The Microbial Oxidation of Methanol

THE ALCOHOL DEHYDROGENASE OF *PSEUDOMONAS* SP. M27

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1. No primary hydrogen acceptor other than phenazine methosulphate has been found for the alcohol dehydrogenase from *Pseudomonas* sp. M27. 2. None of a wide range of vitamins or cofactors has any effect on the activity of the enzyme. 3. The enzyme is far less sensitive to metal-chelating agents and thiol reagents than are other alcohol dehydrogenases. 4. Methanol is oxidized at least as fast as other alcohols by this enzyme and its well-defined substrate specificity is different from that of other alcohol dehydrogenases. Only primary alcohols are oxidized; the general formula for an oxidizable substrate is $R \cdot CH_2 \cdot OH$, where R may be H or $R'\cdot CH_2$ or $\begin{matrix} R' \\ \diagdown \\ \quad C{:}CH \\ \diagup \\ R'' \end{matrix}$. 5. Whole organisms oxidize only those alcohols that are oxidized by the isolated enzyme.

Anthony & Zatman (1964*a*, *b*) have described an alcohol dehydrogenase that differs from other alcohol dehydrogenases in the following ways: activity is independent of nicotinamide nucleotides, it catalyses the oxidation of methanol at a high rate, it requires ammonia (not NH_4^+ ions) or methylamine as activator and phenazine methosulphate is required as primary hydrogen acceptor. The enzyme was originally isolated from *Pseudomonas* sp. M27, a pink organism capable of aerobic growth on methanol or other C_1 compounds as sole source of carbon and energy. The enzyme is also present in the following related organisms: *Pseudomonas* AM1, *Pseudomonas methanica*, *Pseudomonas extorquens* and *Protaminobacter ruber* (Johnson & Quayle, 1964).

The present paper describes the well-defined substrate specificity of this enzyme and also the effect of potential inhibitors, cofactors and hydrogen acceptors on enzyme activity.

A preliminary report of some of this work has been published (Anthony & Zatman, 1964*c*).

MATERIALS AND METHODS

Sodium ethyl mercurithiosalicylate was obtained from Eli Lilly and Co. Ltd. (Basingstoke, Hants.), suramin from Imperial Chemical Industries Ltd. (Alderley Park, Macclesfield, Cheshire) and pyocyanine perchlorate from Mann Research Laboratories Inc. (New York, N.Y., U.S.A.). Mepacrine hydrochloride was a gift from Dr W. A. Sexton of Imperial Chemical Industries Ltd., 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide was a gift from Dr J. W. Lightbown and chlorpromazine was a gift from May and Baker Ltd. (Dagenham, Essex). All other chemicals were obtained from either L. Light and Co. Ltd. (Colnbrook, Bucks.) or British Drug Houses Ltd. (Poole, Dorset).

Unless otherwise stated, the methods used for growth of organisms and for preparation of enzyme were those described by Anthony & Zatman (1964*a*,*b*). The various experiments used either the crude ultrasonically prepared extract of methanol-grown organisms or the dialysed protein fraction precipitating between 65 and 80% saturation with $(NH_4)_2SO_4$ (after removal of nucleic acids from crude ultrasonically prepared extract with protamine sulphate); the dialysis was carried out against 500vol. of 0·05M-sodium–potassium phosphate buffer, pH7·0, for 1hr. at 0°.

Spectrophotometric assay. The cuvettes (10mm. light-path) contained the following, in a total volume of 3ml.: 0·3m-mole of tris–HCl buffer, pH9·0; 16μmoles of methanol; 0·33μmole of PMS*; 0·13μmole of 2,6-dichlorophenol-indophenol; 45μmoles of NH_4Cl. The reference cuvette contained deionized water. Enzyme solution was blown in from a pipette and the rate of reduction (ΔE_{600}/min.) taken as twice the change in extinction at 600mμ (E_{600}) occurring between 15 and 45sec. after the addition of enzyme. The amount of enzyme used did not exceed that which would cause a rate of change of E_{600} of 0·3/min.

Manometric assay. The reaction mixture for the usual manometric assay contained the following, in a total volume of 3ml.: 0·3m-mole of tris–HCl buffer, pH7·5 or pH9·0; 0·66μmole of PMS; 45μmoles of NH_4Cl; substrate (15μmoles of methanol for routine assay); enzyme. Either the PMS or the substrate was added from the side arm of a

* Abbreviation: PMS, *N*-methylphenazonium methosulphate (phenazine methosulphate).

conventional Warburg vessel and the rate of O_2 uptake was measured at 30° between 3 and 8min. after tipping. Air was the gas phase in all experiments.

Effect of various vitamins and cofactors on enzyme activity. The manometric assay system was used; PMS was added from the side arm after preincubation of enzyme in the reaction mixture containing the cofactor for 30min. at 30°, during which time the O_2 uptake was measured. Four test systems were used: (1) dialysed $(NH_4)_2SO_4$ fraction (0·075mg. of protein) assayed at pH9·0 in the presence of NH_4Cl; (2) crude ultrasonically prepared extracts (4mg. of protein) assayed at pH7·5 in the presence of NH_4Cl; (3) crude ultrasonically prepared extracts (1mg. of protein) assayed at pH9·0 in the presence of NH_4Cl; (4) crude ultrasonically prepared extracts (5mg. of protein) assayed at pH9·0 in the absence of NH_4Cl. No O_2 uptake occurred before the addition of PMS in any of these systems.

Alternative primary hydrogen acceptors. Various substances were tested as alternatives to PMS as primary hydrogen acceptor in the manometric assay system at pH7·5 or pH9·0, with large amounts of enzyme [$(NH_4)_2SO_4$ fraction (0·5mg. of protein)]. The hydrogen acceptor under test was added from the side arm; 0·2ml. of a 1% (w/v) solution or suspension was used, except for pyocyanine where 1ml. of a 0·1% (w/v) solution was used. Anaerobic experiments in evacuated Thunberg tubes were based on the same reaction mixture but with one-quarter of the concentration of hydrogen acceptor. In attempts to replace PMS in the spectrophotometric assay 0·1ml. of 1% solutions of the potential hydrogen acceptors were used except for methylene blue, resazurine, pyocyanine and Janus green; 0·1ml. of 0·05% solutions were used for these dyes as they absorb light at 600mμ. Menaphthone and menaphthone bisulphite were tested only in the manometric assay.

Effect of metal-chelating agents, thiol reagents and other potential inhibitors on enzyme activity. These were tested with the $(NH_4)_2SO_4$ fraction (0·06mg. of protein) by using the manometric assay at pH9·0. Methanol and PMS were added from the side arm after preincubation of the remainder of the system at 30° for 30min. It was not possible to test the following substances because they reacted spontaneously with the assay system (usually with the PMS) in the absence of enzyme: sodium diethyldithiocarbamate, 8-hydroxyquinoline, sodium iodoacetate, hydrogen peroxide, sodium iodosobenzoate, suramin, protocatechualdehyde, hydroxylamine.

Substrate specificity. (*a*) Enzyme experiments. To avoid false positive results due to trace impurities in the alcohols used as substrates, the manometric enzyme assay rather than the spectrophotometric assay was used for determining the substrate specificity of the enzyme.

The rate of oxidation of a wide range of alcohols was tested in the manometric assay at pH9·0. Enzyme [$(NH_4)_2SO_4$ fraction (0·06mg. of protein)] was added from the side arm and the rate of O_2 uptake measured. Although the rate was measured in the first 10min. it was recorded over 30min. to eliminate the possibility of impurities giving misleading results. About 200μmoles of soluble liquid alcohol, 0·01ml. of undiluted insoluble liquid alcohol or a few milligrams of finely powdered solid alcohol were used. For the purpose of this paper an insoluble alcohol is defined as an alcohol that cannot be prepared as a 2% (v/v or w/v) solution at room temperature.

(*b*) Experiments with suspensions of whole organisms. Oxidation of various alcohols by washed organisms was measured by conventional manometric techniques at 30° with air as gas phase. The main compartment of each vessel contained 0·05M-phosphate buffer, pH7·0, inhibitor when required and substrate [0·01ml. of undiluted insoluble alcohol or 0·2ml. of 2% (v/v) solution of soluble alcohol]; washed organisms equivalent to 4mg. dry wt. were added from the side arm; the total volume was 3·0ml. The centre well contained 0·2ml. of 10% (w/v) KOH and a filter-paper wick (for CO_2 absorption).

RESULTS

Purified enzyme preparations (dialysed ammonium sulphate fractions) are invariably completely inactive in the absence of an ammonium salt or methylamine in the assay system. Crude ultrasonically prepared extracts, however, are sometimes active without these additions, and such activity is usually between 5 and 7% of the corresponding activity with added ammonium chloride (Anthony & Zatman, 1964*b*). Preparations of crude ultrasonically prepared extracts showing such activity were occasionally used in the following work.

Effect of various vitamins and cofactors on enzyme activity. None of the following substances (per 3ml. of reaction mixture) had any effect on enzyme activity in the four test systems described in the Materials and Methods section: thiamine hydrochloride (50μg.), folic acid (10μg.), *p*-aminobenzoic acid (50μg.), biotin (0·1μg.), vitamin B_{12} (0·2μg.), calcium pantothenate (50μg.), nicotinic acid (50μg.), pyridoxine hydrochloride (50μg.), pyridoxal hydrochloride (50μg.), pyridoxal phosphate (50μg.), riboflavine (50μg.), riboflavine 5′-phosphate (50μg.), FAD (50μg.), Difco yeast extract (100μg.).

Alternative primary hydrogen acceptors. It has been shown (Anthony & Zatman, 1964*b*) that PMS cannot be replaced as primary hydrogen acceptor by NAD, NADP, cytochrome *c* or ferricyanide. The present work with large amounts of purified enzyme [ammonium sulphate fraction (0·5mg. of protein)] has shown that none of the following substances was able to replace PMS as primary hydrogen acceptor at pH7·5 or pH9·0 in the manometric assay (oxygen uptake), or anaerobically in Thunberg tubes (reduction of acceptor), or in the spectrophotometric assay (reduction of 2,6-dichlorophenol-indophenol): pyocyanine, methylene blue, 2,6-dichlorophenol-indophenol, benzyl viologen, methyl viologen, Janus green, phenosafranine, neutral red, resazurine, menaphthone (menadione), menaphthone bisulphite.

Effect of metal-chelating agents, thiol reagents and other potential inhibitors on enzyme activity. The results in Table 1 show the relative insensitivity of this enzyme to such reagents compared with other alcohol dehydrogenases (Sund & Theorell, 1963).

Table 1. *Effect of metal-chelating agents, thiol reagents and other potential inhibitors on the rate of methanol oxidation by the purified alcohol dehydrogenase of* Pseudomonas *sp. M* 27

The manometric assay was used. The gas phase was air, and the temperature 30°. The main compartment contained 0·3m-mole of tris–HCl buffer, pH 9·0, 45μmoles of NH_4Cl, inhibitor and enzyme [$(NH_4)_2SO_4$ fraction (0·06mg. of protein)]; the side arm contained 0·66μmole of PMS and 15μmoles of methanol. The total volume was 3·0ml. The vessels were shaken at 30° for 30min. before tipping. The rate of O_2 uptake in the control (no inhibitor) was 270μl./hr.

Inhibitor	Inhibition (%)
Metal-chelating agents	
Potassium cyanide (10mM)	0
Sodium azide (10mM)	16
$\alpha\alpha'$-Bipyridyl (5mM)	9
o-Phenanthroline (5mM)	30
EDTA (1mM)	0
EDTA (50mM)	33
Hydroxyethylethylenediaminetriacetic acid (10mM)	13
Thiol reagents	
Iodoacetamide (10mM)	0
p-Chloromercuribenzoate (1mM)	0
Sodium phenylmercuric acetate (1mM)	0
Sodium ethylmercurithiosalicylate (Merthiolate) (10mM)	16
N-Ethylmaleimide (10mM)	17
Ferric chloride (1mM)	15
Cupric chloride (1mM)	33
Mercuric chloride (1mM)	60
Other potential inhibitors	
2-*n*-Heptyl-4-hydroxyquinoline *N*-oxide (0·1mM)	0
Phenylhydrazine (0·2mM)	0
Aminopterin (1mM)	0
Isonicotinic acid hydrazide (10mM)	0
Mepacrine hydrochloride (10mM)	29
Chlorpromazine (10mM)	14
Sodium borate (10mM)	14
Sodium acetate (10mM)	0
Sodium formate (10mM)	0
Formaldehyde (30mM)	0

The inhibition by mepacrine might indicate the involvement of a flavine in the action of this enzyme but the concentration necessary for inhibition is very high. The results obtained with EDTA and phenylhydrazine are of particular interest. The oxidation of methanol by suspensions of whole organisms was completely inhibited by mM-EDTA and by 0·1mM-phenylhydrazine (Anthony & Zatman, 1964*a*) but these substances have little effect on the isolated enzyme; similar results were obtained with crude ultrasonically prepared extract in the presence and absence of ammonium chloride at pH 7·0 (phosphate buffer) or pH 9·0. These results suggest that EDTA and phenylhydrazine inhibit methanol oxidation by affecting some site other than the alcohol dehydrogenase. Some exploratory experiments on the effect of EDTA on dye reduction by whole organisms indicate that this substance does not act by preventing entrance of methanol into the organism; under anaerobic conditions in Thunberg tubes whole organisms reduce 2,6-dichlorophenol-indophenol at pH 7·0 in the presence of methanol and PMS and this dye reduction is not inhibited by 5mM-EDTA.

Substrate specificity of the alcohol dehydrogenase. (*a*) Enzyme experiments. The results in Table 2 demonstrate the well-defined substrate specificity of this alcohol dehydrogenase. In all cases the presence of enzyme, PMS and ammonium chloride was necessary for oxidation to occur. The present data show that only primary alcohols are oxidized and the general formula for an oxidizable substrate is $R\cdot CH_2\cdot OH$, where R may be H or $R'\cdot CH_2$ or R' R'' C:CH (R' and R'' can be the same or different, or be H). The only exceptions are the amino alcohols and their *N*-substituted derivatives, and the insoluble solid alcohols; the inactivity of these amino alcohols as substrates might correlate with the requirement for ammonia or methylamine shown by this enzyme.

(*b*) Competition between methanol and other alcohols. Methanol (15μmoles) was incubated with enzyme [ammonium sulphate fraction (0·12mg. of protein)] in the manometric assay mixture at pH 9·0 in the presence or absence of 0·1ml. of undiluted ethanol (about 1·7m-moles), butan-1-ol (about 1·1m-mole) or nonan-1-ol (about 0·6m-mole); after incubation for 10min. formaldehyde was measured by the method described by Anthony & Zatman (1964*b*). No formaldehyde was detected in vessels containing ethanol or butan-1-ol, and only 50% of that formed in the presence of methanol alone was found in vessels containing nonan-1-ol. In concomitant manometric experiments, the presence of the methanol had no effect on the rates of oxygen uptake associated with the oxidation of ethanol and butan-1-ol. These results correlate with the competition *in vivo* between ethanol and methanol in *Pseudomonas* sp. M27 shown by Anthony & Zatman (1964*a*). On the other hand, similar manometric enzyme experiments showed that the alcohols that were not oxidized by the enzyme had no effect on the rate of oxidation of methanol.

These results indicate that if an alcohol can be bound by the enzyme it is also oxidized, and that alcohols which are not oxidized cannot be bound.

Table 2. *Substrate specificity of the purified alcohol dehydrogenase of* Pseudomonas *sp. M* 27

The manometric assay was used as described in the Materials and Methods section. The rate of O_2 uptake in the presence of methanol was 200 μmoles/hr./mg. of protein.

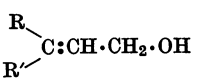
Primary alcohols (general formula $R \cdot CH_2 \cdot OH$)

Most of the following are oxidized

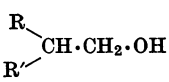
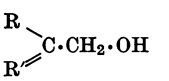
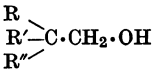
Unsubstituted *normal* aliphatic alcohols

$CH_3 \cdot OH$ or $R \cdot CH_2 \cdot CH_2 \cdot OH$	% of rate with methanol
Methanol	100
Ethanol	100
Propan-1-ol	80
Butan-1-ol	83
Pentan-1-ol	50
Hexan-1-ol	50
Heptan-1-ol	54
Octan-1-ol	48
Nonan-1-ol	60
Decan-1-ol	65
Undecan-1-ol	60
Dodecan-1-ol*	0
Tetradecan-1-ol*	0
Octadecan-1-ol*	0

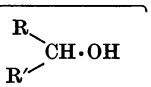
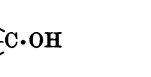
Other alcohols

$R \cdot CH_2 \cdot CH_2 \cdot OH$	% of rate with methanol
2-Chloroethanol	80
2-Bromoethanol	65
3-Chloropropan-1-ol	75
Ethane-1,2-diol	83
Propane-1,3-diol	80
2-Methoxyethanol	85
2-Ethoxyethanol	83
2-(2′-Hydroxyethoxy)-ethanol	60
2-Phenylethanol	90
3-Phenylpropan-1-ol	68
3,5,5-Trimethylhexan-1-ol*	0
2-Aminoethanol	0
3-Aminopropan-1-ol	0
2-Methylaminoethanol	0
	
Allyl alcohol (prop-2-en-1-ol)	81
Crotyl alcohol (but-2-en-1-ol)	52
Geraniol	33
Vitamin A alcohol*	0
Cinnamyl alcohol	92

None of the following is oxidized

$R \cdot C \equiv C \cdot CH_2 \cdot OH$
Propargyl alcohol (prop-2-yn-1-ol)
But-2-yne-1,4-diol

2-Methylpropan-1-ol
2-Methylpentan-1-ol
2-Ethylhexan-1-ol
Propane-1,2-diol
Glycerol
Serine

Glycollate
Dihydroxyacetone
Benzyl alcohol

2,2,2-Trifluoroethanol
2,2,3,3,3-Pentafluoropropan-1-ol
2,2,3,3,4,4,4-Heptafluorobutan-1-ol
Tris

Secondary and tertiary alcohols


Propan-2-ol
Butan-2-ol
Octan-2-ol
Cyclohexanol
Lactate

2-Methylpropan-2-ol

* A solid alcohol that is insoluble in water.

(*c*) Oxidation of alcohols by washed suspensions of methanol-grown *Pseudomonas* sp. M27. With the exception of the solid alcohols, all the alcohols in Table 2 were tested as substrates for oxidation by washed suspensions of methanol-grown organisms. As shown in Table 3, apart from 3-phenylpropan-1-ol, all the alcohols that were oxidized by the isolated enzyme were also oxidized, without a lag period, by whole organisms. 3-Phenylpropan-1-ol was not oxidized by whole organisms but was shown to inhibit the oxidation of both methanol and formaldehyde. That this inhibition is probably due to a physical effect of the insoluble alcohol on the organism is supported by the observation that none of the insoluble alcohols was oxidized by the whole organisms when the amount used was raised from 0·01ml. to 0·1ml. of the undiluted alcohol; the higher concentration of these alcohols also inhibited methanol oxidation. Only one alcohol (nonan-1-ol) was tested with the isolated enzyme at this higher concentration and its rate of oxidation was unaffected.

The alcohols that were not oxidized by the isolated enzyme were not oxidized by whole organisms, nor did they affect the rate of methanol oxidation by whole organisms [with 0·05ml. of a 1% (v/v) solution of methanol, and 0·2ml. of a 2% (v/v) solution of soluble or 0·01ml. of insoluble alcohol].

Table 3. *Oxidation of alcohols by washed suspensions of methanol-grown* Pseudomonas *sp. M 27*

The conventional manometric method was used. The gas phase was air, and the temperature was 30°. The centre well contained 0·2ml. of 10% (w/v) KOH; the main compartment contained 0·05M-phosphate buffer, pH 7·0, and substrate [0·2ml. of 2% (v/v) soluble alcohol or 0·01ml. of undiluted insoluble alcohol, indicated by * in the Table]; the side arm contained washed methanol-grown organisms equivalent to 4mg. dry wt. The control value for methanol oxidation was −108μl. of O_2/mg. dry wt./hr. (corrected for endogenous value −11).

Unsubstituted *normal* aliphatic alcohols	% of rate with methanol	Other alcohols	% of rate with methanol
Ethanol	64	2-Chloroethanol	48
Propan-1-ol	48	2-Bromoethanol	65
Butan-1-ol	49	3-Chloropropan-1-ol	56
*Pentan-1-ol	50	Ethane-1,2-diol	31
*Hexan-1-ol	20	Propane-1,3-diol	65
*Heptan-1-ol	20	2-Methoxyethanol	61
*Octan-1-ol	24	2-Ethoxyethanol	44
*Nonan-1-ol	40	2-(2′-Hydroxyethoxy)ethanol	27
*Decan-1-ol	45	2-Phenylethanol	73
*Undecan-1-ol	29	*3-Phenylpropan-1-ol	0
		Allyl alcohol (prop-2-en-1-ol)	29
		Crotyl alcohol (but-2-en-1-ol)	65
		Cinnamyl alcohol	63
		*Geraniol	12

Anthony & Zatman (1964*a*) showed that 5mM-EDTA had no effect on the oxidation of formaldehyde, formate, lactate or glyoxylate by washed organisms, but that the oxidation of methanol or ethanol was completely inhibited. By using the system described in the Materials and Methods section, it has been shown that the oxidation of all the alcohols in Table 3 by washed organisms was completely inhibited by mM-EDTA.

DISCUSSION

The results obtained with the isolated enzyme and with whole organisms indicate that one enzyme catalyses the oxidation of all the alcohols in the substrate range and that this enzyme is the alcohol dehydrogenase whose properties are discussed in this paper. Throughout this work many experiments have been carried out with ultrasonically prepared extracts and enzyme preparations in an attempt to demonstrate reduction of nicotinamide nucleotides due to the presence of an alcohol dehydrogenase of the type found in yeast or liver; all such experiments have yielded negative results.

The present findings further emphasize the difference between the alcohol dehydrogenase of *Pseudomonas* sp. M 27 and other alcohol dehydrogenases; the latter have been reviewed in detail by Sund & Theorell (1963). Zinc atoms and thiol groups are undoubtedly involved in the binding of coenzyme or substrate or both to the liver and yeast enzymes; in contrast, it appears that metal atoms and thiol groups are relatively unimportant in the action of the NAD-independent alcohol dehydrogenase of *Pseudomonas* sp. M 27. Further, the enzyme from this organism oxidizes only primary alcohols and their steric configuration appears to be more important in determining whether or not they are oxidized than is the presence of atoms or groups producing electron-displacement effects.

Contrary to the conclusion of Johnson & Quayle (1964) on the physiological role of this enzyme, the rate of oxygen uptake associated with methanol oxidation by crude ultrasonically prepared extracts of *Pseudomonas* sp. M 27 (measured in our manometric assay system) is sufficient to account for the rate observed in whole organisms. Washed suspensions of methanol-grown *Pseudomonas* sp. M 27 take up oxygen in the presence of methanol at about 20μmoles/hr./mg. of protein, and crude ultrasonically prepared extracts of these organisms take up oxygen at about the same rate in the manometric assay system at pH 9·0. The measurements of Johnson & Quayle (1964) were made under suboptimum conditions for this enzyme, i.e. low pH and low concentrations of ammonia and phenazine methosulphate.

De Ley & Kersters (1964) have reviewed the oxidation of alcohols by the acetic acid bacteria and have demonstrated that they contain NAD-independent alcohol dehydrogenases with a wide substrate range. It will thus be of interest to determine whether the acetic acid bacteria contain an alcohol dehydrogenase of the type found in *Pseudomonas* sp. M 27.

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