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Studies in Detoxication

34. THE METABOLISM OF CHLOROBENZENE IN THE RABBIT. ISOLATION OF DIHYDRODIHYDROXYCHLOROBENZENE, *p*-CHLOROPHENYLGLUCURONIDE, 4-CHLOROCATECHOL GLUCURONIDE AND *p*-CHLOROPHENYL-MERCAPTURIC ACID

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Baumann & Herter (1877) observed that when the urine of animals dosed with naphthalene was acidified and heated, free naphthalene was liberated. Later Bourne & Young (1933, 1934) suggested that this naphthalene arose from a labile precursor, possibly a dihydronaphthol. Eventually reduced derivatives of polycyclic hydrocarbons were isolated from the urine of animals receiving naphthalene (Young, 1946, 1947), anthracene (Boyland & Levi, 1935, 1936) and phenanthrene (Boyland & Wolf, 1948). These reduced derivatives were shown to be *trans*-1:2-dihydro-1:2-diols in the cases of naphthalene and anthracene (Booth & Boyland, 1949). In the case of phenanthrene, Boyland & Wolf (1948) have produced evidence for the formation of two diols, and there is information which suggests that diols are also metabolites of carcinogenic hydrocarbons such as benzpyrene (Weigert, 1945; Boyland & Weigert, 1947).

The evidence just summarized suggests that the formation of reduced diols may be a general metabolic reaction of polycyclic hydrocarbons, but as yet there is no proof that such a reaction also occurs during the metabolism of monocyclic benzene derivatives. Porteous & Williams (1949) and Garton & Williams (1949) expressed the view that a diol may be a metabolite of benzene on the grounds that benzene gives rise to more catechol than does phenol. It has also been suggested by Smith & Williams

(1949) that aniline forms reduced derivatives *in vivo*.

In the cases of naphthalene and anthracene the corresponding mercapturic acids were isolated as well as the diols. It appeared to us, therefore, that we would be more likely to achieve success in isolating a diol if we selected for study a benzene derivative known to give rise to a mercapturic acid. Chlorobenzene is such a derivative. The present paper deals, in part, with the isolation and characterization of a diol formed in the rabbit from chlorobenzene. *p*-Chlorophenylmercapturic acid was also isolated and further characterized.

The quantitative results of the preceding paper (Spencer & Williams, 1950) showed that the oxygen conjugates of chlorobenzene metabolism were far more important quantitatively than the mercapturic acid. Although the metabolism of the halogenobenzenes was studied as early as 1879 (Baumann & Preusse, 1879, 1881) the nature of these major metabolites has not, until now, been elucidated. Baumann & Preusse, from qualitative evidence not supported by actual isolation, suggested that both monohydric and dihydric halogen-substituted phenols were formed. We shall show that the major phenolic metabolite of chlorobenzene is, contrary to expectation, 4-chlorocatechol. The expected *p*-chlorophenol is quantitatively only a very minor metabolite.

EXPERIMENTAL

Dihydrodihydroxychlorobenzene

Isolation of the diol. Chlorobenzene (12 g., b.p. 132°), emulsified with water, was fed by stomach tube to six rabbits. The urine (1300 ml.) was collected for 48 hr., adjusted to pH 7.5, with a few drops of 2*N*-NaOH and then continuously extracted with ether for two periods of 8 hr. The extracts were washed with two 30 ml. portions of *N*-NaOH to remove phenols and then with 30 ml. water and finally dried with anhydrous Na₂SO₄. The ether solution was evaporated at room temperature to a syrup which was dissolved in warm benzene. After treatment with charcoal, the benzene solution was filtered and cooled to 0°. After 24 hr. the needle-shaped crystals were filtered and recrystallized from benzene. The yield of pure material was 4.5 mg. (i.e. 0.029% of the dose). None was isolated from the urine collected on the third day.

In other experiments, the yields were 11 mg. (0.03% of dose) from 27 g. and 5 mg. (0.007%) from 51 g. of chlorobenzene. The 3:4-dihydro-3:4-dihydroxychlorobenzene formed colourless needles very soluble in ether and ethanol, and moderately soluble in water and warm benzene. The product melted at 129–130° (uncorr.) and showed $[\alpha]_D^{25} - 413^\circ \pm 10^\circ$ (c, 0.025 in water). (Found: C, 49.1; H, 4.7; Cl, 24.1. C₆H₈O₂Cl requires C, 49.1; H, 4.8; Cl, 24.2%.)

The blue colour of a solution of potassium triacetosmate in glacial acetic acid (Criegee, Marchand & Wannowius, 1942) was not changed by addition of a glacial acetic acid solution of the diol. This result suggests that the diol is the *trans*-isomeride (cf. Booth & Boyland, 1949; Cook, Loudon & Williamson, 1950).

Fig. 1. The ultraviolet absorption spectrum of 3:4-dihydro-3:4-dihydroxychlorobenzene in water (full line) and its dehydration product, *p*-chlorophenol, in acid (---) and in alkali (-.-.-) (see Table 1 for position of maxima).

Absorption spectrum of the diol. All absorption spectra in this work were measured with a Unicam Quartz Spectrophotometer Model S.P. 500. The diol showed a single band with max. 264 mμ. (ϵ_{max} , 3030) in water (Fig. 1). This suggested that it was a $\Delta^{1:3}$ -cyclohexadiene derivative. $\Delta^{1:3}$ -cyclohexadienes have been shown to have an absorption

maximum in the region 256–265 mμ. with an ϵ_{max} range of 2500–9900 (Booker, Evans & Gillam, 1940). The compound was unlikely to be a $\Delta^{1:4}$ -cyclohexadiene derivative, since $\Delta^{1:4}$ -cyclohexadiene shows no absorption bands above 210 mμ. (cf. Braude, 1945).

Dehydration of the diol by acid. Since the diol appears to be a $\Delta^{1:3}$ -cyclohexadiene derivative, it could be either 2:3-dihydro-2:3-dihydroxy- (I) or 3:4-dihydro-3:4-dihydroxy-chlorobenzene (II). Badger (1949) has shown that when diols of the polycyclic hydrocarbons are dehydrated, the hydroxyl group remaining on the aromatic hydrocarbon is located on the carbon atom most activated in electrophilic substitutions. On these grounds the diol I should give rise to *o*-chlorophenol (III) and the diol II to *p*-chlorophenol (IV) on dehydration. Furthermore, the dehydration should show

the characteristics of a monomolecular reaction. Thus a decision between structures I and II can be obtained by identification of the chlorophenol formed by dehydration. An indication was first obtained by the use of 2:6-dichloroquinone chlorimine (Gibbs's reagent). This reagent gives a strong blue colour with *o*- and *m*-chlorophenols at alkaline reaction in 2 min. *p*-Chlorophenol in equivalent concentrations gives a blue colour of the same intensity with this reagent only after an hour. According to Ettinger & Ruchhoff (1948), the colour given by *p*-chlorophenol with Gibbs's reagent has the same absorption maxima as that given by phenol; this suggests the slow removal of the *para*-chlorine atom. Gibbs's reagent does not as a rule give colorations with *para*-substituted phenols. An alkaline solution of our diol did not give a colour with Gibbs's reagent, even on standing for 48 hr. at room temperature. After heating the diol solution for 2 min. with 0.1 *N*-HCl, cooling and neutralizing, a blue colour developed slowly and was fairly intense after 2 hr. This suggested that the diol gives rise to *p*-chlorophenol on heating with acid.

Spectroscopic identification of *p*-chlorophenol. Before identification of the chlorophenol produced by dehydration of the diol could be undertaken, it was necessary to show that the *o*-, *m*- and *p*-chlorophenols could be distinguished spectroscopically. The spectra of these three phenols were therefore determined in 0.1 *N*-HCl and in 0.1 *N*-NaOH. As has already been shown by Doub & Vandenberg (1947), the spectrum of the *p*-chlorophenol anion is different from that of *p*-chlorophenol, and therefore the determination of the spectra in alkali as well as in acid serves as a check. The spectra of the chlorophenols are shown in Fig. 2 and the relevant data are given in Table 1.

A solution of the diol in 0.1 N-HCl was heated on the water bath and after the reaction was complete, determination of the absorption spectrum showed that the band at 264 $m\mu$. had been replaced by two bands corresponding exactly to those of *p*-chlorophenol in acid. The solution was now made alkaline (0.1 N with respect to NaOH) and the spectrum redetermined. The spectrum now corresponded to that of the *p*-chlorophenol anion (Fig. 1 and Table 1). It was thus clear that the diol was a 3:4- and not a 2:3-diol.

Fig. 2. The ultraviolet absorption spectra of the *o*-, *m*- and *p*-chlorophenols; full lines in 0.1 N-HCl; broken lines in 0.1 N-NaOH (see Table 1 for position of maxima).

Table 1. Absorption spectra of chlorophenols and their anions compared with the chlorophenol from the diol

Chlorophenol	In 0.1 N-HCl		In 0.1 N-NaOH	
	$\lambda_{\max.}$	$\epsilon_{\max.}$	$\lambda_{\max.}$	$\epsilon_{\max.}$
Ortho	< 210	> 6,610	236.5	8,300
	274.5	1,980	294	3,780
Meta	212	6,250	238.5	9,700
	274.5	1,720	292	3,460
Para*	222	8,900	244	11,700
	280	1,600	298	2,600
Compound from diol	222	8,900	245	11,600
	280	1,575	298	2,600

* In ethanol; $\lambda_{\max.}$ 225, 284 $m\mu$.; $\epsilon_{\max.}$ 8930, 1775.

Rate of dehydration of the diol. As already pointed out, the dehydration process should be a monomolecular reaction. The constants of the dehydration reaction were conveniently calculated from spectroscopic data, that is from the change in the spectrum during conversion of the diol to *p*-chlorophenol. In acid solution *p*-chlorophenol shows a maximum absorption at 222 $m\mu$., whereas the diol, at this wavelength, shows almost minimum absorption (the minimum is actually at 228.5 $m\mu$.). The value of $E (\log I_0/I)$ at 222 $m\mu$. at any moment during the dehydration is due to the absorption of the remaining diol and that of the *p*-chlorophenol formed, and E_t can be expressed by the equation

$$E_t = (x - y) \epsilon_D + \frac{128.5y \epsilon_P}{146.5},$$

where x = original amount of diol in g.mol./l.; y = amount of diol in g.mol./l. dehydrated in time t ; E_t = extinction at time t ; $\epsilon_D = 1145$ = molecular extinction of the diol at 222 $m\mu$.; $\epsilon_P = 8900$ = molecular extinction of *p*-chlorophenol at 222 $m\mu$.; and 128.5 and 146.5 are the molecular weights of *p*-chlorophenol and the diol, respectively. E_t can be measured on the spectrophotometer, x is known and hence y can be calculated from the above equation. Percentage dehydration can be calculated from x and y .

Fig. 3. Rate of dehydration of the dihydrodihydroxychlorobenzene by N- and 0.1 N-HCl at 98°; inset, at room temperature by N-HCl.

Table 2. Velocity constant for the dehydration of dihydrodihydroxychlorobenzene

(The velocity constant k is calculated from the equation $k = 2.3 \log \{a/(a - x)\}/t$, where $a = 100\%$, and $x = \%$ dehydration.)

Time t (min.)	Percentage dehydration (x)	Velocity constant (k)
5	39.5	0.0100
10	64.7	0.0104
15	79.6	0.0106
25	94.4	0.0092

Table 3. Dehydration of dihydrodihydroxychlorobenzene by hydrochloric acid

Temp. (°)	Concn. of acid (N)	Time of dehydration, t	
		50 % dehydration	100 % dehydration
98	1	1.2 min.	10 min.
98	0.1	6.7 min.	35 min.
19	1	48 hr.	120 hr.

Rates of dehydration were measured at 98° in 0.1 N- and N-HCl and at room temperature in N-HCl as follows. Portions of diol solution (0.5 ml. containing 0.248 mg./ml.) were pipetted into five small tubes equipped with reflux condensers and placed quickly in a boiling-water bath. To each tube was added 2 ml. of 1.25 N-HCl already at the temperature of the water bath, the effective normality of the HCl thus being 1. After a given time a tube was cooled to 0° by plunging into an ice bath. The contents were now poured

into a 10 ml. flask, the tube and condenser being washed with N -HCl and the washings added to the flask. After making up to 10 ml. with N -HCl, E_{222} , at 222 $m\mu$, and at about five other wavelengths from 220 to 225 $m\mu$, was measured in the spectrophotometer against a blank of N -HCl. The experiment was repeated with 0.1 N -HCl. At room temperature the solution was simply read periodically in the spectrophotometer. The curves obtained are given in Fig. 3. In Table 2 the velocity constants for the dehydration of the diol by 0.1 N -HCl at 98° have been calculated using the equation for a monomolecular reaction. With N -HCl at 98°, the change was too rapid for accurate determination of the velocity constant. The rate of dehydration at room temperature in N -HCl also fitted the equation for a monomolecular reaction. The times taken for complete dehydration are given in Table 3. The compound appears to be stable in water at room temperature.

Oxidation of the diol with periodic acid. Further evidence that the diol was the α -glycol of $\Delta^{1:3}$ -cyclohexadiene was obtained by a spectroscopic study of the action of periodic acid. This acid oxidizes specifically 1:2-glycols to dialdehydes stoichiometrically (cf. Jackson, 1944), and it is to be expected that the chlorobenzene-diol would be converted by periodic acid to a *cis-cis*-chloromuconic dialdehyde thus:

Muconic acid with its four conjugated chromophores ($\text{O}=\text{C}-\text{C}=\text{C}-\text{C}=\text{C}-\text{C}=\text{O}$) has an intense absorption band at about 260 $m\mu$ with molecular extinction coefficients of 20,000–27,000 (Porteous & Williams, 1949; see also Braude, 1945), and it is to be expected that the dialdehyde with the same chromophoric system would have a similar absorption. Recently, Karrer, Eugster & Perl (1949) have prepared muconic dialdehyde (m.p. 117°) and showed it to have an absorption maximum at 269 $m\mu$ (from their published graph, ϵ_{max} is about 28,000). This is presumably the *trans-trans* isomer.

An ethanolic solution of the diol (0.5 ml. containing 0.15 mg. diol) was treated with 0.5 ml. HIO_4 solution (14.8 mg. KIO_4 in 3 ml. 2 N - H_2SO_4 made up to 25 ml. with distilled water), which contained 1.5 equivalents of HIO_4 , and made up to 10 ml. The spectrum of the solution was observed at intervals until no further change occurred, i.e. for 1 hr. The control cell in the spectrophotometer contained a similar solution with 0.5 equivalent of HIO_4 but no diol. In high concentrations HIO_4 shows appreciable absorption below 250 $m\mu$, but in the concentrations used in this experiment its absorption was almost negligible. Iodic acid which is formed during the reaction was shown, in a separate experiment, to be transparent at the wavelengths relevant to the present experiment. The initial and final absorption curves are given in Fig. 4; the intermediate curves bore no relation to either and thus the rate of reaction could not be followed spectroscopically. The extinctions for the final curve were calculated on the assumption that the product of the reaction was β -chloromuconic dialdehyde. One band was observed in the region 200–350 with a peak at 281 $m\mu$ and ϵ_{max} 21,400. This result strongly supports the view that a muconic aldehyde

type of structure is produced. The peak due to the diol at 264 $m\mu$ had completely disappeared.

Fig. 4. Ultraviolet absorption spectrum of the periodic acid oxidation product (full line; λ_{max} 281, ϵ_{max} 21,400) of dihydrodihydroxychlorobenzene (broken line). Molecular extinction coefficient calculated on the assumption that the oxidation product is chloromuconic dialdehyde.

Fig. 5. Ultraviolet absorption spectrum of *p*-chlorophenylmercapturic acid in ethanol (λ_{max} 262.5 $m\mu$ with ϵ_{max} 11,200; $\sim$ 217 $m\mu$, ϵ , 7600).

Isolation of *p*-chlorophenylmercapturic acid

During the continuous ether extraction of acidified chlorobenzene urine *p*-chlorophenylmercapturic acid passed into the ether phase. On evaporation of the ether, the mercapturic acid crystallized out from the oily residue. The oil was washed away with ether and the acid crystallized repeatedly from hot water. It formed colourless needles, m.p. 153°, $[\alpha]_{\text{D}}^{20} - 20^\circ$ (c. 0.27 in ethanol). (Found: equivalent by titration, 274. Calc. for $\text{C}_{11}\text{H}_{13}\text{O}_3\text{NClS}$, equivalent 273.7.)

The specific rotation of this compound has not been previously recorded, but Jaffé (1879) records m.p. 153–154°. The absorption spectrum of the mercapturic acid in ethanol was determined (Fig. 5). It differed from the spectra of the other *p*-chloro-substituted derivatives studied in this paper in that it showed a large band at 262.5 m μ . ($\epsilon_{\max}$ 11,200), whereas the other compounds (Figs. 2 and 7) showed a low band at about 280 m μ . ($\epsilon_{\max}$ about 2000). Chlorobenzene itself has an absorption band at 263.5 m μ ., but with a very low extinction ($\epsilon_{\max}$ 190) (Doub & Vandenbelt, 1947).

Phenols of chlorobenzene urine

Preparation of reference compounds. 4-Chlorocatechol, 4-chlororesorcinol or chloroquinol (1 g.) in 20 ml. 2.5N-NaOH was shaken for 30 min. with 3.8 g. toluene-*p*-sulphonyl chloride in 30 ml. acetone. After 2 hr. the mixture was poured into water and the crystalline precipitate (yield, 95% theory) collected. 4-Chlorocatechol di-toluene-*p*-sulphonate formed colourless prismatic crystals from ethanol, m.p. 117°. (Found: C, 53.3; H, 3.8; C₂₀H₁₇O₆S₂Cl requires C, 53.0; H, 3.8%.) 4-Chlororesorcinol di-toluene-*p*-sulphonate formed colourless plates from ethanol, m.p. 66–66.5°. (Found: C, 53.5; H, 4.0%.) Chloroquinol di-toluene-*p*-sulphonate was sparingly soluble in hot ethanol, acetone, ether, light petroleum and water and formed flat diamond-shaped crystals from dioxan, m.p. 186°. (Found: C, 52.4; H, 3.8%.)

Other reference compounds are described in the preceding paper (Spencer & Williams, 1950).

Isolation of 4-chlorocatechol and *p*-chlorophenol. Chlorobenzene (10 g.) was fed to six rabbits and urine (950 ml.) collected for 2 days. The pH of the urine was adjusted to 5 with a few drops N-HCl and the solution continuously extracted with ether (8 hr.) until the extracts no longer gave a colour with Gibbs's reagent (2,6-dichloroquinone chlorimine). After drying over anhydrous Na₂SO₄, the ether was evaporated *in vacuo* to a reddish brown oil which gave a green catechol colour with FeCl₃, a blue colour with the Gibbs's reagent and a negative naphthoresorcinol test. The oil was now extracted with 30 ml. hot water. Most of the oil dissolved, but a small oily residue (*A*) remained. The aqueous solution was filtered and cooled. The crystals which separated were identified as *p*-chlorophenylmercapturic acid (m.p. 151°). The filtrate from these was extracted with ether until the FeCl₃ test on the aqueous layer was negative (3 hr.). Evaporation of the ether extract gave an oil which was dissolved in 4 ml. 2.5N-NaOH and then treated with 0.7 g. toluene-*p*-sulphonyl chloride in 6 ml. acetone. After keeping overnight, the mixture was poured into 100 ml. water. The crystalline precipitate on recrystallization from ethanol gave 106 mg. of 4-chlorocatechol di-toluene-*p*-sulphonate, m.p. and mixed m.p. 116°.

The small residue (*A*) was dissolved in 2 ml. 2.5N-NaOH and treated with 0.4 g. *p*-toluenesulphonyl chloride in 3 ml. acetone, and as above, there was obtained 9 mg. of 4-chlorophenyl-toluene-*p*-sulphonate, m.p. 70°, mixed m.p. 70–71° with authentic 4-chlorophenyl toluene-*p*-sulphonate (m.p. 71°). No other phenols were detected.

Ether extraction of mildly hydrolysed chlorobenzene urine.

Isolation of 4-chlorocatechol glucuronide. The urine, after extraction of the free phenols, was warmed on the water bath to expel ether and then made 0.5N with respect to HCl by the addition of conc. HCl. It was then heated for 20 min. on the boiling-water bath. After cooling it was continuously extracted with ether for 12 hr., i.e. until the FeCl₃ test was

negative. Evaporation of the ether left a reddish brown syrup, which gave a strong green FeCl₃ test, a blue colour with Gibbs's reagent and a strongly positive naphthoresorcinol test. The syrup was now extracted on the water bath with 75 ml. water. The aqueous extract was centrifuged from the undissolved oil (*B*) and allowed to cool to 0°, whereby crystals of *p*-chlorophenylmercapturic acid separated. The aqueous extract also contained 4-chlorocatechol, *p*-chlorophenol and a glucuronide. The phenols were removed by continuous extraction for 4 hr. with CHCl₃, and on evaporation of the CHCl₃ extract a reddish-brown oil was obtained. This oil partly dissolved in water (25 ml.) leaving a small oily residue (*C*), and the aqueous solution, which now contained 4-chlorocatechol and small amounts of *p*-chlorophenol, was brought to pH 6.7 with 0.2M-acetate buffer. 4-Chlorocatechol was separated as the Pb salt from this solution by precipitation with an equal volume of saturated basic lead acetate. The precipitate was collected, Pb removed with H₂S and the chlorocatechol extracted with ether. The 4-chlorocatechol was eventually isolated as the di-*p*-toluenesulphonate (yield, 4.37 g.; m.p. and mixed m.p. 116°). The filtrate from the lead acetate precipitation, was freed of Pb by H₂S and extracted with ether. The ether on evaporation gave a small oily residue (*D*).

The aqueous extract after removal of the 4-chlorocatechol was evaporated to dryness to give a yellowish acidic glucuronide gum. This was dissolved in 25 ml. ethanol and methylated twice with ethereal diazomethane. Removal of the ether gave the methylated product as a thick straw-coloured neutral syrup which gave no colour with FeCl₃ or Gibbs's reagent but a strong naphthoresorcinol reaction. (Found: OMe, 21.6%.) The gum was dissolved in 15 ml. methanol, saturated with dry NH₃ at 0°, and, after standing for 3 hr. at 0°, the solution was filtered and evaporated to dryness *in vacuo*. The solid residue was crystallized from hot water and 2.85 g. of needles, m.p. 218°, [α]_D²¹ – 113° (c, 0.2 in acetone), were obtained. (Found: OMe, 8.0. Calc. for C₁₃H₁₆O₇NCl, 2H₂O; OMe, 7.8%.) It was identified as 4-chloro-2-methoxyphenyl- β -D-glucuronidamide dihydrate by comparison (mixed m.p. 218°) with a specimen of this compound prepared by Dodgson & Williams (1949).

The small oily residues *B*, *C* and *D* (see above) were dissolved in 5 ml. 2.5N-NaOH and treated with 1 g. of toluene-*p*-sulphonyl chloride in 8 ml. acetone. On pouring the product into water, a crystalline precipitate was obtained. This was recrystallized from ethanol and then fractionally crystallized. All fractions, however, were 4-chlorophenyl toluene-*p*-sulphonate, m.p. and mixed m.p. 71° (yield 53 mg.) depressed on admixture with 2-chlorophenyl toluene-*p*-sulphonate.

Ether extraction of strongly acid hydrolysed chlorobenzene urine. The extracted mildly hydrolysed urine, after warming to remove ether, was now made 3N with respect to HCl and boiled under reflux for 2.5 hr. After cooling it was exhaustively extracted with ether. Evaporation of the ether gave a dark-red oil which deposited crystals of *p*-chlorophenylmercapturic acid on standing 2 days. The oil was separated from the crystals by trituration with ether and 4-chlorocatechol, and *p*-chlorophenol were separated by lead acetate precipitation and converted into toluene-*p*-sulphonates. The yields were 250 mg. of 4-chlorocatechol toluene-*p*-sulphonate and 61 mg. of 4-chlorophenyl toluene-*p*-sulphonate which were identified by mixed melting points.

The yields of the two phenols obtained during each of the three ether extractions are given in Table 4.

Table 4. Amounts of 4-chlorocatechol and *p*-chlorophenol isolated as toluene-*p*-sulphonates from chlorobenzene urine

Phenol	In free state	Percentage of dose isolated	
		After acid hydrolysis with 0.5N-HCl*	3N-HCl†
4-Chlorocatechol (A)	0.26	10.8	10.5
<i>p</i> -Chlorophenol (B)	0.04	0.21	0.24
Ratio A/B	6.5	51	44

* Ethereal sulphate fraction. † Glucuronide fraction.

The glucuronides of chlorobenzene urine

Chlorobenzene (12 g.) was fed to six rabbits, and over the following 2 days 1350 ml. of urine was collected. The basic lead acetate precipitate was prepared and Pb removed with

Fig. 6. Ultraviolet absorption spectrum in water of the glucuronide gum from chlorobenzene urine (full line; $\lambda_{\max}$ 277 $m\mu$, E_{1cm} 0.17; inflexion at 215 $m\mu$, E_{1cm} 0.54) and that of the gum after hydrolysis on the water bath with 0.5N-HCl (broken line; $\lambda_{\max}$ 280 $m\mu$, E_{1cm} 0.165; $\sim$ 216 $m\mu$, E_{1cm} 0.53). The values of E_{1cm} are relative and not absolute; c unknown.

H₂S in the usual manner. Evaporation of the Pb-free filtrate *in vacuo* at 35° yielded 11.3 g. of a dark glucuronide gum. The gum was dissolved in 75 ml. ethanol and left at 0° overnight, whereby a sludge of inorganic matter separated. This operation was twice repeated and eventually 8.1 g. of purified glucuronide gum was obtained (ash, 0.9%; ethereal sulphate, 0.88%). This gum gave no colour with FeCl₃, but on acid hydrolysis gave a strong green colour. With Gibbs's reagent a strong blue colour was obtained indicating the presence of a free phenolic group. The product showed $[\alpha]_D^{25} - 43^\circ$ (c , 0.6 in water) and after mild hydrolysis $[\alpha]_D^{21} - 39^\circ$. Its ultraviolet absorption spectrum indicated that it was an aromatic glucuronide (Fig. 6), there being a

band at 277 $m\mu$. and an inflexion at 215 $m\mu$. The spectrum was hardly changed by mild acid hydrolysis and appeared to be almost identical in shape with those of 4-chlorocatechol and 4-chloro-2-methoxyphenylglucuronidamide, but not with that of *p*-chlorophenylglucuronide (Fig. 7).

Isolation of 4-chlorocatechol glucuronide. 4-Chlorocatechol glucuronide can be extracted from the glucuronide gum by ether extraction at strongly acid reaction. At first it was thought that this compound was an artifact, but the absorption spectrum of the gum ruled this out and showed that 4-chlorocatechol glucuronide did occur as such in the gum.

The gum (6.4 g.) was dissolved in 200 ml. water and the solution made 0.5N with respect to HCl. It was then continuously extracted with ether (18 hr.). On evaporation of the ether, a gum was obtained which was methylated in methanol with ethereal diazomethane. Evaporation of the ether gave a neutral gum which is, presumably, 4-chloro-2-methoxyphenylglucuronide methyl ester. (Found: OMe, 19.4; C₁₄H₁₇O₈Cl requires OMe, 17.8%.) The ester was converted to the amide with methanolic NH₃ and 3.06 g. of 4-chloro-2-methoxyphenyl- β -D-glucuronidamide dihydrate

Fig. 7. Ultraviolet absorption spectrum of 4-chlorocatechol (full line; $\lambda_{\max}$ 282.5 $m\mu$, $\epsilon_{\max}$ 2880; $\sim$ 215 $m\mu$, ϵ 5970), 4-chlorocatechol glucuronidamide dihydrate (broken line; $\lambda_{\max}$ 280, 224.5 $m\mu$ with $\epsilon_{\max}$ 2480, 9250) and *p*-chlorophenylglucuronide monohydrate (— · — · —; $\lambda_{\max}$ 219.5, 275.5 $m\mu$ with $\epsilon_{\max}$ 10,700 and 930). Solvent, water.

was isolated, m.p. and mixed m.p. 218°, $[\alpha]_D^{25} - 113.2^\circ$ (c , 0.2 in acetone). (Found: OMe, 7.6. Calc. for C₁₃H₁₆O₇NCl. 2H₂O; OMe, 7.8%.) Dodgson & Williams (1949) have quoted $[\alpha]_D^{20} - 96^\circ$ for this glucuronide when dissolved in 50% (v/v) acetone-water. Further investigation has shown that the rotation of this glucuronidamide varies with the proportion of acetone and water. A redetermination of the rotation of Dodgson & Williams's sample in pure acetone gave $[\alpha]_D^{25} - 113.5^\circ$ (c , 0.2 in acetone). It has also been found that the glucuronidamide is much more soluble in acetone-water mixtures than in either of the pure solvents. In water its solubility was <56 mg./100 ml., in acetone at 22° 225 mg./100 ml., and in 50% acetone-water >620 mg./100 ml.

Isolation of *p*-chlorophenylglucuronide. The aqueous solution remaining after extraction of 4-chlorocatechol glucuronide (see preceding paragraph) was neutralized with 10% (w/v) NaOH and evaporated to dryness *in vacuo* at 35°. The residue was extracted with absolute ethanol and impurities removed by allowing the ethanolic solution to stand before filtering.

Evaporation of the ethanol gave a yellowish gum which was methylated in methanol with ethereal diazomethane. After filtering the methylated solution, it was evaporated giving a pale-yellow gum which is presumably *p*-chlorophenylglucuronide methyl ester. (Found: OMe, 11.6. Calc. for $C_{15}H_{14}O_7Cl$; OMe, 10.2%.) The gum was treated with methanolic NH_3 at 0°, yielding eventually 109 mg. of *p*-chlorophenylglucuronidamide, m.p. 244–246° (mixed m.p. 244–246°), $[\alpha]_D^{25} - 84.9^\circ$ (c, 0.12 in methanol). The authentic glucuronidamide has m.p. 245–246° and $[\alpha]_D^{24} - 85.6^\circ$ (in methanol) (Spencer & Williams, 1950). It was further identified by conversion to the triacetyl amide. (Found: $COCH_3$, 29.6. Calc. for $C_{18}H_{20}O_9NCl$; $COCH_3$, 30.1%), which had m.p. and mixed m.p. 157–159°, $[\alpha]_D^{25} - 10^\circ$ (c, 0.1 in ethanol). The authentic triacetyl *p*-chlorophenylglucuronidamide has m.p. 159° and $[\alpha]_D^{21} - 10.7^\circ$ (c, 0.3 in ethanol) (Spencer & Williams, 1950).

DISCUSSION

The present work shows that the major metabolites of chlorobenzene in the rabbit are the monoglucuronide and ethereal sulphate of 4-chlorocatechol, and *p*-chlorophenylmercapturic acid. The figures given in Table 1 of the preceding paper (Spencer & Williams, 1950) suggest that they are excreted in roughly equal amounts, i.e. 25 : 27 : 20, respectively. The minor metabolites are *p*-chlorophenol and 3:4-dihydro-3:4-dihydroxychlorobenzene, as indicated below:

The most striking facts which have emerged from this work are that chlorobenzene, like polycyclic hydrocarbons, is 'perhydroxylated', that *p*-chlorophenol is, quantitatively, only a very minor metabolite, that most of the chlorobenzene oxidized appears as 4-chlorocatechol and that oxidation is more extensive than cysteine conjugation.

Dihydrodihydroxychlorobenzene. The isolation of this diol gives us the first recorded instance of partial saturation of monocyclic benzene derivatives *in vivo*. The experimental evidence shows that it is a 3:4-diol. Theoretically, the 3:4-diol can occur in six forms which include two pairs of enantiomorphs (V and VI, VII and VIII) and two racemates. The potassium triacetylosmate test suggests that it is a *trans*-diol

(VII or VIII). The metabolic diols of naphthalene and anthracene have already been shown to be *trans*-diols (Booth & Boyland, 1949; Cook *et al.* 1950).

The high rotation ($[\alpha]_D^{25} - 413^\circ$) of our diol suggests optical purity, but as the amount of material available was small we were unable to prove this. The amount of diol isolated from the urine was very small (maximum yield 0.03 % of dose), and we believe that the urine did not contain much more because the amount of *p*-chlorophenol isolated was also very small. The total amount of *p*-chlorophenol isolated, which includes that in the free state, that formed after acid hydrolysis and that isolated as the glucuronidamide amounted to 0.5 % of the dose. This *p*-chlorophenol could arise by acid dehydration of the diol by acid hydrolysis of the glucuronide and etherealsulphate of *p*-chlorophenol. *p*-Chlorophenylglucuronide could arise from a monoglucuronide of

the diol. Since the amount of *p*-chlorophenol isolated was small then it follows that the amounts of its precursors including the diol must be small.

4-Chlorocatechol. The occurrence of 4-chlorocatechol as the major metabolite of chlorobenzene may be of considerable theoretical importance metabolically, for it recalls to mind such problems as the biological conversion of phenylalanine to catechol derivatives such as adrenaline and noradrenaline. The glucuronide isolated proved to be 4-chloro-2-hydroxyphenylglucuronide identical with that formed on feeding 4-chlorocatechol (Dodgson & Williams, 1949). This compound could arise by (a) oxidation of *p*-chlorophenylglucuronide, (b) dehydrogenation of the 4-glucuronide of chlorobenzene-3:4-diol, or

(c) conjugation of metabolically formed 4-chlorocatechol as indicated in the formulae.

We feel, however, that the first mechanism (a) is unlikely, because Garton & Williams (1949) have shown that injected phenylglucuronide is not oxidized to catechol. No decision regarding (b) and (c) is as yet possible and both mechanisms may be involved.

Now 4-chlorocatechol could arise by further oxidation of *p*-chlorophenol, by dehydrogenation of 3:4-dihydro-3:4-dihydroxychlorobenzene or possibly by dehydration of a tetrol, as indicated in the following formulae:

On feeding *p*-chlorophenol to rabbits, it was found that very little was converted to chlorocatechol (Spencer & Williams, 1950), and we feel that chlorocatechol is more likely to be the product of the *in vivo* dehydrogenation of a diol. In this connexion Porteous & Williams (1949) (see also Garton & Williams, 1949) have already suggested that the catechol formed during the metabolism of benzene could arise in other ways than from phenol. In fact, a diol could be the precursor of a monophenol by dehydration and of a catechol by dehydrogenation.

The conversion of a diol to a monophenol is a known chemical reaction. At the moment we know nothing concerning the metabolism of diols and their conversion to catechols.

p-Chlorophenylmercapturic acid. The next point which deserves discussion is the position of *p*-chlorophenylmercapturic acid in the metabolism of chlorobenzene and its relation to the oxidation products. In the past, suggestions have been made that the oxygen- and sulphur-conjugates may be related to each other in that one form of conjugation might be the precursor of the others. From this point of view three possible paths of metabolism for the halogenobenzenes can be postulated thus:

- (a) halogenobenzene → hydroxylated compounds → mercapturic acids;
- (b) halogenobenzene → mercapturic acid → hydroxylated compounds;
- (c) halogenobenzene $\begin{cases} \text{mercapturic acid} \\ \text{hydroxylated compounds.} \end{cases}$

The first path is based on the views of Sherwin (1922) (see also Rhode, 1923), who suggested that halogenophenols were intermediates in the conversion of halogenobenzenes to mercapturic acids. Our experiments with *o*-, *m*- and *p*-chlorophenols (Spencer & Williams, 1950) and those of Coombs & Hele (1926, 1927) on the fate of chloro- and iodo-phenols in the dog and pig suggest that, if the hydroxylated compounds are phenols, then this path is unlikely, i.e. phenols are not precursors of mercapturic acids. The hydroxylated compounds, however, could be diols of reduced halogenobenzenes, but as we have no information concerning the metabolic fate of diols, the possibility that diols may be precursors of sulphur-conjugates, although unlikely, cannot be ruled out.

The second path (b), in which mercapturic acids are possible precursors of phenols, was implied in the remarks of Hopkins (1907), who suggested that ethereal sulphates might arise by the oxidation of more complex compounds such as mercapturic acids. On this point Quick (1937) has stated that: 'The union of cysteine with the hydrocarbon might be the first and only step available to the organism for attacking the unsubstituted aromatic ring and that as a further reaction the mercapturic acid is replaced by a hydroxyl group.'

The literature on the fate of mercapturic acids in the body is somewhat confused. The early experiments of Baumann & Preusse (1881) and Marriot & Wolf (1907) seem to indicate that little *p*-bromophenylmercapturic acid fed by mouth to dogs appeared in the urine. The work of Rose, Shiple & Sherwin (1924) suggests that mercapturic acids are oxidized to ethereal sulphates in the rabbit (see also Sherwin, Shiple & Rose, 1927; Shiple, Muldoon & Sherwin, 1924). However, Coombs & Hele (1927)

and Lawrie (1931) from their experiments on *p*-chloro-, *p*-bromo- and *p*-iodo-phenylmercapturic acids, concluded that mercapturic acids are not oxidized *in vivo*. It seems likely, however, that mercapturic acids are end products derived from the unacetylated *S*-arylcyteines and it may be better to write the suggested path (b) as

A number of *S*-phenylcysteine derivatives have been administered to animals, but in most cases less than 50% of the material administered has been accounted for (Stekol, 1938, 1939, 1941, 1946; Zbarsky & Young, 1943, 1934; Hele & Pirie, 1931; Witter, 1945) and no investigation as to whether these compounds give rise to phenols has been carried out. It is clear that new quantitative data on the metabolic fate of arylcysteine derivatives are needed, especially in view of the findings of Binkley, Anslow & du Vigneaud (1942), Binkley (1944) and Anslow & du Vigneaud (1947) that the carbon to sulphur link can be split biologically, the process involving phosphate bond transfer.

metabolites described by Weigert & Mottram (1946). It is thus possible to postulate on these grounds a scheme showing how both the sulphur- and oxygen-conjugates of chlorobenzene could arise from a common precursor.

Chlorobenzene could react with a protein (*P*) containing two sulphhydryl groups—*P*(SH)₂—or a protein containing disulphide groups—*PS*₂—to form a product which might then give rise to a mercapturic acid or, by hydrolysis, to a diol. The latter could then give rise to monohydric and dihydric phenols as visualized in the following scheme. The ideas arising from this scheme are now being pursued.

In the third path (c) the sulphur- and oxygen-conjugations are visualized as separate processes (Baumann, 1883). It is, however, possible that both types of conjugation arise from a common precursor which is not the halogenophenylcysteine mentioned above. There is now much evidence available in the literature which suggests that many types of organic compounds combine with sulphhydryl (SH) groups in the body (see Bournsnel, 1948; Needham, 1948; Thompson, 1948; Dixon, 1948). These SH groups may occur in enzymes, proteins and such substances as glutathione. The work of Crabtree (1944, 1945, 1946) on the action of hydrocarbons and bromobenzene on the glutathione content of mouse skin suggests that the first reaction undergone by these

SUMMARY

1. The fate of chlorobenzene in the rabbit has been studied in detail and several metabolites isolated and identified.
2. Chlorobenzene has been shown to undergo 'perhydroxylation' *in vivo*, by isolation of crystalline 3:4-dihydro-3:4-dihydroxychlorobenzene (yield, 0.03%), the structure of which was proved spectrophotometrically by conversion to *p*-chlorophenol.
3. The rate of dehydration of the diol by acid has been studied spectrophotometrically and the process shown to be a monomolecular reaction.

4. *p*-Chlorophenylmercapturic acid has been isolated, and its optical rotation and absorption spectrum recorded.

5. The main metabolite of chlorobenzene is shown to be 4-chlorocatechol which was isolated from the ethereal sulphate fraction and as its glucuronide, 4-chloro-2-hydroxyphenylglucuronide.

6. *p*-Chlorophenol and its glucuronide were also isolated, but were shown to be a very minor meta-

bolites of chlorobenzene amounting to about 0.5% of the dose.

7. The above findings have been discussed and a hypothesis presented relating mercapturic acid formation with oxidation during chlorobenzene metabolism.

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