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Disulfiram Prevents Acetaminophen Hepatotoxicity in Rats

L. Jørgensen[1], P. Thomsen[1] and H. E. Poulsen[2]*

[1]Department of Medicine A, Laboratories of Experimental Pathology and Department of Pathology, Rigshospitalet, University of Copenhagen, and [2]Department of Pharmacology, University of Copenhagen, 20 Juliane Mariesvej, DK-2100 Copenhagen Ø, Denmark

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Abstract: Hepatic necrosis due to an oral acetaminophen overdose (4.25 g/kg b.wt.) was prevented by pretreatment with disulfiram 100 mg/kg, given for 3 weeks or as a single dose. Twenty-four hours after acetaminophen the impairment of hepatic function, measured as prothrombin index, and the depletion of hepatic glutathione were prevented. Hepatic cytochrome P-450 levels were unchanged but cytochrome P-450 mediated p-nitroanisole demethylation was reduced by disulfiram pretreatment. Disulfiram pretreatment reduced 24 hour urinary excretion of acetaminophen-mercapturate and -cysteine while excretion of -sulfate and -glucuronide was unchanged. After 72 hours acetaminophen induced hepatic necrosis were prevented. Identical observations were made in animals pretreated with disulfiram for 3 weeks. Five hours after acetaminophen overdose its irreversible binding to hepatic proteins was not changed. After 24 hours, however, it was increased in animals pretreated with a single disulfiram dose and unchanged in animals pretreated for 3 weeks. The protective mechanism of disulfiram after acetaminophen overdose is not mediated via a change in overall irriversible binding of acetaminophen to hepatic protein.

Acetaminophen overdose leads to hepatic necrosis in man (Davidson & Eastham 1966) and in animals (Mitchell *et al.* 1974) presumably by a mechanism including formation and macromolecular binding of a toxic arylating metabolite arising from cytochrome P-450 oxidation (Mitchell *et al.* 1973a, b, & 1974; Jollow *et al.* 1973; Potter *et al.* 1973). The toxic action can be abolished by inactivation of the toxic metabolite by administration of substances, e.g. N-acetylcysteine, which stimulate the synthesis of hepatic glutathione (Lauterburg *et al.* 1983) and inactivate the toxic metabolite. Hepatocellular damage from acetaminophen also can be modulated by substances influencing cytochrome P-450 activity (Mitchell *et al.* 1973a & 1974; Jollow *et al.* 1973; Potter *et al.* 1973; Mitchell *et al.* 1973b). According to the above theory stimulation of P-450 should increase the hepatotoxicity whereas inhibition should reduce it. In an earlier study we were not able to confirm the increased hepatotoxicity from inducing P-450 with phenobarbital (Poulsen *et al.* 1985a) and raised questions about the covalent binding theory. In the present study we evaluated the effect of cytochrome P-450 inhibition by disulfiram on the hepatotoxic action of acetaminophen, for further reexamination of the covalent binding theory.

Materials and Methods

Female Wistar rats weighing 190–227 g (Møllegårds Avlslaboratorium) fed Altromin® pellets and given water *ad libitum* were divided into three groups. After an overnight fast, acetaminophen 4.25 g/kg b.wt. (300 mg/ml suspended in tragacant gum 0.2%), corresponding to LD10 (Poulsen *et al.* 1985a) was given by gastric tube at 8 a.m. Prior to treatment with acetaminophen, groups of animals were pretreated with disulfiram 100 mg/kg b.wt. by stomach tube (as a suspension of disulfiram 20 mg/ml in carboxymethylcellulose): Group I twice a week for three weeks with the disulfiram vehicle alone, group II with a single dose of disulfiram 18 hours before acetaminophen administration, and group III was given disulfiram twice a week for three weeks, the last dose was given 18 hours before acetaminophen. Twenty-four hours after acetaminophen overdose animals were anaesthesized with diethyl ether and aortic blood was diluted with an equal volume of isotonic saline for determination of prothrombin index by Owren's method (Owren & Aas 1951). The liver was removed, homogenized and assayed for liver protein (Groves *et al.* 1968) total glutathione (Tietze 1969), cytochrome P-450 (Omura & Sato 1964) and microsomes were prepared for estimation of p-nitroanisole demethylation (Schoene *et al.* 1972).

For irreversible binding assay ^{3}H-acetaminophen (New England Nuclear; Corp. specific activity 2.6 Ci/mmol) was purified immediately prior to use by high pressure liquid chromatography (HPLC) (the system is described below).

Immediately before administration of acetaminophen overdose about 100 μCi ^{3}H-acetaminophen was added and well mixed with the unlabelled acetaminophen. The time course of covalent binding was determined on groups of two animals examined 2, 6, 9, 24, 36 and 48 hours after acetaminophen. The animals were pretreated with disulfiram vehicle. At the time of maximum binding, i.e. after 24 hours (fig. 1), covalent binding was estimated in groups of 4–6 animals pretreated as described above (I, II and III). In three other groups of four animals (treated as I, II and III) the labelled acetaminophen was given intraperitoneally immediately after the oral overdose in order to estimate the irreversible binding to hepatic protein during the first 5 hours.

The procedure for estimating irreversible binding was as follows. All animals were anaesthesized with diethyl ether, bleed from the aorta, and their liver homogenized in a Potter Elvehjelm glass teflon homogenizer in 20 ml of isotonic KCl. In aliquots (2 ml) of the homogenate protein was precipitated with 10% TCA/0.02 M HCl and centrifuged at 10,000 × g. The precipitate was washed three times with 10% TCA and once with methanol, heptane, acetone and diethyl ether. No further radioactivity could be removed by repeating this procedure. The radioactivity of the remnant was then counted in a Packard Tricarb model 4530 scintillation counter with automatic correction for quench from the spectral index of external standard, and taken to represent covalent binding to hepatic protein (Jollow *et al.* 1973). Hepatic protein was determined in homogenate by the method of Groves *et al.* (1968).

Acetaminophen metabolism and liver histology was studied in

*To whom correspondence should be directed.

Table 1.

The effect of disulfiram treatment on hepatic function, cytochrome P-450 and its activity, and hepatic glutathione.

Group	Prothrombin index Arbitrary units	Cytochrome P-450 in microsomes nmol/g liver	Total glutathione µmol/g liver	p-N-panisole demethylation nmol/min. and g liver
I (not pretreated)	0.55±0.04	50.7±1.33	3.61±0.45	530±38
II	0.82±0.09*	54.5±0.97	5.08±0.60*	254±18**
III	0.90±0.03**	48.4±1.04	8.09±0.60**	316±24**

Each group consists of 9 animals, values are mean±S.E.M.

Group I was treated with disulfiram vehicle twice weekly prior to acetaminophen 4.25 g/kg b.wt.
Group II was treated with disulfiram 100 mg/kg b.wt. 18 hrs prior to acetaminophen 4.25 g/kg b.wt.
Group III was treated with disulfiram 100 mg/kg b.wt. twice weekly prior to acetaminophen 4.25 g/kg b.wt.

* indicate $P < 0.05$ compared to group I.
** indicate $P < 0.01$ compared to group I.

three groups pretreated as group I, II and III described above consisting of 5, 6 and 6 animals, respectively. They were housed individually in metabolism cages, and urine was collected over dry ice for 24 hours. Quantitation of acetaminophen and its metabolites was performed on a HPLC system consisting of a Waters M 6000 pump, a Waters M 440 UV-detector with fixed wavelength of 254 nm and a 30 cm µ Bondapack C18 reverse phase column as described earlier (Poulsen *et al.* 1985a & b). The metabolites were quantitated from standard curves of reference metabolites, kindly donated by Sterling-Winthrop, Sweden. After 72 hours these animals were anaesthesized with diethyl ether, liver tissue was fixed in formaldehyde, embedded in paraffin and stained with haematoxylin and eosin for conventional light microscopy. Necrosis was read blind with regard to treatment. The number of necroses was estimated in 50 lobules from each animal and given as per cent. For confluent necroses the size was registered as layers of necrotic cells around the lumen of central vein: 1–2 = +, 2–4 = ++ and >4 layers = +++.

Differences between the three groups were tested with Bartlett's test for homogeneity of variance prior to one way analysis of variance. Further differences within the groups were then tested with Student's t-test; P-values less than 0.05 were considered statistically significant.

Results

Hepatic function, measured as the prothrombin index after 24 hours, was less depressed from acetaminophen overdose after a single disulfiram dose ($P < 0.05$) than after the acetaminophen dose without pretreatment. Further protection was observed when disulfiram was given for 3 weeks prior to the acetaminophen overdose (table 1).

Twenty-four hours after acetaminophen hepatic cytochrome P-450 was not changed from the disulfiram pretreatment ($P > 0.05$) while p-nitroanisole demethylation was equally depressed ($P < 0.05$) from a single dose and from three weeks pretreatment (table 1).

Twenty four hours after acetaminophen hepatic glutathione was depressed as expected in animals pretreated with the disulfiram vehicle (Mitchell *et al.* 1973b; Poulsen *et al.* 1985a & b). Disulfiram pretreatment prevented in part the decrease in hepatic glutathione concentration, which after 3 weeks disulfiram pretreatment corresponded to that of normal animals (Mitchell *et al.* 1973b; Poulsen *et al.* 1985a & b; Milandri *et al.* 1980; Tietze 1969) (table 1). The difference in hepatic glutathione concentration between animals pretreated once and for three weeks did not reach statistical significance ($P > 0.05$).

Light microscopy revealed confluent centrilobular necrosis 72 hours after the acetaminophen (table 2) in animals not pretreated with disulfiram. Animals pretreated with disulfiram once or for three weeks showed no centrilobular necroses after acetaminophen (table 2). In 8 out of 17 animals distinct small hepatic necrosis was found without relation to the central vein or treatment. These small necroses are seen in control animals in many studies and are not considered toxicologically important.

Disulfiram pretreatment decreased the urinary excretion

Table 2.

Hepatic necrosis 72 hours after acetaminophen overdose.

Treatment	Animal no.	Confluent necroses size	Confluent necroses %
I (not pretreated)	1	+++	100
I (not pretreated)	2	++	88
I (not pretreated)	3	+	6
I (not pretreated)	4	++	58
I (not pretreated)	5	++	72
II	6	0	0
II	7	0	0
II	8	0	0
II	9	0	0
II	10	0	0
II	11	0	0
III	12	0	0
III	13	0	0
III	14	0	0
III	15	0	0
III	16	0	0
III	17	0	0

The groups were treated as described in table 1. Confluent liver cell necroses was counted in 50 liver lobules and given as per cent. For confluent necrosis the size was registered as layers of necrotic cell around the lumen of central vein graded 1–2 layers = +, 2–4 layers = ++, and >4 layers = +++.

Table 3.

The effect of disulfiram on acetaminophen metabolism.

	Acetaminophen				
Treatment group	mercapturate	cystein	sulfate	glucuronide	unchanged
I (not pretreated)	12.3±5.7	0.23±0.06	23.0±9.7	48.7±3.7	15.8±8.6
II	4.8±1.1*	0.07±0.01*	24.7±7.4	60.2±6.4	10.3±1.5
III	5.2±1.2*	0.07±0.04*	22.7±6.7	60.7±6.2	11.4±2.3

The groups were treated as described in table 1. The animals were housed individually in metabolic cages for 24 hours and urine collected over dry ice. The values are the mean±S.E.M. of 5,6 and 6 animals of the groups given as percent of the total amount excreted during the 24 hours.

* indicate $P<0.05$ compared to group I.

of acetaminophen mercaptuate and cysteine conjugates ($P<0.05$). There was no difference between animals given one dose and pretreatment for 3 weeks. The excretion of acetaminophen, acetaminophen-sulfate and acetaminophen-glucuronide conjugates was identical in the three groups (table 3), although the group treated with disulfiram had a statistically insignificant higher excretion of glucuronide. The recovery of acetaminophen during the first 24 hours was 20–30% of the dose. This is in accordance with earlier experiments with similar treatment, where urine had to be collected for 96 hours to recover the dose (Poulsen *et al.* 1985; Colin *et al.* 1986). In order to compare the groups each metabolite is given as % of the dose recovered in urine during the first 24 hours.

After 24 hours irreversibly binding of ^{3}H-acetaminophen was at maximum (fig. 1). At this time irreversible binding was increased ($P<0.05$) in animals pretreated once with disulfiram and unchanged in animals pretreated for 3 weeks. Covalent binding after 5 hours was not different in animals in groups I, II and III (table 4).

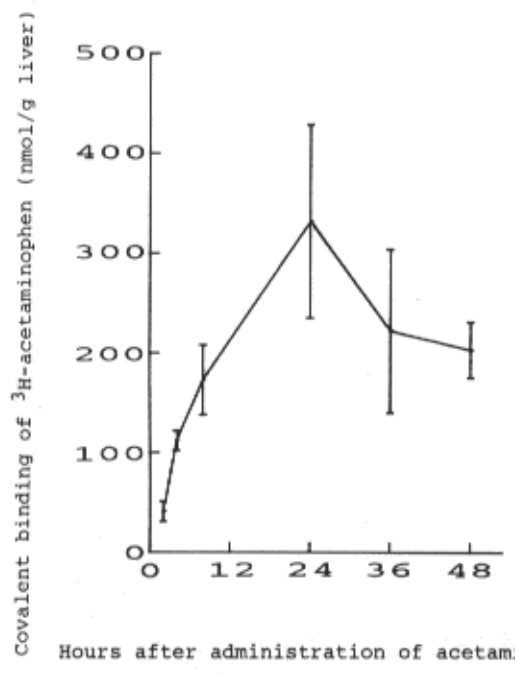

Fig. 1. Time course of irriversible hepatic protein binding of acetaminophen 4.25 mg/kg b.wt. given by stomach tube. Points and bars indicate mean and S.D. of groups of two animals.

Discussion

This study demonstrates that hepatotoxicity from acetaminophen is prevented by disulfiram pretreatment. This applies to histopathological changes as well as to functional disturbances. Long term pretreatment, i.e. treatment for 3 weeks, and single dose pretreatment were both effective in preventing the centrilobular necroses. Prevention of the hepatic functional disturbances were more complete after long term disulfiram treatment. This is in accordance with the observation in man that the effect of disulfiram on hepatic enzymes is maximal after some days treatment (Loft *et al.* 1986).

Hepatic glutathione is regarded crucial in the detoxification of the supposed toxic metabolite of acetaminophen (Mitchell *et al.* 1973a). It is depleted proportionally with the acetaminophen dose (Mitchell *et al.* 1973a, b & 1974; Poulsen *et al.* 1985) and is not influenced by disulfiram treatment in normal animals (Milandri *et al.* 1980). The disulfiram dose was chosen from that study because it produces a considerably reduction in cytochrome P-450 activity, presumably partly by competitive inhibition. In vehicle-treated animals acetaminophen caused the expected depletion of hepatic glutathione, which was in part prevented in animals pretreated with a single disulfiram dose and was not seen in animals pretreated for 3 weeks. In accord-

Table 4.

In vivo irriveible binding of ^{3}H-acetaminophen to hepatic protein after 5 and 24 hours.

Group	5 hours $10^{-4}\times$dose	24 hours nmol/g liver
I (not pretreated)	7.62±0.97 (4)	240±36 (6)
II	5.42±0.62 (4)	431±85* (6)
III	5.27±1.01 (4)	200±79 (4)

The three groups were treated as described in table 1. Numbers are mean±S.E.M., the number of animals is given in ( ).

* indicate $P<0.05$ compared to group I, one sided Student's t-test.

ance we found inhibited *in vitro* cytochrome P-450 activity, measured as p-nitroanisole demethylation, and reduced urinary excretion of acetaminophen-cysteine and -mercapturate. These data suggest that the mechanism of disulfiram protection is by inhibition of cytochrome P-450, resulting in reduced formation of the toxic intermediate, presumably a benzoquinoneimine (Corcoran *et al.* 1980). However, we were not able to find the expected decreased irreversible binding of acetaminophen to hepatic proteins. This is not easily compatible with reduced formation of the toxic metabolite from inhibited formation by cytochrome P-450 oxidation.

In non-pretreated animals irreversible binding of acetaminophen to hepatic protein was about 250–300 nmol/g liver, corresponding to about 1–2 nmol/mg protein since the protein concentration in rat liver is about 200 mg/g liver (fig. 1 and table 4). In accordance covalent binding of acetaminophen found by Jollow *et al.* (1973) amounted to about 1–2 nmol/mg protein in mouse liver. In their studies cobalt chloride was used to inhibit P-450 and at variance with the present study reduced covalent binding was observed. Recently, however, they have reevaluated cobalt chloride protection in hamsters (Roberts *et al.* 1986) and found that it not only inhibits P-450 but also increases the protective capacity of glutathione and enhances the non toxic pathway of glucuronidation. Peterson *et al.* (1983) used cimetidine to inhibit P-450 in mice, and found unchanged covalent binding in accordance with the present study. On the contrary, they found that the glutathione depletion was unchanged. They suggested that the current theories regarding production of acetaminophen-induced liver damage require reexamination.

Covalent binding of acetaminophen has also been studied after stimulation of P-450 with various inducers. Pessayre *et al.* (1980) found unchanged irreversible binding of acetaminophen after induction with phenobarbital, but increased binding after induction with 3-methyl cholanthrene. We have reported earlier that phenobarbital acellerates rather than enhances acetaminophen hepatotoxicity (Poulsen *et al.* 1985). Also Devalia *et al.* (1982) have demonstrated hepatotoxicity from acetaminophen in isolated hepatocytes without relation to its covalent binding.

Cysteamine was originally reported to protect against acetaminophen hepatotoxicity (Mitchell *et al.* 1973a), and the effect attributed to its sulfhydryl nucleophilic properties. Miller & Jollow (1986) reexamined the mechanism of cysteamin protection and found that the proportion of the dose of acetaminophen which is converted to the toxic metabolite was not decreased in the presence of cysteamine, that it appeared to inhibit P-450, and that this effect is not sufficient to explain the antidotal effect.

These data raise questions about unspecific irreversible binding of a reactive metabolite as the mechanism for acetaminophen-induced liver damage. We conclude that disulfiram protects against acetaminophen induced hepatic damage without changing overall covalent binding of acetaminophen to hepatic protein.

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References

Colin, P., G. Sirois, S. Chakrabati: Effects of route of administration on the dose-dependent metabolism of acetaminophen in rats: relation with its toxicity. *Arch. Int. Pharmocodyn.* 1986, **281**, 181–191.

Corcoran, G. B., J. R. Mitchell, Y. N. Vaishnav & E. C. Horning: Evidence that acetaminophen and N-hydroxyacetaminophen form a common arylating intermediate N-acetyl p-benzoequinoneimine. *Mol. Pharmacol.* 1980, **18**, 536–542.

Davidson, D. G. D. & W. N. Eastham: Acute liver necrosis following overdose of paracetamol. *Brit. Med. J.* 1966, **2**, 497–499.

Devalia, J. L., R. C. Ogilvie & A. E. M. McLean: Dissociation of cell death from covalent binding of paracetamol by flavones in a hepatocyte system. *Biochem. Pharmacol.* 1982, **31**, 3745–3749.

Groves, W. E., F. C. Davis & B. H. Sells: Spectrophotometric determination of microgram quantities of protein without nucleic acis interference. *Analyt. Biochem.* 1968, **22**, 195–210.

Jollow, G. J., J. R. Mitchell, W. Z. Potter, D. C. Davis, J. R. Gilette & B. B. Brodie: Acetaminophen-induced hepatic necrosis II. Role of covalent binding *in vivo*. *J. Pharmacol. Exp. Therap.* 1973, **187**, 195–202.

Lauterburg, B. H., G. B. Corcoran & J. R. Mitchell: Mechanism of action of N-acetylcysteine in the protection against hepatotoxicity in rats *in vivo*. *J. Clin. Invest.* 1983, **71**, 980–991.

Loft, S., J. Sonne, H. Pilsgaard, M. Døssing & H. E. Poulsen: Inhibition of antipyrine elimination by disulfiram and cimetidine: the effect of concommitant administration. *Brit. J. Clin. Pharmacol.* 1986, **21**, 75–77.

Milandri, M., H. E. Poulsen, L. Ranek & P. B. Andreasen: Effect of long-term disulfiram administration on rat liver. *Pharmacology* 1980, **21**, 76–80.

Miller, M. G. & D. J. Jollow: Acetaminophen Hepatotoxicity: Studies on the mechanism of cysteamine protection. *Toxicol. Appl. Pharmacol.* 1986, **83**, 115–121.

Mitchell, J. R., D. J. Jollow, W. Z. Potter, D. C. Davis, J. R. Gilette & B. B. Brodie: Acetaminophen-induced hepatic necrosis I. Role of drug metabolism. *J. Pharmacol. Exp. Therap.* 1973a, **187**, 185–194.

Mitchell, J. R., D. J. Jollow, W. Z. Potter, J. R. Gilette & B. B. Brodie: Acetaminophen-induced hepatic necrosis IV. Protective role of glutathione. *J. Pharmacol. Exp. Therap.* 1973b, **187**, 211–217.

Mitchell, J. R., S. S. Thorgeirsson, W. Z. Potter, D. J. Jollow & H. Kaiser: Acetaminophen induced hepatic injury. Protective role of glutathione in man and rationale for therapy. *Clin. Pharmacol. Therap.* 1974, **16**, 676–684.

Omura, T. & R. Sato: The carbon monoxide binding of liver microsomes. *J. Biol. Chem.* 1964, **27**, 2570–2578.

Owren, P. A. & K. Aas: The control of dicumarol therapy and the quantitative determination of prothrombin and proconvertin. *Scand. J. Clin. lab. Invest.* 1951, **3**, 201–208.

Pessayre, D. J. Wandscheer, B. Cobert, R. Level, C. Degot, A. M. Batt, N. Martin & J. P. Benhamou: Additive effects of inducers and fasting on acetaminophen hepatotoxicity. *Biochem. Pharmacol.* 1980, **29**, 2219–2223.

Peterson, F. J., R. G. Knodell, N. J. Lindeman & N. M. Steele: Prevention of acetaminophen and cocaine hepatotoxicity in mice by cimetidine treatment. *Gastroenterol.* 1983, **85**, 122–129.

Potter, W. Z., D. C. Davis, J. R. Mitchell, D. J. Jollow, J. R. Gilette & B. B. Brodie: Acetaminophen-induced hepatic necrosis III. Cytochrome P-450-mediated covalent binding *in vitro*. *J. Pharmacol. Exp. Therap.* 1973, **187**, 203–210.

Poulsen, H. E., A. Lerche & N. T. Petersen: Phenobarbital induction does not potentiate hepatotoxicity but accelerates liver cell necrosis from acetaminophen overdose in the rat. *Pharmacology* 1985a, **30**, 100–108.

Poulsen, H. E., A. Lerche & N. T. Petersen: Potentiated hepatotoxicity from concurrent administration of acetaminophen and allyl alcohol to rats. *Biochem. Pharmacol.* 1985b, **34**, 727–731.

Roberts, S. A., V. F. Price & D. J. Jollow: The mechanism of cobalt chloride-induced protection against acetaminophen hepatotoxicity. *Drug Metab. Disp.* 1986, **14**, 25–33.

Schoene, B., R. A. Fleischmann, H. Remmer & H. F. Oldenhausen: Determination of drug metabolizing enzymes in needle biopsies of human liver. *Eur. J. Clin. Pharmacol.* 1972, **4**, 65–73.

Tietze, F.: Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissue. *Analyt. Biochem.* 1969, **27**, 502–522.