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EVIDENCE FOR THE ACTIVE RENAL SECRETION OF *S*-PENTACHLOROPHENYL-*N*-ACETYL-*L*-CYSTEINE BY FEMALE RATS

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Abstract—Male and female rats received 50 μmoles of pentachloronitrobenzene/kg by oral intubation daily for seven days. The final excreta were hydrolysed and analysed by electron capture GLC for the presence of pentachlorobenzenethiol and tetrachloro-1,4-benzenedithiol (derived from the equivalent *N*-acetylcysteine conjugates). No differences were found between the sexes for faeces and bile but the urinary excretion of both thiols by females was more than 10-fold greater than males. A similar result for urine was obtained following i.p. administration of a single 20 μmoles/kg dose of *S*-pentachlorophenyl-*N*-acetyl-*L*-cysteine (pentachlorophenyl mercapturate); in addition co-treatment with probenecid did not greatly change excretion by the males but considerably reduced excretion by females. The sex difference in the urinary levels of pentachlorophenyl *N*-acetylcysteine after 40 and 100 μmoles/kg doses of pentachloronitrobenzene was confirmed by h.p.l.c. of the mercapturate and again probenecid inhibited the excretion. Analysis of urine by TLC following a dose of [^{14}C]hexachlorobenzene (8 μCi/kg; 0.67 μmoles/kg) showed that more radioactivity was associated with the mercapturate from female rats than males. The results suggest that *S*-pentachlorophenyl-*N*-acetyl-*L*-cysteine, a metabolite of hexachlorobenzene and pentachloronitrobenzene, may be excreted by an active renal secretion which is particularly developed in female F344 rats.

During investigations of possible sex differences in the metabolism of hexachlorobenzene by rats, excreta were hydrolysed and analysed for the three major metabolites, pentachlorophenol, tetrachloro-1,4-benzenediol and pentachlorobenzenethiol [1]. (Pentachlorobenzenethiol was presumed to be formed during alkaline hydrolysis from pentachlorophenyl *N*-acetylcysteine or other metabolites of pentachlorophenyl glutathione [2–4]). A sex difference was observed in the levels of this compound on analysis of urine, the levels in female urine often being more than 10-fold higher than males. In contrast, there were only small differences in the levels of pentachlorobenzenethiol between male and female faeces and bile [1]. Pentachlorobenzenethiol itself was also detected in greater quantities in female liver than male, suggesting that perhaps the urinary variations between the sexes might be explained by differences in hepatic hydrolysis of pentachlorophenyl glutathione.

However, in a recent publication the urinary excretion of perfluorooctanoate was reported to be very much higher by female rats than by males and evidence was presented implying that this was due to an active renal secretory mechanism in females [5]. Consequently, we have investigated further, sex differences in the excretion of pentachlorophenyl *N*-acetylcysteine. In addition to hexachlorobenzene, we have now demonstrated that the much greater excretion of pentachlorophenyl *N*-acetylcysteine in urine by females than males also occurs with pentachloronitrobenzene (the mercapturate is the major metabolite of this fungicide [6, 7]) and after administration of the conjugate itself. Results from experiments with probenecid, an inhibitor of active secretion in the kidney [8, 9], are consistent with the proposition that this lipophilic acid is excreted by an active mechanism which is particularly developed in female rats.

MATERIALS AND METHODS

Chemicals. Organic chemicals were obtained from the following sources; hexachlorobenzene (Organic Analytical grade), BDH Chemicals Co. (Poole, U.K.), methyl pentachlorobenzenethiol, Cambrian Chemical Co. (Croydon, U.K.); *N*-acetyl-*L*-cysteine, Aldrich Chemical Co. (Gillingham, Dorset, U.K.); probenecid, Sigma Chemical Co. (Poole, U.K.); acivicin was a gift from Dr. J. P. McGovren (Upjohn Co., Kalamazoo, MI, U.S.A.) and >99% pure pentachloronitrobenzene was a gift from Dr. J. R. P. Cabral (I.A.R.C., Lyon, France). Toluene was 'Distol' pesticide grade, Fisons Co. (Loughborough, U.K.) [^{14}C]Hexachlorobenzene (12 mCi/mmole) was purchased from California Bionuclear Corp. Sun Valley, CA., U.S.A. and *p*-aminol[^{3}H]hippurate (306 mCi/mmole) from Amersham International, U.K.

S-Pentachlorophenyl-*N*-acetyl-*L*-cysteine (pentachlorophenyl mercapturate) was prepared by the reaction of pentachloronitrobenzene (1 mmole) with *N*-acetyl-*L*-cysteine (1 mmole) in 20 ml of acetonitrile/triethylamine/water (8/1/1 by vol) for 2 hr [7] and purified by chromatography on dry column silica gel (Merck, Darmstadt, Germany) by

elution with benzene/acetic acid (3/1 v/v). The conjugate was identified by electron impact (70 eV) and chemical ionization (isobutane) mass spectrometry, infrared and ultraviolet spectroscopy, by its melting point (227–229°) and by similar analyses of its methyl ester [4, 10].

Methyl tetrachloro-1,4-benzenedithiol was formed by the reaction of methyl pentachlorobenzenethiol (0.6 mmoles) in pyridine (10 ml, with NaHS in ethylene glycol (100 mg in 3 ml) for 10 min under reflux [11]. After extraction the mixture was purified by dry column silica gel chromatography eluting with chloroform/hexane (1/10 v/v). Two products (10 : 1 w/w) were isolated and further purified by TLC. The melting point (84–87° [11]) and the mass spectrum ($M^{+\cdot}$ m/z 292) of the major product confirmed it as the expected methyl tetrachloro-1,4-benzenedithiol. The unknown minor product possessed a similar mass spectrum and melting point (81–83°) and was presumed to be isomer. On methylation the isomers were separable by GLC (5% OV-210) (retention time of unknown 0.87 of 1,4-isomer) and possessed different m.p.s. 78–81° and 132–134° respectively (literature, 1,4-isomer 133–134° [11]; 132–134° [12]; 136.5–137.5° [13]) but very similar, although not identical, mass spectra ($M^{+\cdot}$ m/z 306). ^{1}H n.m.r. (60 M Hz) of the methylated isomers both gave one singlet, unknown 2.513 p.p.m.; 1,4-isomer 2.498 p.p.m. relative to trimethylsilane (methyl pentachlorobenzenethiol, 2.481 p.p.m.). The most likely structure for the methylated unknown is dimethyl tetrachloro-1,2-benzenedithiol.

Animals and treatments. Male and female F 344/N (10–12 weeks old) rats were bred in these laboratories and fed pellet diet MRC 41B. They were given [^{14}C]hexachlorobenzene and pentachloronitrobenzen dissolved in arachis oil (20 μmoles/ml) by oral intubation. Pentachlorophenyl *N*-acetylcysteine as its ammonium salt dissolved in 2% glycerol formal-physiological saline (4 μmoles/ml), probenecid as the ammonium salt in saline (0.5 mmoles/ml) and sodium *p*-amino[^{3}H]hippurate in saline (0.25 μCi/μmole; 20 μmoles/ml) were given by i.p. injection. Urine, faeces and bile were collected as previously described [1] and stored at −20° until analysed.

Analyses. Urine, faeces and bile were hydrolysed, and pentachlorobenzenethiol and tetrachloro-1,4-benzenedithiol extracted as previously described [1] except that toluene was used for all extractions. The thiols were methylated with diazomethane in diethyl ether and estimated by electron capture GLC as before [1] or by using a Varian Vista 6000 chromatograph fitted with a 2 m × 2 mm i.d. column packed with 3% OV-210 coated on Gas Chrom Q (100–120 mesh) operating at 170° with a N_2 flow of 30 ml/min.

For the estimation of pentachlorophenyl *N*-acetylcysteine by h.p.l.c., 0.5 ml portions of urine were acidified with 1.5 ml of 0.2 N HCL and extracted twice with 2.5 ml of dichloromethane. These extracts were then combined, the solvent removed under N_2 and the residue dissolved in 0.5 ml of methanol. The solutions were analysed on a Spherisorb ODS 5μ 25 cm × 4.6 mm i.d. column with two LDC/Milton Roy Constametric III pumps. A three tier linear gradient system from water to 95% methanol at a flow rate of 1 ml/min controlled by an Apple II microcomputer was used to elute the mercapturate which was detected with a Spectromonitor III model 1204A variable wavelength u.v. monitor at 217 nm.

Radioactivity experiments. To detect radioactivity in pentachlorophenyl *N*-acetylcysteine after [^{14}C]hexachlorobenzene metabolism, urine (0.5 ml) plus 0.5 mg of the mercapturate in 0.1 ml of methanol was warmed to ensure complete mixing then acidified with 0.2 N HCl (1.5 ml). The mixtures were extracted twice with ethyl acetate (5 ml), the combined extracts reduced in volume and subjected to TLC on silica gel (solvent, benzene/acetic acid 3/1 v/v). The appropriate band was then removed and radioactivity determined in 0.4 ml of methanol and 10 ml of Instagel (Packard Instrument Co. Inc.) using a Searle Analytic Inc. Mark III liquid scintillation system. Faeces were homogenised in water (1 : 10 w/v) and treated similarly.

The excretion of radioactivity in the urine after treatment with 4-amino[^{3}H]hippurate was performed by counting 0.1 ml of urine in the liquid scintillation system described above.

Results are quoted as means ± S.E.M. Statistical significance between groups was assessed by Student's *t*-test.

RESULTS

Metabolism of pentachloronitrobenzene. Pentachlorophenyl *N*-acetylcysteine is a major metabolite of pentachloronitrobenzene in rats [6, 7]. On alkaline hydrolysis it is converted to pentachlorobenzenethiol which can be quantitated by electron capture GLC (Fig. 1) [1, 2, 4]. Male and female rats were given 50 μmoles of pentachloronitrobenzene/kg daily for seven days to determine whether the sex difference in urinary levels of pentachlorobenzenethiol observed after hexachlorobenzene treatment would also be seen after metabolism of this analogue. Only pentachlorobenzenethiol and tetrachloro-1,4-benzenedithiol (presumably derived from a double glutathione substitution) were estimated, other minor

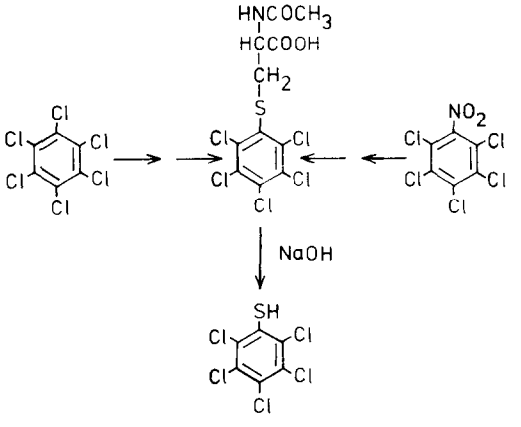

Fig. 1. Formation of *S*-pentachlorophenyl-*N*-acetyl-*L*-cysteine from hexachlorobenzene and pentachloronitrobenzene *in vivo* and its conversion to pentachlorobenzenethiol by alkaline hydrolysis.

Table 1. Analysis of the excreta from rats administered pentachloronitrobenzene*

Sex	Pentachlorobenzenethiol	Tetrachloro-1,4-benzenedithiol (μmoles/24 hr/kg)	Total
Urine			
Male	1.6 ± 0.3	0.2 ± 0.1	1.8 ± 0.4
Female	20.9 ± 0.3†	4.0 ± 0.3†	24.9 ± 0.4†
Faeces			
Male	6.1 ± 1.1	17.2 ± 3.1	23.3 ± 4.2
Female	6.7 ± 0.3	11.2 ± 1.4	17.9 ± 2.0

* Male and female rats were given 50 μmoles of pentachloronitrobenzene/kg by oral intubation daily for 7 days. After the last dose 24 hr samples of urine and faeces were collected, hydrolysed and analysed as described in Materials and Methods. Results are mean ± S.E.M. (N = 4 per group).

† Significantly different from males $P < 0.001$. Combined urinary and faecal excretions were males 25.1 ± 4.5 and females 42.1 ± 2.3 μmoles/24 hr/kg ($P < 0.025$).

metabolites such as pentachloroaniline were ignored for the purpose of this study (Table 1). Urinary excretion of these glutathione derived metabolites by females was considerably higher than males (> 10-fold) but faecal excretion was very similar. Total excretion was only slightly more by females (1.7-fold). These findings were very similar to those previously obtained with hexachlorobenzene although the absolute amounts of metabolites were much higher with pentachloronitrobenzene [1]. The disubstituted product was apparently more readily excreted via the faecal route than pentachlorophenyl mercapturate and this was confirmed by analysing bile collected immediately following the 24 hr period shown in Table 1. There were no significant differences between the sexes i.e. pentachlorobenzenethiol 12.0 ± 5.4 and 13.0 ± 3.3 nmoles/hr/kg body weight and tetrachloro-1,4-benzenedithiol 78.1 ± 30.1 and 97.1 ± 11.5 nmoles/hr/kg in males and females respectively. Only trace amounts of a compound which could be tetrachloro-1,2-benzenedithiol were detected in excreta. Very low levels of pentachlorobenzenethiol were found in urine without prior hydrolysis.

Influence of acivicin on pentachloronitrobenzene metabolism. A difference between male and female rats in the urinary excretion of the glutathione derived conjugate but none in faecal and bile excretion implies a difference either in clearance through the kidneys or perhaps in export from the liver perhaps following differential hydrolysis. Acivicin is a potent inhibitor of liver γ-glutamyl transpeptidase the first enzyme in the hydrolysis of glutathione and its conjugates [14]. Two 0.5 mmoles/kg doses of acivicin at 12 hr intervals (at this dose the enzyme in the liver should be greatly inhibited [15]) did have a small effect on the urinary excretion of the metabolites/24 hr after a single dose of 40 μmoles of pentachloronitrobenzene/kg to female rats. Excretion was 66% of rats not given acivicin, but this was not considered to be of enough significance to account for the male/female difference and so the results are not presented here.

Administration of S-pentachlorophenyl-N-acetyl-L-cysteine and effect of probenecid. Male and female rats received pentachlorophenyl *N*-acetylcysteine by i.p. injection (20 μmoles/kg) and the 24 hr urine was hydrolysed as before. The amounts of pentachlorobenzenethiol detected were 14-fold greater in female urine than in urine from males (Table 2). Further metabolism of the mercapturate was slight. Probenecid, an inhibitor of active renal transport of organic acids [16], was administered before rats received the mercapturate and again after 12 hr (i.e. two 0.5 mmoles/kg doses). There was little change in the excretion by male rats despite the diuretic action of probenecid, but excretion by females was considerably reduced. These results suggest that the sex difference occurs at the renal stage and that female rats secrete the conjugate by an active mechanism which is of much lower importance in the male. If the difference between the sexes was due to active reabsorption by male rats, then probenecid would be expected to have had no effect on females and perhaps increase urinary excretion by males since the drug also inhibits uric acid reabsorption [16].

Direct evidence for a sex difference in the excretion of the mercapturate. All the previous studies were carried out by analysing urine for the presence of pentachlorobenzenethiol following hydrolysis of

Table 2. Comparison of the elimination in the urine of *S*-pentachlorophenyl-*N*-acetyl-*L*-cysteine by male and female rats and the influence of co-treatment with probenecid

Sex and treatment	Pentachlorobenzenethiol (μmoles/24 hr/kg body wt)
Male	0.62 ± 0.21
Male + probenecid	0.54 ± 0.07
Female	8.74 ± 2.14†
Female + probenecid	1.47 ± 0.24‡

Male and female rats received an i.p. dose of pentachlorophenyl *N*-acetylcysteine (20 μmoles/kg body wt) as described in Materials and Methods. Some animals were also given 0.5 mmoles of probenecid/kg body wt at −0.5 hr and after 12 hr. Controls received vehicle alone. Subsequent urine samples were then hydrolysed and analysed for the presence of pentachlorobenzenethiol. Results are means ± S.E.M. (N = 4).

† Significantly different from males $P < 0.01$.

‡ Significantly different from females not receiving probenecid $P < 0.025$.

Table 3. Sex difference in the excretion of radioactivity in the urine associated with *S*-pentachlorophenyl-*N*-acetyl-*L*-cysteine after a dose of [^{14}C] hexachlorobenzene*

Day	Pentachlorophenyl-*N*-acetylcysteine (dpm/24 hr/kg) Male	Female
1	10,940 ± 2,300	28,370 ± 1,900‡
2	3,300 ± 950	16,150 ± 4,400†
3	1,650 ± 720	5,600 ± 1,820
5	100 ± 20	3,150 ± 340§

* Male and female rats that had been fasted overnight received a single oral dose of [^{14}C] hexachlorobenzene (8 μCi/kg body weight; 0.67 μmoles/kg) dissolved in arachis oil (2.5 ml/kg). Urine was collected every 24 hr. Radioactivity associated with pentachlorophenyl-*N*-acetylcysteine after TLC of extracts was determined as described in Materials and Methods. Results are means ± S.E.M. (N = 4). † Significantly different from males $P < 0.05$, ‡ $P < 0.005$, § $P < 0.001$.

excreta. For direct evidence of the excretion of the mercapturate as a metabolite of hexachlorobenzene, male and female rats received [^{14}C]hexachlorobenzene (8 μCi/kg body weight; 0.67 μmoles/kg) and the radioactivity content of pentachlorophenyl *N*-acetylcysteine in excreta was followed over 5 days. There was no consistent difference between the sexes on analysis of faeces but radioactivity in the mercapturate isolated from female urine was always higher than in that from males (Table 3).

In further experiments, the presence of pentachlorophenyl *N*-acetylcysteine in urine was estimated by h.p.l.c. following 40 and 100 μmoles/kg doses of pentachloronitrobenzene. A marked difference between the sexes was observed (Table 4). The high level of pentachlorophenyl *N*-acetylcysteine in the urine of females given 100 μmoles/kg of pentachloronitrobenzene was significantly reduced after two 0.5 mmoles/kg doses of probenecid as before (Table 4).

Excretion of p-*amino*[^{3}H]*hippurate.* Rats were given a dose of *p*-amino[^{3}H]hippurate (100 μmoles/kg) to determine whether a sex difference in urinary excretion also occurred with this naturally occurring acid. Excretion by females after 24 hr (98.7 ± 5.1 μmoles/kg) was only slightly more than by males (78.4 ± 5.0 μmoles/kg; $P < 0.05$). This is in agreement with previous studies since a small sex difference in the reabsorption of *p*-aminohippuric acid in the kidneys has been reported [17].

DISCUSSION

During studies to determine possible sex differences in the metabolism of hexachlorobenzene, levels of pentachlorobenzenethiol in female urine after alkaline hydrolysis were many times that in males whereas such a distinction was not observed on examination of bile and faeces [1]. We have now demonstrated that this difference can also be observed after dosing rats with pentachloronitrobenzene. It was assumed that the major portion of the pentachlorobenzenethiol detected in urine was derived from pentachlorophenyl *N*-acetylcysteine during hydrolysis (Fig. 1). This was conclusively proven by a radiochemical experiment with [^{14}C]hexachlorobenzene and by quantitation of the mercapturate itself by h.p.l.c. following pentachloronitrobenzene metabolism.

Pentachlorophenyl *N*-acetylcysteine is presumably derived *in vivo* from the glutathione conjugate. The first enzyme involved in the hydrolysis of glutathione and its conjugates, γ-glutamyl transpeptidase, has been shown to be of higher activity and of different distribution in the livers of female F344 rats than the males (Dr. M. Manson, personal communication). Thus is seemed possible that partial hydrolysis of pentachlorophenyl glutathione in the livers of females might be linked to the sex difference in urinary excretion, perhaps by faster loss from hepatocytes. However, this now seems unlikely since acivicin, an inhibitor of γ-glutamyl transpeptidase,

Table 4. Sex difference in the urinary excretion of *S*-pentachlorophenyl-*N*-acetyl-*L*-cysteine as a metabolite of pentachloronitrobenzene*

Treatment	Pentachlorophenyl *N*-acetylcysteine (μmoles/24 hr/kg body wt) Male	Female
Pentachloronitrobenzene (40 μmoles/kg)	0.44 ± 0.06	10.1 ± 0.35†
Pentachloronitrobenzene (100 μmoles/kg)	0.96 ± 0.07	27.6 ± 1.30†
Pentachloronitrobenzene (100 μmoles/kg) + probenecid	0.19 ± 0.04	5.18 ± 2.6‡

* Male and female rats received a dose of pentachloronitrobenzene dissolved in arachis oil (20 μmoles/ml) by oral intubation. Urine was collected and analysed for the presence of pentachlorophenyl *N*-acetylcysteine (pentachlorophenyl mercapturate) by h.p.l.c. as described in Materials and Methods. Probenecid was administered as described in Table 2. Results are means ± S.E.M. for 4 or 3 (probenecid experiments) animals.

† Significantly different from males $P < 0.001$. ‡ Significantly different from females not treated with probenecid $P < 0.001$.

at a dose known to greatly inhibit the liver enzyme, had only a small effect on excretion in female urine.

The possibility that the difference between the sexes instead occurred at the renal stage was explored by giving rats pentachlorophenyl *N*-acetylcysteine by i.p. injection and then analysing the urines for pentachlorobenzenethiol. Again a clear sex difference was observed. In addition, probenecid, an inhibitor of renal active transport of organic acids [8, 16], caused a marked decrease in the urinary excretion of the mercapturate by females yet had little effect on excretion by males. These results are very similar to those reported for perfluorooctanoate, the clearance of this compound in female rats with cannulated bladders being considerably greater than the inulin clearance whereas in males it was less [5]. Probenecid greatly inhibited excretion by females but not males. Hanhijarvi *et al.* [5] have proposed that this can be explained by an active secretory mechanism for perfluorooctanate in the kidney tubule of female rats which only poorly operates in males. The excretion of pentachlorophenyl *N*-acetylcysteine thus seems to occur in a similar manner.

A more active secretory mechanism in females could be a partial explanation for the sex differences in the excretion of carnitine [18] and the mercapturate formed from paracetamol [19]. A sex difference in the excretion of 1-aminocyclohexane carboxylic acid, of the magnitude observed for perfluorooctanoate and pentachlorophenyl *N*-acetylcysteine, has been described [20]. In this case the authors suggested that the most likely explanation was a high rate of reabsorption in the male kidney tubule since the acid did not bind to plasma proteins (unlike perfluorooctanoate) and might be expected to be filtered initially at the glomerula in both sexes. Small sex differences in the excretion of *p*-aminohippurate and α-aminoisobutyrate have been ascribed to the same mechanism [17, 21]. However, it is difficult to understand how this hypothesis could explain the effects of probenecid on the excretion of perfluorooctanoate and pentachlorophenyl *N*-acetylcysteine.

If there is an active secretory mechanism for lipophilic organic anions in rat kidneys controlled by sex hormones it seems likely that it has evolved to cope with endogenous metabolites produced by females. For instance, sulphation of cortisol is 6 to 8-fold higher in liver cytosol from female rats than from males [22]. This has been offered as explanation for the absence in male rat urine of the sulphate of tiaramide (an anti-inflammatory agent), whereas substantial quantities have been isolated from female urine (23). However, the operation of a renal secretory mechanism for organic anions that is particularly developed in female rat kidneys could also contribute to the apparent sex difference in sulphate conjugation as judged by urine analysis.

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