Biochemical Pharmacology, Vol. 34, No. 20, pp. 3729–3733, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00

ACETAMINOPHEN METABOLISM BY THE PERFUSED RAT LIVER TWELVE HOURS AFTER ACETAMINOPHEN OVERDOSE

HENRIK E. POULSEN,* ANETTE LERCHE and LENE T. SKOVGAARD

Department of Medicine A and Laboratories of Experimental Pathology, Rigshospitalet and Statistical Research Unit, University of Copenhagen, DK 2100 Copenhagen, Denmark

(*Received* 30 *September* 1983; *accepted* 28 *May* 1985)

Abstract—The effect of a toxic dose of acetaminophen on the hepatic conjugations of acetaminophen was studied in single pass perfused livers from rats given acetaminophen overdose 12 hr prior to perfusion and from control rats. Four different acetaminophen concentrations (0.1–6 mmol/l) were used in each perfusion. Glucuronidation of acetaminophen was increased and sulfation of acetaminophen occurred at an unchanged rate in acetaminophen damaged livers as compared to control livers. Hepatic glutathione concentrations declined to about 0.4 μmol/g liver during perfusion, possibly due to excretion of glutathione to perfusion medium, but in spite of this the formation of glutathione conjugates was increased with acetaminophen concentrations increasing up to about 5 mmol. We conclude that decreased sulfation, glucuronidation and glutathione conjugation in the liver is not present in the early development of acetaminophen-induced hepatic damage.

The metabolism of acetaminophen (paracetamol) has been extensively studied since it was demonstrated that massive overdose resulted in hepatic necroses [1]. The drug is mainly metabolized in the liver by glucuronidation and sulfation [2], a few percent of a dose is metabolized by cytochrome P-450 presumably to an arylating metabolite, which is detoxified by glutathione [3–5].

According to the hypothesis an overdose of acetaminophen leads to incomplete detoxification of the arylating metabolite following depletion of hepatic glutathione. Covalent binding of the arylating metabolite to hepatic proteins is thought to initiate the processes leading to hepatic necrosis. If the necrosis is extensive liver functions are severely reduced. In that situation elimination of acetaminophen has been found to be slower than in healthy individuals [6]; however, also plasma levels of acetaminophen are higher. The slower elimination of acetaminophen is thought to originate from reduction of hepatic metabolism in parallel with the hepatic damage but could also be due to capacity limited hepatic metabolism at the high acetaminophen concentration levels. The purpose of the present study was to examine these two possibilities.

MATERIALS AND METHODS

Chemicals. Acetaminophen–glucuronic acid, –sulfate, –cysteine and –mercapturic acid conjugates were kindly supplied by Sterling Winthrop, Stockholm, Sweden. All other chemicals were the best quality commercially available.

Experimental. Female Wistar rats (180–250 g) were fasted for 12 hr. Acetaminophen (300 mg/ml suspension in 0.2% tragacant gum), 4.25 g/kg body weight, or a corresponding volume of the vehicle was given by gastric tube, and fasting continued. Twelve hours after treatment single pass perfusions were performed with livers from six rats given acetaminophen and five rats given the corresponding volume of vehicle (control).

Hepatic glutathione concentrations in non-perfused livers from 11 animals were determined 12 hr after treatment with the vehicle (N = 5) and acetaminophen (N = 6). The treatment was performed after fasting the rats for 12 hr; fasting was continued after treatment.

Perfusion. Each of the livers was perfused *in situ* with a semisynthetic medium consisting of Tyrode buffer [7] and bovine erythrocytes as described before [8]. Flow was 10 ml/min. In perfused livers from the animals pretreated with acetaminophen, residual acetaminophen was washed out during 20 min of perfusion without acetaminophen in the medium for this initial period. According to Pang *et al.* [9–11] steady state of acetaminophen metabolism is achieved after 20 min of perfusion with acetaminophen. This was confirmed in an experiment where the liver was perfused for 120 min with acetaminophen, 5.5 mmol/l (Table 1).

The livers were perfused with four increasing concentrations of acetaminophen (0.1, 0.2, 0.3 and 6.0 mmol/l) each for a period of 30 min. During the last 10 min of each period five samples were taken from hepatic in- and outlet. Immediately after the last sample in the last period a biopsy was taken for determination of hepatic glutathione and for quantitation of hepatic acetaminophen and acetaminophen metabolite concentrations.

The bile secreted following the initial washout period was collected via a catheter inserted into the bile duct for each of the periods.

Galactose, 5 mmol/l, was added to the perfusion medium to evaluate the metabolic viability of the preparation according to Keiding *et al.* [12], pH and

* Author to whom reprint requests should be addressed.

Table 1. Steady state conditions of acetaminophen metabolism in the isolated perfused rat liver

Time after perfusion with acetaminophen*, min	11	21	31	41	51	61	71	81	91	101	111	121
Acetaminophen sinusoid concentration† (mmol/ml)	5.50	5.48	5.49	5.38	5.44	5.51	4.46	5.44	5.40	5.43	—	5.39
Acetaminophen sulfate concentration‡ (nmol/ml)	27.9	29.1	31.1	30.7	29.1	29.9	26.7	29.1	28.3	27.5	28.3	25.5
Acetaminophen glucuronide concentration‡ (nmol/ml)	27.4	32.3	26.7	25.7	25.7	27.4	27.2	27.7	28.2	30.8	30.3	28.0

* Perfusion flow 10 ml/min, acetaminophen conc. 5.5 mmol/l.
† According to the parallel tube model [17].
‡ Measured in hepatic outlet.

oxygen tension and saturation was determined (ABL auto-analyser, Radiometer, Denmark) for estimation of the oxygen consumption of the liver.

A blind study without a liver was performed, by connecting the inlet and outlet tubes directly, with acetaminophen (0.1, 0.2, 0.3 and 5 mmol/l) in four periods of 30 min each. The acetaminophen concentration remained constant in each period and acetaminophen metabolites could not be detected in the outlet medium.

Analysis. Acetaminophen and its conjugates were analysed according to Knox and Jurand [13] as described earlier [14]. Galactose [15] and glutathione [16] were measured spectrophotometrically by the methods indicated.

RESULTS

The elimination of acetaminophen following the two treatments versus the formation of acetaminophen metabolites is given in Table 2 for each of the four periods as mean ± S.D. During the first three periods the mass balance, i.e. elimination vs metabolite production, was not different from null ($P > 0.05$). In period four elimination of acetaminophen was greater than metabolite production though not statistically significant in livers from animals dosed with acetaminophen 12 hr prior to perfusion ($P > 0.05$). The extraction ratio, i.e. acetaminophen concentration difference across the liver divided by the inlet concentration, and the corresponding sinusoid acetaminophen concentration [17] is given in Table 3. There were no statistically significant differences between the two treatments, but the extraction ratio decreased with increasing acetaminophen concentrations.

The sum of acetaminophen metabolites excreted into bile was identical following the two treatments and amounted to about 11% of the total formation (range 7.6–13%) in periods 1, 2 and 3, and to 22% in the fourth period. The difference between the periods is not statistically significant ($P > 0.005$).

Formation of acetaminophen glucuronide increased with increasing acetaminophen concentrations (Table 4). At the two lowest and the highest concentration formation of glucuronide was higher in the damaged livers as indicated in the table.

Formation of acetaminophen sulfate was identical perfusing the livers after the two treatments as indicated in Table 5. Formation increased with increasing acetaminophen concentrations at the three lower concentrations, but in 8 of the 11 perfusions the

Table 2. Elimination (E) and metabolite formation (M) from acetaminophen by the perfused rat liver (nmol/min)

	Control		12 hr after overdose	
	E	M	E	M
Period 1	252 ± 97	310 ± 87	395 ± 899	388 ± 47
Period 2	377 ± 60	402 ± 106	486 ± 889	601 ± 83
Period 3	574 ± 326	606 ± 285	724 ± 175	769 ± 83
Period 4	3319 ± 1543	1031 ± 577*	2600 ± 2350	1460 ± 279

Values are mean ± S.D. for five (control) or six (acetaminophen overdosed) perfusion periods of 10 min. M is metabolites excreted into perfusion medium and bile.
* Indicate E different from M, $P < 0.05$.

Table 3. Sinusoid acetaminophen concentrations and extraction ratio

Perfusion period	Treatment	Extraction ratio (%)	Sinusoid acetaminophen conc. (mmol)
1	(control)	20.3 ± 7.7	0.116 ± 0.016
1	(d)	28.6 ± 2.2	0.116 ± 0.013
2	(control)	18.7 ± 3.5	0.208 ± 0.014
2	(d)	21.6 ± 1.2	0.200 ± 0.015
3	(control)	15.8 ± 4.2	0.345 ± 0.031
3	(d)	20.6 ± 2.9	0.313 ± 0.027
4	(control)	5.9 ± 1.4	5.86 ± 1.37
4	(d)	3.9 ± 0.7	6.04 ± 0.65

Data are mean ± S.E.M. of five perfusions with livers from control rats (control), and of six perfusions with livers from rats given an acetaminophen overdose 12 hr prior to the perfusion (d).

highest concentration led to lower sulfate conjugation.

The formation of the glutathione conjugates of acetaminophen increased from the lowest to the highest acetaminophen concentration, the results are given in Table 6. In some perfusions, small amounts of acetaminophen–cysteine and acetaminophen–mercapturic acid were found, the formation of these conjugates is included in the values for acetaminophen–glutathione given in the table. The formation of acetaminophen–glutathione in livers 12 hr after overdose was greater than in control livers in all steady-state periods, but the differences between the two groups do not reach statistical significance ($P > 0.05$). The total sum of acetaminophen–glutathione excreted from the liver during the 120 min of perfusion with acetaminophen and the concentration of acetaminophen–glutathione in the liver after perfusion, i.e. the total formation of acetaminophen–glutathione, was 0.367 ± 0.062 μmol/g liver (mean ± S.E.M.) in control livers. The corresponding value 12 hr after overdose was greater, 0.790 ± 0.146 μmol/g liver (mean ± S.E.M., $P < 0.05$), indicating increased glutathione conjugation 12 hr after acetaminophen overdose.

Hepatic glutathione decreased in acetaminophen pretreated animals, decreased further during the perfusion, all being at a lower level in perfused livers from animals given an overdose 12 hr prior. Data are given in Table 7.

During each perfusion pH was constant 7.4, the oxygen consumption was higher than 25 μmol per min and gram liver, and the galactose elimination was 1.89 ± 0.16 μmol/min (mean ± S.E.M.). There was no difference between the damaged livers and the non-damaged livers ($P > 0.05$, Student's *t*-test). Bile flow did not vary significantly during the four periods ($P > 0.05$, one way analysis of variance). There was no difference in bile flow between livers perfused 12 hr after overdose (8.7 ± 1.3 μl/min, mean ± S.E.M.), and control livers (6.0 ± 1.4 μl/min, $P > 0.05$, Student's *t*-test).

Table 4. Glucuronidation of acetaminophen by the perfused rat liver

	Control (nmol/min)	12 hr after overdose (nmol/min)
Perfusion period 1	66.0 ± 13.9	115.1 ± 5.0*
Perfusion period 2	119.1 ± 20.6	216.9 ± 15.4*
Perfusion period 3	216.0 ± 56.2	313.1 ± 15.0
Perfusion period 4	607.9 ± 161.9	947.1 ± 69.1†

Values are given as mean ± S.E.M. from perfusing livers from five control and six rats given acetaminophen (4.25 g/kg BW) 12 hr prior to perfusion. The sinusoid acetaminophen concentrations are given in Table 3. Included in the values is biliary excretion of the metabolite.

* Indicate $P < 0.05$ for comparison with control perfusions.

† Indicate $P < 0.01$ for comparison with control perfusions.

DISCUSSION

Acetaminophen overdose leads to hepatic necrosis. Following oral administration identical to the administration used in the present study, hepatic necrosis develops after 48–72 hr [14]. Coagulation necrosis, however, develops delayed compared to the functional deterioration, and at the time interval of fully developed necrosis, hepatic regeneration has formed new hepatocytes compensating lost function. In the present study we chose an interval of 12 hr after the overdose to study acetaminophen metabolism. After that interval hepatic regeneration has not yet started, and the functional impairment is at maximum as indicated by the fall in prothrombin index and the rise in serum alanine aminotransferase activity as earlier reported from our laboratory [14, 18, 19]. Furthermore, we have demonstrated

Table 5. Sulfation of acetaminophen by the perfused rat liver

	Control (nmol/min)	12 hr after overdose (nmol/min)
Perfusion period 1	231.5 ± 26.3	251.1 ± 21.8
Perfusion period 2	278.8 ± 29.2	345.8 ± 26.4
Perfusion period 3	373.1 ± 70.0	416.2 ± 30.8
Perfusion period 4	283.7 ± 49.1	409.8 ± 39.5

Data are as described in Table 4. The values 12 hr after overdose are not statistically different from control values.

Table 6. Glutathione conjugation of acetaminophen by the perfused rat liver

Steady-state period (10 min)	Acetaminophen sinusoidal conc. (mmol/l) (N = 11)	Glutathione conjugation (nmol/min/g liver)	
		Control (N = 5)	12 hr after overdose (N = 6)
1	0.116 ± 0.010	1.69 ± 0.34	3.34 ± 0.71
2	0.204 ± 0.010	2.12 ± 0.26	6.05 ± 1.63
3	0.328 ± 0.020	2.42 ± 0.49	5.99 ± 1.51
4	5.96 ± 0.58	5.33 ± 1.94	9.69 ± 1.75
Sum of period 1 + 2 + 3 + 4		11.56 ± 1.95	25.07 ± 4.60*
Total formation during entire perfusion, 120 min (μmol/g liver)		0.367 ± 0.062	0.790 ± 0.146*

For each of the four steady-state periods and the sum of the four periods (i.e. 10 min steady-state periods, *not* including the 20 min used to obtain steady state) the glutathione conjugation of acetaminophen is given as mean ± S.E.M. of six perfusions with livers from animals given acetaminophen overdose 12 hr earlier and of five perfusions with livers from control animals. Acetaminophen concentrations [17] are given as mean ± S.E.M. of the 11 perfusions, values for each group are given in Table 3.

* Indicate $P < 0.01$ vs control.

that hepatic glutathione, in this model, is depleted after 3–6 hr [14], which leaves sufficient time for the functional deterioration to occur.

Glucuronidation is dependent upon the enzymatic activity of UDP-glucuronyl transferase [20] and the intracellular concentration of UDP-glucuronic acid [21]. The basis for the increased glucuronidation of acetaminophen in damaged livers when perfused 12 hr after the overdose is not clear from the present study. Acetaminophen might, like several other drugs [22], induce UDP-glucuronyl transferase and/or increase the concentration of UDP-glucuronic acid, however, the time interval of 12 hr is too short to result in considerably increased activity from induction. The increased glucuronidation might also reflect acetaminophen induced hepatic damage to membranes of the endoplasmatic reticulum, leading to a partial destruction of the conformational or compartmental constraint of UDP-glucuronyl transferase [20], a phenomenon also suggested for carbon tetrachloride-induced activation of the glucuronidation of 1-naphthol by the perfused rat liver [23]. The data on glucuronidation of acetaminophen is in accordance with our earlier finding of unchanged urinary excretion *in vivo* of acetaminophen glucuronide after acetaminophen overdose [14] and in livers damaged by combined treatment with acetaminophen and allyl alcohol [19].

Table 7. Hepatic glutathione in perfused and non-perfused livers 12 hr after acetaminophen overdose

	Glutathione concentration (μmol/g liver)	
	Control (N = 5)	12 hr after overdose livers (N = 6)
Non-perfused	3.88 ± 0.11	1.15 ± 0.14*
Perfused	1.41 ± 0.06†	0.46 ± 0.06*†

Values are given as mean ± S.E.M.

* Denotes P values less than 0.01 for comparison to corresponding group of untreated livers.

† Denotes P values less than 0.01 for comparison to the corresponding group of non-perfused livers.

Sulfation, at the concentrations investigated, was unchanged 12 hr after acetaminophen overdose. The perfusion medium used did not contain sulfate or cysteine. It has been shown, that omission of sulfate from the perfusion medium stops sulfation [24, 25]. In the present work our results are unexpected on this point, but sulfation could be supported at a high rate by release of sulfate from the erythrocytes in the semisynthetic medium. Acetaminophen sulfate formation decreased with the highest acetaminophen concentration in damaged livers as well as in control perfusions. This high concentration was always used in the last period of perfusion. However, perfusion with this high concentration for a considerable period, 120 min, resulted in a constant concentration of acetaminophen sulfate in the hepatic outlet, and in the 11 perfusions constant galactose elimination indicated unchanged metabolic activity by the criteria of Keiding *et al.* [12]. This indicates that reduced acetaminophen sulfate formation results from high acetaminophen concentrations rather than from an early damage introduced by prolonged perfusion with acetaminophen.

At the highest acetaminophen concentrations elimination of acetaminophen was greater than metabolite production. This was not observed at the lower acetaminophen concentrations. Why elimination is greater than metabolite production cannot be determined, but possibly results from several factors. The isolated perfused liver always seems to 'sweat', at the high concentrations acetaminophen could be eliminated by this route. A slight deviation

from the assumed steady state conditions assumed may also contribute and finally it is possible that metabolites other than the ones measured are formed.

Utilization of glutathione at the highest acetaminophen concentrations might lead to a depletion of cysteine thereby reducing the formation of activated sulfate, PAPS, necessary for sulfation of acetaminophen, catalysed by sulfotransferases [26]. For other substrates sulfation has been demonstrated to show substrate inhibition [28], and using medium containing sulfate and cysteine, mobilization of PAPS is not limiting for acetaminophen sulfation [27, 28], as it might be in our studies using medium without sulfate and cysteine. It remains undecided whether the reduced sulfate formation at the high acetaminophen concentration is due to substrate inhibition or reduced formation of activated sulfate. However, our experiments without cysteine probably are relevant for the *in vivo* situation, where plasma is depleted for sulfate [29].

The conjugates resulting from glutathione conjugation could only be found in the bile, mainly as acetaminophen–glutathione, but also as –cysteine and –mercapturate. Glutathione conjugation increased with increased acetaminophen concentrations. Hepatic glutathione *in vivo* is reduced to about 1–2 μmol/g liver [5]. In the damaged livers further depletion of hepatic glutathione was observed at the end of perfusion, possibly due to excretion of oxidized glutathione to the medium [30]. This might also explain why the formation of acetaminophen–glutathione in untreated livers, 0.367 ± 0.062 μmol/g liver, is not great enough to account for the fall in hepatic glutathione from 3.88 ± 0.11 μmol/g liver to 1.41 ± 0.06 μmol/g liver during perfusion (Table 7).

In spite of a lower hepatic concentration of glutathione, we observed a greater formation of acetaminophen–glutathione in damaged livers compared to non-damaged livers. The true cellular threshold for glutathione conjugation with acetaminophen has been suggested to be about 2 μmol/g liver [5]. Our study indicates that the cellular threshold of glutathione for conjugation with acetaminophen is not obtained at concentrations of about 0.5 μmol/g liver. Furthermore, in the perfused liver glutathione depletion is thought to result from washout of oxidized glutathione [30] and probably occurs equally across the liver lobule. After acetaminophen overdose, however, depletion of glutathione is probably more pronounced in the centrilobular parts, suggesting that the true cellular threshold at which glutathione levels limit conjugation with acetaminophen is even lower than observed in the present study.

In conclusion we find that in the isolated liver perfused 12 hr after an acetaminophen overdose glucuronidation and glutathione conjugation of acetaminophen are slightly increased while sulfation is unchanged. This indicates that the prolonged elimination of acetaminophen observed *in vivo* after an overdose is due to reduced metabolism at the high acetaminophen concentrations rather than reduced capacity as part of the hepatic damage from acetaminophen overdose.

Acknowledgements—Sterling Winthrop, Sweden, generously supplied the acetaminophen metabolites. We are grateful to Mrs. B. Korsholm and Mrs. H. Wennike for skilful technical assistance. This work was supported by the Brøchner-Mortensen Foundation, the Foundation of 31 December 1977 and the Michaelsen Foundation.

REFERENCES

1. L. F. Prescott, N. Wright, P. Roscoe and S. S. Brown, *Lancet* **1**, 519 (1971).
2. D. J. Jollow, S. S. Thorgeirrson, W. Z. Potter, M. Hashimato and J. R. Mitchell, *Pharmacology* **12**, 2251 (1974).
3. J. R. Mitchell, D. J. Jollow, W. Z. Potter, D. C. Davis, J. R. Gillette and B. B. Brodie, *J. Pharmac. Exp. Ther.* **187**, 185 (1973).
4. D. J. Jollow, J. R. Mitchell, W. Z. Potter, D. C. Davis, J. R. Gillette and B. B. Brodie, *J. Pharmac. Exp. Ther.* **187**, 195 (1973).
5. J. R. Mitchell, D. J. Jollow, W. Z. Potter, J. R. Gillette and B. B. Brodie, *J. Pharmac. Exp. Ther.* **187**, 211 (1973).
6. J. A. H. Forrest, J. A. Clemments and L. F. Prescott, *Clin. Pharmacokin.* **7**, 93 (1982).
7. H. Schimassek, *Life Sci.* **11**, 629 (1962).
8. H. E. Poulsen, H. Vilstrup and S. Keiding, *Scand. J. clin. Lab. Invest.* **44**, 155 (1984).
9. K. S. Pang and J. R. Gillette, *J. Pharmac. Exp. Ther.* **207**, 178 (1978).
10. K. S. Pang and J. A. Terrel, *Biochem. Pharmac.* **30**, 1959 (1981).
11. K. S. Pang, L. Walter, M. G. Horning and K. K. Chan, *J. Pharmac. Exp. Ther.* **222**, 14 (1982).
12. S. Keiding, H. Vilstrup and L. Hansen, *Scand. J. clin. Lab. Invest.* **40**, 355 (1980).
13. J. H. Knox and J. Jurand, *J. Chromatography* **142**, 651 (1977).
14. H. E. Poulsen, A. Lerche and N. T. Pedersen, *Pharmacology* **30**, 100 (1985).
15. G. Kurz and K. Wallenfels, in *Methoden der enzymatische Chemie* (Ed. U. Bergmeyer), p. 1243. Verlag Chemie, Weinheim/Bergstrasse (1970).
16. F. Tietze, *Anal. Biochem.* **27**, 502 (1968).
17. S. Keiding, S. Johansen, K. Winkler, T. Tønnesen and N. Tygstrup. *Am. J. Physiol.* **230**, 1302 (1970).
18. H. E. Poulsen, A. Lerche and N. T. Pedersen, *Biochem. Pharmac.* **34**, 727 (1985).
19. H. E. Poulsen, P. Petersen and H. Vilstrup, *Eur. J. clin. Invest.* **11**, 161 (1981).
20. G. J. Dutton and B. Burchell, in *Prog. Drug Metab.* Vol. 2 (Ed. J. W. Bridges and L. F. Chasseaud). Wiley, New York (1975)
21. G. Otani, M. Mahnud, Abou-El-Makarem and K. W. Bock, *Biochem. Pharmac.* **25**, 1293 (1976).
22. J. B. Watkins and C. D. Klaassen, *Drug Met. Disp.* **11**, 37 (1983).
23. P. V. Desmond, R. James, S. Schenker, J. F. Gerkens and R. A. Branch, *Biochem. Pharmac.* **30**, 993 (1981).
24. G. J. Mulder and K. Keulemans, *Biochem. J.* **176**, 959 (1978).
25. J. G. Conway, F. C. Kauffman, T. Tsukada and R. G. Thurman, *Molec. Pharmac.* **25**, 487 (1984).
26. R. D. Sekura and W. B. Jacoby, *J. biol. Chem.* **254**, 5658 (1979).
27. P. Moldeus, *Drug Met. Dis.* **7**, 416 (1979).
28. R. Grafstrom, K. Ormstad and P. Moldeus, *Biochem. Pharmac.* **28**, 3573 (1979).
29. J. H. Lin and G. Levy, *J. Pharmac. Exp. Ther.* **219**, 14 (1981).
30. G. M. Bartoli and H. Sies, *FEBS Lett.* **86**, 89 (1978).