in blood	*and*	microsomal lipid diene conjugation (OD_{235})
promethazine conc. in liver	*and*	promethazine conc. in blood
desmethyl-PM conc. in liver	*and*	desmethyl-PM conc. in blood
microsomal lipid	*and*	microsomal lipid diene conjugation (OD_{235}) (6 h only),[a] microsomal protein
cytochrome P_{450}	*and*	aminopyrine *N*-dimethylase (6 h only)
RNA[b]	*and*	liver protein[b]
plasma GOT activity	*and*	plasma GPT, LDH activities
Correlations observed at significance level $p = 0.05$–0.01		
CCl_4 concentration in liver	*and*	CCl_4 concentration in blood
square root of CCl_4 conc. in liver	*and*	microsomal lipid diene conjugation (OD_{235})
$CHCl_3$ concentration in liver	*and*	glucose 6-phosphatase (3 h only)[a]
cytochrome P_{450}	*and*	glucose 6-phosphatase
DNA[b]	*and*	microsomal lipid diene conjugation (OD_{235})[a]
RNA[b]	*and*	aminopyrine *N*-demethylase
microsomal lipid	*and*	glutathione[a,c]

[a] Correlation coefficient *negative*
[b] RNA, DNA and liver protein determined at + 6 h only
[c] *Within* treatment groups only

the PM-induced delay in the absorption of CCl_4 during the first 3 h of intoxication[5] these results are as expected. However, the pitfalls of using arbitrarily chosen measurement times for screening procedures (e.g. + 6 h in these examples) are apparent.

Despite the use of a CCl_4 dose of only 1.3 mmol kg^{-1} and very short intoxication periods (3 or 6 h), a wide variety of expected toxic effects were still observed. For example, clear effects on cytochrome P_{450}, glucose 6-phosphatase, lipid peroxidation, relative liver weight, plasma enzyme levels and hepatic triglyceride levels were apparent and the use of this small dose of CCl_4 enabled some differentiation to be achieved in the time of appearance of these toxic effects, and in the sensitivity of the methods used.

Although no significant increases in malonaldehyde were apparent at either 3 or 6 h, diene conjugation in microsomal lipid extracts was significantly increased at both time points (*Figure 2*). This result indicates not only the early onset of significant lipid peroxidation even at this low CCl_4 dose, but also the apparent superior sensitivity of the diene conjugation method over the thiobarbituric acid reaction for determination of lipid peroxidation *in vivo*. A determination *in vivo* of this type is complicated considerably by the continuing metabolism of both the diene conjugates[21] and malonaldehyde (see Ref. 1 for review). Apparent differences in sensitivity of the methods as a measure of lipid peroxidation may thus arise (for example) if the products of the peroxidation are metabolized at different rates, allowing accumulation of one product but not the other. The peroxidative damage is accompanied by destruction of glucose 6-phosphatase and cytochrome P_{450} but not the aminopyrene-*N*-demethylase. This suggests that the damage results as an immediate consequence of CCl_4 activation: metabolic activation of CCl_4 to form highly reactive $CCl_3^{\cdot}$ and $CCl_3O_2^{\cdot}$ free radicals[22]. The enzymes may thus be destroyed directly by $CCl_3^{\cdot}$ free radical attack, or indirectly by peroxidative breakdown of their lipid environment, or by some combination of such and other mechanisms.

In contrast to these early changes, increases in liver triglyceride content first appeared at + 6 h, no changes having been observed in either treatment group at + 3 h. Using a much higher dose of CCl_4 (25 mmol kg^{-1}) a large increase in liver triglyceride is found at + 4 h[23]. Our results using the low dose of CCl_4, therefore distinguish the more sensitive parameters (diene conjugation, glucose 6-phosphatase, etc.) from the increase in triglycerides and the release of enzymes into the blood.

The overall intracellular roles of glutathione (GSH) are manifold and this substance is an essential cellular constituent that may function as a natural, water-soluble free-radical scavenger. It is interesting therefore that concentrations of GSH in the liver were significantly increased by the administration of CCl_4 despite the fact that GSH is in fact at lower concentrations in the centrilobular region than elsewhere in the liver lobule[24]. It may be speculated that GSH accumulates rapidly in the cell as defensive response to the toxic insult. Alternatively, it may be that the acid-soluble, DTNB-positive material measured here is not, in fact, GSH, though GSH does normally represent the major proportion of cellular thiols. Using a higher CCl_4 dose, Docks and Krishna[25] found no change in hepatic GSH at 2 h.

An extensive statistical analysis of the data revealed some strong correlations (*Table 2*). The correlations between liver and blood concentrations of CCl_4, $CHCl_3$, PM and DPM support the contention that the corresponding liver concentrations may be estimated from analysis of blood samples obtained from living animals. This will greatly facilitate the adjustment of dose regimens to provide any desired liver concentrations of either the toxic agent or modifying agents such as Promethazine.

The significant correlations found between cytochrome P_{450} and both *N*-demethylase and glucose 6-phosphatase, and between plasma GOT and both plasma GPT and LDH activities, were expected. However, the most interesting correlations determined in this study were associated with the liver $CHCl_3$ concentrations at any given time, which is an indirect measure of the overall rate of conversion of CCl_4 to the highly reactive $CCl_3^{\cdot}$ free radical. For example the liver concentration of $CHCl_3$ correlated strongly with lipid peroxidation *in vivo* (as measured by diene conjugation of microsomal lipid, E_{235}). Slater and Sawyer[26] demonstrated that CCl_4-induced microsomal lipid peroxidation *in vitro* correlates with the square-root of the CCl_4 concentration in the incubation medium. This was interpreted as evidence for the role of CCl_4 (presumably *via* $CCl_3^{\cdot}$) as an initiator of the chain-reaction lipid peroxidation process. This interpretation is supported by the *in vivo* results since significant correlations were also found to exist at a given time between the square-root of liver CCl_4 concentrations and both diene conjugation OD_{235} and liver $CHCl_3$ concentrations.

That the peroxidation process is a significant aspect of the initial toxicity of CCl_4 is demontrated by the fact that a strong correlation with a significant *negative* coefficient already existed at + 6 h between the microsomal lipid content and the extent of lipid diene conjugation; microsomal lipid breakdown is extensive as shown in the studies of Benedetti *et al.*[27].

The use of $CHCl_3$ concentrations as a measure of the extent of the metabolic activation of CCl_4 is also supported by the strong correlations, again with significant *negative* coefficients, found at + 3 h between $CHCl_3$ liver concentrations and both cytochrome P_{450} and glucose 6-phosphatase. The lack of correlation at + 6 h is considered to be due to the fact that $CHCl_3$ concentrations reach a steady-state situation within 1–2 h of CCl_4 administration[5], whereas the membrane-bound enzymes will continue to be destroyed by the progressive peroxidative destruction of surrounding

lipid, and through attack by free radicals and breakdown products.

Some hepatoprotective activity of PM has been confirmed by these studies. However, a significant part of the protective actions of PM during the first 3 h may be attributed to its effect in producing a consistent 2 h delay in the absorption of CCl_4 (*Table 1* and Ref. 5).

Hence, 'protection' is observed at +3 h *versus* (for example) destruction of cytochrome P_{450} and glucose 6-phosphatase as well as increases in liver weight, diene conjugation and glutathione content, in part because a smaller fraction of the administered CCl_4 dose has reached the liver at this time. Delaying the absorption of CCl_4 permits the build up of high concentrations of PM in the liver *before* significant amounts of CCl_4 are absorbed. PM thereby promotes optimal conditions for its role as free radical scavenger according to the principle[28] that, to be effective a scavenger must be 'at the right place at the right time and in the right concentration'. The ability of PM to scavenge the primary free radical $CCl_3^{\cdot}$ has been questioned following the pulse radiolysis studies of Packer *et al.*[29] but it will unquestionably scavenge very effectively the peroxy radical, $CCl_3OO^{\cdot}$ (Ref. 22), which is more reactive in initiating lipid peroxidation.

The synergistic effects of PM and CCl_4 in reducing the body temperature of rats at both low and high doses of CCl_4 (*Figure 1*) may also be of particular significance to the protective action of PM. Temperature reduction can be made so great (for example, following spinal cord transection) that protection against CCl_4-mediated liver injury is virtually complete (see Ref. 30 for a review). Marzi *et al.*[31] reported that a chlorpromazine-induced reduction in body temperature of 5°C prevented CCl_4-induced hepatic necrosis at + 24 h. Although the temperature reductions following single and repeated-administration of PM recorded here are more modest, the long-lasting 2–3°C reduction in core temperature can be expected to lower considerably the general metabolic rate of the rat and contribute to a substantial reduction in the rate of metabolism of CCl_4.

These studies have attempted to clarify the association between some biochemical parameters commonly used as screening tests in the CCl_4 hepatotoxicity model. By employing a 'least effective' dose of the toxin and modifying the toxic response with a standard hepatoprotective agent, the secondary effects of the toxic reaction have been effectively minimized permitting progress to be made towards elaborating the early sequence of events that follow the initial toxic cleavage of CCl_4.

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