Biochemical Pharmacology, Vol. 24, pp. 37–42. Pergamon Press, 1975. Printed in Great Britain.

THE EFFECT OF DIET ON THE TOXICITY OF PARACETAMOL AND THE SAFETY OF PARACETAMOL–METHIONINE MIXTURES

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(*Received* 26 *March* 1974; *accepted* 30 *May* 1974)

Abstract—Rats fed low protein or 25% yeast diets are highly susceptible to the lethal and hepatotoxic effects of oral paracetamol (*N*-acetyl-*p*-aminophenol, acetaminophen). Phenobarbitone pre-treatment makes the protein-depleted animals even more sensitive. The liver injury produced is patchy in distribution and is best measured by relating plasma enzyme activity to the area of liver involved. Glutathione levels in the liver fall similarly after yeast and 3% casein + phenobarbitone diets, but the yeast fed animals are more sensitive to paracetamol, in spite of lower cytochrome P-450 levels. Addition of methionine to the oral paracetamol dose protects against death and liver injury, and it is suggested that this may be a useful technique for making paracetamol safe against the danger of overdose.

Paracetamol (acetaminophen, *N*-acetyl-*p*-aminophenol) is a useful analgesic antipyretic drug, available without prescription in many countries. In gross overdosage, it produces liver necrosis and in the last few years it has become a fashionable drug for suicide and suicidal gestures in the United Kingdom [1, 2].

Metabolism of paracetamol is mostly carried out in the liver and leads to urinary excretion of sulphate and glucuronide conjugates [3].

Mitchell *et al.* [4, 5] have shown that a small proportion of the paracetamol is metabolised by the mixed function oxidase system, centred on cytochrome P-450 in liver microsomes, and that this fraction of metabolism leads first to depletion of liver glutathione, and then covalent binding of less than 1 per cent of the dose of paracetamol to liver proteins. The implication is that the P-450 system produces a reactive metabolite which is responsible for cell injury.

Low protein diets reduce liver glutathione levels [6], and so would tend to increase sensitivity to paracetamol. But such diets also reduce cytochrome P-450 levels [7, 8], leaving glucuronidation and sulphate conjugation pathways unaltered or increased [9]. This would tend to reduce the amount of paracetamol going into the "toxic" pathway, and might make the protein depleted animal resistant to paracetamol, just as it is resistant to CCl_4 poisoning [10].

The present experiments were designed to see which of these factors predominated and whether diet might have any effect on the toxicity of paracetamol.

MATERIALS AND METHODS

Animals and Diets. Male Wistar rats (A. Tuck & Son, Rayleigh, Essex, U.K.) weighing 120–200 g at the time of dosing were housed in mesh floored cages. Food and water were available *ad lib.* except for fasted groups.

Phenobarbitone was given as a solution containing 1 mg of sodium phenobarbitone/ml in distilled water as the sole source of drinking water, as previously described [7].

Purified diets were made up to any desired protein/fat/carbohydrate/calorie composition by adding casein, fat, maize starch or cellulose to a basal supplement which comprised 35 per cent of the diet. The basal supplement and full diets were made to the composition given in Table 1. Basal supplement was made up in batches of up to 10 kg. The basal supplement can be stored at 4° for many weeks, and to make up a diet of any required fat, protein or carbohydrate composition then requires only a few minutes.

Some rats were given a diet containing 25 per cent yeast powder [11]. This latter diet is deficient in selenium, vitamin E and methionine. All the diets were fed for at least one week before dosing with paracetamol.

Dosing. Paracetamol was made up as a suspension in distilled water to contain up to 300 mg/ml paracetamol. Tragacanth B.P., 10 mg/ml was added to reduce settling of the suspension. The slurry was given by stomach tube (No. 3EG Jaques Plastic Catheter, W. Warne & Co., Barking, Essex, England), under light ether anaesthesia. Phenobarbitone solutions were replaced by water after paracetamol dosing.

Chemicals. Paracetamol (*N*-acetyl-*p*-aminophenol) was bought from Sigma Chemical Co., London. Other chemicals were of analytical grade or best laboratory grade, and obtained from British Drug Houses, Ltd, Poole, Dorset.

Tissue samples and assays. Rats were killed by exsanguination under ether anaesthesia and the livers removed and rapidly cooled in ice cold saline. Cytochrome P-450 was measured in a 1% homogenate as previously described [8]. Glutathione levels were mea-

Table 1. A system of semi-synthetic diets

(a) Basal supplement: 35% of diet	
Sucrose	250 g
Corn starch	65 g
Choline chloride	5 g
α-Tocopherol acetate	0·45 g
Fat soluble vitamins (Adexolin-Glaxo Ltd, Greenford, England)	1·5 ml
Water-soluble vitamins	5 ml
Salts (Glaxo Ltd.) DL6	30 g
(b) Example: 3% Casein–5% Olive oil diet	
Casein (low vitamin content, Fison Scientific, Loughborough)	30 g
Olive oil (B.P.)	50 g
Corn starch (Corn Flour, Brown & Polson CPC Ltd, Esher, Surrey)	570 g
Basal supplement	350 g

The water-soluble vitamin suspension is made up in 10% sucrose so that 5 ml contain the amounts for 1 kg diet, as follows: thiamine, 10 mg, riboflavin, 16 mg, nicotinic acid 100 mg, pyridoxine 10 mg, calcium pantothenate 20 mg, inositol 200 mg, *p*-aminobenzoic acid 100 mg, folic acid 2 mg, menaphthone 10 mg, vitamin B_{12} 0·1 mg [7].

All the vitamins and choline are first mixed with sucrose, added gradually, till a homogenous yellow mix is obtained. Next the starch is added, and the salt mixture is added last and mixed briefly, since too intimate contact would allow iodides in the salts to react destructively with other components.

In making up fat-containing diets the fat is first mixed with any starch, protein, or cellulose powder required. Fats may require warming before mixing. When this 65% of the diet has mixed adequately the final 35% basal supplement is added.

sured by the method of Ellman [13], after metaphosphoric acid precipitation of 0·5 ml of a 10% liver homogenate. Glutathione and cytochrome P-450 levels were sometimes measured in pieces of liver which were weighed, covered with KCl–Tris buffer (150 mM and 20 mM, pH 7·4 at 0°), and stored at −25° for up to 7 days, since this procedure was found not to alter the values obtained in comparison with fresh liver. Liver fat and water content, potassium and sodium content and plasma isocitrate dehydrogenase activity were measured as described earlier [10, 11].

LD_{50} determinations were made and calculated by the method of Weil [12], using at least four groups of four rats for each value.

RESULTS

Effects of diets. Rats fed the 3% Caesin 5% Olive oil diet remain healthy and maintain constant body wt for 6 weeks or more after some weight loss in the first few days. Even the protein free diets allow survival with slow loss of body weight, for many weeks. This is presumably due to the high vitamin content and palatability of these diets, which prevent the early loss of condition and rapid decline and death often described in rats fed unsuitable mixtures.

The rats fed yeast diet gained weight slowly (*ca* 1 g/day), and remained in good condition for 3 weeks or more. The liver necrosis of simultaneous Vitamin E and selenium deficiency was not seen in these rats since they had not been depleted of Vitamin E before weaning [14].

Changes in the liver after feeding diets. Table 2 shows that the amount of cytochrome P-450/g of liver is decreased by feeding low protein diet. Phenobarbitone treatment increases P-450 levels in both stock and 3 per cent Casein fed rats.

Glutathione levels are not altered by phenobarbitone but sharply decreased by feeding low protein diet for 7 days or more.

The yeast diet leaves P-450 levels unaltered, but reduces glutathione content.

Lethality of paracetamol. When the lethal effects of paracetamol are examined in the light of these measurements, it is found that phenobarbitone brings the LD_{50} down by a factor of more than two. Rats fed the low protein diet are highly sensitive to paracetamol. Evidently, the reduced glutathione levels increase sensitivity by a factor greater than the expected protection afforded by low P-450 levels [4, 5].

Table 2. Effect of diet and phenobarbitone on the lethality of paracetamol, and on cytochrome P-450 and glutathione content of the liver*

Diet	Cytochrome P-450 (nmoles/g liver)	Glutathione (μmoles/g liver)	Lethality of paracetamol LD_{50} (g/kg)	(95% Limits)
Stock pellets (41B)	40 ± 9	6·9 ± 1·7	5·2	(4·6–6·0)
Stock pellets (41B) + Phenobarbitone	142 ± 35	7·6 ± 1·0	2·0	(1·7–2·4)
3% Casein 5% Olive oil diet	23 ± 5	2·2 ± 0·2	2·1	(2·0–2·3)
3% Casein 5% Olive oil diet + Phenobarbitone	81 ± 15	2·8 ± 0·3	0·9	(0·8–1·0)
25% Yeast diet	37 ± 3	2·8 ± 0·5	0·4	(0·2–0·7)
25% Yeast diet + Phenobarbitone	101 ± 16	4·0 ± 0·1	—	—
25% Yeast diet + α-Tocopherol	—	—	>1·0	—

* LD_{50} values were determined on at least four groups of four rats and calculated by the method of Weil [12]. Other results are expressed as the mean of at least five determinations on different animals ± one standard deviation.

When P-450 levels are raised by giving the protein depleted rats phenobarbitone, the sensitivity to paracetamol is increased even more.

Rats fed the yeast diet are the most sensitive group. The disastrous effect of paracetamol on these animals could not be predicted from their P-450 and GSH levels. Addition of selenium (1 ppm) to the diet gave no protection and while α-tocopherol somewhat reduced mortality, it did not decrease the incidence of liver necrosis.

The lethal effects of oral paracetamol were always accompanied by signs of severe liver injury (naked eye and histological appearance of liver necrosis, increased water and sodium content of liver, raised serum enzyme activity). This is in contrast to the results reported by Mitchell *et al.* [4, 5], who found deaths without liver injury after intra-peritoneal injection of large amounts of paracetamol.

Variability of liver injury. A striking finding was that in any group of rats treated with an LD_{50} dose and killed at 24 or 48 hr, there would be individual animals with no sign of liver injury at all, while other animals had some lobes showing massive necrosis, some lobes with centrilobular necrosis, and adjacent areas showing no injury whatever. Still other animals, especially those that died, had massive necrosis of all lobes of the liver. The variability was found in all dietary groups and also after intraperitoneal injection of paracetamol.

Liver injury was not detectable 7 hr after dosing, but was well developed at 18 hr. Deaths were almost confined to the time between 10 and 48 hr after dosing, in all groups except those fed yeast diet many of which died between 6 and 8 hr after paracetamol.

Figure 1 shows a plot of plasma enzyme activity 24 hr after an oral dose of 1 g/kg paracetamol in rats fed stock diet + phenobarbitone (i.e. half of an LD_{50} dose). It can be seen that rats with no visible liver necrosis had normal enzyme activity (less than 5 nmoles min^{-1} ml^{-1}) and as the amount of liver assessed to be involved, increased, so the enzyme activity rose. The percentage of liver involved was assessed by weighing the visibly affected portions of the basis of the very striking speckled white appearance of the liver seen in the affected portions.

Histological examination showed that those parts of the liver assessed as normal macroscopically, showed at most a few patches of inflammatory cells. The pale speckled appearance was histologically centrilobular necrosis with very marked swelling of cells. Massive haemorrhagic necrosis was seen as blotched red and yellow patches to the naked eye.

When 100 per cent of the liver was involved this included both instances where each lobule had a small patch of centrilobular cell loss, and times where much of the liver was destroyed by massive necrosis. The latter process was usually accompanied by ICD activities over 1000. Rats dying with paracetamol liver necrosis invariably had plasma ICD levels over 2000 nmoles ml^{-1} min^{-1}.

Quantitative measurement of liver injury. Table 3 shows some of the data gathered to permit dose–response curves to be constructed to measure the effect of altering the diet on the response to paracetamol.

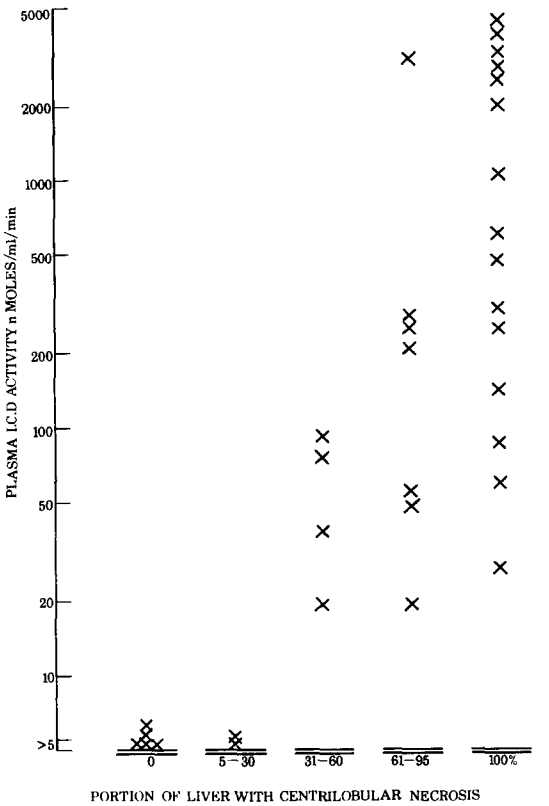

Fig. 1. Plasma isocitrate dehydrogenase activity related to the proportion of liver seen to be affected in rats given 1 g paracetamol/kg body wt and killed 24 hr later. All rats were pretreated with phenobarbitone and fed stock pellets. No samples were taken from rats dying before 24 hr.

Mortality, proportion of animals showing liver necrosis, and mean plasma ICD values allow us to infer that a dose of 1 g/kg given to a rat maintained on stock pellets has the same effect as between 0·25 and 0·5 g/kg paracetamol in a rat given phenobarbitone with pellet diet. Feeding the yeast diet makes paracetamol highly lethal, and potent in causing liver necrosis, and while α-tocopherol reduces lethality, it permits survival to 24 hr of rats whith very severe liver necrosis.

Rats fed 3% Casein diet + phenobarbitone were more sensitive than stock rats, and less sensitive than yeast fed rats.

In other experiments it was found that 0·25 g/kg caused severe injury in yeast fed rats, but no injury to the 3% Casein fed animals. Addition of selenium to the yeast diet gave no protection against liver necrosis, but addition of methionine to the diet gave a marked protection against the lethal and hapatotoxic effects of paracetamol.

Effect of methionine and cysteine. When cysteine is injected together with intraperitoneal paracetamol,

Table 3. The effect of diet on signs of liver injury after paracetamol*

Diet	Treatment	Paracetamol dose (g/kg)	Mortality (deaths/n dosed)	Survivors: No liver necrosis	Survivors: Any visible liver necrosis	Survivors: Plasma ICD geometric mean and 1 S.D. range (nmoles ml^{-1} min^{-1})
Stock (41B)	—	1·0	0/13	10/13	3/13	11 (7–41)
Stock	+ Phenobarbitone	1·0	6/36	3/30	27/30	148 (15–1440)
Stock	+ Phenobarbitone	0·5	0/8	3/8	5/8	33 (4–300)
Stock	+ Phenobarbitone	0·25	0/4	4/4	0	2 (1–3)
3% Casein	+ Phenobarbitone	0·5	0/4	0	4/4	34 (7–166)
Yeast diet	—	0·5	8/13	2/5	3/5	44 (8–240)
Yeast diet	+ α-Tocopherol	0·5	3/12	1/9	8/9	330 (33–3300)
Yeast + Methionine diet	—	0·5	0/8	7/8	1/8	2 (1–6)

* Rats were fed the diets for 1 week before dosing. Na Phenobarbitone was given as 1 mg solution as drinking water, and removed after paracetamol dosing. The 3% Casein diet was made up with 5% Olive oil. α-Tocopherol acetate was given as two oral doses of 45 mg 24 and 48 hr before paracetamol. Methionine was added to the yeast diet at 8 g/kg diet. Rats were killed 24 hr after dosing with paracetamol.

Isocitrate dehydrogenase (ICD) values were measured, and the logarithms of the values taken, and mean ± 1 S.D. values calculated. The antilog of this geometric mean ± 1 S.D. is in the figure given in the ICD column.

animals are protected against liver necrosis [4, 5]. Table 4 shows that some protection is found even when cysteine is injected as late as 4 hr after oral paracetamol. When methionine is mixed with paracetamol, and the mixture given orally, liver injury is completely prevented when the methionine is added in the ratio of 30% to the paracetamol; 15% (not shown) or 10% are still very effective, while 5% methionine is ineffective. At 15% methionine similarly reduces the injury caused by 2 g/kg paracetamol in phenobarbitone treated rats, whilst 25% cysteine is also effective.

Figure 2 shows the alteration of lethality produced whan a paracetamol–methionine mixture (4:1 by weight) is given in increasing doses. Instead of finding an LD_{50} of about 2 g/kg, most rats given paracetamol–methionine mixture survived even 7·5 g/kg.

DISCUSSION

Two points emerge from this work. One purely practical is that paracetamol can be made safe as a therapeutic agent by the inclusion of 10 per cent of methionine. The second point is illustrated in Fig. 3. Figure 3 shows the metabolic pathways open to the paracetamol molecule. It is seen that the drug may be conjugated with sulphate or glucuronidated to yield 'safe' products. Alternately, it may enter the pathway of oxidation by the P-450 system, to yield toxic metabolites. The choice is presumably dictated by the relative activities of the two systems. The toxic metabolities, in turn, may react with either GSH (or cysteine) or with cellular macromolecules [4, 5, 22].

It is clear that each choice can be influenced by nutritional and/or environmental factors. For

Table 4. The effect of sulphur amino acid on liver injury induced by paracetamol (1 g/kg oral) in rats fed stock diet and pretreated with phenobarbitone*

Dose of S amino acid	Route dose	Time after paracetamol (min)	Plasma ICD	(One S.D. range)	Any visible necrosis	P
Nil			148	(15–1440)	27/30	—
Cysteine HCl	i.p. 300 mg/kg	60	9	(1–79)	6/8	<0·01
Cysteine HCl	i.p. 300 mg/kg	120	2	(1–4)	3/4	<0·001
Cysteine HCl	i.p. 300 mg/kg	240	16	(1–200)	3/4	0·1 > P > 0·05
Methionine	Oral 300 mg/kg	0	3	(2–3)	0/4	<0·01
Methionine	Oral 100 mg/kg	0	8	(1–48)	2/4	<0·02
Methionine	Oral 50 mg/kg	0	1300	(240–7000)	4/4	>0·1

* Dosing with paracetamol and S-amino acids was carried out and liver injury assessed 24 hr later as described in Methods.

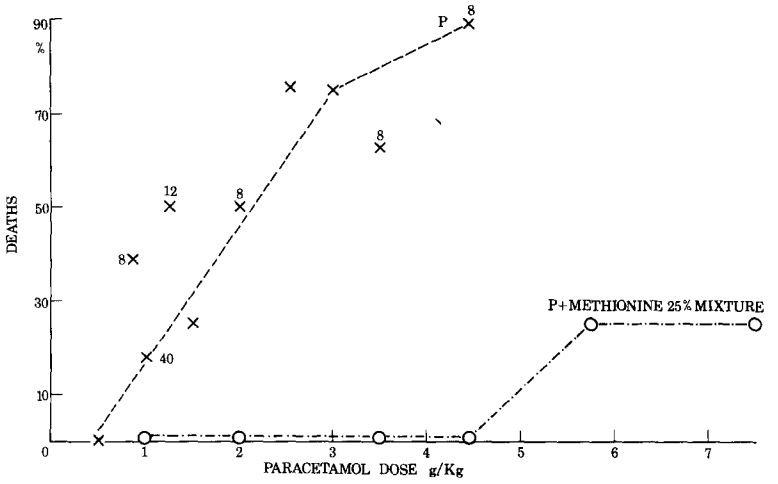

Fig. 2. Lethal effect of oral paracetamol and its prevention by methionine. All the rats were fed stock pellets and pretreated with phenobarbitone. Deaths were noted for 1 week after dosing except for the 40 rats given 1 g/kg where deaths were recorded only in the first 24 hr. Paracetamol or paracetamol–methionine mixture (4 g paracetamol plus 1 g methionine) were given orally under light ether anaesthesia. Each group contained 4 rats except where a greater number is noted. Phenobarbitone was removed after dosing in all groups.

example, a low protein diet causes a marked increase in the toxicity of paracetamol, despite the low P-450 levels and high glucuronyl transferase activity induced by the diet. The smaller amount of the toxic metabolite formed is presumably able to act because of the simultaneous reduction of GSH levels. We can describe as 'critical points' those places in the metabolic pathways followed by paracetamol where there is a choice of a safe pathway, or a pathway leading to cell injury.

For comparison with paracetamol, the possible fates of CCl_4 and DMN are also shown in Fig. 3. The critical point for CCl_4 toxicity is at the point of divergence where molecules are either breathed out harmlessly, or trapped in the P-450 system. If P-450 levels are kept

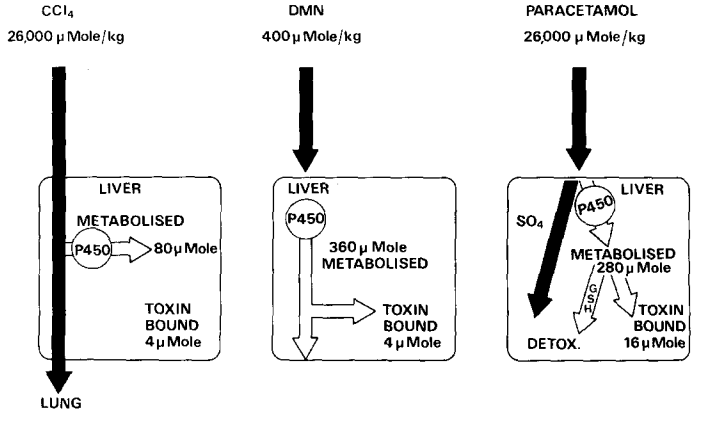

Fig. 3. Cytochrome P-450 linked enzymes as critical points of toxicity. The estimated fate of an LD_{50} dose of three poisons is represented. The amounts indicated as bound to protein and other macromolecules give the order of magnitude, only. For CCl_4 about 80 μmoles/kg rat is metabolised to CO_2 [16, 17] and 4 μmoles/kg rat (i.e. per 40 g liver) is bound to the liver [18]. For DMN 90% is metabolised in the liver, and 10% of this becomes incorporated into protein [19, 20]. However, most of this is in normal amino acids synthesized from the formaldehyde split during DMN demethylation. About 1/10th of the protein counts are found in abnormal spots, accounting for 1% of the total dose [21]. For paracetamol, 90% or more is metabolized to glucuronide and sulphate conjugates, an unknown amount goes into the P-450 pathway and reacts with glutathione. (The 40 g of liver per kg rat would contain 280 μmoles glutathione which could react stochiometrically.) The 8 g liver protein per kg animal has 2 nmoles paracetamol/mg protein attached to it [22].

constant with inducers, then low protein diets do not affect toxicity.

In contrast, we can see no critical point in the metabolism of dimethylnitrosamine (DMN). The total quantity of alkylating metabolite that is formed, depends only on the dose. Variations in P-450 associated enzyme activities will only alter the rate at which such metabolites are formed [8]. The quantity of injury produced by a low dose of DMN (30 mg/kg) is independent of the rate of metabolism (McLean and Day, unpublished work). Inducers like phenobarbitone should influence toxicity only if the induced enzyme stands at a critical point in the metabolism of the toxic compound.

Acknowledgement—Part of this work were supported by grants from the British Nutrition Foundation and the Medical Research Council.

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