DISCUSSION

The formation of cellulose from ethylene glycol by way of glycolaldehyde, as now described, is of interest principally from the fact that, so far as the authors are aware, it constitutes the first instance in which the transformation of this aldehyde to a higher carbohydrate has been established experimentally by use of living cells. The only previously recorded observation of the conversion of ethylene glycol to glycolaldehyde by a micro-organism appears to be that of Goepfert & Nord (1942) who isolated glycolaldehyde, in the form of its dimeric derivative, from a culture of a *Fusarium* species on a glycol medium. The bacterial conversion of glycerol, via dihydroxyacetone (or glyceraldehyde) to cellulose, is likewise of interest, since glyceraldehyde has not hitherto been identified as a metabolite in reactions leading to cellulose.

SUMMARY

1. A strain of *Acetobacter acetigenum*, isolated from East African vinegar, formed cellulose from

all of eighteen carbohydrates, or alcohols related to carbohydrates, which were submitted to its action.

2. By its ability to convert to cellulose the α - and β -methyl-D-glucosides, three pentoses, erythritol and ethylene glycol, respectively, the new organism showed itself to be enzymically more active than *Acetobacter xylinum* which, in previous studies of cellulose formation (Tarr & Hibbert, 1931), proved unable to utilize these substances for this purpose.

3. During the formation of cellulose from ethylene glycol and from glycerol there were formed glycolaldehyde and glyceraldehyde, respectively, and these were characterized as their osazones.

4. It was shown that *A. acetigenum* Henneberg, *A. pasteurianum* (an atypical strain) and *A. kützingianum* Hansen can each synthesize cellulose when grown on suitable media.

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Studies in Detoxication

37. METABOLISM OF BENZENE. EXAMINATION OF THE GLUCURONIDE FRACTION OF RABBIT URINE AFTER ADMINISTRATION OF BENZENE. ISOLATION OF PHENYLGLUCURONIDE

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In 1881 Schmiedeberg obtained a gum from the urine of dogs fed with benzene and concluded that because the gum gave phenol and tests for glucuronic acid, it contained phenylglucuronide, a compound which had not then been described and which was isolated for the first time by Külz in 1890 from the urine of rabbits fed with phenol.

No glucuronide has, hitherto, been isolated from urine after feeding benzene. The urine passed after feeding benzene will be referred to in this paper as benzene urine. Porteous & Williams (1949b) examined the ethereal sulphate fraction of benzene urine and found it to contain phenol, catechol, quinol and hydroxyquinol. We have now examined the

glucuronide fraction and find it to contain mainly phenol with only small amounts of polyphenols. Phenylglucuronide was isolated from this fraction as the crystalline amide and triacetyl methyl ester.

EXPERIMENTAL

Preparation of reference compounds

Phenylglucuronide methyl ester. Phenylglucuronide (m.p. 160°) was prepared by feeding rabbits with phenol (yield 6.1 g. from 5 g. phenol) according to Porteous & Williams (1949a). The glucuronide (4 g.) dissolved in methanol (20 ml.) was methylated with ethereal diazomethane. Evaporation of the solvent gave β -phenyl-D-glucuronide methyl ester (3.9 g.) as a pale-yellow gum which was crystallized with great difficulty from ether-methanol. It formed colourless prisms m.p. 153° and $[\alpha]_D^{25} - 63^\circ \pm 1^\circ$ (c, 1 in methanol). (Found: OMe, 10.6. $C_{13}H_{16}O_7$ requires OMe, 10.9 %.) The compound readily decomposes on heating to about 80°, forming a dark acidic gum.

Triacetyl β -phenyl-D-glucuronide methyl ester. The above ester (2 g.) in 10 ml. pyridine was treated with acetic anhydride (8 ml.) at 5°. After keeping the mixture for an hour, it was poured into an excess of water whereby a pale-yellow viscous gum separated which crystallized on keeping at 0°. The crystals were dissolved in $CHCl_3$ (25 ml.) and the solution was washed several times with 2N-HCl and finally with water. After drying the solution over anhydrous Na_2SO_4 and then evaporating, the triacetyl methyl ester (2.8 g.) was obtained as a crystalline solid. Recrystallization from aqueous ethanol gave the compound as colourless needles (2.1 g.) m.p. 116° and $[\alpha]_D^{25} - 33^\circ \pm 0.5^\circ$ (c, 1 in $CHCl_3$), -32.5° (c, 1 in ethanol). (Found: OMe, 7.5. $C_{15}H_{22}O_{10}$ requires OMe, 7.6 %.)

Phenylglucuronidamide. The above triacetyl methyl ester (1 g.) was dissolved in methanol (10 ml.) and the solution saturated with dry NH_3 . After keeping for an hour, the solution was diluted with 50 ml. water and on standing a crystalline solid (340 mg.) separated. The β -phenyl-D-glucuronidamide was recrystallized from aqueous acetone giving colourless sheaves of needles, m.p. 256° (decomp.) and $[\alpha]_D^{25} - 70^\circ \pm 5^\circ$ (c, 0.1 in methanol). (Found: C, 53.8; H, 5.6; N, 5.0. $C_{12}H_{15}O_6N$ requires C, 53.5; H, 5.6; N, 5.2 %.) The amide was only sparingly soluble in hot water, methanol and ethanol, but readily soluble in hot aqueous acetone.

Triacetyl phenylglucuronidamide. The above amide (100 mg.) was suspended in pyridine (5 ml.) and treated with acetic anhydride (3 ml.) at 5°. The amide dissolved in 2 hr. at room temperature and then the mixture was poured into water (100 ml.). No crystals separated. The solution was extracted with $CHCl_3$ and the extract washed with 2N-HCl and water, and then dried with anhydrous Na_2SO_4 . Evaporation of the $CHCl_3$ left a crystalline solid which on recrystallization from 10 % aqueous ethanol gave the triacetyl amide as colourless needles, m.p. 158° and $[\alpha]_D^{25} - 33^\circ \pm 1^\circ$ (c, 1 in $CHCl_3$). (Found: C, 54.5; H, 5.3. $C_{18}H_{21}O_9N$ requires C, 54.7; H, 5.4 %.) The compound was readily soluble in ethanol but not in water.

Isolation of phenylglucuronide derivatives from benzene urine

The glucuronide gum. Five rabbits were fed a total of 50 g. of benzene in 6 days and the basic lead acetate precipitate of the urine (1.4 l.) was prepared in the usual manner.

After removal of the Pb with H_2S and concentration of the resulting solution, the glucuronide gum was obtained. The gum was dissolved in the minimum of water and the solution poured into ethanol (400 ml.). The precipitate formed was filtered off and the filtrate evaporated to dryness *in vacuo* at 30–35°. The residue was again dissolved in the minimum of water and poured into ethanol. In this way, the glucuronide gum was separated into an ethanol-soluble fraction (A) and an ethanol-insoluble fraction (B). The latter appeared to be an inorganic salt of the glucuronide. B was therefore dissolved in 20 ml. N- H_2SO_4 and poured into excess ethanol. The precipitate of inorganic sulphates was removed by filtration and the ethanolic solution of the glucuronide adjusted to pH 4 with Na_2CO_3 solution and again filtered. The filtrate was then evaporated *in vacuo* at 30–35° to a gum. The yield of purified glucuronide gums was A, 7.2 g. and B, 4.8 g. A contained no ethereal sulphate, but B contained small amounts.

Isolation of triacetylphenylglucuronide methyl ester. The above gums A and B were dissolved separately in the minimum quantity of methanol and methylated with an ethereal solution of diazomethane. After keeping overnight, the solution was filtered and the insoluble material dissolved in methanol and again methylated. The insoluble material left after the second methylation gave only a weak naphthoresorcinol reaction and was therefore discarded. Evaporation of the solvents gave the methylated glucuronide as pale-yellow gums, A yielding 6 g. and B 3.8 g. The two methylated gums were now separately acetylated in pyridine with 10 equiv. of acetic anhydride. After keeping overnight at 0°, the mixtures were poured into water. The semi-crystalline material which formed was filtered off, dissolved in $CHCl_3$ and washed with 2N-HCl followed by water. After drying over anhydrous Na_2SO_4 , the $CHCl_3$ was evaporated and the residue recrystallized from aqueous ethanol. Gum A yielded 0.85 g. of triacetylphenylglucuronide methyl ester, m.p. and mixed m.p. 116° and $[\alpha]_D^{25} - 33^\circ$ (c, 1 in ethanol) and gum B 1.1 g., m.p. 116°.

The residues from the isolation of the crystalline triacetyl methyl ester from both A and B were dissolved in methanol (20 ml.) and the solution saturated with ammonia at 0°. Gum A yielded 40 mg. of phenylglucuronidamide, m.p. and mixed m.p. 256°, and gum B 135 mg. On acetylation both these amides yielded triacetylphenylglucuronidamide, m.p. and mixed m.p. 158°.

The total yield of crystalline phenylglucuronide derivatives isolated from the glucuronide fraction of benzene urine corresponded to 1 % of the dose. No other glucuronide was found.

Examination of the glucuronide fraction for dihydrodi-hydroxybenzene. Direct ether extraction of benzene urine at pH 7 failed to yield any material which spectroscopically might be a benzene-diol. The glucuronide fraction from the urine of rabbits which had collectively received 54 g. of benzene was therefore prepared. It was purified by dissolution in water and pouring into ethanol as above. Insoluble inorganic material which was precipitated was discarded. The purified gum after drying *in vacuo* at room temperature had $[\alpha]_D^{25} - 48^\circ$ (c, 1 in ethanol). Spectroscopic examination indicated that it was mainly phenylglucuronide and there was no pronounced absorption in the 260 m μ . region which might be indicative of the presence of a hexa-1:3-diene derivative.

Hydrolysis of the 'benzene' glucuronide with β -glucuronidase. A preparation of β -glucuronidase from ox spleen, purified by

precipitation between the limits of 1.25 and 1.75 M-(NH₄)₂SO₄ was prepared. The activity of the preparation was determined according to Spencer & Williams (1951). The 'benzene' glucuronide gum (2 g.) was dissolved in 400 ml. of 0.2 M-acetate buffer of pH 5.2 and treated with 60 ml. of β -glucuronidase solution containing 22,500 *p*-chlorophenyl units (1 *p*-chlorophenyl unit of β -glucuronidase activity is defined as that amount of the enzyme which liberates 1 μ g. of *p*-chlorophenol from 1.5×10^{-2} M solution of *p*-chlorophenylglucuronide in 0.2 M-acetate buffer at pH 5.2 and 37° in 1 hr.). After incubation for 24 hr. a further 15,000 units (40 ml.) of enzyme extract was added and incubation continued for a further 3 days. The mixture was now adjusted to pH 7 with N-NaOH and continuously extracted with ether for 18 hr. Evaporation of the ether gave a dark residue consisting mainly of phenol. The phenol was removed by dissolving the residue in water (25 ml.) and evaporating. The phenol-free residue was shown by paper chromatography to contain catechol, quinol and hydroxyquinol. The ultraviolet absorption spectrum of the residue was the same before and after heating with N-HCl for 30 min. at 95°, and no phenol was produced by the acid treatment. Thus no evidence was obtained that a benzene-dihydrodiol occurred in the 'benzene' glucuronide.

Examination of the 'benzene' glucuronide by paper chromatography. The glucuronide gum from benzene urine often contained traces of ethereal sulphates which might give rise to the small amounts of catechol, quinol and hydroxyquinol found in the glucuronide gum. Accordingly, 2 g. of the glucuronide gum were heated with 25 ml. N-HCl at 95° for 30 min. to hydrolyse any ethereal sulphates present and then exhaustively extracted with ether. The extract was freed of phenol by evaporation and the residue chromatographed on paper using benzene (40), acetic acid (50) and water (10) as solvents (cf. Bray, Thorpe, & White 1950; for other solvents for separating phenols, see Bate-Smith, 1949; Evans, Parr & Evans, 1949). The chromatogram, on treatment with a solution of diazotized *p*-nitraniline and alkali according to Bray *et al.* (1950), showed the presence of catechol, quinol and hydroxyquinol, but not of resorcinol, pyrogallol or phloroglucinol. *R_F* values under our conditions and the colours given with diazotized *p*-nitraniline and alkali are quoted in Table 1. The residual glucuronide solution was

chromatograms it was concluded that the benzene glucuronide contained roughly 1 % of each of catechol and quinol glucuronides and about 0.2 % of hydroxyquinol.

DISCUSSION

Porteous & Williams (1949*a, b*) examined the ethereal sulphate fraction of benzene urine and proved that it contained phenol, and the polyphenols catechol, quinol and hydroxyquinol. Although phenol is a major component of this fraction, the polyphenols are also present in considerable amounts and probably constitute about a half of the total phenols of the ethereal sulphate fraction. Our present examination of the glucuronide fraction of benzene urine shows that it is almost entirely made up of phenylglucuronide, the polyphenols occurring only in very small amounts. There is therefore a considerable quantitative difference in the nature of the phenols of the ethereal sulphate and of the glucuronide fractions of benzene urine. At the moment there seems to be no clear explanation of this difference, unless we conclude that phenylsulphuric acid under some conditions is readily oxidized to polyphenols whereas phenylglucuronide is not. Garton & Williams (1949), however, found that neither of these compounds on injection into rabbits was appreciably oxidized to polyphenols. However, it does appear that the formation of polyphenols *in vivo* is in some way connected with sulphur compounds.

Porteous & Williams (1949*a*) suggested that dihydrodihydroxybenzene might be a precursor of some of the phenols of benzene urine. Although a similar compound has been isolated from chlorobenzene urine (Smith, Spencer & Williams, 1950), we have been unable to detect it in benzene urine. Attempts to synthesize 1:2-dihydro-1:2-dihydroxybenzene in this laboratory have been unsuccessful.

SUMMARY

1. The glucuronide fraction from the urine of rabbits dosed with benzene has been examined and shown to be almost entirely phenylglucuronide which was isolated as its crystalline triacetyl methyl ester.

2. Detailed examination of the glucuronide fraction using β -glucuronidase and paper chromatography showed that this fraction contained only very small amounts of the polyphenols, catechol, quinol and hydroxyquinol. The dihydrodiol of benzene was not detected.

3. The methyl ester, triacetyl methyl ester, amide and triacetyl amide have been prepared from phenylglucuronide.

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Table 1. *R_F* values and colour reactions of the di- and tri-hydroxybenzenes

(Solvent benzene (40), acetic acid (50) and water (10); chromatograms run for 15 hr. on Whatman no. 1 paper.)

Compound	<i>R_F</i> value	Colour given with diazotized <i>p</i> -nitraniline and Na ₂ CO ₃
Catechol	0.68	Violet-green
Resorcinol	0.67	Orange
Quinol	0.66	Grey-green
Pyrogallol	0.60	Brown-pink
Hydroxyquinol	0.56	Violet
Phloroglucinol	0.50	Orange-brown

now made 5 N with respect to HCl and heated for 1 hr. at 95° to hydrolyse glucuronides. The extraction with ether, separation of phenol and paper chromatography was repeated as above, and again catechol, quinol and hydroxyquinol were detected. By colour reactions in solution and on paper

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Studies in Detoxication

38. THE METABOLISM OF BENZENE. (a) THE DETERMINATION OF PHENYLMERCAPTURIC ACID IN URINE. (b) MERCAPTURIC ACID EXCRETION BY RABBITS RECEIVING BENZENE

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We have shown that some 60 % of a dose of benzene administered to rabbits can be accounted for by oxidation to phenols (Porteous & Williams, 1949*a, b*) and by elimination in the unchanged state (Parke & Williams, 1950). Benzene, however, is also excreted as phenylmercapturic acid and Zbarsky & Young (1943) have proved this by the isolation of the mercapturic acid in a maximum yield of 0.37 % of the dose from the urine of rats dosed with benzene. Although Witter (1945) failed to isolate phenylmercapturic acid from the urine of rabbits receiving benzene, Drummond & Finar (1937) have suggested that these animals also excrete this conjugate. In the present paper we shall show that rabbits do, in fact, excrete phenylmercapturic acid and that, determined by the quantitative methods developed here, the amount excreted is about 1 % of the dose.

EXPERIMENTAL

Preparation of phenyl-L-cysteine derivatives. Phenyl-L-cysteine has been synthesized by Zbarsky & Young (1943), but we were unable to reproduce their conditions exactly. The present method has produced rather better yields. L-Cystine (5 g.) was reduced by boiling for 2 hr. in 100 ml. $\text{N-H}_2\text{SO}_4$ with 3 g. of Zn dust, a few pieces (total, 1 g.) of granulated Zn (A.R.) being added periodically. The solution was filtered hot and, in order to facilitate the formation of the cuprous mercaptide, 1 ml. of conc. HCl was added (cf. Pirie, 1931). The hot solution was now treated with freshly precipitated Cu_2O (3.5 g. in 20 ml. water) which slowly dissolved on shaking to give a green solution. When the addition of the oxide is near completion the mercaptide may tend to come out of solution as a white precipitate, but this can readily be redissolved by adding a little $2\text{N-H}_2\text{SO}_4$. Excess Cu_2O should be avoided, since it tends to co-precipitate

with the mercaptide as a dark-brown spongy solid. To the solution of cuprous mercaptide cooled to 0° , there was added a solution of diazotized aniline (8 g.) in $2\text{N-H}_2\text{SO}_4$ (120 ml.). A brisk evolution of N_2 occurred with the formation of a buff precipitate. After 30 min. at 0° , the solution was heated at $60-70^\circ$ for 30 min., filtered and extracted with ether (50 ml.) to remove phenol. The resulting green solution was saturated with H_2S to remove Cu and, after filtration of the copper sulphide, the solution was boiled *in vacuo* to remove excess H_2S . The solution was then neutralized with conc. NH_3 solution followed by glacial acetic acid (5 ml.) and after keeping at 0° overnight, the precipitated phenyl-L-cysteine was collected by filtration. At this stage, the compound was heavily contaminated with cystine which was largely removed by repeated dissolution in N-NaOH (charcoal) and precipitation with 2N-acetic acid . The material was finally recrystallized from 50 % aqueous ethanol in which cystine is very sparingly soluble (Clarke & Inouye, 1931). The product (2.2 g.) was obtained as white glistening plates, m.p. $180-181^\circ$; $[\alpha]_D^{25} + 10^\circ$ (c, 1 in 0.1N-NaOH). (Zbarsky & Young (1943) give m.p. $170-172^\circ$ and $[\alpha]_D^{25} + 11^\circ$ in 0.1N-NaOH .) A sample prepared by debromination and deacetylation of biosynthetic *p*-bromophenylmercapturic acid (Zbarsky & Young, 1943) had m.p. $180-181^\circ$ and $[\alpha]_D^{25} + 10^\circ$ (c, 1 in 0.1N-NaOH).

p-Bromophenyl-L-cysteine was similarly prepared from cystine (5 g.) and diazotized *p*-bromoaniline (7.5 g.) (yield, 2.6 g.). It formed colourless, glistening plates, m.p. 193° (not depressed by a sample, m.p. 194° , prepared by deacetylation of biosynthetic *p*-bromophenylmercapturic acid according to Baumann & Preusse, 1881), and $[\alpha]_D^{25} + 20^\circ$ (c, 1 in 0.1N-NaOH).

p-Chlorophenyl-L-cysteine. This compound has not been previously described, although the DL-form (m.p. 202°) has been synthesized by Behringer & Fackler (1949). It was prepared from cystine (5 g.) and diazotized *p*-chloraniline (5.5 g.) as described above for phenylcysteine. The yield was 2 g. after recrystallization from 50 % ethanol. It formed colourless glistening elongated plates, m.p. 190° , $[\alpha]_D^{25} + 18^\circ$