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# PARACETAMOL METABOLISM AND TOXICITY IN ISOLATED HEPATOCYTES FROM RAT AND MOUSE

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**Abstract**—Hepatocytes isolated from mouse and rat catalyzed the formation of glucuronide, sulphate, glutathione and cysteine conjugates of paracetamol. These metabolites were separated by high pressure liquid chromatography. 1. Sulphation had higher affinity for paracetamol than glucuronidation in hepatocytes from both mouse and rat, whereas glucuronidation had higher capacity. The maximal rate of glucuronidation was similar in hepatocytes from both species, the rate of sulphation was, however, several-fold less in hepatocytes from mouse. 2. Formation of the glutathione conjugate was directly correlated with loss of intracellular glutathione (GSH). The rate of glutathione conjugate formation increased about three times in rat hepatocytes after phenobarbital treatment. This induced rate was, however, only half of that in hepatocytes from control mouse. In both species the reaction was saturated only at very high paracetamol concentrations. The rate of formation of the cysteine conjugate was very low compared to the other reactions. 3. Only hepatocytes isolated from mouse lost integrity, measured as increased permeability of the cell membranes, upon incubation in the presence of paracetamol.

Paracetamol is a widely used analgesic and antipyretic drug which in normal dosage is considered safe and nontoxic. However, taken in large overdose it produces acute hepatic necrosis [1–3]. Given in large doses the drug produces similar lesions in laboratory animals [4–6], although considerable species variation exists [7]. Species like the mouse and the hamster have been shown to be highly susceptible to toxic effects of paracetamol [4, 5] whereas the rat is quite resistant [6].

Paracetamol is primarily metabolized to nontoxic conjugates by direct conjugation with glucuronic acid and sulphate [8]. In addition, paracetamol presumably undergoes cytochrome P-450 dependent *N*-hydroxylation [4, 9, 10]. The *N*-hydroxy derivative rapidly undergoes spontaneous degradation to *N*-acetyl-*p*-benzoquinone, generally believed to be the reactive species of the drug [5, 7]. At moderate doses of paracetamol the cell is protected by its content of reduced glutathione which traps the reactive species [5, 7, 11] and is excreted as mercapturic acid and cysteine derivatives [8, 12]. At high doses, however, glutathione is depleted and covalent binding of reactive species to cellular macromolecules occurs [5, 9, 11], which is considered to be the ultimate cause of the cell damage [9, 12].

Most investigations of metabolism and toxicity of paracetamol have been performed *in vivo*. In metabolic studies this system has its obvious limitations due to difficulties in controlling parameters such as absorption, tissue binding and excretion which might play a significant role in the fate of the administered drug. In addition, studies of fast reaction sequences and kinetic measurements are difficult to perform.

Isolated hepatocytes, combining the advantage of an *in vitro* system with most properties of an *in vivo* system, have during the last few years proven useful in studies on drug metabolism, both cytochrome P-450 dependent oxidation reactions [13] and subsequent conjugation reactions [14], as well as on drug toxicity [15, 16], thus offering a more flexible system.

Metabolites derived from paracetamol have been identified by thin layer chromatography, requiring radioactive labeled drug and more recently by high pressure liquid chromatography (HPLC). Howie *et al.* described a HPLC method detecting sulphate, glucuronide, *N*-acetylcysteine acid and cysteine conjugates directly from human urine samples [17], whereas Buckpitt *et al.* reported a similar method for isolating glutathione, cysteine and *N*-acetylcysteine conjugates formed from radioactive labeled paracetamol in microsomal incubations [18].

The purpose of the present study was to perform a detailed analysis of the formation of the different metabolites from paracetamol in hepatocytes isolated from mouse and rat, using a modified HPLC method for isolation of the glucuronide, sulphate, glutathione, cysteine and *N*-acetylcysteine conjugates. The results are discussed with regard to the toxicity of paracetamol in the different species.

## MATERIALS AND METHODS

Male Sprague–Dawley rats, 200–250 g, and male NMRI mice, 20–25 g, were used. Phenobarbital-treated animals were given sodium phenobarbital i.p. at a daily dose of 80 mg/kg for three days.

Microsomes were prepared from phenobarbital-treated rats according to Ernster *et al.* [19]. Microsomal incubations were performed in a 50 mM Tris–HCl buffer, pH 7.4, containing a NADPH generating system, microsomes (2 mg/ml), paracetamol (10 mM) and cysteine, *N*-acetylcysteine or GSH (5 mM).

Hepatocyte isolation from both rat and mouse was performed by collagenase perfusion as previously described [20, 21]. The yield of each preparation was

$2–4 \times 10^8$ cells per liver from rat and $2–5 \times 10^7$ cells per liver from mouse, as measured by counting the final cell suspension in a Buerker chamber. Immediately after isolation the cells excluded both trypan blue and NADH (90–100%).

Incubations were performed at 37°C in rotating round bottom flasks [21] or in shaking 25 ml Erlenmeyer flasks, under a 95% $O_2$ – 5% $CO_2$ atmosphere at a cell concentration of $1–2 \times 10^6$ cells/ml. The incubation medium was a Krebs–Henseleit buffer, pH 7.4, supplemented with 25 mM Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid), penicillin (100 IU/ml), heparin (10 IU/ml), horse serum (17.5%) and an amino acid mixture [22].

Metabolites of paracetamol were analyzed using high pressure liquid chromatography by a method modified after Howie *et al.* [17]. Perchloric acid (0.5 ml, 3 N) was added to 1 ml of incubate to precipitate the protein. After centrifugation, the supernatant was used for metabolite analysis immediately or after storage at − 20°. No degradation of the formed paracetamol metabolites was observed after 3 months of storage. An aliquot (10–30 μl) of the supernatant was injected onto a reversed phase μ-Bondapak $C_{18}$ column (Waters Associates Inc., Massachusetts, USA) and the metabolites were isolated by gradient elution using a Spectra Physics model 3500 liquid chromatograph equipped with a variable UV detector and a Spectra Physics System 1 integrator. The gradient system was solvent A, 1% aqueous acetic acid and solvent B, 1% aqueous acetic acid–methanol–ethylacetate (90:15:0.1). The initial concentration was 75% A and 25% B. A linear gradient was started 7 min after injection and finished at 99% B (sweep time 15 min). The flow rate was 2 ml/min and the eluent was monitored spectrophotometrically at 250 nm. Quantification of the metabolites was accomplished using paracetamol as a standard since the molar extinction coefficients of the metabolites and paracetamol are essentially the same [17]. In early experiments 4-fluorophenol was used as an internal standard but has been omitted since repeated injections of paracetamol onto the column were found to be very reproducible.

Intracellular level of GSH was estimated by the method of Saville [23]. Measurements were performed on $10^6$ cells, reharvested by gentle centrifugation (80 *g*). One ml of 6.5% trichloroacetic acid was added to the reharvested cells and, following centrifugation, a 0.5 ml aliquot of the supernatant was used for analysis.

Penetration of NADH into the cells was measured using the lactate dehydrogenase latency test [20, 21]. An aliquot of a well-mixed hepatocytes suspension was diluted 20-fold in Krebs–Henseleit buffer containing 2% albumin. NADH (0.1 mM final concentration) and pyruvate (0.76 mM final concentration) were then added. The rate of NADH oxidation was recorded at 340 nm and 100% activity was obtained after lysis of the cells by addition of Triton X-100 (0.5% final concentration).

Collagenase was obtained from Boehringer/Mannheim GmbH, Mannheim, Federal Republic of Germany. Bovine serum albumin fraction V was purchased from Sigma Chemical Co., St. Louis, Missouri, USA. Paracetamol metabolite standards were obtained from the chemical division, Sterling Winthrop, Newcastle, England. All other chemicals were of analytical grade and obtained from local commercial sources.

## RESULTS

*Identification of metabolites.* It was possible to separate five different paracetamol metabolites using the HPLC method described in the Methods section (Fig. 1A and B). The five metabolites were the sulphate, glucuronide, cysteine, glutathione and *N*-acetylcysteine conjugates. The sulphate-, glucuronide- and cysteine conjugates were identified using synthesized standards, the glutathione and *N*-acetylcysteine conjugates from incubations of paracetamol with microsomes in the presence of NADPH, $O_2$ and GSH or *N*-acetylcysteine. In the isolated hepatocytes three major metabolites were formed, the glucuronide, the sulphate and the glutathione conjugate (Fig. 1A). Only small amounts of the cysteine conjugate and no *N*-acetylcysteine conjugate were detected. The unmarked peak eluted after paracetamol in Fig. 1B is an unidentified compound present in the incubation medium and is present also at zero time in the absence of paracetamol.

*Metabolite formation in hepatocytes isolated from control and phenobarbital-treated rat.* The metabolic disposition of paracetamol in the rat *in vivo* has previously been shown to change after phenobarbital treatment [8]. The percentage excreted as the glucuronide was enhanced as well as that excreted as the mercapturic acid conjugate. Similar results were obtained with isolated hepatocytes. Thus, the metabolic pattern of paracetamol in hepatocytes isolated from phenobarbital-treated rat was changed compared to control hepatocytes (Table 1). Glucuronide formation was enhanced about two-fold whereas sulphate conjugation was unaffected. Obviously, the formation of the reactive metabolite also increased since the amount of glutathione conjugate formed was almost three times that in hepatocytes from control rats.

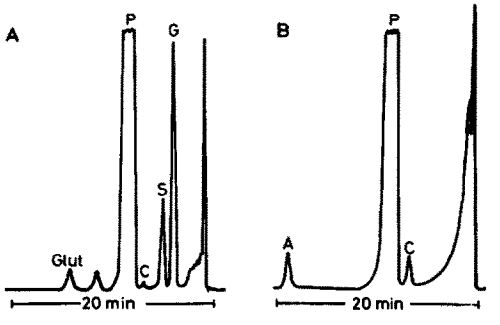


Fig. 1. A. High pressure liquid chromatogram from injection of 10 μl sample from incubation of paracetamol with hepatocytes isolated from phenobarbital-treated rat. G—paracetamol glucuronide, S—paracetamol sulphate, C—paracetamol cysteine, P—paracetamol, Glut—paracetamol glutathione. B. High pressure liquid chromatogram from injection of 10 μl sample from incubation of paracetamol with liver microsomes from phenobarbital-treated rats in the presence of NADPH, $O_2$, cysteine and *N*-acetylcysteine. C—paracetamol cysteine, P—paracetamol, A—paracetamol-*N*-acetylcysteine.

Table 1. Metabolite formation of paracetamol in hepatocytes isolated from control and phenobarbital-treated rats

| Pretreatment | Glucuronide conjugates | Sulphate conjugates | Glutathione conjugates |
|---|---|---|---|
| | nmol/$10^6$ cells/30 min | | |
| Control | 38.8 ± 1.3 | 24.0 ± 1.5 | 3.9 ± 0.4 |
| Phenobarbital | 74.2 ± 2.1 | 23.6 ± 0.7 | 12.6 ± 0.5 |

Incubations were performed in the presence of 10 mM paracetamol (10 μmole/$10^6$ cells) as described in 'Materials and Methods'. Values represent means ± S.E.M. of 2–4 different hepatocytes preparations.

*Effect of dose on metabolite formation in hepatocytes from mouse and rat.* The metabolite pattern varied with the concentration of paracetamol in hepatocytes isolated from rats treated with phenobarbital (Fig. 2). At low concentration, 0.25 mM, there was about equal conjugation with sulphate and glucuronic acid. However, sulphate conjugation was saturated at 1 mM paracetamol, whereas glucuronidation was not maximal until 5 mM concentration. At 25 mM concentration there was a slight decrease of both conjugation reactions. The formation of glutathione conjugate was increased with increasing paracetamol concentration and not saturated until a concentration of 25 mM paracetamol was reached. This reaction was not linear with time at higher paracetamol concentrations probably due to the decreased GSH concentration (see Figs. 4 and 5).

In hepatocytes isolated from mouse, glucuronidation was saturated at 5 mM, sulphate conjugation already at 0.25 mM and formation of the glutathione conjugate at a concentration of 25 mM paracetamol (Fig. 3). As in rat hepatocytes, the formation of glutathione conjugate was not linear for any length of time at the higher drug concentrations. Compared to hepatocytes from phenobarbital-treated rats, the rate of glucuronidation in hepatocytes from non-pretreated mouse was about 50 per cent whereas sulphation was only about 10 per cent. However, the initial rate of glutathione conjugate formation was about twice as fast as in rat hepatocytes. Comparison with hepatocytes from control rats reveals an even greater difference in this reaction whereas glucuronidation is almost equal (see Table 1).

*Long term incubations of hepatocytes in the presence of paracetamol.* Hepatocytes were incubated for 5 hrs in the presence of paracetamol (Figs. 4 and 5) and metabolite formation, GSH level and cell membrane integrity was monitored. Hepatocytes from both mouse and rat incubated in the absence of paracetamol have a constant high level of GSH and the cell membrane shows no sign of loss of integrity ([15], Fig. 5). Hepatocytes from phenobarbital-treated rats were incubated in the presence of 10 mM paracetamol, a concentration saturating both glucuronidation and sulphation. The formation of these

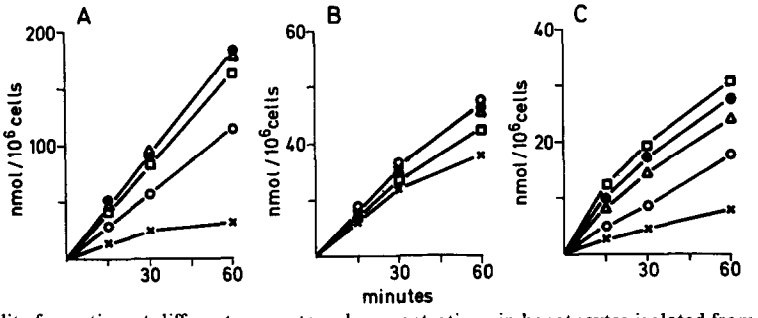


Fig. 2. Metabolite formation at different paracetamol concentrations in hepatocytes isolated from phenobarbital-treated rat. Incubations were performed as described in Methods using 2 × $10^6$ cells/ml of incubate.
A. Glucuronide conjugate
B. Sulphate conjugate
C. Glutathione conjugate
Paracetamol concentrations: ×, 250 μM; ○, 1 mM; △, 5 mM; ●, 10 mM, □, 25 mM. One experiment typical of three.

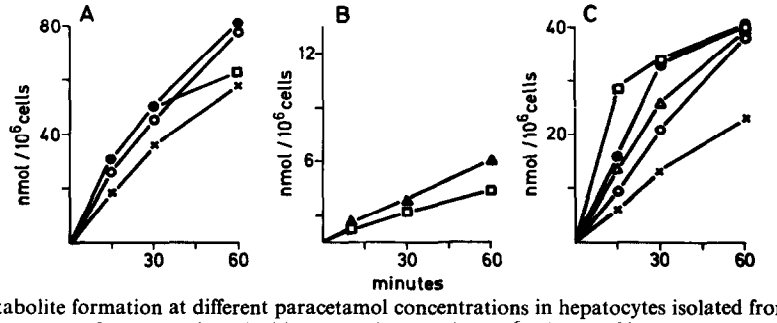


Fig. 3. Metabolite formation at different paracetamol concentrations in hepatocytes isolated from mouse. Incubations were performed as described in Methods using 2 × $10^6$ cells/ml of incubate.
A. Glucuronide conjugate
B. Sulphate conjugate
C. Glutathione conjugate
Paracetamol concentrations: ×, 250 μM; ○, 1 mM; △, 5 mM; ●, 10 mM; □, 25 mM; ▲, 250 μM—10 mM, see B. One experiment typical of three.

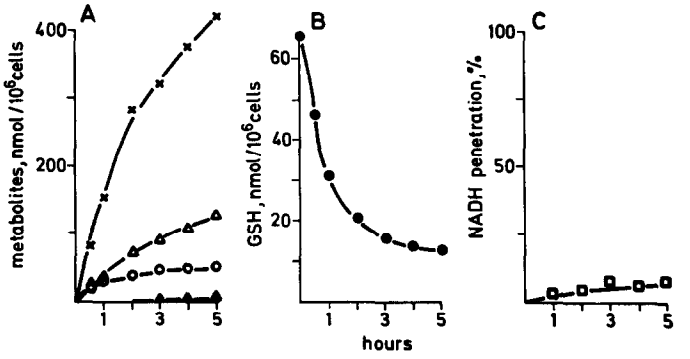


Fig. 4. Paracetamol metabolite formation, glutathione levels and cell viability in hepatocytes from phenobarbital-treated rat incubated in the presence of paracetamol (10 mM). Incubations were performed in rotating flasks using $10^6$ cells/ml of incubate as described in Methods.
A. Metabolite formation; ×, Glucuronide conjugate; △, Sulphate conjugate; ○, Glutathione conjugate; ▲, Cysteine conjugate
B. Glutathione level
C. Cell viability: Intactness of cell membrane estimated by NADH penetration. One experiment typical of three

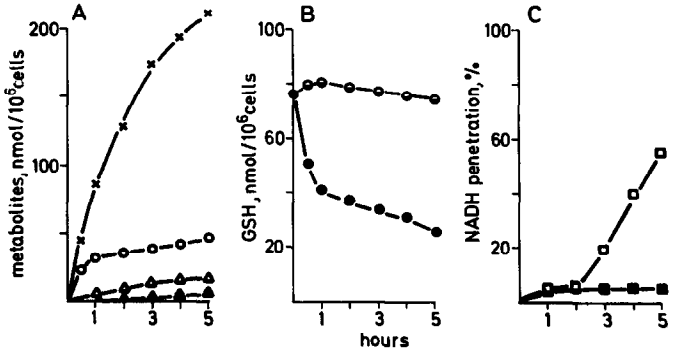


Fig. 5. Paracetamol metabolite formation, glutathione levels and cell viability in hepatocytes from mouse incubated in the presence and absence of paracetamol (2 mM). Incubations were performed in rotating flasks using $10^6$ cells/ml of incubate as described in Methods.
A. Metabolite formation; ×, Glucuronide conjugate; △, Sulphate conjugate; ○, Glutathione conjugate; ▲, Cysteine conjugate.
B. Glutathione level; ●, presence of paracetamol; ⊖, absence of paracetamol.
C. Cell viability: Intactness of cell membrane estimated by NADH penetration; □, presence of paracetamol; ■, absence of paracetamol. One experiment typical of three.

two conjugates was linear for at least two hours. The formation of the glutathione conjugate was initially rapid but decreased after one hour of incubation. This conjugate formation was reflected in a decrease in the intracellular GSH. If one compares these two events one finds almost a direct correlation between the amount of conjugate formed and the loss of GSH. During incubation the GSH level dropped to about 20 per cent of control but even after 5 hr there was no sign of any loss in cell viability. This was not true for mouse hepatocytes which after three hours exhibited an increasing permeability of the cell membrane and after 5 hr more than 50 per cent of the hepatocytes were damaged (Fig. 5). The rate of glutathione conjugate formation was more rapid than in the rat hepatocytes and started levelling off already after 30 min of incubation. As in the rat hepatocytes, the formation of this conjugate corresponded to a decrease in intracellular GSH. The amount of GSH, which in the mouse hepatocytes is somewhat higher than in the hepatocytes from phenobarbital-treated rat, did not decrease more than about 60 per cent in 5 hr. Despite this fact of a high level of GSH, these cells were damaged. In hepatocytes from both species very low but detectable amounts of cysteine conjugate were formed, but no *N*-acetylcysteine conjugate formation was observed.

## DISCUSSION

The results presented demonstrate the use of isolated hepatocytes in studies of the biotransformation of a toxic drug, paracetamol, with simultaneous detection of its toxic effect. The toxic effect of paracetamol as indicated by loss of cell membrane integrity was demonstrated in hepatocytes isolated from mouse. In agreement with *in vivo* data [7] hepatocytes isolated from rat were shown to be very resistant to paracetamol toxicity.

In hepatocytes isolated from both rat and mouse, paracetamol formed four detectable metabolites, a glucuronide, a sulphate, a glutathione and a cysteine

conjugate. It was possible using the HPLC method to separate these conjugates directly after incubation and detect them by u.v. absorption in less than nmol quantities.

Studies on metabolite formation are more favorably performed with isolated hepatocytes than *in vivo*. For instance, data on metabolite formation *in vivo* are generally expressed as per cent excreted as different metabolites of the dose given. This gives information about the total amount of metabolites formed but not about the rate of metabolism or about the $K_m$ and $V_{max}$ of the reactions. As demonstrated in the present investigation, there are differences in the affinity of paracetamol for the different reactions in hepatocytes from both rat and mouse. Sulphate conjugation was saturated at low paracetamol concentration whereas the cytochrome P-450 dependent *N*-hydroxylation, if one assumes this reaction to be reflected by the glutathione conjugate formation, was saturated at much higher concentrations. The fact that sulphate conjugation is saturated at lower concentrations than glucuronidation (cf. Fig. 2) may afford an alternative explanation to the observed decreased proportion excreted as sulphate conjugate *in vivo* with increasing paracetamol dose. This has previously been ascribed to depletion of sulphate [8]. The plateau of excreted sulphate and glucuronide conjugates found in rats by Davies *et al.* [12] at high doses of paracetamol could then also be explained by saturation of these reactions at these paracetamol concentrations.

There were two major differences between the hepatocytes isolated from phenobarbital-treated rats and control mice concerning metabolism of paracetamol. Sulphate conjugation in mouse hepatocytes was found to be only about 10 per cent of that in hepatocytes from phenobarbital-treated rat. However, the formation of the glutathione conjugate was at least doubled in mouse hepatocytes, this reaction reflecting formation of the reactive metabolite. The affinity of paracetamol for cytochrome P-450 is apparently very low in both species since the formation of the glutathione conjugate was not saturated until 25 mM concentration of paracetamol.

If one assumes a low rate of resynthesis of GSH, there was an almost direct correlation between GSH depletion and formation of glutathione conjugate in hepatocytes from both rat and mouse. This indicates very low further metabolism of the formed glutathione conjugate in the hepatocytes which is also evident from the fact that very little cysteine conjugate and no *N*-acetylcysteine conjugate was formed in 5 hr of incubation. These results suggest that further metabolism of the glutathione conjugate of paracetamol, at least in the rat and mouse, mainly occurs in organs other than the liver. It is also not clear yet whether the cysteine conjugate is actually derived from the glutathione conjugate or from direct conjugation of the reactive metabolite with intracellular cysteine.

In conclusion this study has demonstrated large differences in affinity of paracetamol for glucuronide, sulphate and glutathione conjugate formation. Comparison of the metabolite patterns in hepatocytes from rat and mouse revealed very low sulphate conjugation in hepatocytes isolated from mouse as compared to those isolated from the rat, whereas the formation of the glutathione conjugate was faster. It remains to be established whether the low rate of sulphate conjugation in addition to the more rapid formation of the reactive metabolite, as reflected by the formation of the glutathione conjugate, might be of importance regarding the susceptibility of mouse hepatocytes to the toxic effect of paracetamol. This study using isolated hepatocytes as a model is presently being extended to investigations of the mechanisms behind the toxic effect of paracetamol and of the protective action of the different antidotes used in paracetamol overdose treatment.

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