The Aromatic Alcohol Dehydrogenases in *Pseudomonas putida* N.C.I.B. 9869 Grown on 3,5-Xylenol and *p*-Cresol

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Whole cells of *Pseudomonas putida* N.C.I.B. 9869, when grown on either 3,5-xylenol or *p*-cresol, oxidized both *m*- and *p*-hydroxybenzyl alcohols. Two distinct NAD⁺-dependent *m*-hydroxybenzyl alcohol dehydrogenases were purified from cells grown on 3,5-xylenol. Each is active with a range of aromatic alcohols, including both *m*- and *p*-hydroxybenzyl alcohol, but differ in their relative rates with the various substrates. An NAD⁺-dependent alcohol dehydrogenase was also partially purified from *p*-cresol-grown cells. This too was active with both *m*- and *p*-hydroxybenzyl alcohol and other aromatic alcohols, but was not identical with either of the other two dehydrogenases. All three enzymes were unstable, but were stabilized by dithiothreitol and all were inhibited with *p*-chloromercuribenzoate. All were specific for NAD⁺ and each was shown to catalyse conversion of alcohol into aldehyde.

In the metabolism of both 3,5-xylenol and *p*-cresol by *Pseudomonas putida* N.C.I.B. 9869 there is oxidation of a methyl to a carboxy group before attack on the ring itself (Hopper & Taylor, 1975). An intermediate in the biological oxidation of a methyl group is an alcohol, which is converted into the corresponding aldehyde by an alcohol dehydrogenase. The alcohol is formed by hydroxylation of the methyl group and the initial hydroxylases for 3,5-xylenol and *p*-cresol in this organism are very different, each specific for a particular orientation of methyl and hydroxy groups (Keat & Hopper, 1978). 3,5-Xylenol-grown cells can, however, attack *p*-cresol, apparently because of gratuitous induction of a *p*-cresol methylhydroxylase.

In this paper a study of the alcohol dehydrogenases that catalyse the second step in the oxidation of 3,5-xylenol and *p*-cresol is described.

Materials and Methods

Maintenance and growth of organism

The organism was maintained and grown as described by Keat & Hopper (1978).

Preparation of extracts

Extracts were prepared as described by Keat & Hopper (1978). To decrease NADH oxidase activity crude extracts were centrifuged at 150000*g* for 1 h at 4°C and the supernatants used.

Enzyme assays

NAD⁺-dependent aromatic alcohol dehydrogenases were assayed spectrophotometrically by follow-ing the production of NADH at 30°C in 1 cm-light-path cuvettes containing in 1 ml of 50 mM-glycine/NaOH buffer, pH 9.6, 0.4μmol of NAD⁺, 4μmol of substrate and enzyme. For all of the aromatic substrates except *p*-hydroxybenzyl alcohol the reaction was followed at 355 nm and a value of 4.39×10^3 litre·mol⁻¹·cm⁻¹ was used for the molar absorption coefficient for NADH. The reaction with *p*-hydroxybenzyl alcohol was assayed at 370 nm and a molar absorption coefficient of 2.86×10^3 litre·mol⁻¹·cm⁻¹ was used for NADH.

NAD⁺-independent *p*-hydroxybenzyl alcohol dehydrogenase was assayed as described by Hopper & Taylor (1977), but with 50 mM-glycine/NaOH buffer, pH 9.6.

Polyacrylamide-gel electrophoresis

Electrophoresis of enzymes was carried out on 6.4% (w/v) polyacrylamide gels by the method of Ornstein & Davis (1964) at pH 8.9. Gels were stained for protein with Amido Black and cleared by washing with 7% (v/v) acetic acid in water. Gels were stained for enzymic activity by placing them in small test tubes (10 cm × 1 cm) containing, in 1.5 ml of 50 mM-glycine/NaOH buffer, pH 9.6, 2μmol of NAD⁺, 2μmol of aromatic alcohol and 1 mg of 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride. The tubes were sealed and incubated at 30°C. Aromatic alcohol dehydrogenase activity was shown by the appearance of a red band. Gels were scanned in a Gilford 2400S spectrophotometer fitted with a 2410S gel transport attachment (Gilford Instruments, Oberlin, OH, U.S.A.).

Isolation of reaction products

Flask contents from whole-cell oxidations were centrifuged at 10000*g* for 10min and the supernatant extracted twice with 5ml of diethyl ether after acidification to pH1 with 5M-HCl. The pooled extracts were dried over anhydrous Na_2SO_4 and then evaporated to dryness.

Reaction mixtures using purified enzymes contained, in 5ml of 50mM-glycine/NaOH buffer, pH9.6, 10μmol of NAD^+, 20μmol of substrate and enzyme (approx. 60μg). These were incubated at 30°C for 45min. The reactions were stopped by acidification to pH1 with 5M-HCl, and the mixtures were each extracted three times with 5ml of diethyl ether. The ether extracts were dried over anhydrous Na_2SO_4 and then evaporated to dryness.

Chromatography

T.l.c. was carried out on Kieselgel GF_{254} (E. Merck, A.-G., Darmstadt, Germany) coated on glass plates to a thickness of 0.25mm and activated at 110°C for 30min. Phenolic compounds were separated by using solvent A [benzene/dioxan/acetic acid (90:25:4, by vol.) (Pastuska, 1961)]. For 2,4-dinitrophenylhydrazones solvent D [benzene/tetrahydrofuran (19:1, v/v) (Byrne, 1965)], solvent E [light petroleum (b.p. 80–100°C)/diethyl ether (7:3, v/v) (Byrne, 1965)] and solvent F [hexane/ethyl formate (4:1, v/v) (Dagley & Gibson, 1965)] were used.

Paper chromatography of phenolic compounds was carried out on Whatman no. 1 paper with the following solvents: solvent B [benzene/acetic acid/water (125:72:3, by vol.) (Smith, 1960)] and solvent C [5% (w/v) sodium formate/formic acid (200:1, v/v) (Smith, 1960)]. Phenolic materials were detected by spraying with diazotized *p*-nitroaniline.

Preparation of 2,4-dinitrophenylhydrazones

These were prepared as described by Keat & Hopper (1978).

Protein determinations

These were carried out as described by Keat & Hopper (1978).

Purification of m-hydroxybenzyl alcohol dehydrogenase rom 3,5-xylenol-grown cells (activity 1)

All solutions contained 1mM-dithiothreitol.

Step 1: preparation of extract. Frozen cells (30g wet wt.) grown on 3,5-xylenol were thawed in 60ml of 50mM-KH_2PO_4/NaOH buffer, pH7.0, and disrupted by sonication. The extract was centrifuged at 40000*g* for 10min at 4°C and the supernatant was centrifuged at 150000*g* for 1h at 4°C.

Step 2: DEAE-cellulose chromatography (1). The extract was dialysed against 16.7mM-KH_2PO_4/NaOH buffer, pH7.0, until its conductivity was the same as fresh buffer. It was loaded on a DEAE-cellulose column (10cm×5cm) equilibrated with 16.7mM-phosphate buffer, pH7.0. After washing the column with 300ml of this buffer, the enzyme was eluted with a linear gradient of KCl constructed from 900ml of the buffer and 900ml of the same buffer containing 0.35M-KCl. Fractions of volume 10ml were collected. Activity 1 was completely separated from activity 2 on this column and fractions with a specific activity greater than 0.7 unit/mg were pooled. The pooled fractions were dialysed against 16.7mM-KH_2PO_4/NaOH buffer, pH7.0, to remove KCl, and the protein was concentrated by loading on a small DEAE-cellulose column (2cm×3cm) and eluting in a small volume of 16.7mM-KH_2PO_4/NaOH buffer, pH7.0, containing 0.5M-KCl.

Step 3: Sephadex G-200 gel filtration. The concentrated protein was further purified by gel filtration in two equal batches by loading on a Sephadex G-200 column (45cm×2.5cm) and eluting with 50mM-KH_2PO_4/NaOH buffer, pH7.0. Fractions of volume 5ml were collected, and fractions 19–28 were pooled.

Step 4: hydroxyapatite chromatography. The protein was dialysed against 1mM-KH_2PO_4/KOH buffer, pH6.8, until the conductivity was that of fresh buffer. It was then loaded on a hydroxyapatite column (6cm×2.5cm) and eluted with a gradient constructed from 125ml of 10mM-KH_2PO_4/KOH buffer, pH6.8, and 125ml of 200mM-KH_2PO_4/KOH buffer, pH6.8. Fractions of volume 5ml were collected, and fractions 37–45 pooled.

Step 5: DEAE-cellulose chromatography (2). The pooled fractions were dialysed against 16.7mM-KH_2PO_4/NaOH buffer, pH7.0, until the conductivity was that of fresh buffer, and then loaded on a DEAE-cellulose column (6cm×2.5cm). The protein was eluted with a linear gradient from 0 to 0.2M-KCl in 300ml of 16.7mM-KH_2PO_4/NaOH buffer, pH7.0. Fractions of volume 5ml were collected and those containing enzyme were pooled and concentrated on a small DEAE-cellulose column as before.

A summary of the purification is shown in Table 1.

Purification of m-hydroxybenzyl alcohol dehydrogenase from 3,5-xylenol-grown cells (activity 2)

All solutions contained 1mM-dithiothreitol. Steps 1 and 2 were as described for activity 1 except that in the purification reported in Table 2 40g wet wt. of frozen cells was thawed in 80ml of buffer and the fractions from the DEAE-cellulose column containing activity 2 with specific activity greater than 1.3 units/mg were pooled.

Step 3: Sephadex G-200 gel filtration. The concentrated protein was loaded on a Sephadex G-200

Table 1. *Summary of the purification of m-hydroxybenzyl alcohol dehydrogenase (activity 1) from Ps. putida N.C.I.B.* 9869 *grown on 3,5-xylenol*

The starting material was 30 g wet wt. of cells. One unit of enzyme is defined as the amount that catalyses the reduction of 1 μmol of NAD^+/min with *m*-hydroxybenzyl alcohol as substrate.

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Recovery (%)	Purification
(1) Centrifuged crude extract	2300	319.3*	0.15	100	—
(2) DEAE-cellulose chromatography (1)	214	197.3	0.92	62	6.1
(3) Sephadex G-200 gel filtration	67.5	147.8	2.19.	46	14.6
(4) Hydroxyapatite chromatography	8.4	95.6	11.38	30	75.9
(5) DEAE-cellulose chromatography (2)	2.9	59.6	20.31	19	135.4

* The portion, from a total of 1774 units, due to activity 1.

Table 2. *Summary of the purification of m-hydroxybenzyl alcohol dehydrogenase (activity 2) from Ps. putida N.C.I.B.* 9869 *grown on 3,5-xylenol*

The starting material was 40 g wet wt. of cells. One unit of enzyme is defined as the amount that catalyses the reduction of 1 μmol of NAD^+/min with *m*-hydroxybenzyl alcohol as substrate.

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Recovery (%)	Purification
(1) Centrifuged crude extract	3600	2752*	0.71	100	—
(2) DEAE-cellulose chromatography	840	1731	2.02	67	2.8
(3) Sephadex G-200 gel filtration	91	1116	12.24	64	17.2
(4) Hydroxyapatite chromatography	5.2	221	43.4	8	61.1

* The portion, from a total of 3175 units, due to activity 2.

column (45 cm × 2.5 cm) and eluted with 50 mM-KH_2PO_4/NaOH buffer, pH 7.0. Fractions of volume 10 ml were collected, and fractions 11–14 were pooled.

Step 4: hydroxyapatite chromatography. The pooled fractions were dialysed against 1 mM-KH_2PO_4/KOH buffer, pH 6.8, until the conductivity was that of fresh buffer and then loaded on a hydroxyapatite column (8 cm × 2.5 cm). Protein was eluted with a linear gradient constructed from 200 ml of 20 mM- and 200 ml of 200 mM-KH_2PO_4/KOH buffer, pH 6.8. Fractions of volume 5 ml were collected, and those with a specific activity greater than 40 units/mg were pooled. The enzyme was concentrated by adding 1 g of dry Sephadex G-25 powder to each 4 ml of solution and, after expansion of the gel (approx. 30 min), removing it by centrifuging.

A summary of the purification is given in Table 2.

Partial purification of m-hydroxybenzyl alcohol dehydrogenase from p-cresol-grown cells

All solutions contained 1 mM-dithiothreitol.

Step 1: preparation of extract. Frozen cells (30 g wet wt.) were thawed in 45 ml of 23 mM-KH_2PO_4/NaOH buffer, pH 7.0, disrupted by sonic disintegration and centrifuged at 120000 g for 1 h at 4°C.

Step 2: DEAE-cellulose chromatography. The supernatant (60 ml) was loaded on a DEAE-cellulose column (20 cm × 5 cm), which was then washed with 300 ml of 23 mM-KH_2PO_4/NaOH buffer, pH 7.0. Enzyme was then eluted with a linear gradient from 0 to 0.4 M-KCl in 1800 ml of the phosphate buffer. Fractions of volume 13 ml were collected, and fractions 48–61 were pooled; the protein was concentrated by using a Millipore Molecular Separation Kit [Millipore (U.K.), London NW10 7SP, U.K.].

Step 3: Sephadex G-200 gel filtration. The concentrated fractions (15.7 ml) were loaded on a Sephadex G-200 column (52 cm × 2.5 cm) and eluted with 23 mM-KH_2PO_4/NaOH buffer, pH 7.0. Fractions of volume 5 ml were collected, and fractions 25–36 pooled.

Step 4: hydroxyapatite chromatography. The pooled fractions were dialysed against 0.5 mM-KH_2PO_4/NaOH buffer, pH 6.8, until the conductivity was that of fresh buffer, and loaded on a hydroxyapatite column (6 cm × 2.5 cm). Enzyme was eluted with a gradient constructed from 200 ml of 10 mM- and 200 ml of 200 mM-KH_2PO_4/NaOH buffer, pH 6.8. Fractions of volume 5 ml were collected, and fractions 34–36 were pooled.

A summary of the purification is given in Table 3. Although this represents a 71-fold purification, the

Table 3. *Summary of the purification of m-hydroxybenzyl alcohol dehydrogenase from Ps. putida N.C.I.B. 9869 grown on p-cresol*

The starting material was 30 g wet wt. of cells. One unit of enzyme is defined as the amount that catalyses the reduction of 1 μmol of NAD^+/min with *m*-hydroxybenzyl alcohol as substrate.

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Recovery (%)	Purification
(1) Centrifuged crude extract	3408	2592	0.76	100	—
(2) DEAE-cellulose chromatography	200	2347	11.73	90	15.4
(3) Sephadex G-200 gel filtration	75.5	1230	16.3	47.5	21.4
(4) Hydroxyapatite chromatography	14.4	774	53.7	30	71

preparation gave at least six bands after electrophoresis on polyacrylamide gels followed by staining for protein. The major protein band coincided with the enzyme when gels were stained for activity with *o*-hydroxybenzyl alcohol, *m*-hydroxybenzyl alcohol, *p*-hydroxybenzyl alcohol or benzyl alcohol itself as substrate, and, from the intensity of staining with Amido Black, it represents approx. 40 % of the total protein.

Chemicals

p-Cresol, *o*-hydroxybenzyl alcohol, DL-1-phenylethanol, octanol, pentan-2-ol, benzaldehyde, *o*-hydroxybenzaldehyde, *p*-hydroxybenzaldehyde, *m*-hydroxybenzoic acid, *p*-chloromercuribenzoate and 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride were from BDH, Poole, Dorset, U.K. 3,5-Xylenol, 1,2-cyclohexanediol, 1,3-cyclohexanediol and 1,4-cyclohexanediol were from Aldrich Chemical Co., Milwaukee, WI, U.S.A. *m*-Hydroxybenzaldehyde, *p*-hydroxybenzoic acid and Amido Black 10B were from Hopkin and Williams, Chadwell Heath, Essex, U.K. Hexahydrobenzyl alcohol, *m*-methylbenzyl alcohol and *p*-methylbenzyl alcohol were obtained from NIPA Laboratories, Pontypridd, Glam., U.K. *m*-Hydroxybenzyl alcohol and *p*-hydroxybenzyl alcohol were from K & K Laboratories, Plainview, NY, U.S.A. Benzyl alcohol was from Koch–Light Laboratories, Colnbrook, Bucks., U.K. Dithiothreitol was from Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K. NAD^+, NADH, $NADP^+$ and NADPH were obtained from Boehringer Corp., London W.5, U.K. Sephadex G-25 and G-200 were from Pharmacia Fine Chemicals, Uppsala, Sweden. Preswollen microgranular DEAE-cellulose DE-52 was from W. R. Balston, Maidstone, Kent, U.K. Bio-Gel HTP hydroxyapatite was from Bio-Rad Laboratories, Richmond, CA, U.S.A.

Results

Oxidation of compounds by whole cells

The oxidation of various aromatic compounds by cells grown on either 3,5-xylenol or *p*-cresol was

measured in a Warburg apparatus and is shown in Fig. 1. None of these compounds was oxidized by cells grown on succinate.

The product from the limited oxidation of *m*-hydroxybenzyl alcohol by *p*-cresol-grown cells was isolated and identified as *m*-hydroxybenzoic acid by t.l.c. in solvent A and by paper chromatography in solvents B and C.

Aromatic alcohol dehydrogenase activities in 3,5-xylenol-grown cells

NAD^+-dependent alcohol dehydrogenase activity towards both *m*-hydroxybenzyl alcohol and *p*-hydroxybenzyl alcohol in crude extracts of cells grown on 3,5-xylenol was demonstrated spectrophotometrically. Extracts also contained the nicotinamide nucleotide-independent dehydrogenase activity towards *p*-hydroxybenzyl alcohol that is catalysed by the *p*-cresol methylhydroxylase (Keat & Hopper, 1978).

Crude extract was centrifuged at 150000*g* for 1 h at 4°C and the supernatant (10 ml, approx. 30 mg of protein/ml) was dialysed against 1 litre of 16.7 mM-phosphate buffer, pH 7.0, for 1 h. It was loaded on a DEAE-cellulose column (4.5 cm × 2 cm) that was then washed with 50 ml of 16.7 mM-phosphate buffer, pH 7.0, followed by elution with a linear gradient constructed from 140 ml of buffer and 140 ml of buffer containing 0.3 M-KCl. Fractions of volume 3.5 ml were collected and assayed for NAD^+-dependent alcohol dehydrogenase with *m*-hydroxybenzyl alcohol as substrate. Two peaks of activity were detected (Fig. 2). The enzyme eluted at the lower KCl concentration is referred to as activity 1 and the other as activity 2.

Stabilization of enzymes from 3,5-xylenol-grown cells

The *m*-hydroxybenzyl alcohol dehydrogenase activity in the centrifuged crude extract of 3,5-xylenol-grown cells was stable for several days at 4°C. However, after separation by ion-exchange chromatography both enzymes rapidly lost activity, with activity 1 being the more unstable. It lost 50 %

of its activity after 24 h at 4°C and only 10 % remained after 6 days, whereas activity 2 lost 10 % after 24 h and 45 % remained after 6 days. There were also some differences in their response to stabilizing agents. Activity 1 was partially stabilized by 10 % (v/v) ethanol, 10 % (v/v) glycerol or 5 mM-NAD⁺, all of which resulted in approx. 60 % retention of activity after 6 days. Acetone (10 %, v/v) or *m*-hydroxybenzyl alcohol (10 %, w/v) had no effect. With activity 2 only 10 % (v/v) ethanol had a slight stabilizing effect and in this case NAD⁺ or acetone resulted in greater loss of activity than in the control. However, both activities were almost completely stabilized by 1 mM-dithiothreitol, and this was included in all enzyme solutions and buffers during subsequent purifications.

Purification of aromatic alcohol dehydrogenases from 3,5-xylenol-grown cells

The two NAD⁺-dependent *m*-hydroxybenzyl alcohol dehydrogenases in 3,5-xylenol-grown cells were each purified as described in the Materials and Methods section. The results from a purification of activity 1 are shown in Table 1 and for activity 2 in Table 2. In each case the value given for activity in crude extract is the portion of the total activity attributable to that enzyme. This is calculated from the relative amounts of each enzyme after separation by ion-exchange chromatography and assumes that each loses the same proportion of activity during this step.
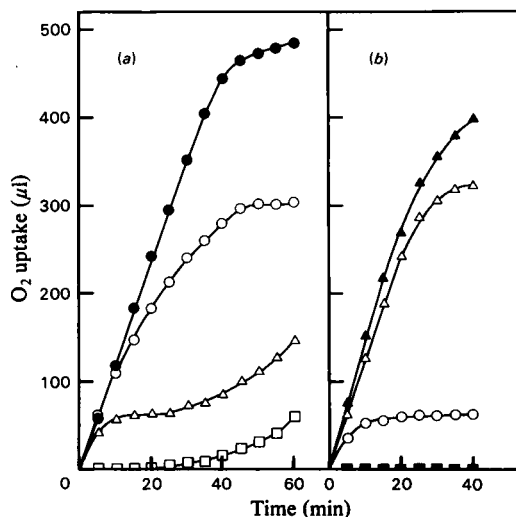

Fig. 1. *Oxidation of compounds by washed cells grown with (a) 3,5-xylenol and (b) p-cresol*
Each Warburg flask contained 1.5 ml of bacterial suspension (5 mg dry wt.) in 50 mM-phosphate buffer, pH 7.0, 0.3 ml of 10 mM-substrate, tipped from the side arm and 0.2 ml of 20% (w/v) KOH in the centre well. Substrates were: ●, 3,5-xylenol; ○, *m*-hydroxybenzyl alcohol; ▲, *p*-cresol; △, *p*-hydroxybenzyl alcohol; ■, *m*-hydroxybenzoic acid; □, *p*-hydroxybenzoic acid. The temperature was 30°C. The gas phase was air. Oxygen uptakes in the absence of substrate [50 μl/h for (a) and 80 μl/h for (b)] have been subtracted.
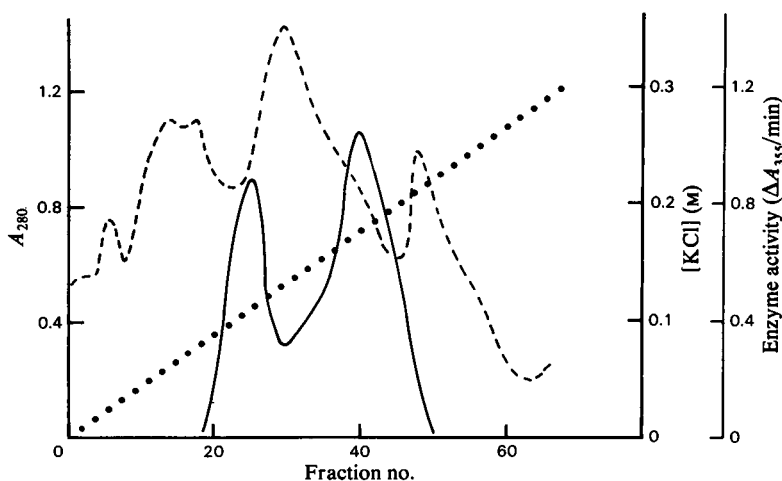

Fig. 2. *Elution of m-hydroxybenzyl alcohol dehydrogenases in extracts from Ps. putida N.C.I.B. 9869 grown on 3,5-xylenol from a DEAE-cellulose column*
Details of the procedures used are given in the text. ——, *m*-Hydroxybenzyl alcohol dehydrogenase activity; ----, A_{280}; ·····, KCl gradient.
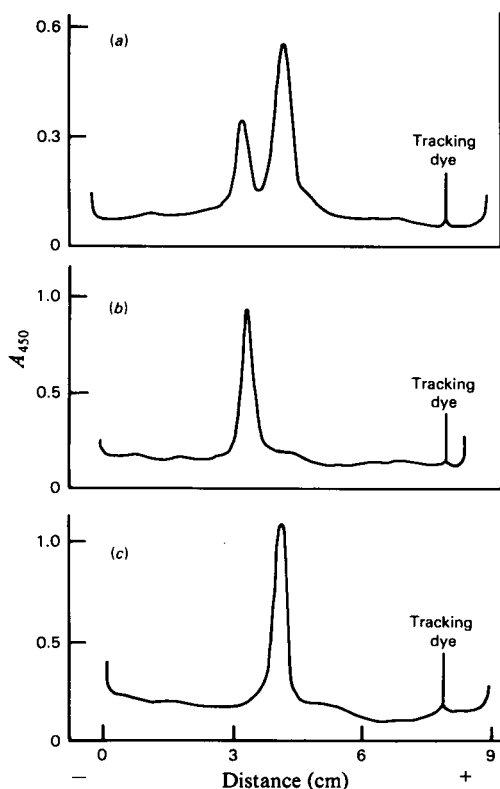
Fig. 3. *Scans of polyacrylamide gels after electrophoresis of m-hydroxybenzyl alcohol dehydrogenase activities* 1 *and* 2
Gels were loaded with: (*a*) $80\mu g$ of crude extract centrifuged at $15000g$ for 1h at 4°C; (*b*) $50\mu g$ of purified *m*-hydroxybenzyl alcohol dehydrogenase activity 1; (*c*) $50\mu g$ of purified *m*-hydroxybenzyl alcohol dehydrogenase activity 2. After electrophoresis gels were stained for enzyme activity and then scanned at 450nm.

Electrophoresis of the purified enzymes on poly-acrylamide gels followed by staining of proteins with Amido Black gave a single band for each dehydrogenase. In both cases the protein band coincided with the bands obtained when gels were stained for enzyme activity with *m*-hydroxybenzyl alcohol, *p*-hydroxybenzyl alcohol or benzyl alcohol as substrate. The two enzymes had different relative mobilities and their positions coincided with the two activities seen after electrophoresis of crude extract followed by activity staining (Fig. 3).

Aromatic alcohol dehydrogenase from p-cresol-grown cells

Crude extract of cells grown on *p*-cresol contained NAD+-dependent dehydrogenase activity towards both *m*-hydroxybenzyl alcohol and *p*-hydroxybenzyl alcohol. Nicotinamide nucleotide-independent activity with *p*-hydroxybenzyl alcohol used phenazine methosulphate as acceptor and could be ascribed to the *p*-cresol methylhydroxylase enzyme (Keat & Hopper, 1978).

Ion-exchange chromatography of crude extract that had been centrifuged at $150000g$ for 1h at 4°C on DEAE-cellulose gave only a single peak of dehydrogenase activity. This activity was rather unstable, with only 16% remaining after 6 days at 4°C. Whereas there was partial stabilization with 10% (v/v) ethanol, the inclusion of 1.0mM-dithiothreitol resulted in complete retention of activity over the 6-day incubation, and this agent was included in all enzyme solutions and buffers during purification.

Purification of aromatic alcohol dehydrogenase from p-cresol-grown cells

The enzyme from *p*-cresol-grown cells was only partially purified as described in the Materials and Methods section. The results are shown in Table 3.

Substrate specificities of aromatic alcohol dehydrogenases

All three dehydrogenases were active with NAD+ as acceptor, but showed no activity when NAD+ was replaced by NADP+ in the standard assay system. A range of alcohols could serve as substrates for the enzymes, although the relative rates varied (Table 4). There was no activity with DL-1-phenylethanol, methanol, ethanol, octanol, pentan-2-ol, 1,2-cyclo-hexanediol, 1,3-cyclohexanediol and 1,4-cyclohexane-diol.

Identification of reaction products

The reaction products were isolated and examined by t.l.c. and by paper chromatography. All three enzymes produced material from *m*-hydroxybenzyl alcohol and *p*-hydroxybenzyl alcohol with the same R_F values in solvents A,B and C as *m*-hydroxy-benzaldehyde and *p*-hydroxybenzaldehyde respectively. The product from *o*-hydroxybenzyl alcohol produced by the enzyme from *p*-cresol-grown cells, corresponded to *o*-hydroxybenzaldehyde in these systems. As benzaldehyde is more volatile than these hydroxybenzaldehydes, it was identified by t.l.c. of its 2,4-dinitrophenylhydrazone. In solvents D, E and F the 2,4-dinitrophenylhydrazone of the benzyl alcohol products corresponded to the derivative of benzaldehyde.

Effect of pH on enzyme activity

The three enzymes showed increasing activity with increase in pH, with a plateau above pH9.5.

Table 4. *Comparison of the activities of the aromatic alcohol dehydrogenases with various substrates*
The rates were measured spectrophotometrically by using purified enzymes with each substrate at a concentration of 4 mM. In each case the rate of NAD^+ reduction was calculated by taking into account absorbance changes due to production of the aromatic aldehydes. Rates are shown relative to that for *m*-hydroxybenzyl alcohol, which is given as 100 for each enzyme.

	Enzyme from 3,5-xylenol-grown cells		Enzyme from *p*-cresol-grown cells
Substrate	Activity 1	Activity 2	
m-Hydroxybenzyl alcohol	100	100	100
p-Hydroxybenzyl alcohol	480	110	147
o-Hydroxybenzyl alcohol	0	12	190
Benzyl alcohol	160	250	131
m-Methylbenzyl alcohol	228	26	106
p-Methylbenzyl alcohol	30	58	81
Hexahydrobenzyl alcohol	0	0	3

Table 5. *Comparison of K_m values of the aromatic alcohol dehydrogenases for various substrates*
Initial velocities were determined spectrophotometrically at 30°C. For the aromatic alcohols the NAD^+ concentration was 0.5 mM and for NAD^+ a fixed concentration of 4 mM-hydroxybenzyl alcohol was used. K_m values were obtained from double-reciprocal plots of velocity against substrate concentration.

	K_m of enzyme from 3,5-xylenol-grown cells (M)		K_m of enzyme from *p*-cresol-grown cells (M)
	Activity 1	Activity 2	
m-Hydroxybenzyl alcohol	1.3×10^{-3}	7.7×10^{-5}	7.6×10^{-5}
p-Hydroxybenzyl alcohol	3.3×10^{-4}	7.7×10^{-4}	1.0×10^{-4}
Benzyl alcohol	3.1×10^{-3}	4.0×10^{-4}	2.6×10^{-5}
NAD^+	1.4×10^{-4}	2.6×10^{-5}	2.8×10^{-5}

Molecular weights

The molecular weights of the enzymes were estimated from their behaviour on a calibrated Sephadex G-200 column by the method of Andrews (1965). The value obtained for activity 1 from 3,5-xylenol-grown cells was 145000 and for activity 2, 122000. The enzyme from *p*-cresol-grown cells had a mol. wt. of approx. 75 000.

Kinetics of aromatic alcohol dehydrogenases

A comparison of the K_m values of the enzymes for various substrates is shown in Table 5.

Inhibition by p-chloromercuribenzoate

There was almost total inhibition of all three enzymes when they were incubated for 5 min at 30°C with 10 μM-*p*-chloromercuribenzoate before addition of substrate in the spectrophotometric assay system. There was no protection against inhibition by including 4 mM-*m*-hydroxybenzyl alcohol, 0.03 mM-NADH, 0.03 mM-NADPH or 0.3 mM-NADP$^+$ before addition of *p*-chloromercuribenzoate. However, inclusion of 0.3 mM-NAD$^+$ resulted in only 45% inhibition of activity 2, although activity 1 and the enzyme from *p*-cresol-grown cells were still completely inhibited under these conditions.

Discussion

In this study of methyl-group oxidation *m*-hydroxybenzyl alcohol has been used as substrate for the alcohol dehydrogenase involved in 3,5-xylenol catabolism. It is an analogue of the true intermediate, 3-hydroxy-5-methylbenzyl alcohol, which is not readily available, and serves well as a replacement, being rapidly oxidized by 3,5-xylenol-grown cells (Fig. 1). Oxygen uptake was greater than that required for metabolism past the point of ring fission. These cells also oxidized *p*-hydroxybenzyl alcohol, but with an initial uptake (approx. 1 μmol of O_2/μmol of substrate) only sufficient for conversion into the acid. However, further oxidation occurred after a lag, which probably represents a period of induction of the enzymes for *p*-hydroxybenzoate metabolism, and the curve for this compound showed a similar lag stage. *p*-Hydroxybenzyl alcohol was also oxidized rapidly by *p*-cresol-grown cells, as would be expected for a catabolic intermediate, but so too was *m*-hydroxybenzyl alcohol (Fig. 1). However, oxygen uptake with the latter compound ceased after consumption of approx. 1 μmol of O_2/μmol of substrate, and *m*-hydroxybenzoic acid was identified as the product. From these preliminary experiments it was clear that cells grown with either 3,5-xylenol or *p*-cresol contain enzyme activities for oxidizing both *m*- and *p*-hydroxybenzyl alcohols.

This was confirmed when cell extracts were assayed, as cells grown on either substrate contained NAD$^+$-dependent dehydrogenase activity towards both of the alcohols and also the NAD$^+$-independent activity with p-hydroxybenzyl alcohol of the p-cresol methylhydroxylases (Keat & Hopper, 1978). The NAD$^+$-dependent dehydrogenase in 3,5-xylenol-grown cells could be separated into two activity peaks by DEAE-cellulose chromatography (Fig. 2) and two activity bands by electrophoresis on polyacrylamide gels (Fig. 3). Although the activities were similar in that both were stabilized by dithiothreitol, inhibited by p-chloromercuribenzoate, used NAD$^+$ but were inactive with NADP$^+$ and oxidized a range of aromatic alcohols, it is clear from a number of properties that they are two distinct enzymes. Besides their separability by electrophoresis or ion-exchange chromatography, their stabilities after separation were not identical, activity 1 being less stable than activity 2; nor were their responses to stabilizing agents other than dithiothreitol the same. The relative rates with aromatic substrates showed marked differences (Table 4), as did some of the K_m values (Table 5), and activity 2 had a slightly lower molecular weight than activity 1. Finally, only activity 2 was partially protected by NAD$^+$ from p-chloromercuribenzoate inhibition.

Only one NAD$^+$-dependent aromatic alcohol dehydrogenase was found in p-cresol-grown cells. Although this has the same common properties listed above for the other two enzymes, there were differences to show that it is not the same enzyme as either of those from 3,5-xylenol-grown cells. It has a much lower molecular weight as judged by its behaviour on a Sephadex G-200 column and it gives a different pattern of activities with various aromatic alcohols (Table 4). In contrast with the other two enzymes, this one gave high activity with o-hydroxybenzyl alcohol and a low rate with hexahydroxybenzyl alcohol. Although this enzyme was not completely purified, the single activity band in the same position on polyacrylamide gels for four of the substrates suggests that it is the only alcohol dehydrogenase present and probably catalyses the reactions with all of the substrates. For all three enzymes the reaction products from oxidation of a number of aromatic alcohols have been identified as the corresponding aldehydes.

In several respects these enzymes resemble the benzyl alcohol dehydrogenase isolated by Suhara et al. (1969) from Ps. putida T-2 grown on toluene. This enzyme, of mol.wt. 110000, used a range of aromatic substrates with a strict requirement for NAD$^+$ as acceptor and was unable to oxidize methanol or ethanol. It too was unstable, but was markedly stabilized by organic solvents such as acetone or ethanol. The enzyme was inhibited by p-chloromercuribenzoate and other thiol-group reagents and protected only a little by NAD$^+$ and

benzyl alcohol. The p-aminobenzyl alcohol dehydrogenase from Mycobacterium tuberculosis too was inhibited by p-chloromercuribenzoate and inactive with methanol or ethanol (Sloane, 1973). This enzyme, however, could use either NAD$^+$ or NADP$^+$ and its rate of oxidation of hexahydrobenzyl alcohol was almost 60% of that with benzyl alcohol itself. NADP$^+$ is the preferred cofactor for the m-hydroxybenzyl alcohol dehydrogenase from Penicillium urticae, in which it is involved in patulin biosynthesis (Forrester & Gaucher, 1972). In contrast with other enzymes mentioned, this one will reduce acetaldehyde besides a number of aromatic substrates. This enzyme was also inhibited by a thiol-group reagent, in this case iodoacetate, and was protected by NADP$^+$ or NADPH.

Broad specificity for aromatic substrates also seems to be shown by the alcohol dehydrogenase in cells of Pseudomonas putida (arvilla) mt-2 grown on m- or p-xylene (Worsey & Williams, 1975). Both of these compounds are degraded by a parallel series of reactions, beginning with oxidation of a methyl group to give a toluic acid. The specificity of the alcohol dehydrogenase activity in extracts was constant for benzyl alcohol, m-methylbenzyl alcohol and p-methylbenzyl alcohol, whatever the growth substrate, suggesting that all three alcohols are metabolized by the same non-specific enzyme. Similarly p-methylbenzyl alcohol was metabolized by cells of Pseudomonas Pxy grown with either m- or p-xylene (Davey & Gibson, 1974). The possibility that catabolism of 3,5-xylenol and p-cresol by Ps. putida N.C.I.B. 9869 involves common enzymes of broad specificity for oxidation of the methyl groups is clearly ruled out. It has already been shown that the initial hydroxylases are not the same (Keat & Hopper, 1978), and, although the NAD$^+$-dependent alcohol dehydrogenases have broad enough specificities to deal with the intermediates of both pathways, again different enzymes are induced by growth on each of the substrates. This is also the case for the aldehyde dehydrogenases (Keat & Hopper, 1975).

Little is known of the control of these methyl-oxidizing systems, but, if the three enzymes hydroxylase, alcohol dehydrogenase and aldehyde dehydrogenase are co-ordinately controlled, then perhaps one of the two alcohol dehydrogenases isolated from 3,5-xylenol-grown cells is associated with the 3,5-xylenol-oxidizing system and the other with the gratuitously induced p-cresol methylhydroxylase found in these cells. There is some indication that such cells also contain two aldehyde dehydrogenases (Keat, 1977), and Worsey & Williams (1975) have suggested that in their xylene-metabolizing organism the alcohol dehydrogenase and aldehyde dehydrogenase at least may be co-ordinately controlled. Confirmation must await further study of the regulatory mechanisms.

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