1,2-Dihydro-1,2-Dihydroxybenzene and Several Other Substances in the Metabolism of Benzene*

By TOKURO SATO, TOMITARO FUKUYAMA, TAEKO SUZUKI
and HARUHISA YOSHIMIWA*

(From the Department of Nutrition and Biochemistry, The Institute of Public Health, Tokyo and the Department of Physiological Chemistry and Nutrition, Faculty of Medicine, University of Tokyo, Tokyo)

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The ratio of catechol to phenol in the urine of rabbits dosed with benzene has been shown by Park and Williams (7) to be about 10 per cent. This is much larger than the ratio which has been shown to be about 0.5~1 per cent by giving phenol to the animals (2). This difference suggests that a greater part of catechol found in the urine of the animals dosed with benzene does not derive from phenol. Enzymatic aromatization of 1,2-dihydro-1,2-dihydroxybenzene (benzeneglycol) has been shown by Ayengar et al. (5) to occur in the rabbit-liver and the trans-form of the substance has been reported by Tomida et al. to be metabolized to catechol mainly and a small amount of unchanged substance, and the cis-form to catechol and a large amount of phenol in the rabbit (4).

In the present study cis- and trans-benzeneglycol have been studied for their detection by paper chromatography and for the metabolism of them in rat-livers. The urine of rabbits dosed with benzene has been studied and a small amount of trans-benzeneglycol and of a glucuronide, which produces trans-benzeneglycol by β-glucuronidase [EC 3.2.1.31] treatment, have been shown by paper chromatography and electrophoresis. Incubating benzene with rat-liver slices a substance which produces the phenylmercapturic acid precursor with rat-kidney slices has been found.

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EXPERIMENTAL

Paper Chromatography — This was carried out on Whatman no. 1 chromatography paper. Ascending chromatograms were developed with the following solvents: 1, butanol saturated with aqueous 2N NH₄ solution; 2, butanol-acetic acid-water (2: 1: 1, by vol.); 3, butanol-acetic acid-water (12:3:5, by vol.) for 15 hours; and with solvent aqueous 0.1N NH₄ solution for 5 hours. The dried chromatograms were examined under ultraviolet light and then treated with one of the following reagents: freshly diazotized p-nitroaniline (0.02% in N HCl) followed by aqueous 10% Na₂CO₃; 0.1M K₂Cr₂O₇-acetic acid (1: 1) followed by 0.1 M AgNO₃ (5); aqueous 2% (w/v) NaIO₄ followed, after 30 minutes, by Schiff's reagent (6); a solution of ninhydrin in butanol (0.4%) and heating the paper to 70°C for 10 minutes.

S-m-sulphate conjugates were detected by radioautography.

Paper Electrophoresis — This was conducted on Whatman no. 1 chromatography paper in 0.03 M borate, pH 10 at the room temperature, with use of 800 volts (20 volts per cm.) and currents between 6 and 20 mA. for 2 hours. This was a modification of the method by Hatanaka et al. (7), and cis-benzeneglycol migrated further than the trans-isomer from minus to positive pole.

Materials — Benzene was distilled before using. β-Glucuronidase [EC 3.2.1.31] (25,000 Fishman unit per g.) was obtained from N. B. C.

Benzeneglycols were gifts from Prof. Nakajima and Dr. Kurihara of Kyoto University. They were synthesized according to the method reported by Nakajima et al. (8, 9).

Method of Sulphate Conjugation — The conjugation were conducted as described previously (10), where three volumes of ethanol were used for deproteinization.
Animal Experiments—Six male rabbits (body wt. approx. 2.5 kg) were injected intraperitoneally with benzene (each 0.5 ml.) in olive oil (0.5 ml.). The urine was collected separately from the feces, pooled and stored at 0°C. The pooled urine was filtered, and activated charcoal (50 g.) was added with stirring. The charcoal was centrifuged off and washed with water (100 ml.) 3 times followed by 2 M NH₃ solution (100 ml.) 2 times. The methanol extracts were evaporated under reduced pressure at 40°C to about 3 ml. The residue thus obtained was extracted with three portions of freshly distilled ether (10 ml. each) for three times. The ether extracts were combined and examined by paper chromatography and electrophoresis. The residue was treated with β-glucuronidase (10 mg. in 1 ml. of 0.5 M phosphate buffer pH 7.4) and the supernatant of rat liver homogenate (0.3 ml.) for 15 hours at 37°C and extracted with ether as described above. Control experiments with the injection of olive oil (0.5 ml.) were run by the same way.

RESULTS

The Properties of Benzene Glycols—They are recognizable by dark absorption under ultraviolet light. The \( R_f \) values of the trans-form are 0.77 in Solvent 1 and 0.91 in Solvent 4. Those of the cis-form are 0.70 and 0.87 respectively. Two dimensional development using the two solvents separates the two isomers at the quantity of about 10 \( \mu \)g. The diazo reaction on paper chromatograms shows red colour which runs a little further from the spot. The spots turn white yellow with the KI-KI₃ Schiffs's reagent and are negative with the ninhydrin and the K₂Cr₂O₇-AgNO₃ reagent.

The Conjugation of Sulphate with Benzene Glycols in Rat Livers—trans-Benzene glycol was chromatographed using Solvent 1 and the dark absorbptive spot under ultraviolet light at \( R_f \) 0.77 was extracted with methanol. The methanol was evaporated under reduced pressure and the residue was used for the experiment.

About 100 \( \mu \)g. of the substance was incubated with shaking in Krebs-Ringer phosphate (pH 7.0) devoid of sulphate (0.2 ml.), containing rat-liver slices (0.1 g.) and SM-sulphate under oxygen at 37°C for 1 hour. The reaction mixture was deproteinized with ethylalcohol and paper chromatographed. The radioautographed film showed a faint spot of phenylsulphate \( (R_f \) 0.70 in Solvent 1 and 0.48 in Solvent 2), and an abundantly sensitized spot of catechol sulphate \( (R_f \) 0.49 in Solvent 1 and 0.46 in Solvent 2).

trans-Benzene glycol was treated with 0.1 N HCl at 100°C for 20 minutes and neutralized with 0.1 N NaOH before the incubation, and the reaction product was phenylsulphate.

trans-Benzene glycol was incubated in the supernatant of the homogenate (0.1 ml.), which was made with 4 volumes of KCl-Ringer-phosphate, pH 7.4, devoid of sulphate, at 60,000×g for 2 hours, with dipotassium salt of ATP (4 mg.) and SM-sulphate. The reaction products were as follows: a spot of phenylsulphate which showed a tailing towards the starting point in Solvent 1, a spot of catechol sulphate and another spot \((R_f \) 0.77 in Solvent 1 and \( R_f \) 0.77 in Solvent 2) which appeared also in the case of the experiments with liver slices though the quantity was small, and the nature of which remains unknown. On adding 100 \( \mu \)g. of NADP to the reaction mixture catechol sulphate was obtained mainly. The diol by hydrolysis with 0.1 N HCl for 1 hour at 100°C produced phenylsulphate after the incubation, and by partial hydrolysis the third substance mainly.

cis-Benzene glycol was experimented just the same way as described above. It produced phenylsulphate and a small quantity of catechol sulphate.

A Metabolite of Benzene in the Ether Extract of the Urine—The urine of rabbits dosed with benzene was treated with charcoal followed by methanol and ether as described in the experimental section. Metabolites in the ether extract were examined with paper chromatography using Solvent 1 and 4 and with paper electrophoresis followed by paper chromatography in Solvent 1. A spot coincident with trans-benzene glycol appeared with the two dimensional development, but cis-benzene glycol was not detected. Dark absorption under ultraviolet light and the colour reactions were
indistinguishable from those with the diol. The sulphate conjugates of the substance using rat liver slices and the supernatant of rat-liver homogenate were indistinguishable chromatographically from those of trans-benzene glycol before and after the acid treatment.

The quantity of the substance was calculated to be less than 1000 µg. from 3 g. of benzene by semiquantitation with the absorption under ultraviolet light of the reference substance, but this was not found in the control experiment using olive oil.

A Metabolite of Benzene in the Ether Extract of Urine after the Treatment with β-Glucuronidase—The residue, after the extraction with ether of the methanol extract, was treated with β-glucuronidase and reextracted with ether. This was examined by paper chromatography and by paper electrophoresis and showed a spot indistinguishable from trans-benzene glycol on the papers and by the sulphate conjugation techniques. This substance was not found in the control experiment and in the residue before treating with β-glucuronidase. The quantity was calculated to be about 500 µg. from 3 g. of benzene. cis-Benzene glycol was not detected. These observations suggest that there was a metabolite which liberated the trans-diol by β-glucuronidase.

The urine was treated with charcoal which was eluted with methanol containing aqueous NH₄ solution and the eluate was evaporated under reduced pressure. The residue was paper chromatographed in Solvent 1. A dark spot at 0.04 and a blue fluorescent spot under ultraviolet light at 0.11 were positive in glucuronide reaction with naphthoresorcinol. The spot at 0.04 was treated in NHCl for 10 minutes and rechromatographed. The spot at 0.04 disappeared and a spot at 0.11, which was positive in the glucuronide reaction, appeared. This suggests that the substance at 0.04 turned to the substance at 0.11, which liberated phenol on hydrolysis with 7 N HCl at 100°C for 3 hour. The substance at 0.04 was treated with β-glucuronidase (10 mg.) for 15 hours in phosphate buffer solution (pH 7.4) and extracted with ether. The ether extract contained a substance indistinguishable from trans-benzene glycol on papers by the properties described before. From these observations the substance at 0.04 appeared to be the glucuronide of trans-benzene glycol.

Metabolites Positive in the Reaction with the K₂Cr₂O₇-AgNO₃ Reagents—L-Phenylmercapturic acid was isolated from rabbits urine (11). It showed the Rₚ value of 0.32 in Solvent 1 and was positive in the reaction with the K₂Cr₂O₇-AgNO₃ reagents, which is used for organic sulphur test, and was negative with the ninhydrin reagent.

The eluate of charcoal treating freshly voided urine of rabbits dosed with benzene showed rather a faint spot at 0.32 but a strongly positive spot at 0.20. On standing the specimen for several days at room temperature the spot at 0.32 became strongly positive and the spot at 0.20 fainter. The spot at 0.20 was rechromatographed after the spot had been treated with the spray of 2 N HCl solution on the paper and a greater part of it turned to the spot at 0.32. The substance at 0.20 appeared to be the phenylmercapturic acid precursor that has been reported by Knight and Young (12). Both substances migrated with the solvent front in Solvents 2 and 3.

Slices of liver (approximately 20 g. wet weight) from male adult rabbits or rats were incubated in Krebs-Ringer phosphate (pH 7.4, 100 ml.) containing benzene (100 mg.), under oxygen for 2 hours at 37°C with shaking. The reaction mixture was treated with charcoal which was extracted with methanol containing aqueous NH₄ solution as reported before (13) and showed a spot, though the quantity was small, at 0.16 in Solvent 1 detecting by positive reaction with the ninhydrin reagent and with the K₂Cr₂O₇-AgNO₃ reagents. The spot was extracted with methanol and evaporated under reduced pressure. The residue was incubated with rat-kidney slices. The incubation showed a spot indistinguishable from the phenylmercapturic acid precursor, by paper chromatography in Solvent 1 tested by the colour reaction. The
substance at 0.16 appeared to be the glutathione compound of the epoxide of benzene that turned to phenylmercapturic acid precursor by kidney slices as reported in the case of the glutathione compounds of the epoxide of naphthalene (14) and the epoxide of 1,2-dihydronaphthalene (15).

**DISCUSSION**

In the present study the properties of benzeneglycols on paper chromatograms have been described together with the behaviour towards conjugation with sulphate in the rat-liver. The diols are reported rather stable under the experimental conditions (7). Phenyl sulphate, which appeared in the supernatant of liver homogenate with the diols, ATP and sulphate, will be explained by the conjugation of sulphate with the diols and subsequent spontaneous dehydration as suggested in the metabolism of 1,2-dihydro-1,2-dihydroxynaphthalene (15) and by the observation by Sims (16) in the chemically synthesized conjugate of the diol with sulphate. The tailing of phenyl sulphate towards the starting line on paper chromatograms appears to show the lability of the conjugate, some of which decomposes in the course of the development in Solvent 1. Catechol sulphate with the supernatant will be explained by the dehydrogenation of the diols by the dehydrogenase of diob, first reported by Ayengar et al. (3) with trace amount of phosphopyridine nucleotides as the cofactor. Nicotinamide-adenine dinucleotide as well as nicotinamide-adenine dinucleotide phosphate have been reported to work as the cofactor to the diol dehydrogenase from rabbit-livers; and the trans-isomers of 1,2-dihydroxy-1,2,3,4-tetrahydronaphthalene and indane-1,2-diol to be more easily dehydrogenated than the cis-isomers (17). These observations have been found adaptable to the enzymatic aromatization of benzeneglycols by the diol dehydrogenase from the livers of various kind of animals (7). The fact that trans-benzeneglycol produces more catechol sulphate than the cis-form appears to be explained by these observations. The nature of the third substance which moves faster than the other two substances in Solvent 1 is difficult to know but it might be the association product of the diols.

A substance which is indistinguishable chromatographically from trans-benzeneglycol has been found in the urine of rabbits dosed with benzene. A metabolite which produces trans-benzene glycol by β-glucuronidase and changes to phenylglucuronide by acid treatment has been concluded to be the glucuronide of trans-benzeneglycol. The abundance of catechol in the urine of the rabbit dosed with benzene compared with that dosed with phenol (1, 2) appears to be explained by the occurrence of trans-benzeneglycol, a part of which is eliminated as free or conjugated form, and a greater part of which as metabolites of catechol and phenol. The occurrence of the cis-form of the diol is difficult to know, but it was not detected at the present stage of the study.

The phenylmercapturic acid precursor has been shown on paper chromatograms. The precursor appears to be formed from the glutathione compound of the epoxide of benzene in the liver, that appears to be changed to the cysteine compound and acetylated in the kidney, as reported in the case of the metabolism of naphthalene (14) and 1,2-dihydronaphthalene (13).

The coexistence of the diol and the phenylmercapturic acid precursor which seems to derive from the glutathione compound of the epoxide of benzene appears to suggest the formation of the epoxide of benzene as the precursor of the substances. Both the trans- and cis-benzene glycol have been shown to produce no muconic acid in the rabbit and the trans-form to produce a small quantity of phenol (4). On the other hand phenol production from benzene has been reported in the microsome-NADPH-O system (18). These observations suggest that there exists a pathway, apart from the diol production, leading to phenol and muconic acid from benzene.

**SUMMARY**

1. Properties of trans- and cis-benzenegly-
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col have been studied by paper chromatography, paper electrophoresis, colour reactions and sulphate conjugating technique. The diols produce phenylsulphate, presumably through dehydration of the conjugate of them with sulphate in the supernatant of animal liver homogenate, and catechol sulphate. trans-Benzene glycol is more easily dehydrogenated to produce catechol sulphate than the cis-diol.

2. A substance indistinguishable chromatographically and electrophoretically from trans-benzeneglycol has been found in the urine of rabbits dosed with benzene.

3. A substance, which is a glucuronide, has been found. It produces a substance indistinguishable from trans-benzeneglycol by the treatment with β-glucuronidase and turns to a stable glucuronide producing phenol by acid hydrolysis.

4. The phenylmercapturic acid precursor has been shown by paper chromatography. Incubating benzene with animal livers, a substance, which produces the phenylmercapturic acid precursor in animal kidney slices, has been found.

5. These observations have been discussed in relation to the epoxide formation in the course of the metabolism of benzene in animal body.

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