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ACUTE PARACETAMOL INTOXICATION OF STARVED MICE LEADS TO LIPID PEROXIDATION *IN VIVO*

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Abstract—Lipid peroxidation was monitored in female mice *in vivo* by the measurement of exhalated hydrocarbons. In liver homogenates *in vitro* lipid peroxidation as determined by malondialdehyde formation, and hepatic total glutathione levels were measured. After a dose of 500 mg/kg i.p. of paracetamol, the hepatic glutathione of fed mice decreased from 61 nmoles/mg liver protein to 30 nmoles/mg, while the animals expired 5 nmoles of ethane/kg·hr. The same dose in starved mice led to a glutathione level of 6 nmoles/mg and an exhalation rate of 125–150 nmoles ethane/kg·hr. *In vivo* determined and post-mortem *in vitro* determined lipid peroxidation correlated with a coefficient of 0.66. If hepatic glutathione was depleted to the same extent by administration of diethylmaleate, no significant lipid peroxidation was found. Our findings demonstrate that the drug-induced depletion of liver glutathione leads *in vivo* to lipid peroxidation, provided that the glutathione level has been diminished by starvation. The data indicate that glutathione depletion alone by other mechanisms does not account for lipid peroxidation. Hence the hepatoprotective role of liver glutathione against drug-induced liver injury has to be reconsidered in detail. This investigation shows a suitable model for such studies.

The drug-induced liver necrosis has been extensively studied in mice and rats using acute paracetamol intoxication as a model system [1–3]. It was shown that this drug depletes the hepatic GSH pool, most probably after its biotransformation. This led to the concept [4], that the binding of the drug's metabolites to intracellular proteins or other macromolecules causes liver injury, provided that the GSH level has been considerably lowered. On the other hand, lipid peroxidation has been discussed as a basic deteriorative mechanism in the chain of events which leads to liver dysfunction [5]. The prevention of lipid peroxidation is intimately connected with the GSH metabolism: hepatic glutathione peroxidase (EC 1.11.1.9) is able to remove H_2O_2 and organic peroxides in the cell compartments where catalase (EC 1.11.1.6) is absent [6, 7], or can reduce in a second line of defence already formed lipidhydroperoxides to the correspondent hydroxy compounds [8]. In both cases, reduced GSH is consumed. Although it has been shown that lipid peroxidation occurs under such conditions *in vitro* [9] and that the extent of peroxidation can be diminished by GSH [10] or other antioxidants [11–13], neither peroxidation nor its prevention have been demonstrated *in vivo*.

The present study was undertaken to find out whether acute paracetamol intoxication leads *in vivo* to lipid peroxidation, and, if this holds true, to relate this event to the actual hepatic GSH level in order to estimate the hepatoprotective potential of the tripeptide.

MATERIALS AND METHODS

Female albino mice of 15 g average weight were adapted for at least 2 weeks to the special diet C 1018 supplied by Altromin, Lage, Germany. This diet was not supplemented as usual with 1 p.p.m. selenium, was low in vitamin E, and contained 4% soybean oil instead of palmitin as fat source.* The animals were injected i.p. with a 0.25 M solution of paracetamol (Fluka) in 0.9% saline as described in [3], or alternatively with 20% diethylmaleate (Fluka) in sesam oil.

Ethane evolution was determined as described in [14] and [15] with some modifications. Single animals were kept in an all-glass system in a 800 ml desiccator using 80 g 'Sodasorb' (Rhein-Pharma-Arzneimittelwerk Planckstadt) for CO_2 adsorption and drying. The equivalent amount of gas was replaced as O_2 from a reservoir at constant pressure. At the times indicated, the air within the desiccator was mixed using a 50 ml syringe, 5 ml were removed with a syringe and applied to a Hewlett-Packard 5750 G gas chromatograph with an integrator through an inlet system at room pressure. With N_2 as carrier gas, a $\frac{1}{8} \times 6$ inch steel column filled with Porapak Q-100-120 mesh (Applied Science Laboratories) was used for resolution of the expired hydrocarbons. Analysis was run isothermally at 70° with flame ionisation detection at the highest sensitivity range. Calibration was performed from 3–40 pmoles ethane using an ethane–N_2 standard supplied by Messer-Grießheim, Duisburg. The recovery of the gases was controlled by allowing untreated animals to breath in a premixed atmosphere for 4 hr; under these conditions the ethane concentration remained constant within ±5%. Since ethane exhalation started at different lag periods of 60–120 min and the animals exhibited impaired respiratory function about 1 hr before death, the linear exhalation within the first hour after the lag phase was taken to calculate the rate.

* This diet was chosen for the following reasons: following earlier observations [24] it was anticipated that feeding a diet with a high amount of unsaturated lipid and low amounts of vitamin E, and a low selenium content (hence low activity of selenium-dependent glutathione peroxidase in the animals) might lead to a manifestation of lipid peroxidation. This assumption has since been experimentally supported [25].

For the *in vitro* determinations, similarly treated groups of animals were killed, the livers were immediately removed and homogenized in ice-cold 3% (w/v) metaphosphoric acid. Aliquots of the supernatants of a 1 min centrifugation in an Eppendorff centrifuge at full speed were withdrawn and used for *in vitro* determination of lipid peroxidation by the thiobarbituric acid method [16]: 500 μl of supernatant were incubated in a total volume of 4 ml containing 5% (w/v) trichloroacetic acid and 0.1% (w/v) thiobarbituric acid (Ega-Chemie) at 95° for 30 min. After centrifugation the extinction at 532 nm was measured against a blank. Calibration was performed using malondialdehyde-bis-diethylacetal supplied by Schuchardt, München. Total glutathione was determined by following the formation of nitrobenzoic acid thiolate from Ellman's reagent at 412 nm in a reaction coupled to glutathione reductase [17]. A linear calibration curve was obtained from 0.1–1 μM using reduced glutathione from Waldhof-Pharma, Aschaffenburg.

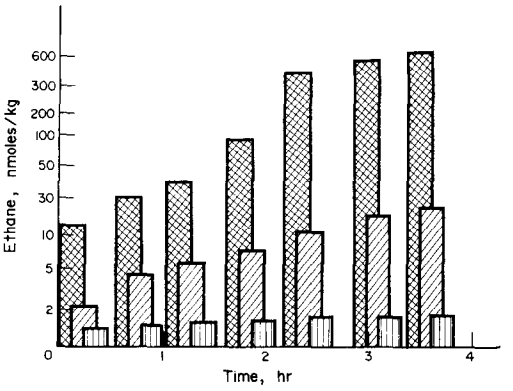

Fig. 1. *In vivo* ethane exhalation of fed (hatched columns) and starved (cross-hatched columns) mice treated intraperitoneally with 500 mg/kg of paracetamol (logarithmic scale). The control animals (striped columns) were injected with isotonic saline. $n = 5$; variation coefficient = 12.5%; the values represent arithmetic means.

RESULTS

In agreement with earlier observations [1–3], treatment of mice with 500 mg/kg paracetamol led to a decrease of the intrahepatic glutathione by 50%, i.e. in these experiments, from 61 nmoles/mg protein to 30 nmoles/mg. However, this was not accompanied by lipid peroxidation: neither were significant amounts of expired hydrocarbons detected *in vivo* (Fig. 1, hatched columns) nor was *in vitro* malondialdehyde formation measured. The animals survived the time of the experiments (>6 hr). Since on the one hand a decrease of the liver glutathione upon starvation [18] and on the other hand a decrease of the lethal dose of paracetamol by starvation [24] was shown in rats, we subjected starved mice to the same treatment. While paracetamol-treated fed mice expired 5 nmoles of ethane/kg·hr, the starved mice showed a maximal ethane exhalation of 150 nmoles of ethane/kg·hr after i.p. injection of 500 mg/kg paracetamol (Fig. 1, cross-hatched black columns). The fed, untreated controls (Fig. 1, striped columns), showed still less ethane production than the fed, treated animals; however, these low concentrations turned out to be not statistically different.

Figure 2 shows the time course of the hepatic total glutathione level during starvation and subsequent paracetamol treatment. While a dose of 500 mg/kg led in fed mice to a decrease of glutathione by 50 per cent, the same dose diminished the concentration of the tripeptide in starved animals by 90 per cent within the first hour.

In Fig. 3, the dose–response relation of ethane evolution vs paracetamol dose is given. The mortality increased from 20 per cent at 300 mg/kg to 75 per cent at 500 mg/kg within 4–6 hr after paracetamol administration. Below 375 mg/kg, the ethane evolution was not significantly different from untreated controls. At 500 mg/kg paracetamol the amount of *in vivo* expired ethane and the residual glutathione level as determined post-mortem in the livers of the animals are inversely related (Fig. 4). Assuming the existence of a threshold, straight lines were calculated. The flat one represents a concentration range where reduction of the glutathione level does not result in lipid peroxidation, whereas the

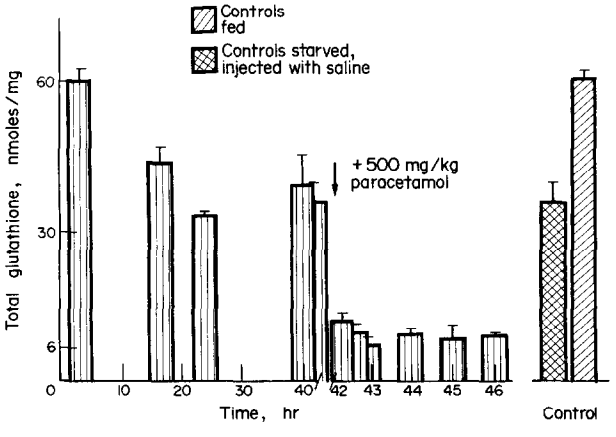

Fig. 2. Concentration of total glutathione determined *in vitro* (nmoles per mg of protein) in mouse liver during starvation and subsequent treatment with 500 mg/kg paracetamol. The data are given as mean values from 3 animals ± S.D.

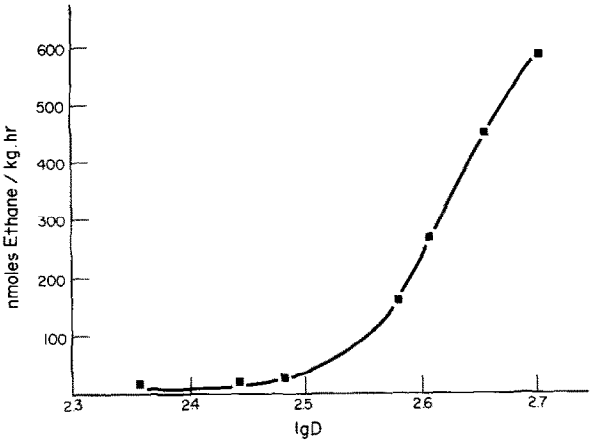

Fig. 3. Dose–response relation between *in vivo* ethane exhalation of starved mice and the amount of paracetamol administered i.p. (logarithm). Each point represents the mean value obtained with 3 animals.

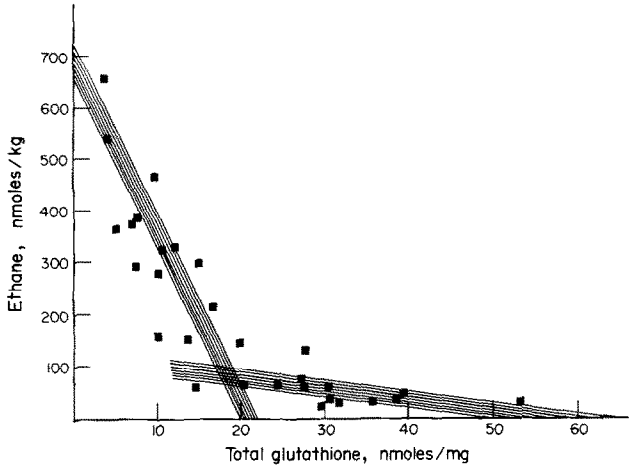

Fig. 4. Relation of *in vivo* expired ethane of starved mice treated i.p. with doses of 225–500 mg/kg paracetamol to the hepatic glutathione content of the same individuals determined *in vitro* 4–6 hr after intoxication. $n = 29$.

steep one shows that in this range both events are inversely proportional. The intersection may thus indicate the minimal hepatic GSH requirement under these conditions, which amounts to 18 nmoles/mg or approx. 3.4 mmoles/l glutathione.

In order to find out whether this minimal GSH level really represents a threshold another series of experiments was performed, in which the hepatic glutathione level was lowered by i.p. administration of the glutathione-*S*-transferase (EC2.5.1.18) substrate diethylmaleate [19].

Table 1 shows that the depletion of hepatic glutathione by diethylmaleate does not result in significant *in vivo* measurable lipid peroxidation in neither fed nor in starved mice.

Some malondialdehyde formation was found at the

Table 1. Effect of i.p. injection of diethylmaleate to fed or starved mice on lipid peroxidation

Dose of diethylmaleate	Starved (hr)	Total liver glutathione (nmoles/mg)	Expired ethane (nmoles/kg·hr)	Malondialdehyde formation in liver homogenate (pmoles/mg)	Number of animals
0	not	61 ± 1.7	<2†	<10†	5
400 mg/kg	not	32 ± 5.5	8 ± 2	25 ± 3	4
400 mg/kg	45	16 ± 5.3	5 ± 0.4	20 ± 2	3
1000 mg/kg	45	6.4*	5.5*	20*	2

Ethane exhalation was measured *in vivo*, malondialdehyde formation and total liver glutathione *in vitro* for 4 hr after injection of the compound. The values are arithmetic means ± S.D.

* Means.

† Below detection limit.

Table 2. Effect of i.p. injection of diethylmaleate (DEM) and of double intoxication by diethylmaleate plus paracetamol to fed and starved mice on lipid peroxidation *in vivo* and *in vitro* and on the hepatic glutathione level

Drug dose	Starved (hr)	Total liver glutathione (nmoles/mg)	Expired ethane (nmoles/kg·hr)	Malondialdehyde formation in liver homogenate (pmoles/mg)	Number of animals
3 × DEM* (400 mg/kg)	48	2.3 ± 1.7	3 ± 1.3	<10§	3
2 × DEM† (400 mg/kg)	not	10.4 ± 5	3 ± 2	44 ± 9	3
2 × DEM (400 mg/kg)+ 1 × 500 mg/kg paracetamol†	not	2.4 ± 1.5	30 ± 10	49 ± 11	4

The values are means ±S.D.
*Injection at 0, 20 and 40 min.
† Injection at 0 and 20 min.
‡ Injection of diethylmaleate at 0 and 20 min; of paracetamol at 50 min.
§ Below detection limit.

end of the 4 hr period of the experiments.

As Table 2 demonstrates, even a depression of the glutathione level of starved animals to 5% by a triple injection of diethylmaleate is not accompanied by the signs of lipid peroxidation. This holds also true for fed animals. Surprisingly, these fed or starved animals survived. Only after additional intoxication with the highest paracetamol dose, 30 nmoles ethane/kg·hr are evolved in fed mice. This corresponds to $\frac{1}{5}$ of the amount which is measured in starved mice not pretreated with diethylmaleate. However, a quantitative comparison seems to be hazardous because the degree of liver function impairment by pretreatment with diethylmaleate is unknown.

Finally, Fig. 5 compares the amount of lipid peroxidation as measured *in vivo* by total ethane evolution with the amount of malondialdehyde formed as determined in liver homogenates after the experiments *in vitro*. It shows that a fairly good correlation exists and that the ethane evolution is an approx. 10 times more sensitive index. In none of our experiments did we detect measurable amounts of propane or pentane, as described by others [23].

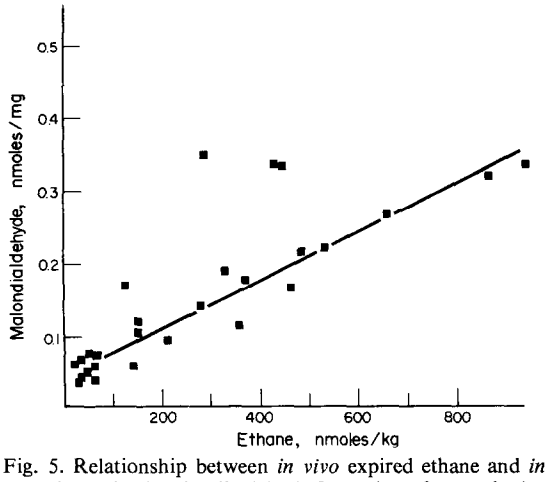

Fig. 5. Relationship between *in vivo* expired ethane and *in vitro* determined malondialdehyde formation of starved mice treated with 225–500 mg/kg paracetamol. $n = 29$. Correlation coefficient $r = 0.66$. Ethane = total amount expired during 4–6 hr depending on the time at which the animals died or were killed. The amount of post-mortem determined malondialdehyde pertains to the same time for each individual.

DISCUSSION

The present study demonstrates that highly-dosed paracetamol intoxication of mice leads in a defined dose–response relation to lipid peroxidation which is manifest *in vivo* and *in vitro*, provided that the animals had been starved prior to the administration of the drug. The hepatodestructive action of the drug has been previously associated with the hepatic glutathione level [4]. Our results show that a more detailed view of the hepatoprotective role of glutathione is needed. It is feasible that the two hepatic glutathione pools with half-lives of 1.7 and 28 hr, as postulated from isotopic labelling studies of the tripeptide in rat liver [18] contribute in a different way to the protection of the organ.

Above 375 mg/kg of paracetamol, significant lipid peroxidation takes place in starved animals. This critical dose coincides with the one described for the increase in the protein binding of the drug's metabolites [2] which was held responsible for the degree of liver lesion [4]. Although it is still unclear which event is the primary attack and what is the target, our findings also lead to the conclusion that paracetamol exerts its toxicity by a dual action, which involves an initial depletion of intrahepatic glutathione and a surplus of a reactive metabolite after its bioactivation by the microsomal P-450-dependent system. It is hardly conceivable that the *N*-acetyl-*p*-hydroxybenzoquinone derivative as the biotransformation product of paracetamol initiates lipid peroxidation. We tentatively propose that either O_2^- or its dismutation product H_2O_2, which have been shown to be generated by the P-450 system [20–22], are the species responsible for the peroxidative attack which reaches its target after the GSH-peroxidase defence mechanism is paralyzed by lack of substrate. In line

with this view is that diethylmaleate, which is exclusively metabolized via glutathione-*S*-transferases, does not cause significant lipid peroxidation, while it completely depletes hepatic glutathione. Although the detailed biochemical background remains to be resolved, this work shows that the drug-induced lipid peroxidation as measured *in vivo* by ethane exhalation seems to be a suitable model for the screening of the action of hepatoprotective or hepatodestructive compounds.

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