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SPECIES DIFFERENCES IN THE HEPATOTOXICITY OF PARACETAMOL ARE DUE TO DIFFERENCES IN THE RATE OF CONVERSION TO ITS CYTOTOXIC METABOLITE

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Abstract—The cytotoxicity of paracetamol and of its putative toxic metabolite, *N*-acetyl-*p*-benzoquinoneimine (NABQI) have been investigated in hepatocytes from hamster, mouse, rat and human liver. Whereas paracetamol readily caused cell blebbing and a loss of viability in hepatocytes from mouse and hamster, human and rat hepatocytes were much more resistant to these effects. In marked contrast, there were no significant differences in the sensitivity of the cells from any species to the toxic effects of NABQI. Glutathione depletion by NABQI and paracetamol correlated very well with the toxic effects of these compounds. It is concluded that species differences in sensitivity to the hepatotoxicity of paracetamol are due almost entirely to differences in the rate of formation of NABQI, and not to any intrinsic differences in sensitivity or in any difference in the fate of NABQI once formed. Further, man appears to be relatively resistant to the hepatotoxic effects of paracetamol, and the results in hepatocytes were confirmed by both *in vitro* and *in vivo* analyses.

Although the antipyretic analgesic paracetamol is relatively safe at normal therapeutic doses [1], in overdose it can cause severe and sometimes fatal hepatic necrosis in man [2]. There are marked species differences in susceptibility to the hepatotoxic effects of paracetamol [3]. The hamster and mouse are very sensitive whereas guinea pig, rabbit and rat are resistant [3]. Similar conclusions on the sensitivity of rat, hamster and rabbit were made on the basis of the cytotoxicity of paracetamol in cultured hepatocytes.

The metabolic basis for the toxicity of paracetamol was elucidated by Gillette, Mitchell and their colleagues [4–8]. The major route of disposition of paracetamol is by conjugation with sulphate and glucuronide [7]. A small proportion of the dose, 5–10%, is oxidised by the cytochrome P-450 dependent mixed function oxidase system to a highly reactive intermediate which readily forms an adduct with reduced glutathione (GSH)‡ [5, 8], a reaction that can be catalysed by glutathione *S*-transferases [9]. The adduct is then further metabolised and excreted in the urine as the mercapturic acid or cysteine conjugate [10]. At higher doses of paracetamol, hepatic GSH is depleted and the reactive metabolite is then free to initiate those events that lead ultimately to cytoxocity [5, 8]. The nature of the reactive metabolite has still not been established beyond doubt but there is now very good evidence that it is *N*-acetyl-*p*-benzoquinoneimine (NABQI) [11–14].

Species differences in sensitivity to the hepatotoxicity of paracetamol have been attributed to differences in the rate of formation of the reactive metabolite [3, 15]. However, this has yet to be demonstrated by studying species differences in the effects of the putative toxic metabolite, NABQI. In addition, the relative sensitivity of man has been difficult to assess [16, 17]. There are several reasons for this. Firstly, the information available on the exact size of the dose and time of drug ingestion are usually unreliable [16]. Secondly, whereas populations of laboratory animals are relatively homogeneous, the human population is heterogeneous in its ability to oxidise drugs [18]. Thirdly, assessment of susceptibility of humans to the hepatotoxic effects of paracetamol inevitably depends heavily upon those patients who present at hospital and who subsequently develop liver damage [19]. There is a widely held belief that man is very sensitive to paracetamol toxicity [20] perhaps even as sensitive as the hamster, with doses of 150 mg/kg producing liver damage. However, this is an estimate of the minimum toxic dose, not of the ED_{50}, a much more difficult parameter to estimate in the human population. In the hamster, the ED_{50} is 250–400 mg/kg [3].

With the availability of a suitable preparation of pure NABQI [12], we have investigated the relative susceptibility of hepatocytes from man, rat, mouse and hamster to both paracetamol and NABQI induced damage. In addition to viability, the extent of plasma membrane blebbing was assessed as was covalent binding of paracetamol to cellular proteins and the GSH content of the cells.

MATERIALS AND METHODS

Materials. Earle's balanced salt solution, calcium- and magnesium-free (EBSS) and foetal calf serum

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‡ Abbreviations used: EBSS, Earle's balanced salt solution; EC_{50}, that concentration of a compound causing 50% of the maximum possible effect; ED_{50}, the dose of a drug required to cause an effect in 50% of the population; EGTA, ethylene glycol-bis-(β-aminoethyl ether)-*N*, *N*, *N'*, *N'*-tetraacetic acid; GSH, reduced form of glutathione; GSSG, oxidised form of glutathione; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid; NABQI, *N*-acetyl-*p*-benzoquinoimine; TCA, trichloroacetic acid.

Table 1. Comparison of hepatocytes isolated from human, rat, mouse and hamster liver

Parameter	Species			
	Human	Rat	Mouse	Hamster
No. of samples*	11	32	4	14
Wt of liver (g)	28.0 ± 15.2†	17.1 ± 2.7	2.1 ± 0.3	5.3 ± 0.5
Size of cannula (outside dia. mm)	1.3–2.1	1.3	1.1	1.3
Perfusate flow rate (ml/min)	60–180	40–60	10	20–30
Collagenase concentration (mg/100 ml)	50	35	20	30
Enzyme perfusion time (min)	49 ± 7	19 ± 5	8 ± 1	10 ± 2
Initial cell viability (% total cells)	60.3 ± 17.3	90.8 ± 3.3	81.6 ± 6.2	90.4 ± 4.1
Cell yield ($\times 10^6$ viable cells/g)	11.9 ± 12.2	46.2 ± 5.6	14.6 ± 4.1	40.8 ± 12.1
Protein content (mg/10^6 cells)	N.D.	1.48 ± 0.26 (5)	1.22 ± 0.06 (3)	1.23 ± 0.06 (3)
Cytochrome P-450 content (pmol/mg protein)	N.D.	253 ± 18 (5)	N.D.	180 ± 45 (3)
GSH content (pmol/10^6 cells)	N.D.	58.8 ± 6.2 (5)	61.0 ± 3.6 (4)	18.2 ± 2.9 (10)

* Values are mean (±SE where indicated). Number of samples studied was as shown or otherwise as indicated in parentheses.

† Weight of resected liver samples.

N.D. Not determined.

were purchased from Gibco Limited, Uxbridge, Middlesex. Gentamicin sulphate, insulin (from bovine pancreas), hydrocortisone-21-hemisuccinate, ethylene glycol-bis-(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), collagenase type I, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid (HEPES), Trypan blue, reduced glutathione (GSH), oxidised glutathione (GSSG), glutathione reductase (type IV, from yeast) 5,5′-dithiobis-2-nitrobenzoic acid, NADPH, EDTA, paracetamol and L-cysteine were obtained from Sigma Chemical Co. Ltd, Poole, Dorset. Pentobarbitone sodium was purchased from May & Baker Ltd, Dagenham, Essex. Packard Instruments, Caversham, Berks, supplied Instagel liquid scintillation cocktail. Radiolabelled paracetamol [ring U-^{14}C], specific activity 19.5 mCi/mmol (98% pure) was obtained from Amersham International Limited, Amersham, Bucks. 3-*S*-Glutathionyl paracetamol was a generous gift from D. Johnson, Sterling Winthrop, Newcastle-upon-Tyne. *N*-Acetyl-*p*-benzoquinoneimine (NABQI) and [ring U-^{14}C]NABQI were synthesised according to the method of Huggett and Blair [21] by the oxidation of paracetamol with freshly prepared silver oxide. NABQI was stored as a solution in chloroform in liquid nitrogen until use. Under these conditions less than 6% of the NABQI was lost over two months [22]. All other reagents were of analytical reagent grade or better.

Human liver samples. Human liver samples were obtained from patients undergoing partial hepatectomy for primary or secondary carcinoma of the liver [23]. Local Research Ethics Committee permission was obtained to use such liver samples for these studies. The method of isolation of hepatocytes, utilising collagenase perfusion, has been described in detail elsewhere [23]. All samples were assessed as non-malignant from histology of a small biopsy taken from the edge of the section used for hepatocyte isolation. All patients were hepatitis B surface antigen negative.

Animals. Male Wistar rats (180–250 g) and male Balb/c mice (18–25 g) were obtained from Olac, Bicester, Oxon. Male golden Syrian hamsters (90–110 g) were supplied by Intersimian, Abingdon, Oxon. Animals were permitted free access to food (PRD Diet from Labsure Animal Diets, Poole, Dorset) and water. They were maintained under constant conditions of heating and lighting.

Isolation of rodent hepatocytes. The isolation of hamster hepatocytes was as previously described [24]. Rat hepatocytes were isolated by an identical technique to that described for the preparation of cells from the hamster. Mouse hepatocytes were isolated by *in situ* perfusion via the portal vein using an Argyle Medicut (Petit Rechain, Belgium) cannula (1.10 mm o.d. × 45 mm). Perfusate was returned to the reservoir via a cannula placed in the inferior vena cava above the diaphragm. Other details of the perfusion were essentially as described for the isolation of hamster hepatocytes [24], with appropriate reductions in scale for the size of the mouse liver. Differences in the conditions of perfusion for the species investigated and a comparison of the hepatocytes thus obtained are shown in Table 1.

Assay of total intracellular glutathione content. Hepatocyte total glutathione content was assayed by the enzymic recycling method originally described by Tietze [25], as modified by Tee *et al.* [24].

Covalent binding of [^{14}C]paracetamol to hepatocyte proteins. The covalent binding of [^{14}C]paracetamol (specific activity 11–870 μCi/mmol) and [^{14}C]NABQI (specific activity, 142 ± 14 μCi/mmol) to hepatocye proteins was determined by a modification [24] of the method of Jollow *et al.* [6]. Proteins were precipitated by the addition of 6.5% (w/v) trichloroacetic acid (TCA), washed twice with TCA and then extracted repeatedly with 80% (v/v) methanol until no further radioactivity could be removed. The protein pellet was digested with sodium hydroxide and aliquots taken for protein determination [26] and liquid scintillation spectrometry [24].

Determination of cell viability and cell blebbing. The viability of the cells was determined from their ability to exclude Trypan blue. Suspensions of hepatocytes were diluted in PBS containing Trypan blue, to give a final concentration of the dye of 0.15% (w/v). Viable cells, those not stained with Trypan

blue and showing a refractile edge, were counted using an improved Neubauer counting chamber (depth 0.1 mm; 1/400 mm^2) with visualisation under phase contrast illumination (10× binocular eyepiece; 10× objective). Two fields, each of 1 mm^2, were counted for each cell preparation. Percentage viability has been expressed relative to the total number of cells present.

In the same preparation as that used for determining cell viability by Trypan blue exclusion, plasma membrane blebbing was evaluated. The number of unstained cells which exhibited blebbing, readily discernible under phase contrast illumination, were counted. Cells showing any type of blebbing were included in the count. Percentage blebbed cells has been expressed relative to the total number of viable cells present.

Experimental procedure. Freshly isolated hepatocytes were resuspended in EBSS to a density of 1.5–2.0 × 10^6 cells/ml. The suspensions were then incubated at 37° with gentle agitation in an oxygen atmosphere in the presence of varying concentrations of NABQI or paracetamol and aliquots of the cell suspensions were removed at various times for assessment of the parameters described above.

Covalent binding of paracetamol to microsomal proteins. The covalent binding of [^{14}C]paracetamol to hepatic microsomal proteins from mouse and human was determined by the method of Jollow *et al* [6]. [^{14}C]Paracetamol was purified by reverse phase HPLC prior to use in these covalent binding experiments, to minimise non-specific background. Hepatic microsomal fraction sufficient to give a final protein concentration of 1 mg/ml was incubated with NADPH, 0.7 mM, $MgCl_2$, 30 mM, potassium phosphate buffer, pH 7.40. 50 mM, and [^{14}C]paracetamol, 0.5 mM, 0.8 μCi, in a final volume of 3 ml (mouse) or 1 ml (human). Samples were incubated for 10 min (mouse) or 30 min (human) at 37° in a shaking water bath and the reaction was terminated by the addition of 5 ml of ice-cold TCA, 1.2 M. Covalent binding of paracetamol was first order with respect to both protein concentration and time of incubation.

Precipitated proteins were recovered by centrifugation at 1000 *g* for 15 min. The protein pellet was washed twice in 5 ml of 0.6 M TCA and then extracted repeatedly with 80% methanol (5 ml) until no further radioactivity could be removed, usually five times. The protein was finally digested in 1 ml sodium hydroxide, 1 M, by incubating for 10 hr at 70°. Aliquots of the digested protein were taken for the determination of protein concentration and for liquid scintillation spectrometry, the latter following digestion with Soluene-350 (Packard) for 2 hr at room temperature. Liquid scintillation spectrometry was performed after the addition of 10 ml Dimilume-30 (Packard).

Determination of glutathione adduct formation in vitro. Microsomal fraction equivalent to 1 mg protein was incubated with NADPH, 2.4 mM, $MgCl_2$, 5 mM, Tris hydrochloride buffer, pH 7.4, 37 mM and paracetamol, 0.45 μCi, from 100 to 1500 μM, in a final volume of 0.25 ml. Samples were incubated for 20 min (mouse), 40 min (rat) or 60 min (human) at 37° in air in a shaking water bath. The reaction was terminated by the addition of sufficient ice-cold perchloric acid to give a final concentration of 3% (v/v). The reaction was first order with respect to both time of incubation and protein concentration.

Following neutralisation of the samples with 0.2 M Tris hydrochloride buffer, pH 7.0, and addition of the reference standard, authentic glutathionyl paracetamol, protein was removed from the samples by centrifugation at 1500 *g* for 10 min. The supernatant was analysed for glutathionyl paracetamol by HPLC, with a Spheri 5 RP8 column (Brownlee Laboratories) fitted to a model UK 6000A pump (Waters Associates). The mobile phase was 0.1 M sodium acetate, pH 4.0, containing, by volume, 2% acetic acid, 0.2% propan-2-ol, 0.2% methanol, 0.2% methoxyethanol and 0.15% perchloric acid. The flow rate was 2 ml/min and the absorbance of the eluate was monitored at 254 nm. One ml fractions of the eluate were collected and to each was added 10 ml Instagel (Packard). Liquid scintillation spectrometry was performed with a Tricarb 2660 (Packard) liquid scintillation spectrometer with interactive quench correction software. Unreacted paracetamol eluted at 4 min and the glutathione conjugate eluted at 2.5 min. There was 0.5 min of baseline separation between the peaks.

RESULTS

There were marked species differences in the cytotoxicity of paracetamol in isolated hepatocytes (Table 2). Mouse and hamster hepatocytes were relatively sensitive whereas hepatocytes from rat and human were resistant. Significant loss of viability with rat hepatocytes became apparent only at the highest concentrations of paracetamol used, 40 mM and above. With human hepatocytes, there was no evidence of cytotoxicity, even with concentrations of paracetamol as high as 50 mM. In marked contrast, hepatocytes from all four species showed very similar sensitivity to the toxic effects of NABQI. This caused a rapid and profound decrease in the viability of the hepatocytes.

Viability profiles were analysed in more detail for hepatocytes from hamster, rat and human with respect to concentration and time (Figs 1–3). The results of this analysis confirmed the conclusions from Table 2. Hamster hepatocytes showed a time- and concentration-dependent decrease in their viability on incubation with paracetamol (Figs 1 and 3). Even at the highest concentration tested, 20 mM, no loss in viability was apparent over the first hour of incubation. Thereafter there was a progressive decrease in cell viability, the extent of which was dictated by the concentration of paracetamol to which the cells were exposed. Thus, after 3 hr incubation with 20 mM paracetamol less than 20% of the cells remained viable. With rat hepatocytes, there was very little effect of paracetamol at concentrations below 50 mM. At the highest concentration, there was a time-dependent decrease in viability although even after incubation for 3 hr, viability was still approximately 70% in these cells. With human hepatocytes, although there was a slight decrease in viability over the 3 hr of incubation, by 13%, at no concentration of paracetamol, even at 50 mM, was

Table 2. EC_{50} values for the cytotoxic effects of paracetamol and NABQI on hepatocytes from different species

Parameter	Compound	EC_{50} value (mM)* Hamster	Rat	Human	Mouse
Cell blebbing	NABQI	0.176	0.152	0.144	N.D.
	Paracetamol	1.00	35.0	35.0	N.D.
Cell viability	NABQI	0.256	0.336	0.392	0.300
	Paracetamol	3.50	>50	>50	10.1

* EC_{50} value is that concentration of the compound producing blebbing in 50% of the viable cells or a 50% decrease in viability from its initial value, respectively. Results are typical of those obtained with at least four different preparations from each species.
N.D. Not determined

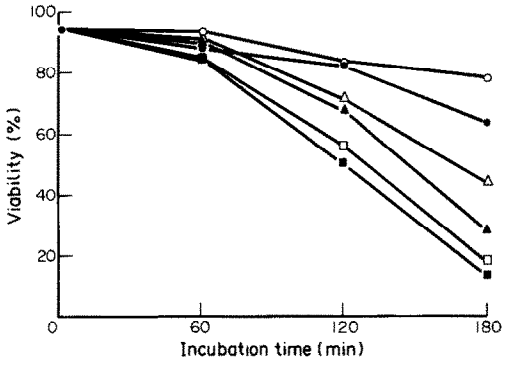

Fig. 1. Effects of paracetamol on the viability profile of freshly isolated hamster hepatocytes. Cells were incubated for up to 3 hr with 1.25 mM (●), 2.5 mM (△), 5.0 mM (▲), 10 mM (□), and 20 mM (■) paracetamol. Control cells (○) were incubated in medium alone. Results are typical of those obtained with at least four different cell preparations.

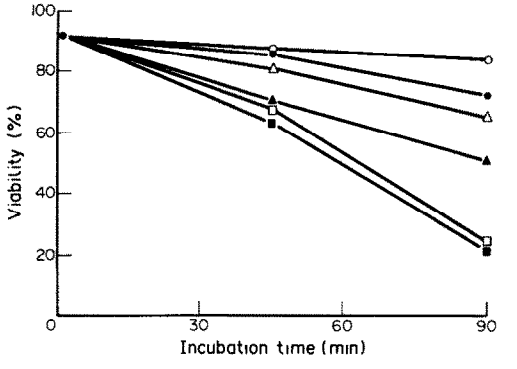

Fig. 2. Effects of NABQI on the viability profile of freshly isolated hamster hepatocytes. Cells were incubated for up to 90 min with 0.04 mM (●), 0.08 mM (△), 0.2 mM (▲), 0.4 mM (□), and 0.8 mM (■) NABQI. Control cells (○) were incubated in medium alone. Results are typical of those obtained with at least four different cell preparations.

there any significant difference in viability between control and paracetamol-treated cells.

Viability profiles were also obtained with NABQI (Figs 2 and 3). In this case, the profiles were very similar in hepatocytes from hamster, rat and human. At the higher concentrations of NABQI, 0.20 mM and above, a significant decrease in viability was apparent within 45 min of incubation and this progressed during the subsequent 45 min of incubation. At lower concentrations of NABQI, some loss of viability was apparent only after 90 min of incubation.

The extent of plasma membrane blebbing was assessed in hepatocytes exposed to paracetamol (Figs 3 and 4). The most obvious effects were apparent with cells from the hamster. There was a concentration-dependent increase in the number of blebbed cells apparent, and this occurred within the first 60 min of incubation. The extent of blebbing progressed throughout the experiment, so that even at the lowest concentration of paracetamol used, 1.25 mM, almost 70% of the hepatocytes were blebbed after 3 hr incubation. Hepatocytes from rat and human showed much less extensive blebbing. At the lowest concentrations of paracetamol, very little increase in blebbing was apparent, but at higher concentrations there was a time- and concentration-dependent increase in the number of blebbed cells visible. One difference between the rat and human hepatocytes was that whereas with the former, blebbing did not increase significantly until after 60 min of incubation, with the human cells there was appreciable blebbing at the higher concentrations of paracetamol within 60 min.

The time course of plasma membrane blebbing in hamster, rat and human hepatocytes exposed to NABQI was very similar in all three species (Fig. 5). Blebbing was apparent within 45 min at all concentrations of NABQI in all three species and, in all cases, at the highest concentration, 0.80 mM, 100% of viable cells were blebbed within 45 min.

EC_{50} values were calculated for the cytotoxicity and cell blebbing caused by paracetamol and NABQI (Table 2). In all three species, a concentration of NABQI between 0.14 and 0.18 mM caused blebbing in 50% of viable cells within 90 min. The EC_{50} for cytotoxicity with NABQI was between 0.25 and 0.4 mM for all four species. Thus, the concentration of NABQI responsible for 50% cytotoxicity is approximately double that required for blebbing. In contrast to the lack of variation in the EC_{50} values for NABQI in the different species, EC_{50} values for paracetamol showed considerable inter-species variability. The hamster was most sensitive to both the blebbing and the cytotoxic effects of paracetamol, with EC_{50} values of 1 mM and 3.5 mM respectively. The mouse was slightly less sensitive, and the rat and human were very much less sensitive, with 35-fold greater EC_{50} values for blebbing. The EC_{50} for

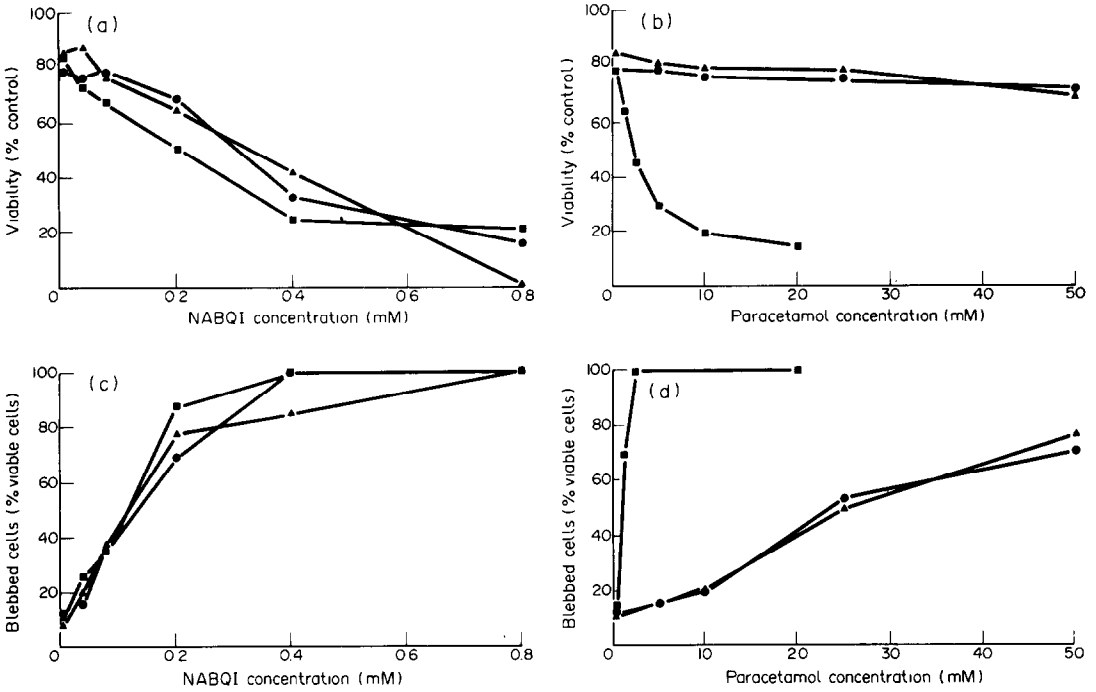

Fig. 3. Concentration–effect curves for viability and plasma membrane blebbing following exposure of freshly isolated hepatocytes to paracetamol or NABQI. (a, b) viability and (c, d) plasma membrane blebbing on exposure to (a, c) NABQI for 90 min or (b, d) paracetamol for 3 hr. Freshly isolated hepatocytes from hamster (■), rat (▲) and human (●) were incubated with up to 0.8 mM NABQI and 50 mM paracetamol respectively. Results are typical of those obtained with four different cell preparations.

cytotoxicity in these species was considerably above the highest concentration of paracetamol tested, which was 50 mM.

The effects of paracetamol and NABQI on glutathione content in a resistant species, the rat, and in a sensitive species, the hamster, were investigated (Fig. 6). Even low concentrations of paracetamol, less than 1.25 mM, caused 90% depletion of the GSH content of hamster hepatocytes. With rat hepatocytes, GSH content was depleted to a maximum of approximately 50% at 10 mM paracetamol and even at 40 mM, GSH content was not further depleted. With NABQI, the decrease in GSH content was similar in both rat and hamster. Depletion of GSH by 95% was achieved at a concentration of 0.2 mM, with lower concentrations causing a proportional decrease. At the higher concentrations of NABQI, the GSH content of the cells was essentially unmeasurable.

The covalent binding of paracetamol and NABQI was compared in hepatocytes from rat and hamster (Fig. 7). Covalent binding of paracetamol to proteins in hamster hepatocytes was significantly greater than that in rat hepatocytes. In both species were was a concentration-dependent increase in covalent binding although, with the hamster, the rate of covalent binding was greater at lower concentrations of the substrate. Covalent binding of NABQI was very similar in rat and hamster hepatocytes and there were no significant differences between the two species. Covalent binding was much more extensive with NABQI than with paracetamol on a molar basis, the difference being between 400- and 800-fold.

The likeliest explanation for the species differences observed in the sensitivity of hepatocytes to the cytotoxic effects of paracetamol was that the rate of its conversion to the reactive metabolite, NABQI, is different. The metabolic conversion of paracetamol to NABQI was therefore estimated by three different methods, two *in vitro* and one *in vivo*.

The kinetics of formation of the glutathione adduct of paracetamol in the presence of excess glutathione were determined with microsomal fractions from mouse, rat and human (Table 3). V_{max} was greatest for the mouse, for the rat it was some 3-fold lower and for the human it was 3-fold lower again. The K_m for mouse liver was 450 μM, the K_m for human liver being some 2-fold greater and that for rat liver some 3- to 4-fold higher than in the mouse. Calculating the intrinsic clearance of paracetamol due to oxidation to the reactive metabolite revealed that human and rat had similar values of approximately 0.25–0.40 μl/min/mg, whereas mouse was some 5- to 10-fold more active. The intrinsic clearance is the activity that would be obtained *in vivo* with non-saturating concentrations of paracetamol.

An alternative measure of the formation of the reactive metabolite of paracetamol is the extent to which it binds covalently to microsomal proteins in the absence of glutathione. These studies were performed with microsomal fractions from human and mouse liver and compared with the published data for rat and hamster liver (Table 4). Human and rat liver showed almost equal activity, with approximately 0.02 nmol residue bound/mg protein/min. Mouse and hamster liver were similar to each other

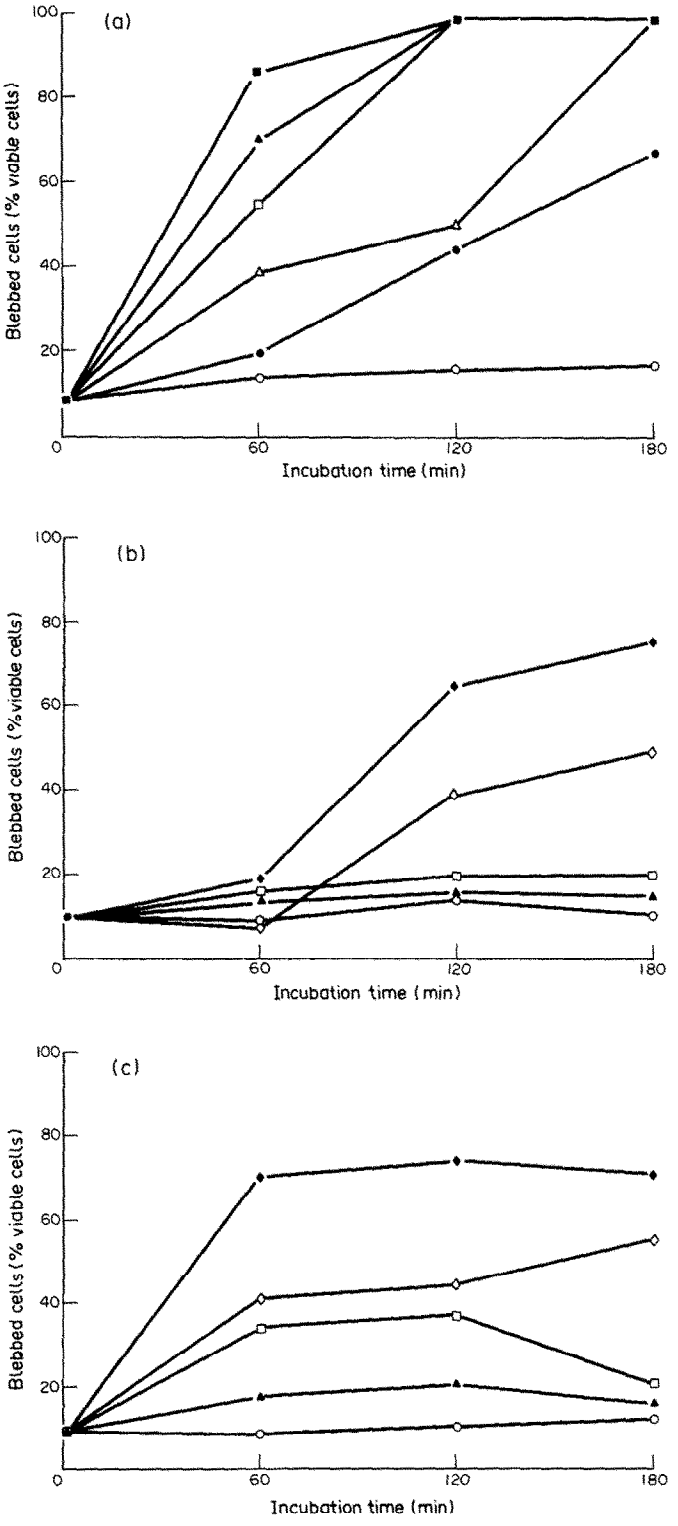

Fig. 4. Plasma membrane blebbing profiles of freshly isolated hepatocytes from (a) hamster, (b) rat and (c) human. Hepatocytes were incubated for up to 180 min with 1.25 mM (●) 2.5 mM (△), 5.0 mM (▲), 10 mM (□), 20 mM (■), 25 mM (◇) and 50 mM (◆) paracetamol. Control cells (○) were incubated in medium alone. Results are typical of those obtained with at least four different cell preparations.

in activity, some 10-fold higher than human and rat liver.

The *in vivo* oxidation of paracetamol to its reactive metabolite was assessed by calculating the partial clearance of paracetamol to the mercapturic acid excreted in urine. The published values for the kinetics of the parent compound and the percentage of the dose eliminated as the mercapturic acid were

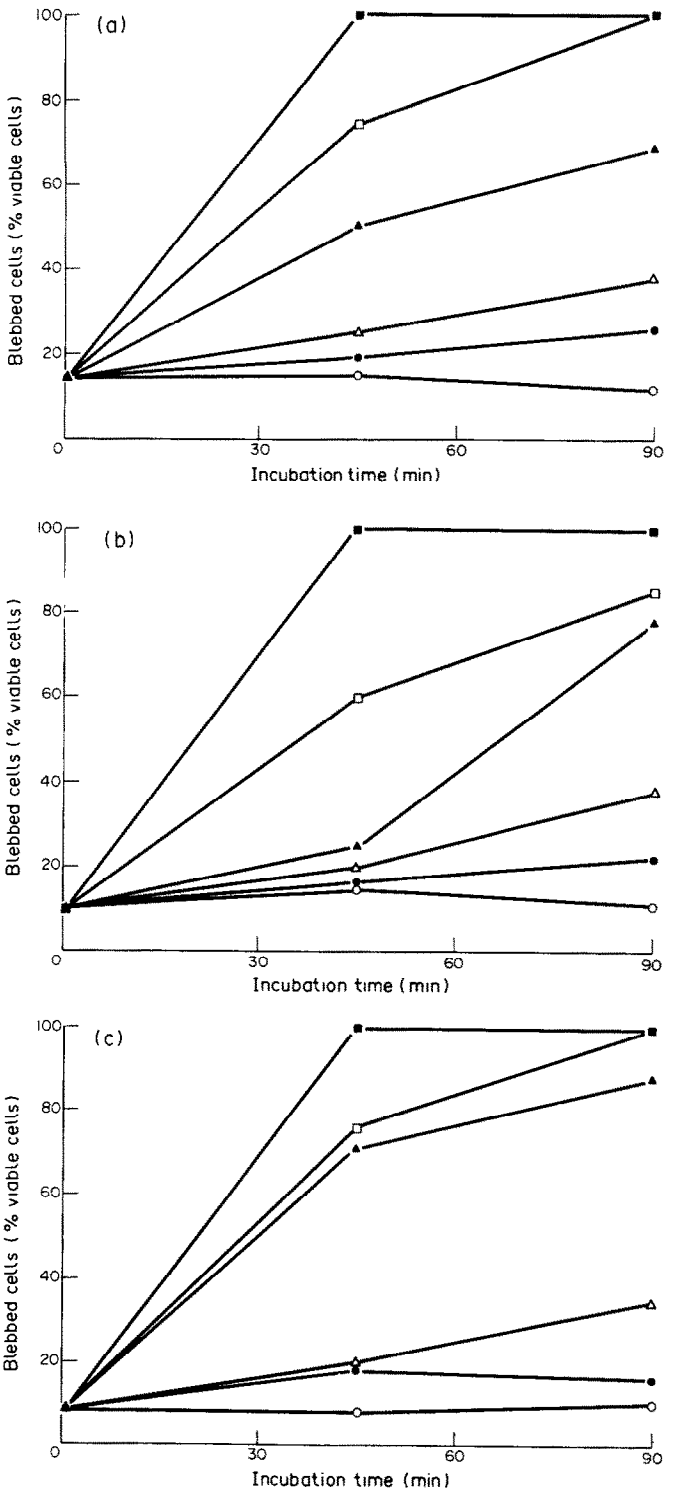

Fig. 5. Effects of NABQI on the plasma membrane blebbing profiles of freshly isolated hepatocytes from (a) hamster, (b) rat and (c) human. Cells were incubated for up to 90 min with 0.04 mM (●), 0.08 mM (△), 0.20 mM (▲), 0.40 mM (□) and 0.80 mM (■) NABQI. Control cells (○) were incubated in medium alone. Results are typical of those obtained with at least four different cell preparations.

used in this calculation (Table 5). Human and rat had comparable activity, with the rat slightly more active. Mouse was far more active than either of the other two species, with clearance to the mercapturic acid some 10–15 times that in the other species.

DISCUSSION

There are marked species differences in the sensitivity of hepatocytes to the cytotoxicity of paracetamol. As expected, rat hepatocytes are very

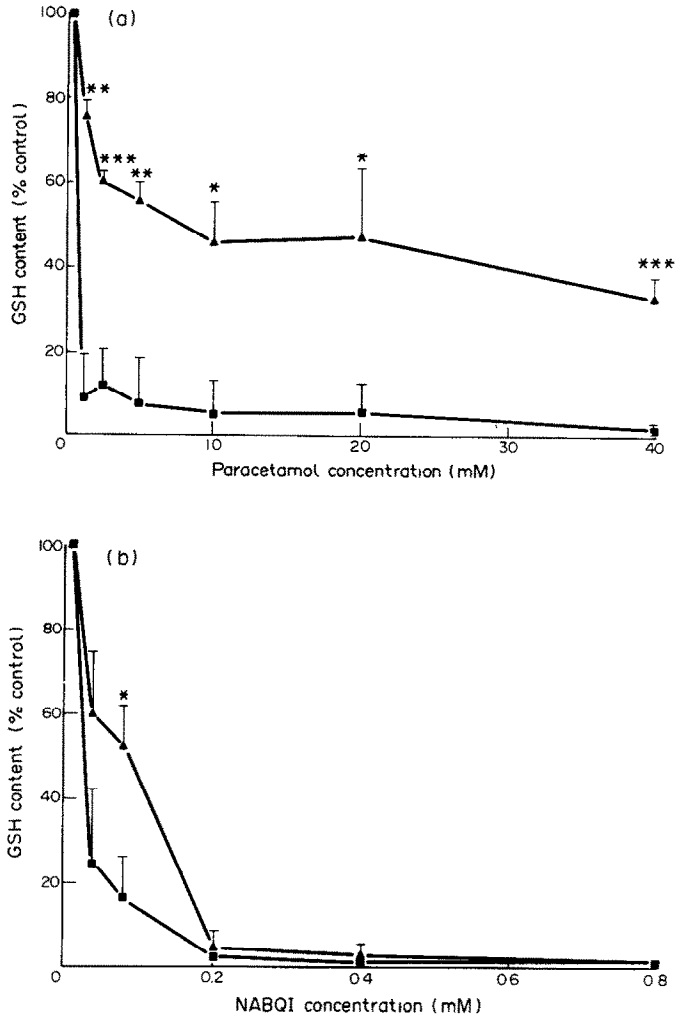

Fig. 6. Effects of (a) paracetamol and (b) NABQI on intracellular glutathione content of rat (▲) and hamster (■) hepatocytes. Freshly isolated cells were incubated with various concentrations of paracetamol and NABQI for 3 hr and 1.5 hr respectively. Results are mean ± SE of three separate cell preparations. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with the results for hamster hepatocytes at the same concentration of the cytotoxin.

resistant whereas mouse and hamster hepatocytes are very sensitive. These results are in agreement with the published data from *in vivo* studies [3] and also, for the rat and hamster, with studies in cultured hepatocytes [15]. This latter system is perhaps not the best in which to investigate species differences in the hepatotoxicity of a compound like paracetamol which depends upon conversion to a reactive metabolite by the cytochrome P-450 dependent mixed function oxidase system [27]. It has been demonstrated by a number of groups that the cytochrome P-450 content [28, 29], and also the complement of different isozymes of cytochrome P-450 [30] show profound changes during primary culture of hepatocytes. The present system, similar to that described by Orrenius and his colleagues [31], in which freshly isolated hepatocytes are used to investigate hepatotoxicity, appears to offer many advantages. Metabolic activity is well maintained at initial levels and these are comparable to those in the microsomal fraction and also *in vivo* [22].

We report here for the first time the relative sensitivity of human hepatocytes to the cytotoxic effects of paracetamol. Somewhat unexpectedly, in view of the prevailing view on human sensitivity to paracetamol [20], human hepatocytes are extremely resistant to the toxicity of this compound and, if anything, are less sensitive than rat hepatocytes. Although there was no overt loss of viability over the duration of the experiment, nevertheless morphological changes were apparent in cells from all species including man. Thus, human hepatocytes do show evidence of damage from paracetamol as indicated by membrane blebbing, but this was not of sufficient severity to cause cell death over the 3 hr of the experiment.

One obvious explanation for these species differences is that the rate of conversion of paracetamol to its hepatotoxic metabolite varies greatly between sensitive and resistant species. If this is so then hepatocytes from all species should be sensitive to the hepatotoxic metabolite, NABQI, as this would

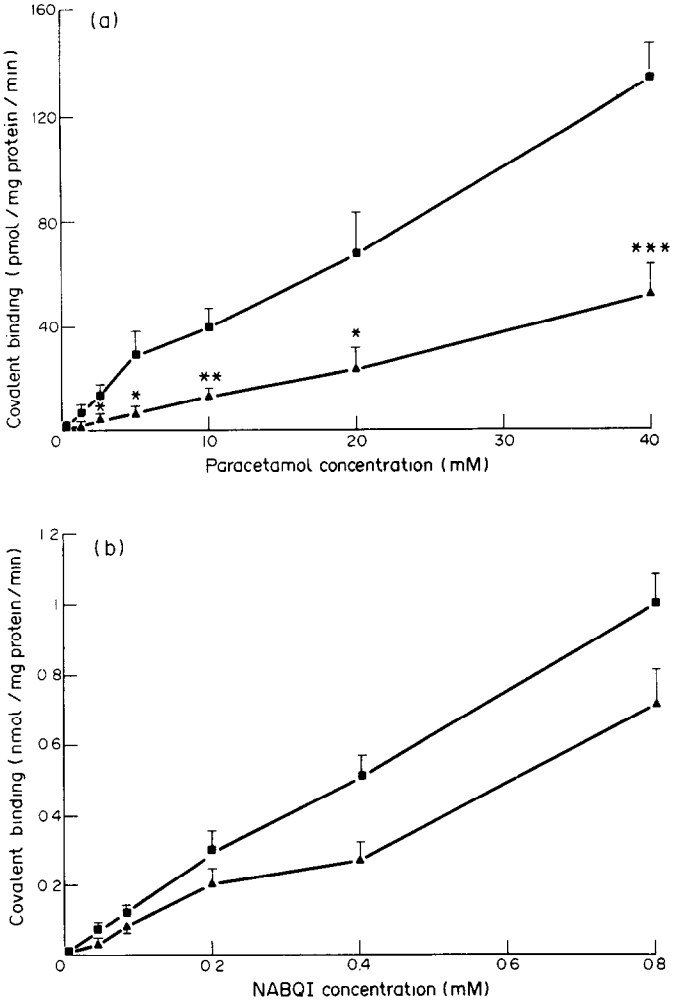

Fig. 7. Covalent binding of (a) paracetamol and (b) NABQI to hepatocellular proteins of freshly isolated rat (▲) and hamster (■) hepatocytes. Cells were incubated with various concentrations of paracetamol or NABQI for 3 hr and 1.5 hr respectively. Results are mean ± SE of three separate cell preparations. * $P < 0.05$, *** $P < 0.01$ compared with the results from hamster hepatocytes at the same concentration of the cytotoxin.

Table 3. Species differences in 3-glutathionyl paracetamol formation *in vitro*

Species	V_{max} (nmol/mg/min)	K_m (μM)	V_{max}/K_m (μl/min/mg)
Mouse (2)	0.900	448	2.009
Human (4)	0.115 ± 0.018*	952 ± 241	0.121
Rat (4)	0.291 ± 0.056	1570 ± 218	0.185

* Values are mean ± SE of number of experiments indicated in parentheses under *species*, except for mouse, for which the results are the average of two determinations.

circumvent differences in the oxidation step. There are two points that should be borne in mind about these and other [32–34] studies in which exogenous NABQI is added directly to cells. Firstly, the local concentration of the compound will be much higher than that generated *in situ* from paracetamol and secondly, exogenous NABQI is presented to the outside of the cell whereas that produced from paracetamol will interact with intracellular sites first. When synthetic NABQI is added to the cells, hepatocytes from all four species show a dramatic decrease in viability, with EC_{50} values varying by a maximum of approximately 1.5-fold. In contrast, EC_{50} values for paracetamol vary by at least 15-fold. Similar results are obtained when plasma membrane blebbing is analysed, hepatocytes from the hamster, rat and human showing EC_{50} values which are almost identical whereas there is a 35-fold difference in EC_{50} values for paracetamol induced plasma membrane blebbing. It thus appears clear that differences in

Table 4. Species differences in the covalent binding of paracetamol to microsomal proteins *in vitro*

Species	N	Covalent binding (nmol/mg/min)	Reference
Human	28	0.0258 ± 0.0023*	[41]
Mouse	3	0.1880 ± 0.0030	[41]
Rat	8	0.0203 ± 0.0040	[27]
Hamster		0.1800	[3]

* Values are mean ± SE, except for hamster for which only the mean value could be provided.

susceptibility to paracetamol-induced cytotoxicity between human and other species are not due to any difference in their sensitivity to NABQI, the putative cytotoxic metabolite of paracetamol. Thus, differences must lie in either the formation of inactivation of NABQI.

Detoxication of NABQI depends upon adduct formation with glutathione [5, 8], a reaction catalysed by glutathione *S*-transferases [9]. Since toxicity does not ensue until GSH is depleted to less than 20% of control values [5, 8], differences in detoxication could arise from differences in either the GSH content of the cells or in the activity of glutathione *S*-transferases. Although hamster hepatocytes have a lower GSH content than those from rat, hepatocytes from the mouse have a similar content to the rat (see Table 1). Yet mouse hepatocytes are almost as sensitive to the cytotoxicity of paracetamol as hamster hepatocytes and are much more sensitive than hepatocytes from the rat. In addition, the glutathione *S*-transferase activity of mouse and hamster is, if anything, greater than that of rat, and the activity of human is much lower than that of mouse and hamster [35]. These activities are the precise opposite of what would be expected if the activity of the glutathione *S*-transferases is important in determining species differences in susceptibility to paracetamol toxicity. It is, of course, possible that a specific form of glutathione *S*-transferase is involved in catalysing adduct formation between glutathione and NABQI. However, there is little evidence for this in the literature and it seems unlikely that the kinetics of the reaction are such that the rate of adduct formation would be a major determinant of species susceptibility.

Thus, the likeliest explanation for species differences in the toxicity of paracetamol lies in the activity of the monooxygenase system responsible for converting paracetamol to NABQI. It might be argued that human hepatocytes are less active than those from other species due to the longer isolation time required to obtain such cells [23]. However, the monooxygenase activity of hepatocytes such as those used in this study compares well to that of microsomal fractions from histologically normal biopsy samples of human liver [22]. In addition, we have determined the rate of oxidation of paracetamol *in vitro* by two other methods. These involve trapping NABQI as the glutathione adduct and measuring its reaction with microsomal proteins in the absence of GSH or other added proteins. Both techniques reveal a major difference between mouse liver on the one hand and human and rat liver on the other. Microsomal fractions from mouse liver are some 5- to 10-fold more active than microsomal fractions from human or rat liver in converting paracetamol to an electrophilic metabolite. This would suggest that the basis for species difference lies in the rate of conversion of paracetamol to NABQI and not in the fate of the NABQI once formed.

We also estimated the clearance of paracetamol to its oxidised metabolite from the amount of the mercapturic acid excreted in urine. We recognise that this is not the only end product of glutathione conjugation of NABQI [10]. Nevertheless, it seems likely that the other possible products, such as the cysteine conjugate, represent a relatively constant proportion of the total adduct formed. In addition, it is assumed that the majority of the material derived from the GSH adduct is excreted in the urine. This certainly appears to be true for the species that have been studied [36, 37]. Hence, measurement of the mercapturic acid should provide an estimate of the relative proportion of the dose of paracetamol being converted to the reactive intermediate. Results of this analysis again revealed that human and rat have similar rates of conversion of paracetamol whereas the mouse is much more active, some 10- to 15-fold, than the other two species.

The foregoing data suggest that the species differences observed in laboratory animals in the hepatotoxicity of paracetamol are due almost entirely to differences in the rate of conversion of paracetamol to its reactive metabolite. The evidence is against any important role for differences in detoxication in contributing to species differences in sus-

Table 5. Species differences in the partial clearance of paracetamol to its mercapturic acid *in vivo*

Species	Total body clearance of paracetamol (ml/min/kg)	% Dose excreted as mercapturic acid	Partial clearance to mercapturic acid (ml/min/kg)
Human	4.1	5.0	0.205
Mouse	25.7	13.3	3.418
Rat	10.5	3.6	0.378

The values in the table for the total body clearance of paracetamol and the % of the dose excreted as the mercapturic acid were obtained or interpolated from data provided in references [4], [7] and [42] and the partial clearance to the mercapturic acid calculated from these values.

ceptibility. Once NABQI is produced, it is equally toxic to cells from all species. The other major conclusion from this is that man is very resistant to the hepatotoxocity of paracetamol. Indeed, careful analysis of the clinical literature would suggest that this is so and that only a small number of individuals taking an overdose of paracetamol develop serious hepatic necrosis [16, 19]. In addition, there are indications for a wide inter-individual variability in the extent to which paracetamol is converted to its reactive metabolite within the human population [38]. The range of activities span that observed in a sensitive species such as the mouse and a resistant species such as the rat. The activity in most people appears to lie towards that of the resistant species, with a smaller number showing much higher rates of conversion. It is not yet clear whether this is genetically determined but there is certainly some evidence that environmental factors, such as induction of a specific isozyme of cytochrome P-450 by ethanol [39], will contribute to increased sensitivity.

There is a very close association between the concentration of either paracetamol or NABQI required to produce maximum depletion of hepatocyte GSH and the onset of cell killing. It is of some interest that the appearance of blebbed cells shows a different relationship to GSH depletion. For example, whereas GSH depletion by NABQI does not exceed 80% with concentrations below 0.2 mM, even the lowest concentration of NABQI produces plasma membrane blebbing, and this shows a concentration-dependent increase. Further, in the rat, paracetamol causes no greater than 60% depletion of GSH at the highest concentration tested. However, there is significant plasma membrane blebbing apparent in rat hepatocytes with increasing concentrations of paracetamol. These results suggest that the relationship between GSH depletion, plasma membrane blebbing and cytotoxicity is more complex than it first appears.

NABQI is both a potent electrophile and a potent oxidising agent [12]. It binds readily to tissue proteins most probably at the sulphydryl group in cysteine residues [40]. However, its toxicity appears more closely related to the oxidation of sulphydryl groups in critical enzymes such as calcium translocases [33]. Thus, there is an interval following the production of NABQI when toxicity can be reversed by thiol-reducing agents such as GSH synthesised from a precursor, e.g. *N*-acetylcysteine [41], or directly by DTT [24]. Since the proportion of NABQI that forms an adduct with tissue proteins and that which oxidises thiol groups will depend upon chemical processes, determination of covalent binding should provide a quantitative index of NABQI formation under most circumstances. There are marked species differences in the extent of covalent binding of paracetamol, hamster hepatocytes showing much more binding than rat hepatocytes. This result is similar to that reported *in vivo* [6, 8] and in microsomal fractions *in vitro* [3]. In contrast, exogenous NABQI binds equally well to tissue proteins in hamster and rat hepatocytes. It may be significant that covalent binding proceeds in the rat, albeit at a lower rate than in the hamster, when the GSH content of these hepatocytes is not depleted below 40% of control values. This is further evidence [24] that gross covalent binding is not the cause of paracetamol cytotoxicity.

In conclusion, we have demonstrated that the major determinant of species differences to the hepatotoxicity of paracetamol is the rate of its conversion to NABQI. In general, man is resistant to the hepatotoxic effects of paracetamol due to a low rate of conversion to the putative toxic metabolite, NABQI, although some individuals do appear to be at increased risk. The reason for their higher activity is not yet clear but it may include a combination of both genetic and environmental factors. Further studies on this aspect of paracetamol toxicity are in progress.

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