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THE ROLE OF INTESTINAL MICROFLORA IN THE FORMATION OF THE METHYLTHIO ADDUCT METABOLITES OF PARACETAMOL

STUDIES IN NEOMYCIN-PRETREATED AND GERM-FREE MICE

M. MIKOV,* J. CALDWELL,† C. T. DOLPHIN and R. L. SMITH
Department of Pharmacology and Toxicology, St. Mary's Hospital Medical School, London W2 1PG, U.K.

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Abstract—The contribution of the gastrointestinal microflora to the formation of methylthio adducts from paracetamol has been studied by comparing the fate of this drug in conventional mice with that in germ-free and neomycin-treated animals. In both germ-free and neomycin-treated mice there was a highly significant reduction in the urinary excretion of 3-methylthioparacetamol, its glucuronic acid and sulfate conjugates and its sulfoxide, with no other systemic alteration to the overall fate of the drug. These data are consistent with the gut flora playing a major role in the *C–S* cleavage of paracetamol-3-cysteine, thereby reducing the excretion of the array of methylthio adducts subsequently formed by tissue enzymes from 3-thioparacetamol, the putative product of the *C–S* cleavage.

It is well known that the intestinal microflora can contribute to the metabolism of a wide range of drugs and other xenobiotics, either by mimicking the actions of tissue enzymes or by carrying out reactions having no counterpart in the animal body [1, 2]. In recent years, there has been considerable interest in the pathways of metabolism of glutathione conjugates [3], which due to their high molecular weight are frequently excreted in the bile and thereby exposed to the enzymes of the gastrointestinal microflora. It has been known for some time that the gut flora can cleave glutathione conjugates to the corresponding *S*-substituted cysteines, and more recently the further bacterial metabolism of these products by cysteine conjugate *C–S* lyase has been demonstrated [4–6]. Although the first report of the formation of a thiol from a cysteine conjugate is now some 40 years old [7], it is only in the past five years that the importance of this pathway as a precursor of the various thiomethyl conjugates formed from a wide range of xenobiotics has been realized [8]. Thus, the thiols produced by the *C–S* lyase reaction are absorbed and methylated by microsomal methyltransferases, giving products which are partly excreted as such, and partly, following *S*-oxidation, as sulfoxide and/or sulfone derivatives [3].

A significant fraction of a dose of the widely used minor analgesic paracetamol is metabolized by a cytochrome P-450-dependent pathway yielding a reactive alkylating species which is inactivated by conjugation with glutathione [9]. This conjugate undergoes extensive biliary excretion [10, 11], and is ultimately excreted in the urine as cysteine, mercapturate and a range of methylthio derivatives [12, 13]. The manner in which these latter are formed is unknown, but it seems likely that the gut flora plays an important role.

In the present study, we report on studies in neomycin-pretreated and germ-free mice which show the significance of the gastrointestinal flora in the formation of methylthio metabolites of paracetamol in this species.

MATERIALS AND METHODS

Compounds. [*ring*-^{14}C]Paracetamol (sp. act. 19.5 mCi/mmol; radiochemical purity > 99%) and [^{35}S]L-cysteine (sp. act. 1260 Ci/mmol; radiochemical purity > 98%) were purchased from Amersham International (Aylesbury, U.K.). Neomycin sulfate and paracetamol were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.). Reference samples of paracetamol metabolites were the kind gift of Sterling–Winthrop Research (Alnwick, Northumberland, U.K.).

Animals and treatments. Two groups each of 8 male CDFI mice (body wt 33 ± 1 g; Charles River Laboratories) were given neomycin sulfate (500 mg/kg, in saline) or saline (10 ml/kg) orally twice daily for 3 days. On the 4th day, each animal was given [*ring*-^{14}C]paracetamol (200 mg/kg; 100 μCi/kg) dissolved in saline by i.p. injection.

In a second experiment, groups of 8 conventional and germ-free male BALB/c mice (both body wt 19 ± 2 g; MRC Laboratory Animal Unit, Carshalton, Surrey, U.K.) were given [*ring*-^{14}C]paracetamol as above.

In a further study, groups of conventional (N = 7)

* Present address: Department of Pharmacology and Toxicology, Medical Faculty Novi Sad, Hajduk Veljkova 3, POB 98, 21000 Nov Sad, Yugoslavia.
† To whom correspondence should be addressed.

and germ-free (N = 4) male BALB/c mice (body wt 20 ± 2 g) were given [^{35}S]L-cysteine (0.01 mg/kg; 120 μCi/kg i.p. followed 1 hr later by paracetamol (200 mg/kg) i.p.

After paracetamol administration, each mouse was kept in an individual metabolism cage (Mini Metabowl, Jencons Ltd., Hemel Hempstead, Herts, U.K.) permitting separate collection of urine and faeces. Each cage was silanized with dimethyl chlorosilane (2% in dichloromethane) to prevent loss of metabolites by adsorption. The 0–8 hr urine was collected in the dark in tubes kept at 0°. At the end of the 8 hr collection period, the cages were washed with 5 ml water, the washings added to the urine and the whole kept at −20° until analysed. The animals had free access to food and water throughout, which in the experiments with germ-free mice were sterilized by autoclaving.

Analytical procedures. Urinary excretion of ^{14}C was determined by liquid scintillation spectrometry and paracetamol and its metabolites separated and quantitated by gradient elution radio HPLC [14]. Urinary excretion of ^{35}S was determined by liquid scintillation spectrometry, using the same procedures as for ^{14}C with correction for loss of counts due to isotopic decay made with reference to the nomogram provided by Amersham International.

Statistical analysis. Results are expressed as mean ± SD, and the significance of differences between the groups assessed by the unpaired 2-tailed Student *t*-test.

RESULTS

Metabolism of paracetamol in the mouse

The 0–8 hr urinary recovery of ^{14}C following administration of ^{14}C-paracetamol to control CDF1

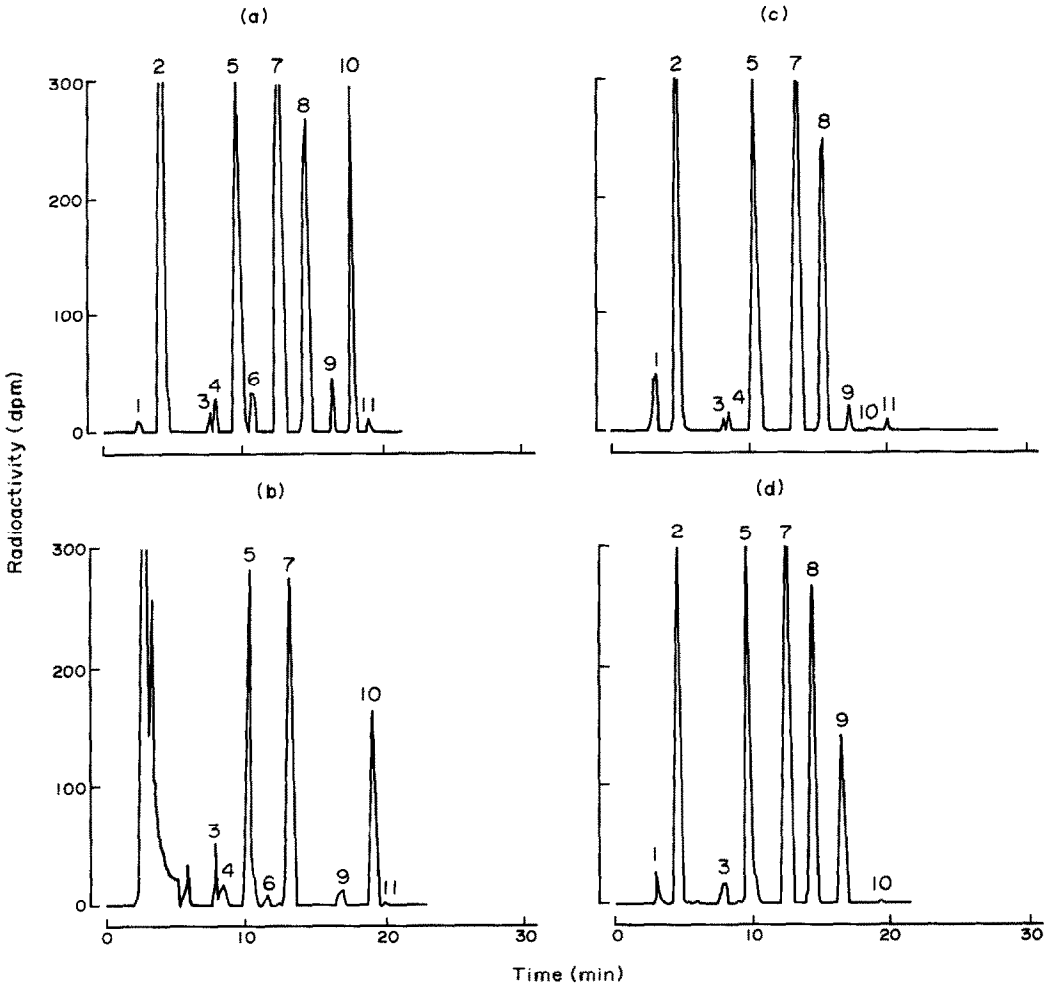

Fig. 1. Radiochromatograms of 0–8 hr urine of mice given paracetamol. (a) ^{14}C-paracetamol (200 mg/kg i.p.) to conventional mice. (b) Paracetamol (200 mg/kg i.p.) and ^{35}S-cysteine (0.01 mg/kg i.p.) to conventional mice. (c) ^{14}C-paracetamol (200 mg/kg i.p.) to conventional mice pretreated with neomycin (500 mg/kg p.o. twice daily for 3 days). (d) ^{14}C-paracetamol (200 mg/kg i.p.) to germ-free mice. Details of HPLC analysis and dosing schedules are given in the text. Identities of radioactive peaks were assigned by comparison of retention times, as follows: 1—unknown; 2—paracetamol (APAP) glucuronide; 3,4—unknown; 5—APAP sulfate; 6—3-methylthio APAP glucuronide; 7—APAP-3-cysteine; 8—free APAP; 9—APAP-3-mercapturate; 10—3-methylthio APAP sulfoxide *and* sulfate; 11—3-methylthio APAP.

Table 1. Urinary metabolites of [*ring*-^{14}C]paracetamol in the mouse: effect of neomycin pretreatment

	% of 0–8 hr urinary ^{14}C as: Control	Neomycin-treated
Paracetamol		
-free	6.6 ± 1.6	6.0 ± 2.1
-glucuronide	57.6 ± 4.8	60.1 ± 4.8
-sulfate	9.9 ± 1.3	11.1 ± 1.1
-3-cysteine	20.6 ± 3.3	21.5 ± 3.6
-3-mercapturate	0.43 ± 0.66	0.14 ± 0.12
Paracetamol 3-methyl-		
-thio	0.14 ± 0.15	0.08 ± 0.09
-thio glucuronide	0.73 ± 0.40	0.08 ± 0.12*
-thio sulfate -sulfoxide	2.83 ± 1.51	0.36 ± 0.56†
Total	3.70 ± 1.9	0.51 ± 0.65†

* $P < 0.002$; † $P < 0.001$.
Figures quoted are means ± SD for groups of 8 animals.

mice was 74 ± 14% of the dose. Gradient radio HPLC analysis revealed the major urinary metabolites to be the glucuronic acid and 3-cysteinate conjugates, accompanied by smaller amounts of paracetamol sulfate, 3-methylthioparacetamol, excreted as such and as its O-glucuronide and O-sulfate, paracetamol 3-methylsulfoxide and unchanged paracetamol. A typical chromatogram is shown in Fig. 1A and quantitative data are presented in Table 1. The presence of sulfur in the peaks assigned to the various thioadducts was confirmed by the coadministration of ^{35}S-cysteine and unlabelled paracetamol: a radiochromatogram of the resulting urine is shown in Fig. 1B, which shows the incorporation of sulfur from ^{35}S-cysteine into the cysteinate, mercapturate and various 3-methylthio metabolites, as well as into paracetamol sulfate.

Effect of neomycin pretreatment

Table 1 compares the fate of ^{14}C-paracetamol in control and neomycin pretreated mice. The overall recovery of ^{14}C in the 0–8 hr urine was unaffected by antibiotic treatment, and the only differences between treated and control mice were seen in the highly significant reductions in the excretion of the various 3-methylthio metabolites. A representative HPLC trace is shown in Fig. 1C, presented to allow comparison with control results.

Fate of paracetamol in germ-free mice

Table 2 compares the fate of ^{14}C-paracetamol in conventional and germ-free BALB/c mice. The total recovery of ^{14}C in the 0–8 hr urine was the same in both groups of mice. There were small but significant differences between the groups in the excretion of paracetamol glucuronide (less in germ-free) and sulfate (more in germ-free). The total excretion of thioadduct metabolites was the same in both groups, but the quantitative aspects of the individual metabolites were very different. Thus, the excretion of the mercapturate is some 5 times higher in germ-free animals and that of the cysteinate is also raised, while the various 3-methylthio metabolites account for far less of the dose in the germ-free mice. A representative chromatogram is shown in Fig. 1D. These changes in the relative proportions of the various thioadduct metabolites were also seen when unlabelled paracetamol was coadministered with ^{35}S-cysteine, and the results of this study are listed in Table 3.

Table 2. Urinary metabolites of [*ring*-^{14}C]paracetamol in conventional and germ-free mice

	% of 0–8 hr urinary ^{14}C as: Conventional	Germ-free
Paracetamol		
-free	7.4 ± 1.1	7.7 ± 1.9
-glucuronide	51.3 ± 3.1	45.5 ± 1.3*
-sulfate	9.4 ± 0.8	13.4 ± 2.1*
-3-cysteine	22.4 ± 2.9	25.7 ± 4.1
-3-mercapturate	0.8 ± 0.5	5.1 ± 2.4*
Paracetamol 3-methyl		
-thio	0.07 ± 0.04	0.03 ± 0.04
-thio glucuronide	1.28 ± 0.56	0.02 ± 0.06*
-thio sulfate -sulfoxide	4.58 ± 1.60	0.21 ± 0.14*
Total	6.21 ± 2.10	0.27 ± 0.21*

* $P < 0.001$ cf. control.
Figures quoted are means ± SD for 8 animals.

Table 3. Incorporation of ^{35}S-cysteine into paracetamol metabolites by germ-free and conventional mice

	% ^{35}S dose in 0–8 hr urine as: Conventional	Germ-free	P
Paracetamol sulfate	7.4 ± 3.7	9.7 ± 2.1	NS
Paracetamol-3-cysteine	8.1 ± 2.1	4.4 ± 0.6	<0.005
Paracetamol-3-mercapturate	0.7 ± 0.6	1.3 ± 0.8	NS
Total 3-methylthio paracetamol	3.7 ± 1.1	0.7 ± 0.6	<0.03
% ^{35}S dose excreted in 0–24 hr urine	33.6 ± 12.9	25.1 ± 10.4	NS

Figures quoted are means ± SD for 5 animals.

DISCUSSION

Paracetamol is metabolized by three major pathways [9], namely conjugation with glucuronic acid and sulfate and metabolic activation by the microsomal mixed function monooxygenases yielding a reactive intermediate which is inactivated by conjugation with glutathione. This glutathione conjugate is extensively transformed prior to elimination [11], and the present data show the contribution of the intestinal microflora to these processes, notably their role in the formation of 3-methylthio-paracetamol.

S-arylglutathiones are metabolised by hydrolysis to *S*-arylcysteines, which may undergo at least 3 distinct fates, *N*-acetylation to give a mercapturic acid, transamination, and *C–S* cleavage [3], the relative extents of which vary greatly between animal

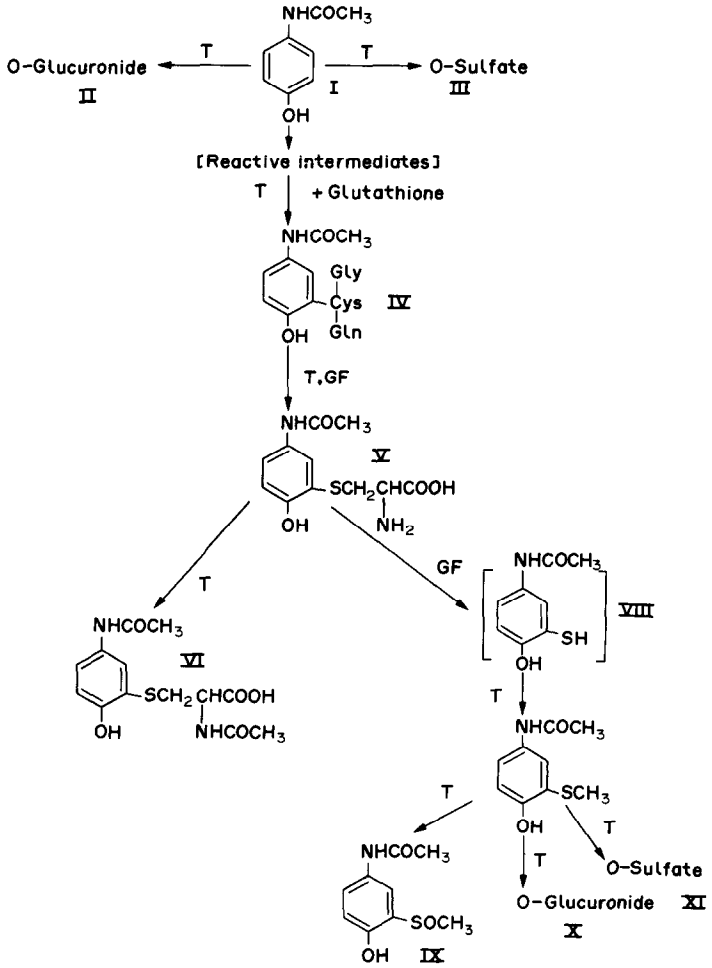

Fig. 2. Proposed pathways of paracetamol metabolism in the mouse, illustrating the roles of intestinal and tissue enzymes in the intestinal and tissue enzymes in the catabolism of the glutathione conjugate. Intermediates shown in square brackets have not been isolated. T = tissue enzymes; GF = intestinal microflora. Key to compounds: I—paracetamol (APAP); II—APAP glucuronide; III—APAP sulfate; IV—3-glutathionyl APAP; V—APAP-3-cysteine; VI—APAP-3-mercapturate; VII—3-thio APAP; VIII—3-methylthio APAP; IX—3-methylthio APAP sulfoxide; X—3-methylthio APAP glucuronide; XI—3-methylthio APAP sulfate.

species [15]. This latter pathway produces a thiol, which is generally then methylated to give a thiomethyl conjugate, which may in turn be *S*-oxidized to the corresponding sulfoxide and sulfone [8]. The tissue localization of the cysteine conjugate *C–S* lyase is problematical: although activity has been demonstrated in various tissues, notably the kidney [16], it seems likely that the major contribution to this reaction is made by the intestinal microflora [3]. The incubation of paracetamol-3-cysteine with the intestinal contents of conventional mice resulted in substantial degradation of the conjugate without producing methylthio metabolites. In the present case, the suppression of excretion of thiomethyl metabolites of paracetamol by pretreatment with the antibiotic neomycin and their virtual absence in germ-free mice very strongly supports this view. The significance of the residual elimination of thiomethyl metabolites in germ-free animals is unknown: it may indicate a minor contribution by tissue enzymes.

Additionally, in germ-free animals, the excretion of paracetamol-3-mercapturate was increased, compensating for the very marked drop in the excretion of thiomethyl metabolites. However, no such rise in the mercapturate was seen in neomycin-treated mice, although the thiomethyl metabolites were similarly reduced. A possible explanation for this could be a modification by neomycin of the reabsorption from the gut of the biliary glutathione-related metabolites of paracetamol.

These findings are thus consistent with the intestinal microflora playing an important role in the metabolism of paracetamol, particularly with respect to the catabolism of the glutathione conjugate, as shown in Fig. 2. It appears that the intestinal microflora plays a major role in the generation of the metabolic precursors of the thiomethyl conjugate and its *S*-oxidation products. Analogy with the fate of other aromatic cysteine conjugates [17] strongly supports both the proposed scheme and the indicated interplay between tissue and microfloral enzymes in the formation of the various excretion products.

It is interesting to consider the possible toxicological significance of the *C–S* lyase pathway for paracetamol-3-cysteinate, in view of the examples to be found in the literature of the metabolic activation of cysteine conjugates by this reaction [18, 19]. 3-Thioparacetamol is a reactive compound [18] which could contribute to the toxicity of the parent drug.

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REFERENCES

1. R. R. Scheline, *Pharmac. Rev.* **25**, 451 (1973).
2. P. Goldman, in *Metabolic Basis of Detoxication* (Eds. W. B. Jakoby, J. R. Bend and J. Caldwell), p. 323. Academic Press, New York (1982).
3. J. E. Bakke and J-A. Gustafsson, *Xenobiotica* **16**, 1047 (1986).
4. D. F. Colluci and D. A. Buyske, *Biochem. Pharmac.* **14**, 457 (1965).
5. J. Nomura, Y. Nishizuka and O. Hayaishi, *J. biol. Chem.* **238**, 1443 (1963).
6. J. C. Saari and M. O. Schultze, *Archs Biochem. Biophys.* **109**, 595 (1965).
7. D. V. Parke and R. T. Williams, *Biochem. J.* **48**, 27 (1951).
8. M. Tateishi, *Drug Metab. Rev.* **6**, 1207 (1983).
9. J. R. Mitchell, D. J. Jollow, W. Z. Potter, D. S. Davis, J. R. Gillette and B. B. Brodie, *J. Pharmac. exp. Ther.* **187**, 185 (1973).
10. A. Malnoe, M. S. Benedetti, R. L. Smith and A. Frigerio, in *Biological Reactive Intermediates* (Eds. D. J. Jollow, J. J. Kocis, R. Snyder and H. Vainio), p. 387. Plenum Press, New York (1977).
11. J. A. Hinson, T. J. Monks, M. Hong, R. J. Highet and L. R. Pohl, *Drug Metab. Dispos.* **10**, 47 (1982).
12. M. W. Gemborys and G. H. Mudge, *Drug Metab. Dispos.* **4**, 340 (1982).
13. A. Warrander, J. M. Allen and R. S. Andrews, *Xenobiotica* **15**, 891 (1985).
14. C. T. Dolphin, J. Caldwell and R. L. Smith, *Biochem. Pharmac.* **36**, 3835 (1987).
15. Y. Tanaka and J. Caldwell (submitted).
16. J. Stevens and W. B. Jakoby, *Molec. Pharmac.* **25**, 761 (1983).
17. W. B. Jakoby and J. Stevens, *Biochem. Soc. Trans.* **12**, 33 (1984).
18. G. H. Lowe and A. Goldblum, *Molec. Pharmac.* **27**, 375 (1985).
19. A. J. Gandolfi, R. B. Nagle, J. J. Soltis and F. H. Plescia, *Res. Comm. Chem. Pathol. Pharmac.* **33**, 249 (1981).