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The Metabolism of Benzene by Bacteria

PURIFICATION AND SOME PROPERTIES OF THE ENZYME *CIS*-1,2-DIHYDROXY-CYCLOHEXA-3,5-DIENE (NICOTINAMIDE ADENINE DINUCLEOTIDE) OXIDOREDUCTASE (*CIS*-BENZENE GLYCOL DEHYDROGENASE)

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1. *cis*-Benzene glycol dehydrogenase was purified to a homogeneous state from a species of *Pseudomonas* grown with benzene as the major carbon source. 2. The enzyme was specific for the *cis*-isomer of its substrate and required NAD^+ as hydrogen acceptor. 3. Partial inactivation of the enzyme, which was observed during purification, could be reversed by the addition of Fe^{2+} and GSH. 4. A molecular weight of 440000 was calculated from data obtained by sedimentation-velocity and diffusion analysis in the ultracentrifuge. Sodium dodecyl sulphate polyacrylamide-gel electrophoresis indicated a subunit of molecular weight 110000. 5. *p*-Chloromercuribenzoic acid and 1,10-phenanthroline were shown to inhibit the enzyme.

With the object of purifying the bacterial enzyme systems that convert benzene into catechol, and determining their mode of action, samples of soil have been screened in our laboratories to isolate organisms that utilize benzene as their major source of carbon and energy. Several strains of *Pseudomonas* and *Arthrobacter* were obtained which grew with benzene added directly to the aqueous culture medium. From these organisms a *Pseudomonas* species was selected which yielded a cell-free system that was stable at 4°C for several weeks.

cis-1,2-Dihydroxycyclohexa-3,5-diene (*cis*-benzene glycol) has been identified as an intermediate in the metabolism of benzene by *Pseudomonas putida* (Gibson *et al.*, 1968). The dehydrogenase enzyme converting this intermediate into catechol was reported to be unstable. Reiner (1972) purified the analogous enzyme, 3,5-cyclohexadiene-1,2-diol-1-carboxylic acid (NAD^+) oxidoreductase (decarboxylating), to a homogeneous state at a high specific activity. We have studied some of the factors which determine the activity of *cis*-1,2-dihydroxycyclohexa-3,5-diene (NAD^+) oxidoreductase (*cis*-benzene glycol dehydrogenase) and have obtained preparations which appear to be essentially pure when examined by disc electrophoresis or in the analytical ultracentrifuge.

Materials and Methods

Isolation, maintenance and growth of the organism

The organism, identified as a *Pseudomonas* species, was isolated from a sample of refinery soil obtained in Holland by elective culture in a simple salts medium containing benzene as the major carbon source. Stock cultures were maintained on nutrient–agar slopes prepared in screw-cap bottles. The organism was subcultured monthly and stored at 4°C.

Aqueous culture medium was prepared, containing: 1g of $(NH_4)_2SO_4$ and 40mg of Bacto-peptone (Difco; from Baird and Tatlock Ltd., Chadwell Heath, Essex, U.K.) per litre of 25mM-potassium phosphate buffer, pH7.2. Concentrated solutions of $MgSO_4,7H_2O$ and $FeSO_4,7H_2O$ were autoclaved separately and added to the bulk of the medium at final concentrations of 400mg/l and 20mg/l respectively. Shake cultures also contained benzene (0.1%, v/v), which was retained in the culture vessels by rubber septum stoppers. These were autoclaved separately and inserted after all additions had been made to the culture flasks.

Large-scale growth of biomass was carried out in Biotec 10 litre fermenters (Biotec AB, Bromma, Sweden) containing 8.5 litres of medium. Air was introduced at a rate of 1 litre/min and the culture was stirred at 600rev./min. Temperature was maintained at 30°C and pH was controlled at 7.2 by the automatic addition of aq. NH_3 solution. Benzene was supplied in the vapour phase by means of a solenoid valve, which alternately switched the incoming air through a container of benzene or via a by-pass.

The benzene concentration was regulated by monitoring its presence in the outflow gas with a microkatharometer (type DK 223; Servomex Controls Ltd., Crowborough, Sussex, U.K.), after removal of CO_2 by passage through a column of soda-lime (BDH Carbosorb self-indicating granules). The output from the katharometer was applied

to a set-point meter controlling the switching of the solenoid valve in a proportional manner. The concentration of benzene in solution was determined initially by its extinction at 254nm, and related to the value selected on the set-point meter scale.

By using this system the maximum concentration of benzene in the culture medium could be maintained at a pre-selected value between 1 and 8mM, the normal concentration used being 2mM. Pressure in the fermenter was 13800N·m^{-2} above atmospheric, owing to the constricting effect of the katharometer on the outflowing gas. Benzene was absorbed after its detection by a column of molecular sieve (Union Carbide type 13X).

Medium in the fermenter was inoculated by the aseptic transfer of 300ml of shake-cultured cell suspension. After 22h the cells were harvested by using an Alfa-Laval centrifugal separator (Alfa-Laval Co. Ltd., Brentford, Middx., U.K.) before maximum cell density was reached. The fermenter vessel was then refilled with sterile medium and the procedure was repeated by using the residue from the previous culture as the inoculum.

Harvested cells were washed by resuspension in chilled 25mM-potassium phosphate buffer, pH7.4, and centrifuged at 4°C at 40000$g_{av.}$ for 30min yielding 15–20g wet wt. of cells per litre of culture medium. After being washed, the cells were stored at −22°C.

Preparation of cell-free extracts

Washed cells (100g) were thawed and resuspended in 100ml of buffer A (25mM-potassium phosphate containing 0.1mM-dithiothreitol, pH7.4). The suspension was cooled in ice and subjected to the maximum output from a Branson Soniprobe ultrasonic disruptor (Dawe Instruments Ltd., London W.3., U.K.). Batches (50ml) were treated for 6×30s each. The probe was cooled in ice between periods of use. Cell debris and unbroken cells were removed by centrifuging at 40000$g_{av.}$ for 1h. The supernatant solution, containing 70–80mg of protein/ml, was decanted. These and all following purification operations were performed between 0° and 4°C. All centrifuging was carried out in an MSE 10×100ml rotor. Fractions obtained during purification were stored at −22°C.

Standard assay

cis-Benzene glycol dehydrogenase activity was determined by using a Pye–Unicam SP.1800 recording spectrophotometer to measure the rate of NADH generation at 30°C, in a 10mm-light-path cuvette.

Reaction mixtures contained an appropriate amount of the enzyme or fraction, 0.7μmol of $FeSO_4$, 0.7μmol of GSH and 2μmol of NAD^+ diluted to a total of 7ml with 25mM-potassium phosphate buffer, pH7.4. The reaction was initiated by the addition of 0.3μmol of *cis*-benzene glycol to 3ml of this mixture. NADH generation was measured by the increase in extinction at 340nm compared with that of a further 3ml of the reaction mixture without substrate. The extinction of *cis*-benzene glycol at this wavelength and concentration was less than 0.001. One unit of activity was defined as the formation of 0.1μmol of NADH/min under these conditions.

Purification of cis-benzene glycol dehydrogenase

Protamine sulphate (0.5g/100g wet wt. of cells) was dissolved in the minimum volume of buffer A and added dropwise with constant stirring to the crude cell-free extract. The suspension was stirred for a further 15min and the precipitate was removed by centrifuging at 40000$g_{av.}$ for 30min. The supernatant was brought to 40% saturation with a saturated solution of $(NH_4)_2SO_4$, which had been adjusted to pH7.4 with aq. NH_3. After the mixture had been stirred for 15min the precipitate was collected by centrifuging at 40000$g_{av.}$ for 30min and was dissolved in the minimum volume of buffer A. Ammonium sulphate was removed by dialysis for 18h against 2 litres of buffer A (giving fraction III). Fraction III was applied to the top of a column (450mm^2×500mm) of DEAE-cellulose (Whatman DE-52; Whatman Biochemicals Ltd., Maidstone, Kent, U.K.) previously equilibrated with buffer A. Proteins were eluted by means of a 0–6% (w/v) linear KCl gradient in 500ml of buffer A. Fractions (9ml) were collected at 30min intervals and 20μl samples from each fraction were assayed for enzyme activity by the standard method. The enzyme was eluted as a single peak between 4%- and 5%-KCl (Fig. 1). Four to six fractions containing the highest specific activity were pooled and concentrated to 8ml by using a Diaflo ultrafiltration cell (Amicon, High Wycombe, Bucks., U.K.) with an XM-50 membrane (giving fraction IV).

Fraction IV was applied to the top of a column (600mm^2×770mm) of Sephadex G-200 (Pharmacia, Uppsala, Sweden) previously equilibrated with 100mM-potassium phosphate buffer containing 0.1mM-dithiothreitol, pH7.4, and was eluted with the same buffer. Fractions (9ml) were collected at 20min intervals and 20μl portions were assayed as above. *cis*-Benzene glycol dehydrogenase activity was found to coincide exactly with the second protein peak (Fig. 2) and four to six fractions of equal specific activity from the centre of this peak were pooled and concentrated to 6ml by ultrafiltration on an XM-50 membrane (giving fraction V).

Protein was measured by the modified biuret method of Layne (1957). A standard curve was prepared by using a previously dried sample of bovine

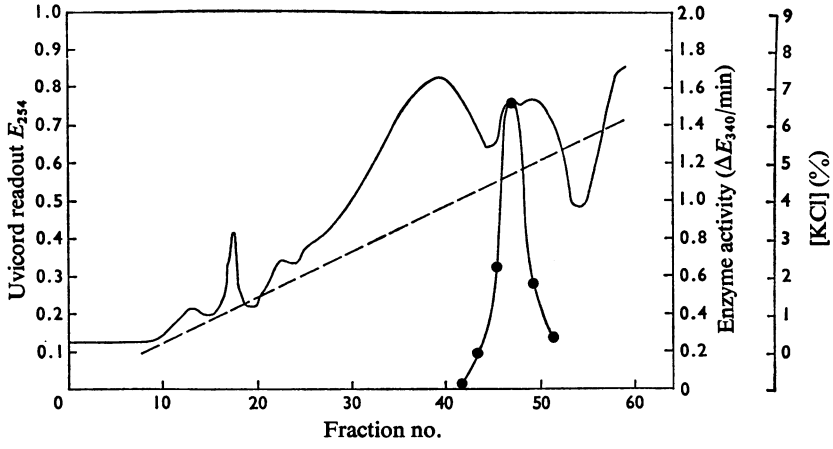


Fig. 1. *Elution of protein from DEAE-cellulose by using a linear* 0–6% (*w/v*) *KCl gradient in* 500 *ml of buffer A*

For details see the Materials and Methods section ●—●, *cis*-Benzene glycol dehydrogenase activity; ——, protein (E_{254}); ----, KCl gradient.

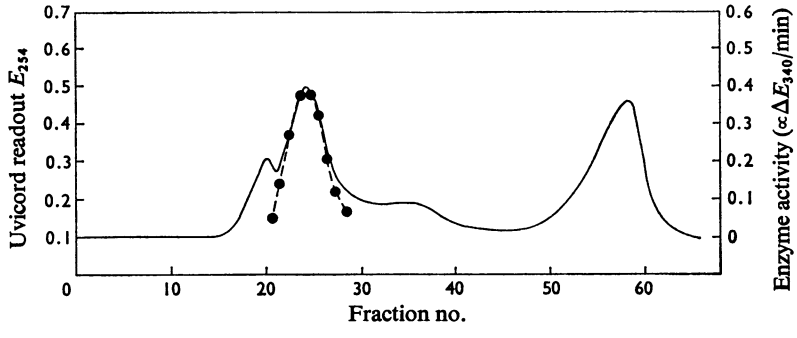


Fig. 2. *Sephadex G-200 chromatography of cis-benzene glycol dehydrogenase*

For details see the Materials and Methods section. ●---●, *cis*-Benzene glycol dehydrogenase activity; ——, protein (E_{254}).

serum albumin (Armour Pharmaceutical Co., Eastbourne, Sussex, U.K.). Details of the purification of *cis*-benzene glycol dehydrogenase are summarized in Table 1.

Ultracentrifuge measurements

A Beckman model E analytical ultracentrifuge (Beckman-RIIC Ltd., Glenrothes, Fife, U.K.) was used to obtain sedimentation-velocity and diffusion data. The sedimentation coefficient (*s*) was determined by using a double-sector cell and the diffusion coefficient (*D*) was calculated by the area–height method (Schachman, 1957) from results obtained at 6000 rev./min and 20°C by using a double-sector capillary synthetic-boundary cell. All measurements of photographic plates were made with a Projectorscope travelling microscope (Precision Grinding Ltd., Mitcham, Surrey, U.K.).

Polyacrylamide-gel electrophoresis

The purified enzyme (fraction V) was examined by polyacrylamide-disc-gel electrophoresis (Shandon Scientific Co. Ltd., London N.W.10, U.K.) by the method of Williams & Reisfeld (1964). Sample and large-pore gels were at pH 5.5 and the small-pore separating gel was at pH 7.5. Tris (1 g/l)–barbitone (5.52 g/l) buffer, pH 7.0, was used in both reservoirs. Electrophoresis was performed at room temperature with a constant current of 4mA/tube until the Bromophenol Blue marker was 5 mm from the lower

Table 1. *Summary of the purification of cis-benzene glycol dehydrogenase*

One unit of enzyme is the amount that catalyses the production of 0.1 μmol of NADH/min at 30°C and pH7.4. For details see the text.

	Stage	Vol. (ml)	Activity (units)	Protein (mg)	Specific activity (units/mg of protein)	Recovery (%)
I	Crude cell-free extract	108	72800	7780	9.3	100
II	Protamine sulphate supernatant	108	79000	7100	11.2	108
III	0–40%-satd.-$(NH_4)_2SO_4$ fraction (dialysed)	37	56500	1960	28.8	78
IV	Conc. DEAE-cellulose eluate	8	18200	168	108.0	25
V	Conc. Sephadex G-200 eluate	6	14300	75	191.0	20

end of the gel. Protein was stained by immersing the gels in 1% (w/v) Amido Black (George T. Gurr Ltd., London N.W.9, U.K.) in 7% (v/v) acetic acid for 1h. Excess of dye was removed by immersion in 7% (v/v) acetic acid for 48h. Relative band densities were measured with a Joyce-Loebl u.v. scanner (Joyce-Loebl and Co., Gateshead, Co. Durham, U.K.).

Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate was performed by the method of Weber & Osborn (1969). Except for the inclusion of 0.1% (w/v) sodium dodecyl sulphate the small-pore gel and buffer described above were used. Protein was stained with Coomassie Brilliant Blue (1.25g in 454ml of aq. 50%, v/v, methanol mixed with 46ml of acetic acid) and gels were destained electrophoretically. Standard marker proteins used for calibration purposes were; β-galactosidase, fumarase, bovine serum albumin, ovalbumin, L-amino acid oxidase and glutamic dehydrogenase.

Fe^{2+} determination

Fe^{2+} was determined by a modification of the 1,10-phenanthroline method of Harvey *et al.* (1955). For this 0.5ml of 3% (w/v) HCl was added to a suitable amount of protein in 1ml of 25mM-potassium phosphate buffer, pH7.4. The mixture was maintained at 80°C for 10min and the precipitated protein was removed by centrifuging for 10min at maximum speed in an MSE bench centrifuge. A portion (1ml) of the supernatant was withdrawn and mixed with 0.5ml of 200mM-potassium biphthalate and 1ml of 0.3% (w/v) 1,10-phenanthroline. The extinction was determined at 512nm after 30min at room temperature (approx. 20°C). A calibration curve was prepared by using a standard solution of $FeSO_4 \cdot (NH_4)_2SO_4$ (1mM) and was shown to be linear over the experimental range of 0–0.4 μmol of Fe^{2+}.

Materials

cis- and *trans*-Benzene glycol were prepared by the methods of Nakajima *et al.* (1956, 1959). Materials used for disc electrophoresis were supplied by Kodak Ltd., Kirkby, Liverpool, U.K. NAD^+, $NADP^+$, GSH, 1,10-phenanthroline, dithiothreitol, protamine sulphate, bovine serum albumin, L-amino acid oxidase, glutamic dehydrogenase and ovalbumin were from Sigma (London) Ltd., Kingston-upon-Thames, Surrey, U.K. *p*-Chloromercuribenzoic acid, catechol, FMN and FAD were from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K. β-Galactosidase and fumarase were from The Boehringer Corp. (London) Ltd., Ealing, London W.5, U.K. All other reagents were supplied by BDH Chemicals Ltd., Poole, Dorset, U.K., except where stated in the text.

Results

Properties of cis-benzene glycol dehydrogenase

Molecular weight. Schlieren patterns obtained with the enzyme during sedimentation are shown in Fig. 3. For a solution of 6.5mg of protein/ml in 100mM-potassium phosphate buffer, pH7.4, $s_{20,w}$ and $D_{20,w}$ were calculated as $11.21 \times 10^{-13} s^{-1}$ and $2.55 \times 10^{-7} cm^2 \cdot s^{-1}$ respectively. The molecular weight was calculated to be approx. 440000, assuming a partial specific volume of 0.749 (Svedberg & Pederson, 1940).

Polyacrylamide-gel electrophoresis. A single major band was obtained when a sample containing 100 μg of protein was subjected to disc electrophoresis (Fig. 4). Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate also showed essentially a single band of mobility 0.335. Comparison with standard molecular weight marker proteins indicated that the subunit of *cis*-benzene glycol dehydrogenase obtained under these conditions had a molecular weight of approx. 110000.

Activation by Fe^{2+}. A loss of enzyme activity (>50%) was experienced after $(NH_4)_2SO_4$ precipitation of the enzyme and subsequent dialysis. Neither FAD nor FMN would restore activity, and incubation of the enzyme with cysteine, GSH or mercaptoethanol was also ineffective. The addition of low concentrations of Fe^{2+} restored activity to the expected value,

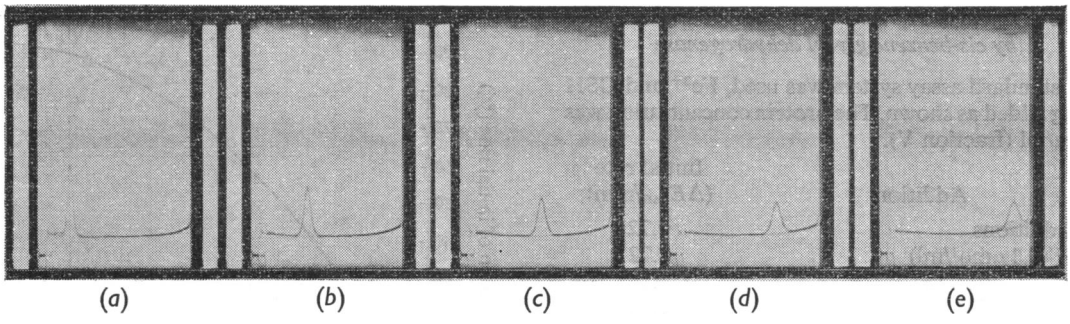


Fig. 3. *Ultracentrifuge pattern for cis-benzene glycol dehydrogenase (fraction V)*

The concentration was 6.5mg/ml in 100mM-potassium phosphate buffer, pH7.4. The photographs were taken at the following times (min) after the rotor had reached two-thirds of operating speed (56000rev./min): (*a*) 12; (*b*) 28; (*c*), 44; (*d*) 60; (*e*) 76. Temperature was 20°C. Sedimentation is from left to right.

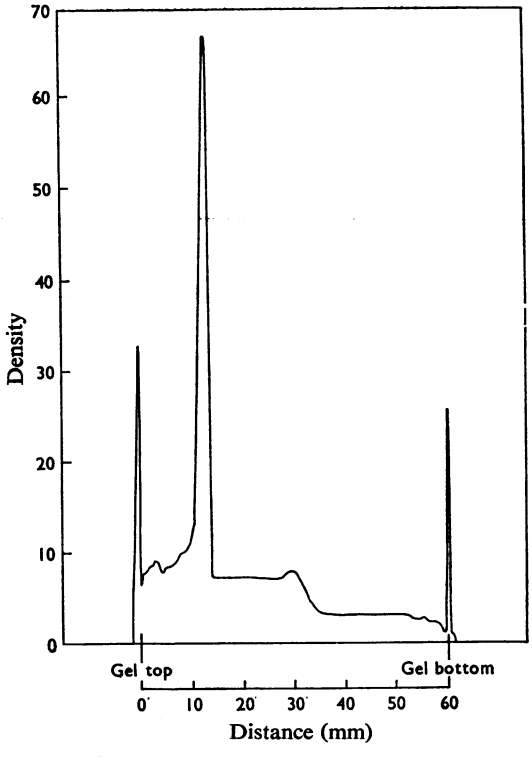


Fig. 4. *Polyacrylamide-gel electrophoresis of cis-benzene glycol dehydrogenase*

Densitometer trace obtained from a stained gel after electrophoresis of 100μg of enzyme (fraction V). For details see the Materials and Methods section.

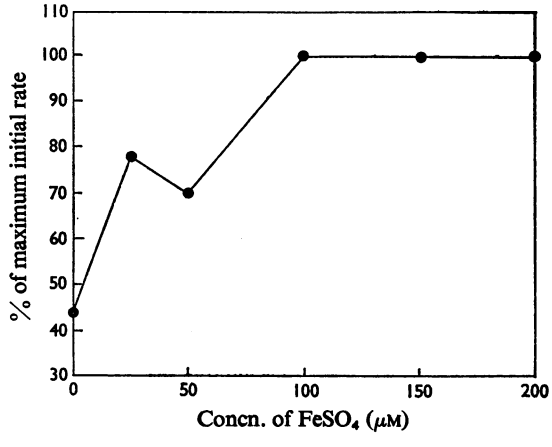


Fig. 5. *Reactivation of the enzyme by Fe^{2+}*

The standard assay was used, the concentration of Fe^{2+} being varied as shown. The protein concentration was 18μg/ml (fraction V).

allowance being made for normal losses in the procedure. Other metal ions tested were Fe^{3+}, Cu^{2+}, Zn^{2+}, Mg^{2+}, Mn^{2+} and Na^{+}, none of which was effective in restoring activity. The optimum Fe^{2+} concentration was 0.1mM in the reaction mixture. No inhibition was apparent up to 0.2mM-Fe^{2+} (Fig. 5). Maximum reactivation was obtained within 5min after the addition of Fe^{2+}, after which the activity declined to the original value over a period of 1h. The time from reactivation to addition of substrate was therefore kept to a minimum and maintained constant for any series of assays. In the presence of

Table 2. *Effect of Fe^{2+} and GSH on NADH production by cis-benzene glycol dehydrogenase*

The standard assay system was used, Fe^{2+} and GSH being added as shown. The protein concentration was 18 μg/ml (fraction V).

Addition	Initial rate (ΔE_{340}/min)
No additions	0.72
GSH (0.1 μmol/ml)	0.72
Fe^{2+} (0.1 μmol/ml)	1.56
Fe^{2+} and GSH (both 0.1 μmol/ml)	1.62

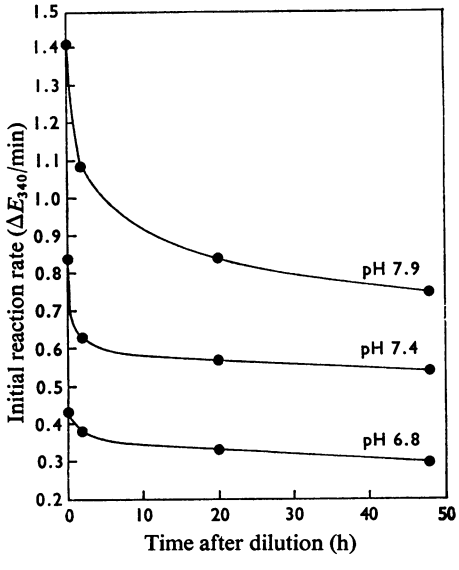


Fig. 6. *Effect of pH on stability of the enzyme at 4°C after dilution with buffer*

A solution of protein (fraction V, 30 μg/ml) was prepared in 25mM-potassium phosphate buffer at each pH value. At the intervals specified, samples were withdrawn and assayed by the standard method.

Fe^{2+}, GSH had a further small stimulatory effect and was always included in assays (Table 2). The residual Fe^{2+} present in purified enzyme preparations before reactivation (fraction V), determined by the 1,10-phenanthroline method (Harvey *et al.*, 1955), varied between 1 and 2mol of Fe^{2+}/mol of enzyme.

Stability. The enzyme was stable for several weeks when stored as a concentrated solution at 0–4°C or when frozen at −22°C. However, if the enzyme was diluted with buffer and stored at 0–4°C (with additions of NAD^+, Fe^{2+} and GSH just before assay)

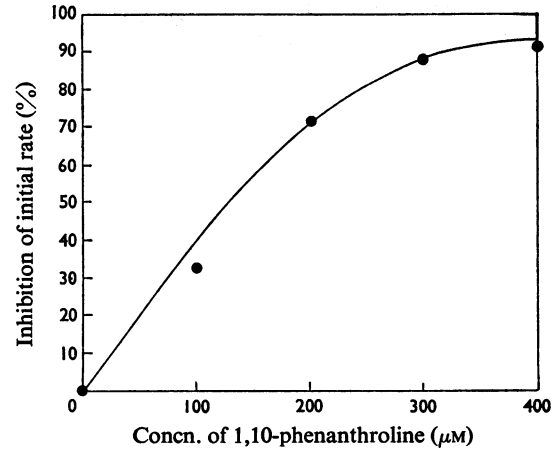


Fig. 7. *Inhibition of the enzyme by 1,10-phenanthroline*

The enzyme was diluted with 25mM-potassium phosphate buffer, pH7.4, and preincubated for 10min with the inhibitor at 30°C. Standard additions of GSH and NAD^+ were then made and the reaction was initiated with 0.3 μmol of *cis*-benzene glycol. The 1,10-phenanthroline concentrations are the final concentrations in the complete reaction mixture. The protein concentration was 18 μg/ml (fraction V).

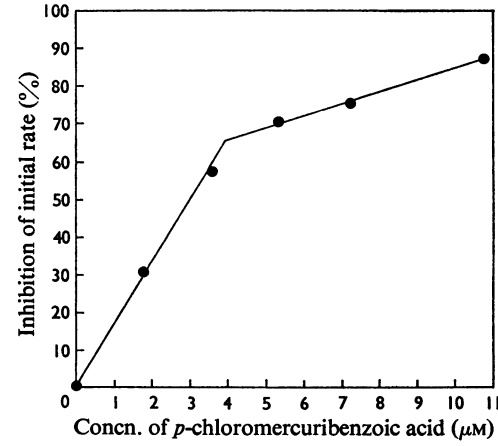
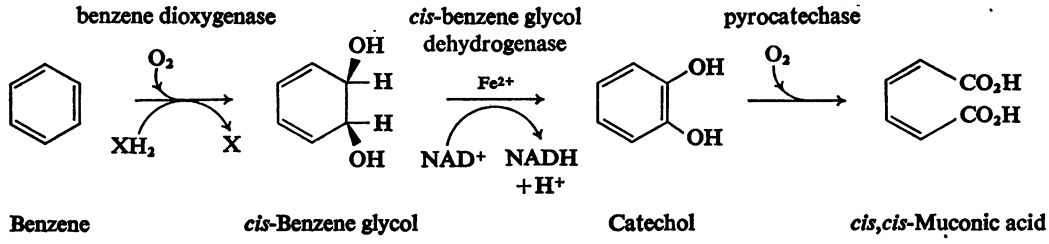


Fig. 8. *Inhibition of the enzyme by p-chloromercuribenzoic acid*

p-Chloromercuribenzoic acid was added to 25mM-potassium phosphate buffer, pH7.4, to achieve a final concentration in the reaction mixture as shown. The enzyme (fraction V, 18 μg/ml) was then added and assayed immediately by the standard method.



Scheme 1. *Intermediates in the conversion of benzene into cis,cis-muconic acid*

there was a sharp fall in activity within the first 5–10h followed by a slower linear loss of activity. After the initial loss of activity the greatest stability was obtained at pH7.4 (Fig. 6).

Effect of pH. The optimum pH for activity of the enzyme in 25mM-potassium phosphate buffer was pH7.9.

Specificity. The enzyme was specific for the *cis*-form of benzene glycol and showed no generation of NADH when incubated with *trans*-benzene glycol in the standard assay mixture. The *trans*-isomer, at substrate concentrations, did not inhibit the metabolism of the *cis*-isomer by the enzyme. $NADP^+$ would not substitute for NAD^+ as a hydrogen acceptor.

Spectrum. The absorption spectrum of the enzyme exhibited one maximum at 280nm and there was no significant absorption in the visible part of the spectrum.

Kinetic measurements. The enzyme preparation from fraction V, which was free of pyrocatechase activity, gave K_m values for *cis*-benzene glycol and NAD^+, determined from double-reciprocal plots (Lineweaver & Burk, 1934) of 0.286mM and 43.5μM respectively. At a protein concentration of 15μg/ml in the reaction mixture $V_{max.}$ was 1.54 (ΔE_{340}/min). One mol of NADH was generated for each mol of *cis*-benzene glycol converted into catechol under standard assay conditions.

Inhibition. The enzyme was progressively inhibited by increasing concentrations of 1,10-phenanthroline and *p*-chloromercuribenzoic acid (Figs. 7 and 8).

Fate of catechol. Gibson *et al.* (1968) confirmed that catechol was produced by the dehydrogenation of *cis*-benzene glycol by $(NH_4)_2SO_4$-treated extracts of *Pseudomonas putida*. The product of ring-fission of catechol in this organism was identified as 2-hydroxymuconic semi-aldehyde. We have obtained a stable preparation of pyrocatechase by concentrating fractions 38–41 (Fig. 1) from the DEAE-cellulose column by ultrafiltration with an XM-50 membrane. The product of ring-fission of catechol by this enzyme had an absorption spectrum characteristic of *cis,cis*-muconic acid, with a maximum at 258nm (Ornston & Stanier, 1966). The full reaction sequence from benzene, which we propose for the organism being studied, is shown in Scheme 1.

Discussion

The enzyme catalysing the conversion of *cis*-benzene glycol into catechol has been shown to consist of four apparently identical subunits. Fe^{2+} were essential for activity and were relatively loosely bound to the enzyme, being partially removed during $(NH_4)_2SO_4$ precipitation and subsequent dialysis, and chelated by low concentrations of 1,10-phenanthroline. Inhibition of the enzyme by *p*-chloromercuribenzoic acid suggests the involvement of thiol groups at the active site.

The enzyme investigated by Reiner (1972) was also shown to consist of four equivalent subunits of mol.wt. 24000. NAD^+ was the only cofactor required by this enzyme, although the author's evidence did not exclude the presence of a tightly bound bivalent cation.

The loose nature of the binding of Fe^{2+} to *cis*-benzene glycol dehydrogenase precluded accurate determination of the iron content of the fully reactivated enzyme. Purified enzyme from fraction V contained between 1 and 2 mol of Fe^{2+}/mol of enzyme, and from the increase in activity obtained on the addition of Fe^{2+} it may be postulated that the fully reactivated enzyme contained 4 mol of Fe^{2+}/mol (i.e. one Fe^{2+}/subunit of mol.wt. 110000).

Catechol formed during the metabolism of benzene in the organism used has been shown to be further oxidized to *cis,cis*-muconic acid. Ornston & Stanier (1966) have elucidated the pathway whereby this compound is converted into succinate and acetyl-CoA in a *Pseudomonas* species. The enzyme system catalysing the initial conversion of benzene into *cis*-benzene glycol remains to be characterized before bacterial utilization of benzene is fully understood.

We are grateful for the assistance of Dr. J. Van Eyk and Mr. J. R. Le Pelley of this laboratory, who isolated the organism used in these experiments. We also thank Mr. J. E. Hawes for the synthesis of *cis*- and *trans*-benzene glycol.

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