# Studies in Detoxication

## 51. THE DETERMINATION OF CATECHOLS IN URINE, AND THE FORMATION OF CATECHOLS IN RABBITS RECEIVING HALOGENOBENZENES AND OTHER COMPOUNDS. DIHYDROXYLATION *IN VIVO*

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Naphthalene, anthracene and phenanthrene are metabolized in rats and rabbits to 1:2-dihydroxy compounds which have been proved to be 1:2-dihydro-1:2-diols (Young, 1950; Boyland, 1950). In the benzene series, an *o*-dihydroxy compound, 4-chlorocatechol, is a major metabolite of chlorobenzene in the rabbit (Spencer & Williams, 1950; Smith, Spencer & Williams, 1950). Many monosubstituted benzenes, e.g. phenol and nitrobenzene (cf. Robinson, Smith & Williams, 1951; Garton & Williams, 1949) also form small amounts of catechols. The addition or substitution of two hydroxyl groups ortho to each other, i.e. *o*-dihydroxylation, appears therefore to be a biological reaction of several aromatic compounds, and may result in the formation of reduced diols of the type mentioned above (this reaction has been called 'perhydroxylation') or of catechol derivatives, in which the aromatic ring is not reduced. Whether these two forms of dihydroxylation are related is not known at present.

The objects of the present work were to develop a quantitative method for the determination of catechols in the urine of rabbits receiving benzene derivatives and to find out whether other halogenated benzenes behaved like chlorobenzene.

### COLORIMETRIC DETERMINATION OF CATECHOLS

The present method is based on the formation of intensely blue-coloured cobalt complexes of catechol derivatives in the presence of strong alkali. The various complexes of catechol with bivalent cobalt have been described by Weinland & Döttinger (1918). These complexes are stable to alkali. The addition of 10N-sodium hydroxide to cobaltous nitrate solution results in the precipitation ultimately of the brown cobaltic hydroxide, but in the presence of 1:2-dihydroxybenzenes a permanent deep blue colour is obtained instead. A similar blue colour, attributed to the tetrahydroxy cobalt ion $[Co(OH)_4]^{2-}$, is obtained by adding a cobaltous salt to excess saturated aqueous potassium hydroxide or sodium hydroxide (Gordon & Schreyer, 1951). The absorption spectrum of the blue cobalt-catechol coloured complex using 4-chlorocatechol is shown in Fig. 1.

The colour reaction was given by all aromatic compounds tested which contained two or more adjacent hydroxyl groups, e.g. 4-halogeno-, 4-nitro- and 4-amino-catechols, adrenaline and noradrenaline. Pyrogallol and hydroxyquinol gave unstable colours. With monohydric and *m*- and *p*-dihydric phenols no colour was obtained. The colour,

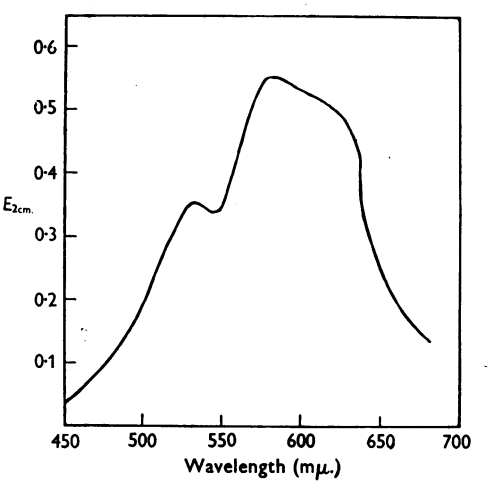


Fig. 1. The absorption spectrum of the blue colour obtained when $Co(NO_3)_2$ reacts with 4-chlorocatechol in the presence of NaOH. Solution prepared by adding 2 ml. of 0·025% aqueous 4-chlorocatechol to 2 ml. of 0·8% aqueous $Co(NO_3)_2.6H_2O$, then adding 4 ml. of 40% NaOH, mixing and centrifuging (see text).

however, is given by glucose, glucuronic acid and tartaric acid since these compounds contain vicinal hydroxyl groups. Glycollic, lactic and uric acids gave very pale colours but urea and furfural did not react. A pale-blue colour which fades completely in 5 min. is also given by phosphoric acid and phosphates.

*The effect of cobalt and alkali concentrations.* The maximum colour was obtained when 12–20 mg. $Co(NO_3)_2$/8 ml. was

used. The concentration of NaOH was important since it affected the blank solution. It was eventually found that using the optimum amounts of cobalt, a final concentration of 5N-NaOH was necessary to produce the maximum colour and the least coloured blank. KOH gives much less colour than NaOH (cf. Gordon & Schreyer, 1951) and whereas ammonia solution gave more highly coloured solutions than NaOH, it also produced more highly coloured blanks. Simultaneously with the production of colour there is a slight precipitation of $Co(OH)_3$ which tends to destroy the colour. The blue colour remained stable for more than 1 hr. if this precipitate was removed by centrifuging within 5 min.

*Preparation of the standard curve.* To a series of 10 × 1 cm. centrifuge tubes, there were added 0·2–2·0 ml. of 4-chlorocatechol solution (25 mg./100 ml. water) and the volume in each tube was made up to 2 ml. with water. Then to each tube 2 ml. of $Co(NO_3)_2$ solution (0·8%, w/v, $Co(NO_3)_2, 6H_2O$ in water) and 4 ml. of 40%, w/v, NaOH were added. The contents of the tubes were mixed by twice inverting and the solutions were centrifuged for 5 min. The extinction at 580 mμ. (slit width, 0·5 cm.) of each solution was then measured in 2 cm. cells in the Unicam S.P. 500 spectrophotometer against a blank solution prepared in the same way as the test solution but replacing the chlorocatechol solution by water. The standard curve was linear for concentrations of 4-chlorocatechol up to 30 μg./ml. A similar curve was obtained with catechol, 4-fluoro-, 4-chloro-, 4-bromo-, 4-iodo- and 4:5-dichloro-catechols. The Lambert-Beer Law was obeyed by each compound and the optical density of the cobalt colour was inversely proportional to the molecular weight of each catechol. From pure solutions the recovery of the catechols was 97–98%.

*Application of the method to urine.* Many substances occur in urine which give a blue colour with $Co(NO_3)_2$ under the conditions described above, notably glucuronic acid which gives a very intense colour. A solution of glucurone, even after heating at 110° for 3 hr. with 3·3N-HCl, still gives an intense colour. The reaction could not, therefore, be applied directly to urine. However, the catechols involved in the present investigation are ether soluble, whereas the interfering substances are not. Total catechols (i.e. free and conjugated) in urine could, therefore, be readily estimated if the urine was first hydrolysed and the catechols then extracted with ether. Free catechols in urine, however, could not be estimated by this method, since certain conjugates of glucuronic acid which also give a blue colour with $Co(NO_3)_2$, e.g. catechol glucuronides, are soluble in ether.

*The recovery of chlorocatechol from urine.* The 24 hr. normal urine from a rabbit was filtered and made up to 100 ml. with water. 4-Chlorocatechol (about 5 mg.) was added to 10 ml. of the diluted urine which was then boiled under reflux condenser on a sand bath for 3 hr. with 5 ml. of conc. HCl. Simultaneously a second 10 ml. of diluted urine without added chlorocatechol was treated in the same way, to provide the blank value for normal urine. After cooling, the solutions were continuously extracted with ether for 4 hr. (This period of extraction was determined in separate experiments.) The ether extracts were evaporated and the residues dissolved in water, filtered and made up to 25 ml. The determination of catechol was then carried out as above on 2 ml. of solution. The recovery of 30–50 mg. 4-chlorocatechol/100 ml. was 96–98%. The blank value was usually small and in thirty-two experiments it varied from 0·75 to 7·1 mg./day (average 3·8 mg.) calculated as catechol.

## CATECHOL FORMATION FROM VARIOUS BENZENE DERIVATIVES

### *Materials and methods*

*Reference compounds.* 4-Chlorocatechol, m.p. 80°, and 4:5-dichlorocatechol, m.p. 105°, were prepared according to Peratoner (1898); 4-fluorocatechol, m.p. 83°, according to Corse & Ingraham (1951); 4-bromocatechol, m.p. 85°, according to Rosenmund & Kuhnhenn (1923) and 4-iodocatechol, m.p. 92°, according to Fourneau & Druey (1934). When 4-iodocatechol in aqueous solution is heated with mineral acids, it is deiodinated to yield catechol and this had to be kept in mind during our experiments on iodobenzene. *o-* and *p*-Chloro-, *p*-bromo-, *o-* and *p*-iodo-phenols were purchased (British Drug Houses Ltd.); *o-*, *m-* and *p*-fluoro-, *m*-chloro-, *o-* and *m*-bromo- and *m*-iodo-phenols were prepared by methods quoted in the literature. These compounds were purified by crystallization and chromatography of ethereal solutions on alumina, and m.p.'s and b.p.'s were checked with the literature.

The following new compounds were prepared by standard methods: *4-fluorocatechol ditoluene-*p*-sulphonate*, m.p. 143°, colourless prisms from ethanol. (Found: C, 55·6; H, 4·1; S, 14·1. $C_{20}H_{17}O_6S_2F$ requires C, 55·1; H, 3·9; S, 14·7%.) *4:5-Dichlorocatechol ditoluene-*p*-sulphonate*, m.p. 201°, prisms from ethanol. (Found: C, 49·7; H, 3·7; S, 12·7. $C_{20}H_{16}O_6S_2Cl_2$ requires C, 49·3; H, 3·3; S, 13·2%.) *4-Bromocatechol ditoluene-*p*-sulphonate*, m.p. 134°, prisms from ethanol. (Found: C, 48·1; H, 3·2; S, 12·4. $C_{20}H_{17}O_6S_2Br$ requires C, 48·3; H, 3·4; S, 12·9%.) *4-Iodocatechol ditoluene-*p*-sulphonate*, m.p. 141°, prisms from ethanol. (Found: C, 44·8; H, 3·2. $C_{20}H_{17}O_6S_2I$ requires C, 44·1; H, 3·1%.) Since the last ester was not easily purified it was more convenient to use *4-iodocatechol dibenzoate*, m.p. 130°, needles from ethanol:ethyl acetate, as a reference compound. (Found: C, 54·1; H, 3·2. $C_{20}H_{13}O_4I$ requires C, 54·1; H, 2·9%.)

*Paper chromatography of the monohalogenophenols.* Johnson, Stein & Weiss (1951) have shown that the separation by paper chromatography of the isomeric monochlorophenols themselves was not possible. This we can confirm. They, however, achieved separation after coupling the phenols with diazotized sulphanilic acid. This method can be applied to the fluoro-, bromo- and iodo-phenols. Johnson *et al.* (1951) applied their coupled phenols to the paper in *iso*butanol, but in the present work ether was used. Ether solutions or extracts of the phenols (varying quantities up to 5 mg. were used) were evaporated to dryness and the residue coupled at 0° with diazotized sulphanilic acid. The product was extracted with ether. The latter extract was then placed drop by drop at the starting point of the chromatogram (Whatman no. 1 paper) and the ether allowed to evaporate. In this way varying quantities of the coupled phenols could be used. The chromatograms were run in the solvent described by Johnson *et al.* (1951). With small quantities of the phenols, the *o*-isomers gave two spots, the *p-* and *m-* one spot each as described by Johnson *et al.* for the chlorophenols. With larger quantities of phenols, the *o*-phenols gave up to five spots, the *p-* four spots and the *m*-phenols usually one but very occasionally two spots. With the bromophenols, for example, the $R_F$ values of the spots in a chromatogram run for 16 hr. were: ortho, 0·24 (orange-yellow), 0·39, 0·58, 0·70 (yellow) and 0·75 (blue); meta, 0·33 (orange-yellow); para, 0·63 (yellow), 0·69, 0·78 and 0·84 (orange-yellow). All the *o*-phenols gave the fast-running

blue spot when they were used in larger quantities. Several control tests were carried out to eliminate possible artifacts. With the *o*- and *p*-phenols the spots were not of the same intensity, since the number which appeared depended on the amount of coupled phenol put on the paper initially. We cannot explain the multiplicity of spots from the authentic *o*- and *p*-phenols which were purified by chromatography, except to suggest, as did Johnson *et al.* (1951), that they may be due to coupling in more than one position at the same time and separately. The *o*- and *p*-phenols were thus readily distinguished from the *m*-phenols. A very rough idea of the amounts of *o*- and *p*-phenols could be made from the size and number of the spots formed.

The urines after feeding the fluoro-, chloro- and bromobenzenes were hydrolysed with 0·5 vol. conc. HCl for 3 hr. In the case of iodobenzene the urine only received mild acid hydrolysis to avoid possible de-iodination of iodophenols by the stronger acid. The hydrolysed urines were then exhaustively extracted with ether, and the extracts evaporated to dryness at room temperature to avoid loss of volatile phenols. The residues were extracted with cold water to remove catechols and the insoluble material was redissolved in ether, filtered and evaporated as before. The residue was then coupled at 0° with diazotized sulphanilic acid, the product extracted with ether and the extract chromatographed as above.

*Animals.* Chinchilla doe rabbits (2–3 kg. wt.) were kept on a constant diet of 70 g. rat cubes/day ('Diet 41'; Associated London Flour Millers Ltd.). All compounds were administered orally, and the urine from each animal was collected and analysed daily for catechol as above.

## RESULTS

The results of the quantitative experiments are given in Table 1. Qualitative tests showed that the catechols were excreted mainly in the conjugated form (cf. Smith *et al.* 1950). Fig. 2 shows the rate of elimination of catechols after single doses of halogenobenzenes. Up to 4 days may be necessary before catechol excretion ceases, although the bulk is excreted on the first 2 days. The peak excretion of catechols occurred most frequently on the first day but sometimes on the second day after feeding. In the case of *o*-dichlorobenzene, the peak excretion always occurred on the second day; the fate of the dichlorobenzenes, however, will be discussed in a future paper. With the phenols, increased catechol excretion only occurred for one day after dosing, and never on subsequent days.

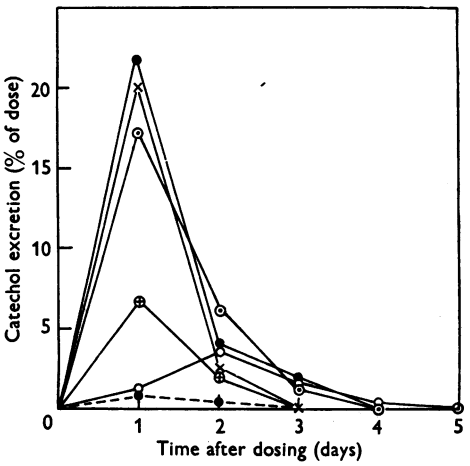


Fig. 2. The excretion of catechols by rabbit no. 17 after dosing with benzene and halogenobenzenes. ●- - -●, Benzene; ●—●, bromobenzene; ×—×, iodobenzene (rabbit no. 21); ⊙—⊙, chlorobenzene; ⊕—⊕, fluorobenzene; ○—○, *o*-dichlorobenzene.

### *Qualitative experiments*

*General treatment of urines.* The urines were collected for 48 hr. The free phenols were removed from the urines by continuous extraction with ether for 10 hr. at pH 5. Phenols present as ethereal sulphates were similarly extracted with ether for 12 hr., after the residual urine had been made 0·5 N with respect to HCl and heated for 0·5 hr. at 100°. The phenols present as glucuronides were similarly extracted after the residual urine had been made 3 N with respect to HCl and boiled for 3 hr. at 100°. Each ethereal extract was dried over anhydrous $Na_2SO_4$ and after removal of the ether, the gummy residues were further fractionated into fractions soluble in cold water (mainly catechols) and soluble in hot water (monophenols and mercapturic acids).

Table 1. *Excretion of catechols by rabbits receiving oral doses (0·5 g./kg.) of halogenobenzenes and chlorophenols*

| Compound fed | Period of excretion of catechols (days) | Catechol excretion (% of dose) | | | Av. |
|---|---|---|---|---|---|
| Benzene | 2 | 1·7 | 2·5 | 3·2 | 2·5 |
| Fluorobenzene | 2 | 8·8 | 12·8 | 13·7 | 11·8 |
| Chlorobenzene | 3 | 24·7 | 27·8 | 28·8 | 27·1 |
| Bromobenzene | 4 | 27·9 | 28·0 | 28·7 | 28·2 |
| Iodobenzene | 2 | 18·2 | 21·3 | 22·9 | 20·8 |
| *o*-Dichlorobenzene | 3–4 | 7·0 | 7·7 | 8·6 | 7·8 |
| *p*-Chlorophenol | 1 | 1·4 | 1·7 | 3·2 | 2·1 |
| *m*-Chlorophenol | 1 | 4·4 | 4·7 | — | 4·5 |
| *o*-Chlorophenol | 1 | 1·3* | 1·5* | 1·7* | 1·5 |
| Phenol | 1 | 0·4* | 0·5† | 0·8* | 0·6 |

* Dose 0·3 g./kg. † Dose 60 mg./kg.

*Fluorobenzene.* The free-phenol fraction from the urine of three rabbits which had received collectively 4 g. of fluorobenzene weighed 150 mg. Colour tests showed that it contained no fluorocatechol (e.g. no green colour with $FeCl_3$), but probably some monofluorophenols (blue colour with 2:6-dichloroquinone chloroimide at pH 8), but crystalline compounds were not isolated. The ethereal sulphate fraction (180 mg.) yielded, after esterification with toluene-*p*-sulphonyl chloride, 4-fluorocatechol ditoluene-*p*-sulphonate (10 mg.), m.p. and mixed m.p. 143°. The glucuronide fraction (1·6 g.) also yielded the same ditoluene-*p*-sulphonate (65 mg.), m.p. and mixed m.p. 142–143°. (Found: C, 55·9; H, 4·1%.)

*Bromobenzene.* From the urine of a rabbit which had received 3 g. of bromobenzene, the free-phenol fraction (100 mg.) yielded 64 mg. of material soluble in cold water containing 4-bromocatechol. A few mg. of 4-bromocatechol ditoluene-*p*-sulphonate (m.p. 128°) were isolated. The fraction insoluble in cold water also yielded a few mg. of *p*-bromophenylmercapturic acid, m.p. 148° (authentic m.p. 153°) and gave colour tests for monobromophenols none of which were crystallized. The part of the ethereal sulphate fraction soluble in cold water (720 mg.) was precipitated with basic lead acetate at pH 9. Removal of the lead with $H_2S$ yielded a solution of 4-bromocatechol from which 298 mg. (3·1% of dose) of the ditoluene-*p*-sulphonate was isolated, m.p. and mixed m.p. 134°. *p*-Bromophenylmercapturic acid (35 mg., m.p. 148°, mixed m.p. 149°) was isolated from the part insoluble in cold water. The glucuronide fraction (1·225 g.) also yielded 4-bromocatechol ditoluene-*p*-sulphonate (310 mg. or 3·2% of dose), m.p. and mixed m.p. 134° (Found: C, 48·6; H, 3·8%); and *p*-bromophenylmercapturic acid (25 mg.), m.p. and mixed m.p. 150°. Monobromophenols were present in all fractions but none could be isolated.

*Iodobenzene.* Iodobenzene (4 g.) was fed to a rabbit and a 96 hr. urine collected. The urine gave a deep-green colour with $FeCl_3$ and a strong naphthoresorcinol test. The free phenol fraction (930 mg.) yielded no crystalline material, but colour tests indicated the presence of a catechol derivative (green colour with $FeCl_3$) and of *p*-iodophenol (blue colour slowly developing at pH 10 with 2:6-dichloroquinone chloroimide after removal of the catechol with basic lead acetate). The ethereal sulphate fraction (4 g.) gave a strongly positive naphthoresorcinol reaction as well as a green catechol reaction with $FeCl_3$. An ether-soluble glucuronide had thus been extracted. The part of this fraction soluble in cold water was extracted with $CHCl_3$ and from this 590 mg. (5·5% of dose) of 4-iodocatechol ditoluene-*p*-sulphonate, m.p. and mixed m.p. 141° (Found: C, 44·6; H, 3·2%) was isolated after esterification. 4-Iodocatechol was also identified as the dibenzoate, m.p. and mixed m.p. 130°. (Found: C, 54·4; H, 3·0%.) No other crystalline compound was isolated from this fraction.

The glucuronide fraction contained free iodine (equivalent to 5·5% of dose) which was probably derived from 4-iodocatechol during the hydrolysis in strongly acidic solution since the 600 mg. of this fraction soluble in cold water yielded, after lead-acetate precipitation and esterification, catechol ditoluene-*p*-sulphonate, m.p. and mixed m.p. with an authentic sample (m.p. 162–163°, Porteous & Williams, 1949) 162° (yield 240 mg. or 2·9% of dose).

*p*-Iodophenylmercapturic acid was isolated from the 2-day urine of three rabbits which had received 2 g. each of iodobenzene. The urine was made N with respect to HCl and extracted three times with 0·2 vol. of $CHCl_3$. The mercapturic acid was then transferred to 2N-$NaHCO_3$ and the bicarbonate extract washed once with ether. The solution was acidified with HCl and the mercapturic acid extracted with $CHCl_3$ which on evaporation yielded 187 mg. of the acid (1·9% of dose). On recrystallization from water (charcoal) 66 mg. of pure *p*-iodophenylmercapturic acid were obtained, m.p. 154° and $[\alpha]_D^{22} - 18° \pm 2°$ in ethanol (*c*, 1). (Found: C, 36·6; H, 3·5; SH after alkaline hydrolysis, 8·9. Calc. for $C_{11}H_{12}O_3NSI$; C, 36·2; H, 3·3; SH, 9·0%.) Parrod (1948) records m.p. 150° and $[\alpha]_{5461} - 11·8°$ and Baumann & Schmitz (1895) m.p. 152–153° and $[\alpha]_D - 10·7°$ in ethanol (*c*, 2·5).

o-*Dichlorobenzene.* Three rabbits were each fed with 1·5 g. of *o*-dichlorobenzene and their urines collected for 24 hr. The pooled urines were boiled under reflux condenser for 3 hr. with 0·5 vol. of conc. HCl and the resulting solution exhaustively extracted with ether. The phenols in the ether were transferred to 20 ml. of 2N-NaOH and the alkaline solution then shaken with 1 g. of toluene-*p*-sulphonyl chloride in 20 ml. of acetone. On dilution with water and fractional crystallization of the product from ethanol, there was obtained 4:5-dichlorocatechol ditoluene-*p*-sulphonate (180 mg.), m.p. and mixed m.p. 201° (Found: C, 49·1; H, 3·6%), and an unidentified ester (260 mg.), m.p. 98°.

That *p*-chlorophenol is a minor metabolite of chlorobenzene was shown by Smith *et al.* (1950) by isolation. This has now been confirmed by paper chromatography and it has also been shown that the other three monohalogenobenzenes form the corresponding *p*-halogenophenols. It was shown (p. 147) that the *o*-phenols, after coupling with diazotized sulphanilic acid, produce a fast-moving blue spot, whereas the *m*- and *p*-isomers do not. This spot was obtained with all halogenobenzene urines when the extracts were used in appropriate amounts. As far as the chromatographic method goes it appears that *o*-phenols are formed in very small amounts. No *m*-halogenophenols were detected in any of the four urines. By comparing the size and number of spots obtained from urine extracts with those obtained with the authentic phenols it was possible to make a very rough estimate of the amounts of phenols present. This was about 2–3% of the dose for the *p*-isomer and not more than about 0·1% for the *o*-isomer.

## DISCUSSION

Chloro-, bromo- and iodo-benzenes each form in rabbits two major metabolites, namely the *p*-halogenomercapturic acid and the 4-halogenocatechol. They therefore differ from most other monosubstituted benzenes such as aniline, acetanilide, nitrobenzene, phenol and cyanobenzene which yield little or no mercapturic acid and only small amounts of the 4-substituted catechols. It was earlier suggested (Smith *et al.* 1950) that the formation of catechols may be connected with mercapturic acid formation. In fact, compounds which are *o*-dihydroxylated *in vivo*, e.g. naphthalene, anthracene, phenanthrene and chloro-, bromo- and iodo-benzenes also give rise to much mercapturic acid. In the case of the three halogenobenzenes mentioned above, catechol and mercapturic acid

formation each account for about 20–30 % of the dose metabolized (Table 2). With *o*-dichlorobenzene there again appears to be a correlation between these two types of metabolites, although each process only accounts for 8 % of the dose. The phenols in Table 1 do not form mercapturic acids and only form small amounts of catechols. However, fluorobenzene differs from all other monosubstituted benzenes in that it forms very little mercapturic acid (3 %), yet it is appreciably *o*-dihydroxylated (about 20 %, Table 2). This suggests that there is no correlation between mercapturic acid formation and *o*-dihydroxylation.

Table 2. *A comparison of the catechol and mercapturic acid outputs of rabbits dosed with various benzene derivatives*

(Values are expressed as percentage of the dose after correction for the amount of the compound which is eliminated unchanged in the expired air. The percentages of the dose eliminated in this way are benzene, 39; fluorobenzene, 44; chlorobenzene, 27; bromobenzene, 6; and iodobenzene, 3 (see Azouz, Parke & Williams, 1952). Data for mercapturic acid excretion are from the same paper.)

| Compound fed | Percentage of dose (corrected) excreted: As catechol derivatives | Percentage of dose (corrected) excreted: As mercapturic acids |
|---|---|---|
| Benzene | 4·1 | 1·3 |
| Fluorobenzene | 21 | 3 |
| Chlorobenzene | 37 | 28 |
| Bromobenzene | 30 | 22 |
| Iodobenzene | 21 | 23 |
| *o*-Dichlorobenzene | 8 | 8* |
| Phenol | 0·5 | 0† |

* Determined iodometrically by Stekol's (1936) method.
† Callow & Hele (1926).

The mechanism of these hydroxylations is at present unknown, but several hypotheses have been put forward (cf. Boyland, 1950). Boyland has suggested that simple addition of oxygen to a double bond to form an epoxide might be the initial reaction. Addition of water to the epoxide could yield a *trans*-dihydrodiol. The latter could then yield a monophenol by dehydration and a catechol by dehydrogenation. Several observations seem to support these possibilities. Epoxides are known to occur naturally, particularly in the carotenoids (cf. Goodwin, 1952) and, recently, heptachlor epoxide has been found as a metabolite of the insecticide heptachlor (a heptachlorotetrahydromethaneindene) in dogs (Radomski & Davidow, 1952). Furthermore, the known natural dihydrodiols of naphthalene, anthracene and phenanthrene (and probably the 3:4-dihydro-3:4-dihydroxychlorobenzene of Smith *et al.* 1950) are *trans*-compounds which would be expected if they were formed by hydration of an epoxide. Dihydrodiols are also readily dehydrated by acids to monophenols whose orientations agree with those found biologically, although Boyland & Wiltshire (1952) did not observe the conversion of 1:2-dihydronaphthalene-1:2-diol to 1-naphthol *in vivo*. Dehydrogenation of dihydrodiols to catechols, however, has not yet been observed chemically or biologically.

In earlier papers from this laboratory (Smith & Williams, 1950; Robinson *et al.* 1951; Smith, 1950) attention was drawn to the similarities between the orientation of biological and of free-radical hydroxylation of benzene derivatives. Recently, Johnson *et al.* (1951) have submitted chlorobenzene to the action of free hydroxyl radicals and found that *o*-, *m*- and *p*-chlorophenols were produced. These results appear to be different from the biological hydroxylation of chlorobenzene where the phenols produced are 4-chlorocatechol, *p*-chlorophenol and *o*-chlorophenol roughly in the ratio of 200–300 : 30 : 1. However, 4-chlorocatechol could be a hydroxylation product of both *m*- and *p*-chlorophenols. *p*-Chlorophenol does form 4-chlorocatechol (Spencer & Williams, 1950). The *m*-phenol probably does so also, but this has not been proved. The *m*-compound yields 2 to 3 times as much catechol as does the *p*-compound (see Table 1). The chlorophenols, however, are rapidly converted to glucuronides and ethereal sulphates (Spencer & Williams, 1950) which are readily excreted and are not likely to be hydroxylated to any large extent (cf. Bray, Humphris, Thorpe, White & Wood, 1952). These phenols may not reach, except in small quantities, the site of hydroxylation which may well be different from that of conjugation. However, the fact that, apart from conjugation reactions, these phenols are converted into catechols could indicate that the catechols are further hydroxylation products of *m*- and *p*-halogenophenols produced by initial hydroxylation of halogenobenzenes. This second hydroxylation would have to take place before conjugation of the monophenol, since conjugates once formed are not further metabolized (Garton & Williams, 1949). For this to happen, however, different sites for hydroxylation and conjugation must be postulated. On these grounds the phenolic metabolites of the halogenobenzenes could be formed by a mechanism similar to free-radical hydroxylation.

## SUMMARY

1. A spectrophotometric method for the determination of ether-soluble catechols in urine has been studied. The method depends on the measurement at 580 m$\mu$. of the blue colour formed between cobalt salts and catechol in the presence of alkali. The colour is given by many 1:2-dihydroxy compounds, including adrenaline.

2. This method has been applied to the determination of catechols formed during the metabolism of fluoro-, chloro-, bromo-, iodo- and *o*-dichlorobenzene in the rabbit. Between 20 and 30 % of the monohalogenobenzenes metabolized are converted into the corresponding 4-halogenocatechols which are excreted mainly in conjugated forms for 2–3 days after dosing. Some 8 % of *o*-dichlorobenzene is dihydroxylated. The isomeric monochlorophenols and phenol are also oxidized to catechols to a small extent (0·5–4 %).

3. The halogenobenzenes also form small amounts (about 2–3 %) of *p*-halogenophenols and traces of the *o*-phenols. These were detected mainly by paper chromatography.

4. The relation between mercapturic acid and *o*-dihydroxylation in these compounds is discussed, but no definite conclusions have been reached.

5. It is shown that the formation of catechols from the halogenobenzenes need not be contrary to the free radical hypothesis of biological hydroxylation, although other explanations are also feasible.

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# Studies in Detoxication

## 52. THE APPARENT DISSOCIATION CONSTANTS OF SOME GLUCURONIDES, MERCAPTURIC ACIDS AND RELATED COMPOUNDS

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Quick (1932) noted that the conjugated products formed during detoxication processes in the body were highly acidic, and suggested that the purpose of conjugation may not primarily be a detoxication mechanism in the sense of rendering a toxic group inert, but a means of converting a weak acid into a strong acid which can be readily eliminated. That conjugated compounds such as glucuronides, hippuric acids, arylsulphuric acids are strongly acidic seems to be generally true but the ionization constants of only a few of these compounds have been measured (cf. Williams, 1947).

## EXPERIMENTAL

*Compounds.* The glucuronides, mercapturic acids and phenylacetylglycine used had been prepared biologically from time to time in this laboratory. Their melting points and rotations were checked with the literature. The hippuric acids and ornithuric acid used were synthetic.

*Measurement of* $pK_a$. The apparent dissociation constants of the acids at 22° were determined in the usual manner from the potentiometric titration curves. Measurements of pH were made with a Cambridge pH meter using a glass electrode. The compound (15–30 mg.), dissolved in freshly boiled distilled water to give an approximately 0·001 M