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EFFECT OF D- OR L-METHIONINE AND CYSTEINE ON THE GROWTH INHIBITORY EFFECTS OF FEEDING 1% PARACETAMOL TO RATS

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Abstract—Rats fed 1% paracetamol in the diet failed to grow and a dose-dependent inhibition of growth was observed and found to be independent of hepatotoxicity.

Addition of 0.5% D- or L-methionine, or L-cysteine to a diet containing 1% paracetamol restored growth. Addition of L-methionine to the drinking water was equally effective. Feeding D-cysteine or sodium sulphate were ineffective. Acute paracetamol toxicity was also prevented by D- as well as by L-methionine.

It is concluded that the inhibition of growth was due to depletion of sulphur amino acids in the course of paracetamol metabolism. This was sometimes followed by episodes of liver cell injury. Since the normal human dosage of paracetamol is up to 4 g/day, which is equivalent to 1% of the diet, the possibility of induction of amino acid deficiency by chronic use of paracetamol in normal dosage is raised.

Rats fed 1% paracetamol in the diet fail to grow or have a reduced growth rate [1–4]. Paracetamol is normally metabolised at the 4(OH) position to glucuronide and sulphate conjugates with some 5% of the paracetamol going to cysteine conjugates at the "3" position following P-450 oxidation steps. Overall the proportion of the paracetamol that is excreted as sulphur compounds varies with species and dose [6]. At therapeutic dosages human beings, like rats, excrete 30–50% or more as sulphate and other sulphur-containing metabolites [7]. When paracetamol is fed as 1% of the diet, that is 6.6 mM of paracetamol per 100 g of diet, it could require most of the sulphur amino acid content of an ordinary diet (3–6 mM/100 g diet) just to produce the sulphate and cysteine required for paracetamol excretion. The depression of growth in rats fed 1% paracetamol is likely to be due to underlying processes, liver injury, sulphur amino acid deficiency, or a combination of the two.

Rats or human beings given an acute overdose of paracetamol develop the well known pattern of centri-lobular liver necrosis subsequent to metabolism of paracetamol by P-450 linked enzymes [9–11]. In some of the rats fed 1% paracetamol in stock diets, episodes of histologically visible liver injury develop after one or more weeks. However, these are sporadic events, while inhibition of growth is produced invariably, and also precedes any visible cell injury. If there were cell injury, this should be prevented by additional L-methionine or L-cysteine, leading to glutathione synthesis and prevention of liver injury from paracetamol metabolites [11]. In contrast, induction of synthesis of P-450 enzymes by giving phenobarbital should make the rats more sensitive to paracetamol feeding if liver injury were the cause of growth failure.

There could be depletion of sulphate leading to increased metabolism of sulphur amino acids to sulphate, and so indirectly causing a methionine/cysteine deficiency. One would expect this to be corrected by feeding sulphate or sulphate sources such as D-cysteine, as well as by feeding the essential L-sulphur amino acids. D-Methionine is well known to be converted to L-cysteine through the cystathionine pathway whereby the sulphur from D-methionine is transferred to L-serine, and the carbon skeleton of the D-amino acid oxidised [8]. As a result D-methionine is equivalent to feeding L-cysteine and cannot be used to distinguish between the various pathways. While D-methionine can supply a large proportion of the sulphur amino acid requirement, D-cysteine cannot be utilised in this pathway and so cannot relieve a deficiency of sulphur amino acids [8]. However, D-cysteine is a good source of intracellular inorganic sulphate for sulphate conjugations and readily reverses the low serum sulphate found in protein-deficient animals. This means that we can distinguish between the role of cysteine as a source of sulphate and its role as a precursor of glutathione or proteins, as has been pointed out by Glazenberg *et al.* [12]. The question remains whether an adequate rate of conversion of D-methionine to L-cysteine is possible [13].

It is well recognised that relative excess of some amino acids can lead to suppression of food intake and a variety of pathological changes such as fatty liver. Depletion of sulphur amino acids by diversion into the sulphate pathway could lead to a relative excess of other amino acids.

Another possibility is that the failure of growth is due to the combination of removal of sulphur amino acids because of sulphate and glutathione utilisation, and this in turn leads to increased diversion of cysteine into the sulphate pathway. The consequent sulphur amino acid deficiency makes the animals sensitive to the hepatotoxic effects of paracetamol

Table 1. Influence of dosage level of paracetamol and L-methionine on growth in rats fed 15% casein diet

Additions to diet		Weight gain	
Paracetamol g/100 g diet	L-Methionine g/100 g diet	g/day (days 0–10)	Body weight (day 26)
—	—	+7.0 ± 0.5	301 ± 16
0.25	—	+6.3 ± 0.4	274 ± 24
0.5	—	+1.9 ± 1.1*	244 ± 17
1.0	—	−3.1 ± 0.5*	—
1.5	—	−4.0 ± 0.5*	105 ± 4
1.5	0.15	−4.4 ± 0.5*	129 ± 6
1.5	0.4	−0.2 ± 1.8*†	203 ± 25
1.5	1.5	+1.5 ± 1.0*†	215 ± 26

Mean ± 1SD for groups of at least four rats.
Significantly different from control (no paracetamol) group. (* $P < 0.01$).
Significantly different from 1.5% paracetamol group. († $P < 0.01$, ‡ $P < 0.05$).
Starting weight, day 0 = 173 ± 12 g.

[11] and there is a continued subacute injury to liver cells. For this to be corrected one would need D- or L-methionine or L-cysteine. Neither inorganic sulphate nor D-cysteine would correct this defect, although they might have an indirect effect by sparing oxidation of L-cysteine to SO_4^{2-}.

In order to investigate these questions we have looked at the effect of sulphate, D- and L-methionine, and D- and L-cysteine, on some acute and chronic effects due to paracetamol.

MATERIALS AND METHODS

Albino rats of the Wistar strain weighing 100–120 g were bought from A. Tuck & Son (Ware, Herts) or Olac (Harlan Olac, Bicester, Oxon). They were fed commercial pellet diets [SDS Maintenance diet (RM1) Special Diets Services, BP Nutrition, PO Box 705, Witham, Essex] containing 14% protein in an expanded pellet, with a sulphur content of 3.8 mM/100 g, mostly sulphur amino acids.

Other rats were fed a semi-purified diet [11] containing 15% casein, either as a powder or pelleted. The sulphur content of this was 2.9 mM/100 g of which 0.4 mM was inorganic sulphate, and the rest cysteine and methionine. Some rats were given Na phenobarbital in their drinking water as a 1 g/l solution (for one week or more) to induce maximal cytochrome P-450 synthesis.

Paracetamol dosage. Rats were given diets containing 1% paracetamol (Sigma Chemicals, London) mixed into the diet. D- and L-cysteine and D-, L- and D,L-methionine were also bought from Sigma. Methionine or cysteine were either mixed into the powdered diet or else put into the drinking water, together with 0.2% ascorbic acid in distilled water to prevent oxidative spoilage. Drinking water was made up freshly each day.

For acute experiments rats were given paracetamol at a dosage of 2 g/kg body weight, orally by gavage, with or without cysteine, 500 mg/kg. The mixture was made into a slurry containing 125 mg paracetamol/ml with 0.5% tragacanth gum and given at a volume of 1.6 ml/100 g body weight. Liver injury and glutathione levels were assessed as previously described [11].

RESULTS

Figure 1 shows that rats fed 1% paracetamol in a 15% casein diet lost weight immediately and failed to gain weight over the following 3 weeks. In comparison the rats fed the 15% casein control diet gained weight steadily at almost the same rate as the rats fed stock "SDS" diet. Histological examination of the livers of the treated rats at the end of the experiment showed only glycogen loss and no cell necrosis, nor were serum enzyme values raised. Table 1 shows the results of a separate experiment. Weight loss was seen at 1.5 and 1% paracetamol, while rats fed 0.5% paracetamol had a significant decrease in growth rate to 2 g/day in comparison with 7 g/day in control rats. At a level of 0.25 g of paracetamol/100 g diet there was no longer a significant inhibition of growth. These effects were to a considerable extent reversed by addition of L-methionine to the diet. Figure 1 shows that the rate of growth in rats fed 1% paracetamol plus 1% methionine was only slightly less than that found in the casein control group after an initial check to growth in the first few days of feeding the new diet. Figure 1 also shows that the addition of 1% sodium sulphate to the diet did not prevent the growth depression due to paracetamol. Table 1 shows that in rats fed 1.5% paracetamol the addition of 1.5% L-methionine permitted some growth, 0.4% methionine reduced the weight loss and 0.15% methionine seemed to have little effect.

One possible reason for the weight loss could have been that the bitter taste of paracetamol might have been sufficient to stop the rats from eating, especially in the first few days. However, one would have to postulate that this bitter taste was somehow masked by the addition of methionine to the diet. Moreover, when methionine was added to the drinking water the effect was the same as addition to the diet in restoring growth. Addition of methionine to drinking water led to a slight check in growth in both control and paracetamol fed animals, as is usual when any change in diet takes place. But thereafter the two groups grew at the same rate (Fig. 2).

Table 2 shows that D-, L-, and D,L-methionine were effective in restoring growth, as was L-cysteine.

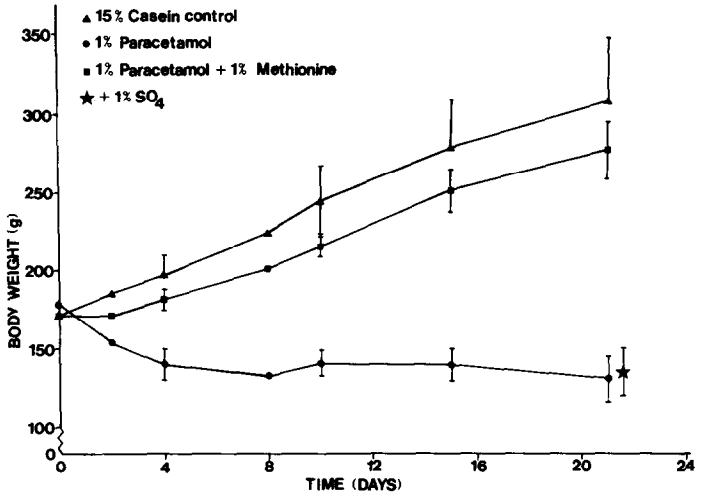

Fig. 1. Effect of feeding diets containing 1% paracetamol with addition of L-methionine or sodium sulphate on the growth of young rats. Male rats were fed on 15% casein diets prepared as described in Materials and Methods, with additions of 1% paracetamol and 1% L-methionine, or 1% sodium sulphate. Four rats per group were housed in cages with mesh bottoms, and fed *ad libitum*. Standard deviations were from 6 to 8% of the means at day 8, and 4 to 13% on day 21.

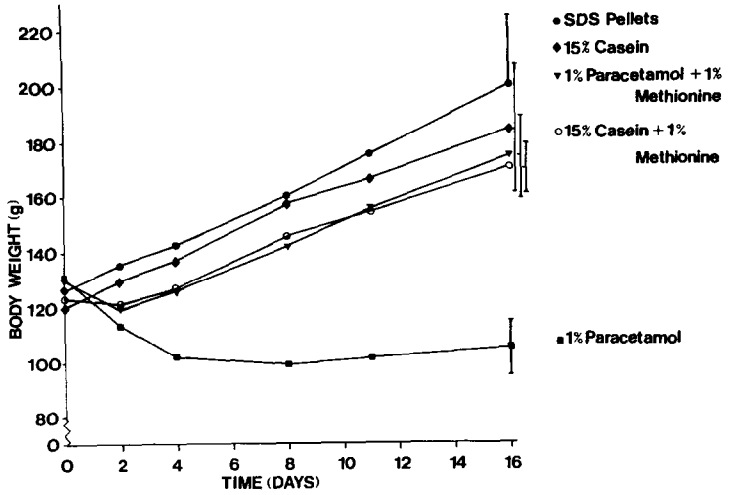

Fig. 2. Effect of giving drinking water containing 1% methionine on growth of rats fed 15% casein and 1% paracetamol in the diet. Male rats were fed stock pellets or 15% casein diets with or without paracetamol. Methionine was given as a 1% solution in the drinking water in distilled water with 0.2% ascorbic acid. Results are given as means for groups of 5 rats. Standard deviations are plotted for day 16; on previous days standard deviations ranged from 3 to 12% of the means.

However D-cysteine was completely ineffective in reversing the growth inhibitory effect of paracetamol in this diet.

Table 3 shows that addition of 1% paracetamol to a stock unpurified diet containing 60% more sulphur amino acid than the casein diet effectively stopped growth for the first 10 days. This was followed by a resumption of growth, though at a reduced rate, in comparison with the control group. It is notable that giving phenobarbital to these rats did not increase the inhibition of growth by paracetamol (phenobarbital alone did not alter growth rates in rats fed stock diet). Histological examination of the liver of these animals showed centrilobular glycogen loss and occasional foci of inflammatory cells, more frequent in the phenobarbital treated rats, but no evidence of extensive liver cell necrosis.

Table 3 also shows that after 10 days of feeding paracetamol in stock diet the glutathione levels in the liver were down to half the control values and not affected by addition of phenobarbital treatment. Water content of the liver, a good indicator of cell injury, was raised in the paracetamol treated rats, but serum enzymes (not shown) were not raised.

Table 2. Effect of D- and L-methionine and D- and L-cysteine on growth of rats fed 1% paracetamol in 15% casein diet

Additions to diet		Weight gain
Paracetamol	Cysteine/methionine	g/ day
Experiment I		(days 0–9)
—	—	+9.3 ± 0.4
1%	—	−3.5 ± 0.9
1%	0.5% D methionine	+3.8 ± 1.7
1%	0.5% L methionine	+5.8 ± 2.0
1%	0.5% D-L methionine	+5.1 ± 1.2
Experiment II		(days 0–7)
—	—	+5.1 ± 0.3
—	0.5% D cysteine	+5.1 ± 0.2
1%	—	−4.0 ± 0.6
1%	0.5% L cysteine	+3.8 ± 1.0
1%	0.5% D cysteine	−4.1 ± 0.4

Results are expressed as mean ± SD for groups of at least four rats.

Table 4 shows that both D- and L-methionine are effective in preventing the acute lethal and hepatotoxic effects of paracetamol overdose in rats pretreated with phenobarbital. Paracetamol, at an oral dose of 2 g/kg, led to death and severe liver injury with raised serum enzymes in the treated rats. Histological examination showed massive liver cell necrosis with some haemorrhage in all the rats not protected by methionine. None of the rats given D- or L-methionine with the paracetamol showed any histological liver damage. There were no deaths when methionine was added to the paracetamol dose in the ratio of 0.5 g methionine per 2 g paracetamol. D- and L-methionine were equally effective and there was no evidence of liver injury as assessed by serum enzymes nor from liver water content. The liver injury had been totally prevented.

DISCUSSION

Paracetamol (1%) is equivalent to 6.6 mM/100 g diet while the S-amino acid content per 100 g of the 15% casein diet is 2.5 mM/100 g of diet. SDS unpurified diet has a greater S-amino acid content, 3.8 mM/100 g. There is essentially no other major source of sulphur in these or other ordinary diets.

One might expect that an intake of paracetamol which could utilise essentially all of the sulphur amino acid content of the diet for paracetamol metabolism should lead either to growth depression (reversible by feeding methionine) or else to an adaptive switch to glucuronide metabolite formation. If only 50% of the S-amino acids were removed one would be left with a sulphur amino acid intake equivalent to an 8% protein diet which is quite sufficient for moderate growth [14]. Young rats continue to grow on diets containing 4% casein (by weight or as a percentage of calories) and grow maximally with diets containing about 15% casein or more. The quantity of inhibition of growth by paracetamol and its reversal by methionine is approximately what would be expected if the sulphur amino acid content of the diet were limiting to growth, and about 50% of the paracetamol intake was converted to sulphur metabolites irrespective of the ensuing sulphur amino acid deficiency.

When paracetamol is fed the ratio of sulphur amino acid to other amino acids is also grossly disturbed. Normally low protein diets with reduced intake of all amino acids, leads to reduced cysteine oxidation [15] with reduced excretion of inorganic sulphate in the urine. It may be that the paracetamol-induced deficiency of sulphur amino acids does not lead to an adaptive reduction cysteine oxidation to sulphate.

One may consider that amino acid imbalance is the underlying cause of the growth inhibition found in rats fed paracetamol. It is no surprise to see that D-methionine is as effective as L-methionine in preventing both acute liver injury and subacute growth depression effects of paracetamol dosage, since it is well known that D-methionine can be converted to L-cysteine. However, it is more surprising to see that neither inorganic sulphate nor D-cysteine permit growth of the animals. D-Cysteine is rapidly oxidised to sulphate [12] and as such would be expected to be a good source of intra-cellular sulphate for conjugation, and so at the very least, spare sulphur amino acids from oxidation to provide sulphate. Even a small reduction in S-amino acid oxidation would be expected to cause an improvement in growth since the addition of small amounts of L-methionine or a small reduction in the paracetamol dosage would cause some amelioration of the effect of paracetamol.

Table 3. Effect of phenobarbital on growth and glutathione and water content of the liver in rats fed 1% paracetamol in unpurified stock diet

	Body weight			Liver composition day 11	
Additions to diet	Day 0	Day 11	Day 25	Glutathione μmol/g liver	Water content g/g fat free dry weight
Paracetamol	102 ± 7	110 ± 13	152 ± 23	3.6 ± 0.3	2.89 ± 0.10
Paracetamol + phenobarbital	102 ± 7	114 ± 11	181 ± 13	3.5 ± 0.5	2.80 ± 0.13
Control (stock diet)	105 ± 7	186 ± 15	303 ± 32	6.8 ± 1.4	2.53 ± 0.06

Results are expressed as mean ± 1 SD for groups of at least four rats.
The diet was unpurified stock powdered diet (SDS) with or without addition of 1% paracetamol. Na Phenobarbital was given as a 1g/l solution in tap water as sole drinking water.

Table 4. Effect of D- and L-methionine on acute toxicity of paracetamol (2 g/kg) in phenobarbital pretreated rats

Treatment	Serum ICD activity mean (range)	Mortality
Paracetamol	3045 (924–4341)	3/4
Paracetamol + D-methionine	1.1 (0.7–1.5)	0/4
Paracetamol + L-methionine	1.1 (1.0–1.5)	0/4
Control (no paracetamol)	1.3 (0.6–2.6)	0/9

Rats were fed stock pellets and pretreated with phenobarbital in the drinking water for 7 days before oral dosage with paracetamol (2 g/kg, 4 rats per group), with and without addition of methionine 500 mg/kg. Plasma isocitrate dehydrogenase (ICD) activity was measured as previously described [11]. Blood samples were taken at 24 hr, or when the animals were moribund.

The major loss of sulphur is into the sulphate metabolite of paracetamol. If feeding sulphate or D-cysteine supplied the need for sulphate we should be left with only a small requirement for L-cysteine for resynthesis of glutathione utilised in reaction with the oxidised metabolites such as the quinone imine, which form only about 10% of the paracetamol output. This small proportion would not cause a serious loss of S-amino acids.

We are left with the possibility that neither inorganic sulphate nor D-cysteine can lead to adequate sulphation, which is contrary to the results reported by Mulder and co-workers [13].

Rats seem to adjust their food intake to their energy requirements [16]. If a diet lacking in essential amino acid is fed, then net protein synthesis and growth stop and the food intake drops [8]. There is a noticeable drop in food intake in the rats fed 1% paracetamol in the diet and this could be due either to the metabolic disorder produced by feeding paracetamol or else one could postulate that paracetamol made the diet so unpalatable that the rats stopped eating and this led to growth failure. However, one would have to postulate that the unpalatability of the diet was reversed by addition of D- or L-methionine to the diet, L-methionine to the drinking water or L-cysteine to the diet, while D-cysteine was ineffective, it is extremely improbable that these effects are all due to changes in palatability.

Another possible cause for growth deficit is that there is a subacute injury to liver cells due to GSH depletion and continued paracetamol dosage but not accompanied by raised serum enzymes or histological change. It is a possibility that requires further investigation.

Since the human diet consists of around 400 g dry weight of food/day, with about 30 mM of sulphur, and the recommended dose of paracetamol is up to 4 g/day (26 mM) the question arises whether long term paracetamol at normal doses could lead to depletion of S-amino acids and effective protein deficiency, glutathione depletion or subacute liver injury. This can only be settled by clinical investigation to see whether human beings can switch paracetamol metabolism towards glucuronidation when faced with S-amino acids deficits.

REFERENCES

1. Wright HN, Chronic toxicity studies of analgesic and antipyretic drugs and congeners. *Toxicol Appl Pharmacol* **11**: 280–292, 1967.
2. Johansson SL, Carcinogenicity of analgesics: long-term treatment of Sprague–Dawley rats with phenacetin, phenazone, caffeine and paracetamol (acetamidophen). *Int J Cancer* **27**: 521–529, 1981.
3. Hiraga K and Fujii T, Carcinogenicity testing of acetaminophen in F344 rats. *Jpn J Cancer Res (Gann)* **76**: 79–85, 1985.
4. Hagiwara A and Ward JM, The chronic hepatoxic, tumor-promoting and carcinogenic effects of acetaminophen in male B6C3F$_1$ mice. *Fundam Appl Toxicol* **7**: 376–386, 1986.
5. McLean AEM and Beales D, Paracetamol as a cause of dietary deficiency of sulphur amino acids. *Br J Pharmacol* **90**: 182P, 1987.
6. Wong BK, Galinsky RE and Corcoran GB, Dissociation of increased sulfation from sulfate replenishment and hepatoprotection in acetaminophen-poisoned mice by N-acetylcysteine stereoisomers. *J Pharm Sci* **75**: 878–880, 1986.
7. Davis M, Labadarios D and Williams RS, Metabolism of paracetamol after therapeutic and hepatotoxic doses in man. *J Int Med Res* **4**: (Suppl.) 40–45, 1976.
8. Harper AE, Benevenga NJ and Wohlhueter RM, Effects of ingestion of disproportionate amounts of amino acids. *Physiol Rev* **50**: 429–558, 1970.
9. Mitchell JR, Jollow DJ, Potter WZ, Davis DC, Gillette JR and Brodie BB, Acetaminophen-induced hepatic necrosis. I. Role of drug metabolism. *J Pharmacol Exp Ther* **187**: 185–194, 1973.
10. Vale JA, Meredith TJ and Goulding R, Treatment of acetaminophen poisoning. The use of oral methionine. *Arch Int Med* **141**: 394–396, 1981.
11. McLean AEM and Day PA, The effect of diet on the toxicity of paracetamol and the safety of paracetamol-methionine mixtures. *Biochem Pharmacol* **24**: 37–42, 1975.
12. Glazenburg EJ, Jekel-Halsema IMC, Baranczyk-Kuzma A, Krijgsheld KR and Mulder GJ, D-Cysteine as a selective precursor for inorganic sulfate in the rat *in vivo*. Effect of D-cysteine on the sulfation of harmol. *Biochem Pharmacol* **33**: 625–628, 1984.
13. Krijgsheld KR, Glazenburg EJ, Scholtens E and Mulder GJ, The oxidation of L- and D-cysteine to inorganic sulfate and taurine in the rat. *Biochim Biophys Acta* **677**: 7–12, 1981.
14. Njaa LR, Weight maintenance and protein intake of the young rat. *Br J Nutr* **19**: 443–457, 1965.

15. Kohashi N, Yamaguchi K, Hosokawa Y, Kori Y, Fujii O and Ueda I, Dietary control of cysteine dioxygenase in rat liver. *J Biochem* **84**: 159–168, 1978.

16. Davidson S, Meiklejohn AP and Passmore R, *Human Nutrition and Dietetics*. Livingstone, Edinburgh, 1959.