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The effect of dichloralphenazone pretreatment on paracetamol hepatotoxicity in mice

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Paracetamol is a widely used minor analgesic which although relatively safe at therapeutic doses, causes acute dose-dependent centrilobular hepatic necrosis after large doses in man and experimental animals [1, 2]. This hepatotoxicity has recently been shown to be due to the formation of a reactive arylating metabolite by the hepatic microsomal enzymes [3]. Induction of these enzymes by pretreatment with phenobarbital markedly increases the hepatotoxicity of paracetamol in experimental animals [4] and there is evidence that this potentiation also occurs in man [5].

Paracetamol is sometimes given in combination with dichloralphenazone (dichloralantipyrine) in preparations for children and dichloralphenazone has been shown to be an inducer of the microsomal enzymes in experimental animals and man [6]. Therefore it was of interest to discover if dichloralphenazone increased the hepatotoxicity of paracetamol in experimental animals.

Adult, male, Balb/c strain mice (18–25 g) obtained from Bantin and Kingman (Hull, U.K.) were used throughout. Before and during all experiments the animals were allowed food and water *ad lib.*

NADPH, glutathione and glutathione reductase (Type II) were obtained from Sigma (London, U.K.) and 5,5′-dithio bis-(2-nitrobenzoic acid) from Aldrich Chemical Co. (Dorset, U.K.) Paracetamol, sodium phenobarbital and dichloralphenazone were of pharmaceutical grade.

Paracetamol was given by gavage in 0.9% saline (pH 11), 24 hr after the last pretreatment dose. Pretreatment involved giving animals 3 doses of dichloralphenazone or phenobarbital at 24 hr intervals. Phenobarbital (75 mg/kg) was given intraperitoneally (i.p.) in 0.9% saline (pH 7.4) as this procedure has previously been shown to potentiate the hepatotoxicity of paracetamol [4]. Unless otherwise stated, dichloralphenazone (130 mg/kg) was given by gavage (p.o) in distilled water as this is the normal route of administration of the drug in humans. Controls received distilled water only.

Histology. Mice were killed by cervical dislocation 24 hr after dosing with paracetamol. Samples were taken from the left lateral (2 pieces), median (2 pieces), right posterior (1 piece) and omental lobes (1 piece) of each liver and fixed in 10% buffered formalin. Paraffin sections of 6 μm thickness were stained with haematoxylin and eosin. Quantitative assessment of liver necrosis was carried out by a modification of the method of Chalkley [7] which was similar to that of Mitchell *et al.* [4] except that 10 random fields (× 400 magnification) were examined per section (giving 60 fields per mouse). The number of necrotic hepatocytes was expressed as a percentage of the total number of hepatocytes.

Hepatic cytochrome P-450. Twenty four hr after the last pretreatment dose the livers were removed from the mice and placed in 5 ml of ice cold potassium phosphate buffer (0.25 M, pH 7.3) containing potassium chloride (0.15 M). The livers were chopped and homogenised and homogenates were centrifuged at 40,000*g* for 15 min at 4°. Each supernatant was recentrifuged at 105,000*g* for 60 min and the microsomal pellet was resuspended in 2.0 ml of phosphate buffer (0.25 M, pH 7.3) containing 30% glycerol (v/v). One millilitre aliquots were diluted with buffer (3.0 ml) and assayed for cytochrome P-450 by the method of Omura and Sato [8]. Protein determinations were carried out by the method of Lowry *et al.* [9] using crystalline bovine serum albumin as the standard.

Hepatic glutathione. Reduced glutathione was determined in liver homogenates by the method of Ellman [10] as previously described [11].

Total glutathione was determined in liver homogenates after reduction of oxidised glutathione with glutathione reductase by incubating 1 ml of liver homogenate in 0.1 M phosphate buffer (pH 7.4) containing 0.5 μmoles of NADPH and 0.5 units of glutathione reductase, at 25° for 15 min.

From Table 1 it can be seen that a 250 mg/kg intragastric dose of paracetamol in untreated mice caused a minimal degree of hepatic necrosis. This dose is within the dose threshold range previously reported for paracetamol hepatotoxicity after intraperitoneal dosing in mice [12]. However, when this threshold dose was given to mice pretreated with dichloralphenazone or phenobarbital, necrosis was markedly increased (Table 1). This effect of phenobarbital has previously been observed in mice [4]. Pretreatment with dichloralphenazone alone had no effect on hepatic histology.

Measurement of hepatic reduced glutathione levels at various times after the administration of paracetamol revealed a rapid depletion within 30 min (25.3 ± 4.5% of initial value), being maximal after 1 hr (15.9 ± 2.4%) as previously described [11]. Although neither phenobarbital nor dichloralphenazone pretreatment significantly increased this depletion,

Table 1. Effect of dichloralphenazone or phenobarbital pretreatment of mice on paracetamol hepatotoxicity

Pretreatment	Mean % necrosis in survivors	Grade of necrosis 1	2	3	4
			% of surviving animals		
Water (4)	2.8% (4)	100	0	0	0
Dichloralphenazone (6)	25.6% (5)	20	20	60	0
Phenobarbital (6)	44.5% (4)	0	25	25	50

Figures in brackets refer to numbers of animals.
Dose of paracetamol: 250 mg/kg, p.o.
Grades of necrosis: 1: 0–10% necrotic hepatocytes
2: 10–25% necrotic hepatocytes
3: 25–50% necrotic hepatocytes
4: > 50% necrotic hepatocytes.

Table 2. Effect of dichloralphenazone or phenobarbital pretreatment of mice on hepatic cytochrome P-450 and paracetamol mediated glutathione depletion

Pretreatment	After pretreatment alone		1 hr after 250 mg/kg paracetamol (p.o.)	
	Cytochrome P-450 (nmoles/mg protein)	Total glutathione (μmoles/g liver)	Total glutathione (μmoles/g liver)	Total liver weight (g)
Distilled water	1.42 ± 0.07 (6)	5.01 ± 0.43 (4)	0.71 ± 0.09 (6)	1.19 ± 0.05 (6)
Phenobarbital	2.97 ± 0.12*(4)	—	0.51 ± 0.09 (6)	1.43 ± 0.05*(6)
Dichloralphenazone (3×130 mg/kg, p.o.)	1.06 ± 0.18 (3)	5.57 ± 0.13 (4)	0.49 ± 0.02+(6)	1.23 ± 0.04 (6)
Dichloralphenazone (3×80 mg/kg, p.o.)	1.26 ± 0.06 (4)	—	—	—
Dichloralphenazone (3×130 mg/kg, i.p.)	1.35 ± 0.04 (3)	—	—	—

* $P < 0.01$. + $P < 0.05$.
Results are mean ± S.E.
Figures in brackets represent numbers of animals.

when total glutathione was measured paracetamol caused a significantly greater depletion in dichloralphenazone pretreated animals than in controls (Table 2). Phenobarbital pretreated animals showed a similar trend although this was not significantly different. Dichloralphenazone pretreatment alone had no effect on the total hepatic glutathione (Table 2).

The minimal effect of dichloralphenazone and phenobarbital pretreatments on glutathione depletion may be due to the threshold dose level of paracetamol used. Thus at this dose level in these studies glutathione is being depleted to about 15 per cent of the control value and previous work has indicated that hepatic glutathione depletion by paracetamol or diethyl maleate reaches a maximum at about 15 per cent of control levels [11]. Therefore pretreatments which induce the microsomal enzymes and thereby increase toxicity may increase the rate of depletion to the minimum level rather than reduce this minimum. Previous observations on the effect of phenobarbital pretreatment on glutathione depletion after paracetamol have indicated that this is indeed the case [11].

The potentiation of paracetamol hepatotoxicity by dichloralphenazone and the increased depletion of total glutathione is consistent with its action as a microsomal enzyme inducer as previously reported in both humans and experimental animals [6]. Consequently cytochrome P-450 levels were measured in the livers of mice pretreated with dichloralphenazone or phenobarbital and compared with those in control animals. The results indicated that cytochrome P-450 levels were not increased by dichloralphenazone, whereas phenobarbital pretreatment caused a significant increase (Table 2) as previously shown in a number of studies [13]. The lack of effect of dichloralphenazone was seen after both intragastric and i.p. dosing. Two different dose levels of dichloralphenazone were given intragastrically but there was no significant difference between the cytochrome P-450 levels (Table 2).

Liver weights were also measured and no effect was observed on this parameter also suggesting that dichloralphenazone had no effect on cytochrome P-450 content whereas phenobarbital caused a significant increase in liver weight (Table 2).

This lack of effect of dichloralphenazone on cytochrome P-450 was surprising in view of previous evidence suggesting that this drug induces microsomal enzyme activity [6]. The results clearly indicate that dichloralphenazone pretreatment increases paracetamol hepatotoxicity but it would seem it does so by some means other than by increasing hepatic cytochrome P-450. Activation of microsomal enzyme activity or a selective induction of a particular form of cytochrome P-450 are possibilities. Inhibition of pathways of paracetamol metabolism alternate to microsomal oxidation such as conjugation, by metabolites of dichloralphenazone seem unlikely as the last dose of dichloralphenazone was given 24 hr before the paracetamol. However, such effects would not be apparent from this study. Investigation of the effect of dichloralphenazone pretreatment on paracetamol metabolism and on microsomal enzyme activity would be necessary to clarify this.

It may be concluded therefore that dichloralphenazone pretreatment increases paracetamol hepatotoxicity in mice. There is an increased depletion of total glutathione after a dose of paracetamol caused by dichloralphenazone pretreatment which indicates that the pretreatment in some way alters the metabolism of paracetamol leading to greater amounts being metabolised to the reactive toxic intermediate or an increased rate of production of this intermediate. This effect would not appear to be due to an increase in hepatic cytochrome P-450 content, however.

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