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SEX DIFFERENCES IN THE METABOLISM OF HEXACHLOROBENZENE BY RATS AND THE DEVELOPMENT OF PORPHYRIA IN FEMALES

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Abstract—Male and female F 344 rats were dosed every other day for 103 days with 50 μmole of hexachlorobenzene (HCB)/kg. Females developed a hepatic porphyria, the urine and liver levels of porphyrins being 40- and 310-fold higher respectively than those of males. Urine was periodically hydrolysed and analysed for the three metabolites pentachlorophenol, 2,3,5,6-tetrachlorobenzene-1,4-diol and pentachlorothiophenol (derived from the mercapturate). The combined urinary excretion of these was greater in females than males, especially during the first 10 weeks. Pentachlorothiophenol was particularly high in female urine. After 103 days this metabolite was slightly less in female faeces than in male's but free hepatic pentachlorothiophenol was 3.6-fold greater. Although total 24 hr excretions of metabolites were higher by females than males and after 7 daily doses of HCB, a difference in this respect was not conclusively proven. However, total pentachlorothiophenol excretion was always significantly greater by females. The male/female ratios for pentachlorophenol and pentachlorothiophenol in bile were identical to those for faeces. Excretion of metabolites by both adult males and females was stimulated by pretreatment with diethylstilboestrol (DES). No sex differences in metabolism were observed with immature rats.

Between 1955 and 1959 many people in south-eastern Turkey developed a chronic porphyria because they had eaten seed wheat which had been treated with hexachlorobenzene (HCB) as a fungicide [1]. Twenty years later some of these people still showed symptoms of poisoning [2]. The condition was very similar to the familial and sporadic forms of *porphyria cutanea tarda*. This disease is characterized by the high urinary excretion of uroporphyrin as a consequence of an inhibition of hepatic uroporphyrinogen decarboxylase [3, 4]. The condition can be reproduced in rats and rabbits fed HCB [5–7] with associated inhibition of the hepatic decarboxylase [8, 9]. Other polyhalogenated aromatic compounds also induce this type of porphyria in humans and laboratory animals, the most potent being 2,3,7,8-tetrachlorodibenzo-*p*-dioxin [10–13].

The development of porphyria in rats dosed with HCB is much slower in males than females [14, 15]. However, urinary excretion of uroporphyrin by males can be greatly increased by repeatedly administering 17 β-oestradiol. This has been proposed as a model for the sporadic form of *porphyria cutanea tarda* in humans which is triggered by oestrogen therapy [16, 17]. Although haem biosynthesis is known to be stimulated by some steroids [18], we have shown that in male rats fed HCB and injected with the oestrogenic drug diethylstilboestrol (DES), the induction of porphyria is due to an increased inhibition of hepatic uroporphyrinogen decarboxylase [19]. Estimations of liver levels of HCB in these experiments led us to suggest that the increased inhibition of the enzyme was linked to stimulation of the metabolism of HCB. According to Koss *et al.* [20, 21], the three main metabolites found after alkaline hydrolysis of urine and faeces are pentachlorophenol, tetrachlorobenzene-1,4-diol (tetrachlorohydroquinone) and pentachlorothiophenol. The last is formed from pentachlorophenyl *N*-acetylcysteine, which has been isolated from rat urine [22]. We have therefore compared the excretion of these compounds by male and female rats dosed with HCB. Overall, females excreted greater quantities of these than males, especially during the earlier stages of treatment. The most distinct difference was in the excretion of pentachlorothiophenol.

MATERIALS AND METHODS

Chemicals. Organic chemicals were obtained from the following sources; HCB (Organic Analytical grade), BDH Chemicals Co. (Poole, U.K.); pentachlorophenol, pentachloroaniline and *N*-acetyl-L-cysteine, Aldrich Chemical Co. (Gillingham, Dorset, U.K.); pentachlorothioanisole (methylthiopentachlorobenzene), Cambrian Chemical Co. (Croydon, U.K.); 2,3,5,6-tetrachlorobenzene-1,4-diol (tetrachlorohydroquinone) and diethylstilboestrol dipropionate, Sigma Chemical Co. (Poole, U.K.). Pentachlorothiophenol was synthesized from pentachloroaniline via the diazonium salt and *O*-ethyl-*S*-pentachlorophenyl xanthate [23]. It was characterized by its mass spectrum ($[M]^+$ *m/z* 280 based on ^{35}Cl) and after methylation by comparison of its thin layer and gas chromatographic, and mass

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spectrometric properties with authentic pentachlorothioanisole. *N*-Acetyl-*S*-(pentachlorophenyl)-cysteine was prepared from pentachloronitrobenzene (> 99% pure, a gift from Dr J. R. P. Cabral) and *N*-acetyl-L-cysteine [24]. The conjugate was crystallized from methanol (m.p. 227–229°, literature 228–230° [25]) and characterized by its thin layer chromatographic mobility and u.v., i.r. and mass spectra [22, 24, 25]. The methyl ethers of pentachlorophenol and tetrachlorobenzene-1,4-diol were prepared by methylation with etheral diazomethane and possessed the appropriate physical properties. All solvents were 'Distol' pesticide grade, Fisons Co. (Loughborough, U.K.).

Animals and treatments. Male and female Fischer (F 344/N) rats were bred in these laboratories and fed pellet diet MRC 41B. They were dosed by oesophageal intubation with HCB (50 μmole/kg; 14 mg/kg) or pentachlorophenol (50 μmole/kg), both dissolved in arachis oil (5.7 mg/ml; weanlings 11.4 mg/ml at one-day intervals in short-term experiments and two-day intervals in the HCB chronic experiment. Diethylstilboestrol dipropionate (20 μmole/kg) dissolved in arachis oil (10 mg/ml) was given to rats by i.p. injection. Controls received oil alone. Urine and faeces were collected over 24 hr using metabolic cages and stored when necessary at −20° before analysis. Bile was collected for 1 hr by cannulation of the bile duct after the rats had been anaesthetized with sodium pentabarbitone (48 mg/kg) dissolved in saline (60 mg/ml). At the end of the experiments rats were killed by decapitation. Median lobes of livers were homogenized in ice-cold distilled water (1:9 w/v). Urine, faeces, bile and liver were analysed as described below.

Estimation of porphyrins. Liver homogenate (0.5 ml) or urine (0.5 ml) was mixed with water (4.5 ml), 60% perchloric acid (0.5 ml) and ethanol (5 ml) and left for 10 min. After centrifugation the porphyrins in the supernatant were estimated by fluorescence spectroscopy using uroporphyrin as a standard [26, 27].

Estimation of metabolites. HCB and its metabolites were extracted from excreta, bile and liver by methods which were adaptations of those of Koss *et al.* [20]. Urine (0.5 ml), water (1.5 ml), 2 M NaOH (1 ml) and ascorbic acid (5 mg as an antioxidant) were heated under N_2 for 3 hr at 70° to hydrolyse conjugates. After the addition of more ascorbic acid the hydrolysates were acidified to pH 1 with conc. HCl and extracted twice with toluene (5 ml). Portions of faeces (about 1 g) were homogenized in 1 M NaOH (1:9 w/v) and then 1 ml of homogenate was mixed with ascorbic acid and 1 ml of 1 M NaOH. After hydrolysis and acidification as above, the samples were extracted with cyclohexane (4 ml) and three times with diethyl ether (4 ml). Bile (0.1 ml) was treated in a similar manner to urine. Liver homogenate (0.5 ml) was diluted to 1 ml with 1 N perchloric acid and extracted twice with toluene (5 ml).

Solvent extracts from each sample were combined and dried over anhydrous Na_2SO_4. Then 1 ml aliquots were mixed with 0.5 ml of a saturated solution of diazomethane in diethyl ether, left for 30 min and analysed by g.l.c. using a Pye 104 instrument fitted with a ^{63}Ni electron capture detector heated to 250°. HCB and the methylated phenols were separated on a column (2 m × 4 mm i.d.) of 5% OV-210 coated on Gas Chrom Q (100–120 mesh) operating at 175° with a gas phase of 48 ml/min of N_2. Retention times relative to HCB were pentachloroanisole 1.27, 2,3,5,6-tetrachloro-1,4-dimethoxy benzene 1.50 and pentachlorothioanisole 2.47. Responses were linear over the range used for quantitation (5–200 pg). Recoveries of HCB, pentachlorophenol, tetrachlorobenzene-1,4-diol and pentachlorothiophenol from 'spiked' samples were 70–100%. Values quoted are expressed as means ± 1 S.E.M. Statistical significance between groups was assessed by Student's *t*-test.

RESULTS

Metabolism of hexachlorobenzene in mature rats during chronic treatment

Young post-pubertal male and female rats (58 and 64–76 days old and 192–219 g and 143–168 g body wt respectively) were given 50 μmole of HCB/kg body wt by oral dosing every two days for 103 days. Samples of urine were collected at intervals and analysed for porphyrins and metabolites of HCB. After 75 days the amounts of porphyrins excreted in female urines began to increase rapidly and by the end of the experiment these animals were very porphyric (Fig. 1). Excretion by males showed little change.

When the urines were hydrolysed, differences between the sexes in the concentrations of pentachlorophenol, tetrachlorobenzene-1,4-diol and pentachlorothiophenol were also apparent. During much of the experiment, excretion of pentachlorophenol was significantly higher by females than males, but then it began to plateau at a lower level (Fig. 2). Urinary levels of the diol, formed by further metabolism of pentachlorophenol, were also higher in females (Fig. 2). By far the most striking result

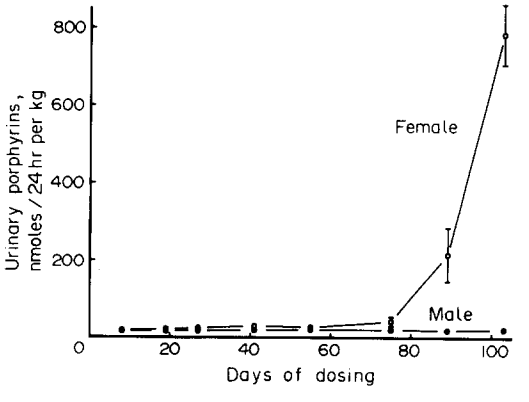

Fig. 1. Excretion of porphyrins in the urines of male (●) and female (○) rats during chronic treatment with HCB. Rats received HCB (50 μmole/kg) every other day for 103 days. Values are means ± S.E.M. ($N = 6$ except for day 103 when only 5 males had survived). Errors not shown were less than the size of the symbol. Female values were not significantly different from male and control male and female values until after 75 days.

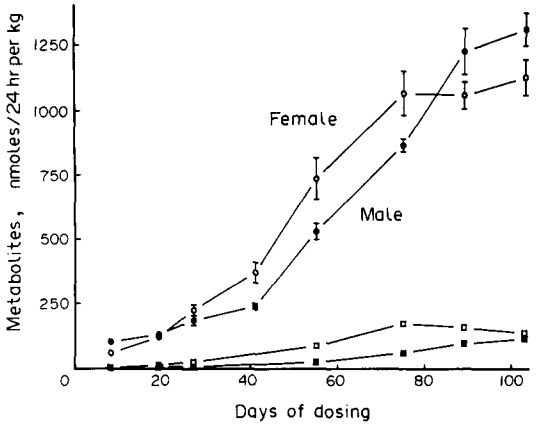

Fig. 2. Time course of the urinary excretion of phenolic metabolites of HCB by male and female rats. Rats received HCB (50 μmole/kg) every other day for 103 days. Pentachlorophenol in male (●) and female (○) and 2,3,5,6-tetrachlorobenzene-1,4-diol in male (■) and female (□) urines were estimated as described in Materials and Methods. Values are means ± S.E.M. for 5 or 6 animals. Errors not shown were less than symbol size.

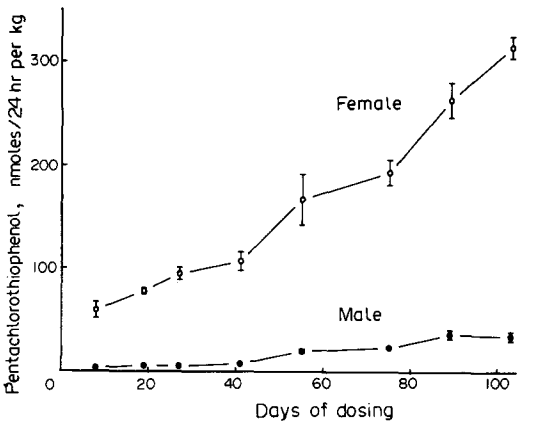

Fig. 3. Time course of the excretion in urine of pentachlorothiophenol during treatment of male (●) and female (○) rats with HCB (50 μmole/kg) every two days for 103 days. Pentachlorothiophenol was estimated after hydrolysis of urine as described in Materials and Methods. Values are means ± S.E.M. for 5 or 6 animals. All female levels are significantly different from those of males, $P < 0.001$. Errors not shown were less than symbol size.

was the consistently greater concentration of pentachlorothiophenol in female urine than that in urine from males (often > 10-fold, Fig. 3).

Male urine contained semen, which might have interfered in the hydrolysis and extraction of metabolites, so giving apparent differences between the sexes. However, the recoveries of the metabolites added to control male and female urines were identical. Also, levels in urines from HCB-treated male rats were unchanged when protein was removed before hydrolysis by heat treatment at 90° for 20 min followed by centrifugation. As an additional check, pentachlorophenyl *N*-acetylcysteine was added to control urines at a concentration (4 μg/ml) approximately equivalent to that estimated in the final urines from dosed females. After hydrolysis and extraction, yields of pentachlorothiophenol were slightly lower from males than from females (77.5 and 88% respectively) but this did not account for the large differences observed in the metabolism experiment. HCB was consistently detected in male urine but was present at far lower concentrations in the urines from females (e.g. the levels on day 75 were 319 ± 35 and 13.1 ± 2.8 nmole/24 hr/kg respectively). The amounts were reduced by the heat treatment described above, presumably because of association with precipitated protein.

Analysis of the faeces from day 103 did not show wide variations between the sexes. Pentachlorophenol was slightly lower in males (316 ± 34 nmole/24 hr/kg) than females (441 ± 57 nmole/24 hr/kg), whereas the thiol analogue was higher (493 ± 25 and 358 ± 34 nmole/24 hr/kg respectively, $P < 0.025$). Excretions of the diol by this route were identical (81 ± 6 and 78 ± 10 nmole/24 hr/kg respectively). At the end of the experiment combined urinary and faecal excretions of the metabolites were not significantly different between males and females, i.e. 2291 ± 116 and 2425 ± 182 nmole/24 hr/kg respectively. However, excretion of pentachlorothiophenol was significantly higher in females (694 ± 44 nmole/24 hr/kg) than males (527 ± 27 nmole/24 hr/kg, $P < 0.025$).

The development of porphyria in females was confirmed on analysis of the livers. Porphyrin concentrations in females were more than 300 times greater than males (Table 1). Although HCB and pentachlorophenol levels were similar in livers of

Table 1. Porphyrins, hexachlorobenzene, pentachlorophenol and pentachlorothiophenol in the livers of rats chronically treated with hexachlorobenzene*

Sex	(*N*)	Porphyrins§	Hexachlorobenzene (nmole/g liver)	Pentachlorophenol	Pentachlorothiophenol
Male	(5)	1.40 ± 0.2	335 ± 20	7.01 ± 0.38	0.55 ± 0.07
Female	(6)	434 ± 77†	339 ± 10	7.90 ± 0.85	1.99 ± 0.30‡

*Rats received HCB (50 μmole/kg) by intubation every two days for 103 days. Porphyrins, HCB and its metabolites in the liver were assayed as described in Materials and Methods. Results are means ± S.E.M.

† Significantly different from males, $P < 0.001$.

‡ $P < 0.005$.

§ Control rats not given HCB had porphyrin concentrations in the range 0.5–0.8 nmole/g.

Table 2. Analysis of bile following hexachlorobenzene treatment*

Sex	Hexachlorobenzene	Pentachlorophenol (nmole/hr/kg body wt)	Pentachlorothiophenol
Male	2.17 ± 0.47	0.83 ± 0.02	2.82 ± 0.21
Female	1.63 ± 0.19	1.01 ± 0.25	1.79 ± 0.12†

* Male and female rats (5 per group) were given 50 μmole of HCB/kg body wt every morning for 7 days. After the last dose the animals were anaesthetized with sodium pentabarbitone and bile collected for 1 hr during the afternoon by cannulation of the bile duct. Bile was hydrolysed and analysed as described in Materials and Methods. Only traces of 2,3,5,6-tetrachlorobenzene-1,4-diol were detected. Results are means ± S.E.M.

† Significantly different from males, $P < 0.005$.

both sexes, the concentration of pentachlorothiophenol, measured without hydrolysis for comparison with previously published work [21, 28], was 3.6-fold higher in females than in males (Table 1).

Biliary excretion of HCB metabolites

Mature male and female rats were administered 50 μmole of HCB/kg daily for seven days. The rats were then anaesthetized and bile collected for 1 hr by cannulation of the bile duct. The ratio of pentachlorothiophenol concentrations in hydrolysed male and female bile (1.6:1, Table 2) was close to those observed in faeces after either one week or long-term treatments with HCB (both 1.4:1, Table 3 and text respectively). A similar consistent relationship was observed for pentachlorophenol, although levels relative to pentachlorothiophenol were decreased.

Metabolism of pentachlorophenol

Adult rats were given pentachlorophenol (50 μmole/kg) daily for seven days and then the urines were analysed for 2,3,5,6-tetrachlorobenzene-1,4-diol. Significantly higher levels were excreted by females than males, i.e. males 315 ± 39, females 993 ± 87 nmole/24 hr/kg body wt ($N = 4$, $P < 0.001$). This confirms the results from HCB experiments in which urinary excretion of the diol was always higher in females.

Excretion of the metabolites of HCB by immature rats

Weanling rats (21 days old and 36 g body wt, $N = 4$ per sex) were given six daily doses of HCB and then the levels of metabolites in their excreta were estimated. No significant differences between the sexes were detected for any of the metabolites and so the results are not presented here. However, values were higher than expected from experiments with adults, and were similar to those observed for adult females dosed with DES (Table 3), i.e. total metabolites, males 998 ± 59, females 1006 ± 62 nmole/24 hr/kg. Only traces of the diol were observed in faeces. Hepatic concentrations of HCB and metabolites were also very similar in the sexes.

Influence of diethylstilboestrol on metabolism of HCB

Young rats (141–178 g) were given four doses of DES (20 μmole/kg body wt) by i.p. injection over 24 days. From the 25th day when they were 77–98 days old, the rats were administered HCB (50 μmole/kg) daily for seven days and then excreta collected and analysed. Animals which received DES grew more slowly than rats given oil alone. This was

Table 3. Analysis of the excreta from rats administered hexachlorobenzene after an initial treatment with diethylstilboestrol*

Sex and treatment	Pentachlorophenol	Tetrachlorobenzene-1,4-diol (nmole/24 hr/kg body wt)	Pentachlorothiophenol
Urine			
Male + oil	151 ± 19	3 ± 1	23 ± 3
Male + DES	190 ± 22	17 ± 2†	158 ± 9†
Female + oil	174 ± 17	16 ± 2‖	142 ± 12§
Female + DES	453 ± 105‡	35 ± 9	176 ± 7‡
Faeces			
Male + oil	85 ± 15	Trace	74 ± 23
Male + DES	160 ± 23‡	Trace	166 ± 33
Female + oil	116 ± 35	Trace	65 ± 4
Female + DES	279 ± 80	Trace	149 ± 13†

* Male and female rats (52–54 and 71–73 days old respectively) were given 20 μmole of DES dipropionate/kg dissolved in arachis oil (10 mg/ml) or oil alone by i.p. injection on days 1, 4, 14 and 24. From day 25 all rats were given 50 μmole of HCB/kg by oral intubation daily for seven 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 means ± S.E.M. ($N = 4$ per group). Significance of differences from rats not given DES were † $P < 0.001$; ‡ $P < 0.05$. Significance of differences from males; § $P < 0.001$; ‖ $P < 0.005$. Total excretions of these metabolites were, male 336 ± 57; male + DES 691 ± 70 ($P < 0.01$); female 513 ± 62; female + DES 1092 ± 175 ($P < 0.025$) nmole/24 hr/kg.

especially noticeable with males whose body and testes weights at the end of the experiment were approximately 77% and 35% respectively of rats which were not treated with the oestrogenic drug. The total excretion of metabolites was less in untreated males than females, although the difference was not statistically different (Table 3). However, total pentachlorothiophenol excretion was significantly higher in females (206 ± 12 nmole/24 hr/kg) than males (97 ± 26 nmole/24 hr/kg, $P < 0.01$). DES not only increased the levels of pentachlorophenol, tetrachlorobenzene-1,4-diol and pentachlorothiophenol in the urine of males, but also in females (Table 3). A similar pattern emerged after analysis of faeces so that total excretions of metabolites were twice those of undosed animals in both sexes. No significant differences were observed in the hepatic level of HCB or metabolites (data not included).

DISCUSSION

By giving rats doses of HCB for 103 days we confirmed that there is a clear difference between the sexes in their susceptibility to the induction of porphyria [14, 15, 29]. At the end of the experiment, concentrations of porphyrins in urine and livers from females were 40- and 310-fold higher, respectively, than males which were similar to control animals. It seemed possible that the susceptibility of females might be associated with a faster metabolism of HCB than males and these investigations were started to explore this possibility.

After the initial reports of the metabolism of HCB to pentachlorophenol by male rats [30, 31], it was shown that, when excreta from female rats were hydrolysed, this phenol, together with 2,3,5,6-tetrachlorobenzene-1,4-diol and pentachlorothiophenol, were the main metabolites that were isolated [20, 21, 32]. Further studies demonstrated the presence of other minor metabolites, formed by additional dechlorination and oxidation steps [33–35]. Based on excretion of these three compounds per kg of body weight, it appears that female rats may metabolize HCB (and pentachlorophenol) more quickly than males, the total excretion of these metabolites by females being 1.5 times higher after a week of daily doses. However, these studies were not conclusive as to this point. In contrast, urinary and total excretion of pentachlorothiophenol was always significantly greater by females than males. This compound is probably formed during hydrolysis from pentachlorophenyl *N*-acetylcysteine, which in turn is derived *in vivo* by dechlorination of HCB and subsequent conjugation with glutathione. The levels of pentachlorothiophenol were particularly high in female urine. The relative amounts of pentachorothiophenol in male and female biles were similar to faecal samples and this suggests that the sulphur-containing metabolite in female urine does not primarily originate by intestinal absorption of the glutathione conjugate excreted in the bile, but by a direct route from the liver. The estimation of 3.6 times more free pentachlorothiophenol in female liver than males at the end of the chronic dosing experiment correlates with the analyses of urine. While these experiments were in progress, Richter *et al.* reported a similar difference between the sexes in the liver levels of this compound after dosing with HCB [28]. The presence of free hepatic pentachlorothiophenol implies that the hydrolytic enzymes required to cleave the glutathione conjugate are active in liver. The enhancement of one of these, γ-glutamyltranspeptidase, is currently considered as a preneoplastic indicator [36]. HCB fed to female rats produces a high incidence of liver cell tumours [37] and the enhancement of γ-glutamyltranspeptidase-positive foci by HCB, especially in female rats, has recently been described [38]. More detailed investigations of the formation of pentachlorophenyl glutathione and its subsequent hepatic fate are obviously required.

The differences between male and female rats only occurred in postpubertal animals. Weanling male and females gave similar urinary and faecal levels of metabolites, although these were higher than the adults. Probably prepubertal animals have a faster rate of metabolism than adults. The high rate of urinary excretion of a sulphur-containing metabolite by mature females and immature animals of both sexes, but not by adult males, resembles findings for paracetamol (acetaminophen) metabolism [39].

In a previous experiment multiple dosing with DES caused a decrease in hepatic concentrations of HCB in male rats fed the compound for many weeks [19]. The suggestion that this was due to increased metabolism of HCB would seem to be supported by the stimulation of metabolite excretion on dosing with the oestrogenic drug. The urinary excretion of pentachlorothiophenol and tetrachlorobenzene-1,4-diol were both increased 5-fold. Interestingly, metabolism in females also appeared to increase on injection with DES so that total excretion of metabolites by both sexes rose to twice that of undosed animals. No differences in hepatic levels were detected, however, perhaps, due to the much shorter time-span of this experiment relative to that previously reported [19]. The mechanism by which DES stimulates HCB metabolism requires elucidation. Most sex differences in the ability of rats to metabolize xenobiotics, and in the hepatic activity of microsomal monooxygenases, are in favour of males. One of the exceptions is ethoxyresorufin-*O*-deethylation [40, 41], which is known to be stimulated in both sexes by oestradiol administration [41]. The involvement of the pituitary in these inductions and other sex differences in drug metabolism has been postulated and explored [40–42].

In the chronic treatment experiment reported here, no difference in hepatic concentrations of HCB between the sexes was detected, but such a difference has been found [28]. This evidence, together with the analyses of the excreta of male and female rats and those of the DES-treated animals, suggests that metabolism of HCB can be controlled by oestrogens, although reflection of this in liver levels of HCB may depend on a variety of factors such as time and redistribution around the body. However, total metabolism is probably of less importance in the induction of porphyria than the stimulation of a particular route which may be only a minor one. Pentachlorophenol and other phenolic metabolites

of HCB do not produce porphyria [43–45] and, despite the consistently greater quantities of pentachlorothiophenol produced by females than males, the formation of the glutathione conjugate itself does not seem to be crucial since pentachlorophenyl *N*-acetylcysteine is a major urinary metabolite of non-porphyrogenic pentachloronitrobenzene [24, 46]. Perhaps the initial dechlorination of HCB before conjugation with glutathione is a key step. Alternatively, a combination of metabolites may be required.

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