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THE EFFECT OF AGE ON PARACETAMOL HEPATOTOXICITY IN MICE

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Abstract— The hepatotoxicity of paracetamol to mice was investigated in relation to age. Measurement of hepatic necrosis, covalent binding of radiolabelled paracetamol to liver protein and hepatic glutathione depletion indicated that paracetamol was less toxic to neonatal mice than to the adult animal. Although the neonatal hepatic glutathione level is considerably less than that of the adult (26 per cent) the levels of P-450 in the neonate are also less (9 per cent). This suggests that in this case the development of the ability to detoxify the toxic reactive metabolite of paracetamol precedes the development of the enzyme system producing it.

Although paracetamol (Tylenol, acetaminophen) is a safe minor analgesic after therapeutic doses, it may cause severe and sometimes fatal hepatic necrosis in both man and experimental animals after large doses [1, 2]. Numerous previous studies have indicated that the hepatotoxicity of paracetamol is due to its metabolism by the microsomal mixed function oxidase system to a reactive arylating species [3, 4]. After a low, non-toxic dose of paracetamol, this reactive intermediate is adequately detoxified by conjugation with hepatic glutathione. When this glutathione is depleted by an excessive dose of paracetamol the reactive intermediate covalently binds to liver macromolecules, resulting in hepatic necrosis [5].

Both the covalent binding of the reactive intermediate to liver macromolecules and the depletion of hepatic glutathione correlate with the necrosis produced by paracetamol [6].

Glutathione therefore fufils a protective role in the liver, by combining with the hepatotoxic intermediates of compounds such as paracetamol and bromobenzene [5, 7] and thereby preventing arylation of nucleophilic sites in hepatocytes. Artificially depleting hepatic glutathione with compounds such as diethyl maleate markedly increases the hepatoxicity of paracetamol and bromobenzene [5, 7]. It has been reported that hepatic glutathione levels are low in neonatal mice [8] but it is well known that the activity of the mixed function oxidase system is also low in neonates. Furthermore, it has been reported that bromobenzene is less hepatotoxic in neonatal rats [9]. Hence it was of interest to compare the hepatotoxicity of paracetamol in mice of various ages to determine the effect of these deficiencies.

MATERIALS AND METHODS

Adult Balb/c strain mice obtained from Bantin & Kingman (Hull, U.K.) were used throughout to produce litters of immature animals. Only male animals were used in the studies described.

[^{3}H]-Paracetamol (generally labelled, sp. act. 3.43×10^3 Ci/mmole;) was obtained from New England Nuclear (Boston, MA). Purity was greater than 99 per cent.

Glutathione was obtained from Sigma (London U.K.) and 5,5′-dithiobis-(2-nitro benzoic acid) from Aldrich Chemical Co. (Dorset, U.K.).

Paracetamol used was of pharmaceutical purity.

Paracetamol was given to animals by intragastric intubation as a solution (20 mg/ml) in 0.9% saline, pH 11 [5]. Animals received 0.2–0.5 ml and controls received vehicle only.

Histology. Animals given various doses of paracetamol were killed 48 hr later and paraffin sections of liver slices, taken from various lobes of liver, were prepared. The sections were stained with haemotoxylin/eosin and quantitative analysis of liver necrosis carried out using a modification of the method of Chalkley [10]. Ten fields of liver sections were examined microscopically (400 × magnification) with a grid of 100 squares superimposed over the field. The degree of necrosis was quantitated as the proportion of necrotic cells to the total number of cells in the grid area and the average necrosis for the ten fields was calculated.

Covalent binding. Mice were given [^{3}H]paracetamol (350 mg/kg; 420 μCi/kg) orally as described. After 4 hr livers and samples of sartorious muscle were removed, minced and homogenised in 2 ml of distilled water. The homogenate was divided into two equal portions and 1 ml of methanol added to each. Precipitated protein was centrifuged off and covalent binding of radiolabel determined as described by Jollow *et al.* [4]. Protein was determined by the method of Lowry *et al.* [11].

Glutathione determinations. Hepatic glutathione levels were determined by the method of Ellman [12] as previously described [5], without modification.

Hepatic cytochrome P-450. Livers were removed from mice, weighed and homogenised in ice cold buffer (1.15% KCl/0.02 M Tris, pH 7.4; 5 ml/g of liver). Homogenates were centrifuged at 40,000 *g* for 15 min at 0–4°. The supernatant was recentrifuged at 100,000 *g* for 60 min at 0–4°. Microsomal pellets were resuspended in cold Tris/KCl buffer with a final protein concentration of 2–5 mg/ml. Protein was determined

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Table 1. Hepatotoxicity of paracetamol and covalent binding of [^{3}H]paracetamol to liver protein *in vivo* in mice of various ages

Age (days)	Dose (mg/kg)	No. of surviving animals	Grade of necrosis % of survivors 1	2	3	4	Covalent binding of [^{3}H]paracetamol to protein nmoles/mg protein Liver	Muscle
3	300	10	90	10	0	0		
8	350	4	50	25	25	0	0.34 ± 0.06 (8)	0.1 ± 0.02 (3)
10	300	7	71	29	0	0		
19	350	6	—	—	—	—	1.19 ± 0.16$^+$ (6)	0.09 ± 0.01 (3)
35	300	8	64	18	18	0		
35	350	2	0	0	0	100	0.84 ± 0.17* (6)	0.05 ± 0.01 (3)

Grades of necrosis: 1 = Less than 10% of hepatocytes; 2 = 10% to 25% of hepatocytes; 3 = 25% to 50% of hepatocytes; 4 = Greater than 50% of hepatocytes.

Animals were given paracetamol orally in aqueous solution (20 mg/ml), 0.2–0.5 ml/animal as described in the text.

Covalent binding was determined 4 hr after administration of [^{3}H]paracetamol, and results are means ± S.E., numbers of animals in brackets. Significant difference from 8 day old mice, *$P < 0.01$; $^+P < 0.001$.

by the method of Lowry *et al.* [11]. The cytochrome P-450 concentration in microsomes was determined by the method of Omura and Sato [13].

RESULTS AND DISCUSSION

The results presented in Table 1 indicate that the neonatal mouse is less susceptible to paracetamol hepatotoxicity than the adult animal. Thus the hepatotoxicity, characterised as a centrilobular necrosis, with anuclear or pyknotic hepatocytes and eosinophilic denegeration was less extensive in the 3, 8 and 10 day old mice than in the 35 day old adult mice.

Previous studies of the mortality, in mice of various ages following doses of paracetamol indicated that neonatal mice were more susceptible [14]. However, the reason for this increased mortality in the neonate was not established. The lower hepatotoxicity of paracetamol in the neonate suggested by the histological data was substantiated by measurement of the covalent binding of radiolabelled paracetamol. This covalent binding of radiolabelled paracetamol to liver protein has previously been shown to correlate with liver necrosis [4]. Thus after a hepato-toxic dose of [^{3}H]paracetamol, covalent binding in the neonate was significantly less than that in adults and 19 day old mice, whereas binding to muscle was not (Table 1).

Table 2. Depletion of reduced glutathione various times after a 500 mg/kg dose of paracetamol given to mice of various ages.

Time after dosing (hr)	Age (days) 4	6	18	35
	Glutathione % Control			
1	83 ± 5	77 ± 3	48 ± 4	42 ± 5
2	91 ± 6	45 ± 5	23 ± 5	15 ± 6
3	77 ± 4	67 ± 6	31 ± 6	33 ± 5
4	—	—	—	21 ± 4

Glutathione is expressed as per cent of control value. Results are the means of at least 2 animals ± S.E. Paracetamol given as a single oral dose in aqueous solution (20 mg/ml) 0.2–0.5 ml/animal.

This was further supported by determination of liver reduced glutathione depletion caused by a toxic dose of paracetamol. Neonatal mice (4-and 6-day-old) suffered significantly less depletion of glutathione at all 3 time points than either 18 day old or adult mice (Table 2). The 18-day old-mice were not significantly different from adults. It can be seen that the extent of depletion increases with age reaching the adult level by 18 days of age.

Therefore, all three parameters investigated: histologically assessed necrosis, covalent binding to protein and glutathione depletion, indicate a reduced susceptibility to paracetamol hepatotoxicity in the neonate. It appears that the mouse reaches adult susceptibility by 18–19 days of age. Investigation of the development of hepatic glutathione (Fig. 1) confirmed previous studies [8], indicating that hepatic glutathione reaches adult levels 10 days after birth.The protection of the neonatal liver by glutathione would therefore be expected to be reduced in comparison with the adult animal. However, measurement of the development of hepatic cytochrome P-450 (Fig. 1) revealed that neonatal levels were only 9 per cent of adult levels, less than the neonatal glutathione level expressed on the same basis (26 per cent). A number of studies have indicated that in both humans and experimental animals neonatal monooxygenase activity is low compared to the adult [15].

Although the activity of the microsomal enzymes was not measured directly in this study, the data presented suggest that the production of the reactive intermediate of paracetamol by the microsomal enzymes may be less in the neonate. The hepatic cytochrome P-450 achieves adult levels by 16 days of age and it is at approx. this age that depletion of glutathione and covalent binding are similar to the adult. This would suggest that the microsomal oxidation of paracetamol also reached adult capacity at 16 days of age as well as the level of cytochrome P-450.

Therefore it would seem that the protective mecha-

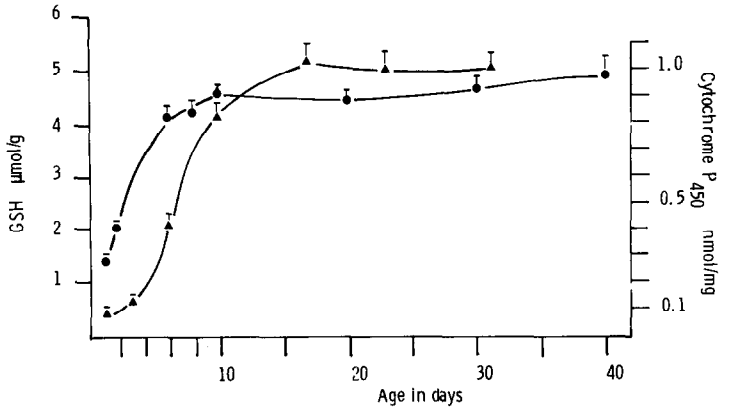

Fig. 1. Development of hepatic reduced glutathione and cytochrome P-450 levels with age in mice. Glutathione ● ; Cytochrome P-450 ▲. Points are means ± S.E. of at least 4 animals.

nism, in the form of hepatic glutathione develops earlier than the enzyme responsible for the production of the toxic metabolite.

The reduced level of glutathione and perhaps lower glutathione transferase activity [16] are thus still sufficient to cope with the smaller amount of reactive metabolite formed.

This differential development may therefore explain at least in part, the reduced toxicity of paracetamol in the neonate and is consistent with previous findings that neonatal rats are less susceptible to bromobenzene hepatotoxicity [9].

The importance of these findings to the human neonate are at present unknown, but it can be seen that the relative amounts of glutathione and cytochrome P-450 will be crucial in determining the overall hepatotoxicity of paracetamol in the human neonate.

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