Microbial Growth on C₁ Compounds

6. OXIDATION OF METHANOL, FORMALDEHYDE AND FORMATE BY METHANOL-GROWN *PSEUDOMONAS* AM1*

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Several studies have been made of the oxidation of C₁ compounds by micro-organisms grown on such compounds as sole source of carbon. In some cases, rather different results have been obtained by different workers. Kaneda & Roxburgh (1959) obtained evidence for an NAD⁺-linked methanol dehydrogenase in methanol-grown *Pseudomonas* PRL-W4, whereas neither Harrington & Kallio (1960) nor Anthony & Zatman (1963) found such an enzyme in methanol-grown *Pseudomonas methanica* or *Pseudomonas* sp. M27 respectively. Instead, Harrington & Kallio (1960) suggested that methanol was oxidized by *P. methanica* '...via a catalase-linked peroxidase to formaldehyde which in turn is oxidized by a substrate specific aldehyde dehydrogenase requiring NAD⁺ and GSH...'. Anthony & Zatman (1963) showed by inhibitor experiments that methanol oxidation by *Pseudomonas* sp. M27 was independent of catalase and instead found a methanol dehydrogenase that could be linked to phenazine methosulphate. It is somewhat unexpected that such closely similar bacteria should display different oxidation pathways for methanol, and to furnish further information on this question we present below a study of the oxidation of C₁ compounds by methanol-grown *Pseudomonas* AM1 and a comparative study of the oxidation of C₁ compounds by three other types of bacteria.

MATERIALS AND METHODS

Growth of the organisms. *Pseudomonas* AM1 was grown on methanol as described by Peel & Quayle (1961). The same medium was used for the growth of *Pseudomonas* *extorquens* (Bassalik, 1913) and *Protaminobacter ruber* (Weaver, Samuels & Sherago, 1938) (N.C.I.B. no. 2879). We are indebted to Dr L. Janota-Bassalik (Institute of Plant Physiology, Warsaw) and Professor J. W. Foster (Department of Microbiology, University of Texas, Austin 12, Texas, U.S.A.) for gifts of cultures of *Pseudomonas* *extor-*

quens and *Pseudomonas methanica* respectively. The latter organism was grown essentially as described by Dworkin & Foster (1956).

Chemicals. NAD⁺, NADH, NADP⁺ and NADPH were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany, cytochrome *c* (horse heart) was from Sigma Chemical Co., St Louis, Mo., U.S.A., and 3-amino-1,2,4-triazole was from the Aldrich Chemical Co. Inc., Milwaukee, Wis., U.S.A. Other chemicals were obtained from British Drug Houses Ltd., Poole, Dorset, AnalaR grade being used when obtainable.

Preparation of buffers. Phosphate buffers were prepared by mixing 0.2M-Na₂HPO₄ and 0.2M-KH₂PO₄ to give the required pH values. The stock buffer solutions were then diluted with water as required. Tris-HCl buffers were prepared by adjusting the pH of 0.2M-tris with HCl and diluting to the required strength with water.

Manometric methods. Oxygen consumption and carbon dioxide evolution were measured at 30° in a conventional Warburg apparatus. Catalase was estimated manometrically by the perborate method of Feinstein (1949).

Preparation of cell-free extracts. Cells were suspended in 2 vol. of buffer and transferred to a French pressure cell (American Instrument Co. Inc., Washington, D.C., U.S.A.) pre-cooled to 0°. The cell was placed in a hydraulic press (Wabash Metal Products Co., Wabash, Ind., U.S.A.) and its contents were extruded through the needle valve at a pressure of 9000–12000 lb./in.². This procedure was repeated and the crude extract centrifuged at 25000g for 10 min. at 2° in the high-speed angle head of a refrigerated centrifuge (model PR-2; International Equipment Co., Boston, Mass, U.S.A.). Alternatively, cell-free extracts were prepared by crushing the frozen bacteria in a Hughes (1951) press, grinding with alumina (McIlwain, 1948), or ultrasonic disintegration. When the last method was used cells were suspended in 2 vol. of buffer cooled to 0° and subjected for 3–4 min. to the output at 25 kcy./sec. of a 600 w Mullard magnetostrictor oscillator.

Purification of aldehyde dehydrogenase. Step 1: preparation of crude extract. Frozen methanol-grown cells (10 g. wet wt.) were thawed and suspended in 25 ml. of ice-cold 10 mM-phosphate buffer, pH 7.0. The cells were ruptured in the French pressure cell and the crude extract was centrifuged at 25000g for 10 min. at 2° to remove unbroken cells and cell debris. All subsequent operations were performed at 2°.

Step 2: protamine sulphate treatment. The protein content of the supernatant was determined and an amount of protamine sulphate (dissolved in 3 ml. of 10 mM-phosphate buffer, pH 7.0) equivalent to one-tenth of the total

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protein content was added slowly with constant stirring. The resultant precipitate was removed by centrifuging and discarded.

Step 3: ammonium sulphate fractionation and dialysis. To the supernatant (37 ml.) was added 33.5 ml. of saturated ammonium sulphate solution with constant stirring, to give 50% saturation. After 10 min. the precipitate was centrifuged and discarded. Saturated ammonium sulphate solution (15.2 ml.) was added to the supernatant, producing 60% saturation, and after 10 min. equilibration the precipitate was again removed by centrifuging and discarded. A third treatment with saturated ammonium sulphate solution (32 ml.) produced 70% saturation. The protein precipitated in this fraction was centrifuged, resuspended in 3 ml. of 5 mM-tris-HCl buffer, pH 7.5, and dialysed overnight against 1.5 l. of the same buffer. The preparation at this stage could be stored at -14° without loss of activity.

Step 4: ion-exchange chromatography. DEAE-cellulose (Whatman DE50) (6 g.) was slurried in 500 ml. of 5 mM-tris-HCl buffer, pH 7.5, and decanted several times to remove fine particles. The slurry was poured into a chromatographic column (30 cm. $\times$ 1.5 cm.) and the column washed with 1.5 l. of the same buffer. The enzyme solution was applied to the top of the column and this was then eluted with an increasing gradient of KCl in 5 mM-tris-HCl buffer, pH 7.5. The gradient was produced essentially as described by Large, Peel & Quayle (1962), except that the first mixing bottle contained 0.5 M-KCl instead of 1 M-KCl. Fractions (3.5 ml.) were collected automatically at a flow rate of 35 ml./hr. Under these conditions, the aldehyde-dehydrogenase activity was eluted as a sharp peak in fractions 20-25 (Fig. 1). The two most active fractions were combined and used to investigate the properties of the purified enzyme. The purification procedure is summarized in Table 1.

Chloride determination. The chloride concentration of the eluted fractions from the column was determined by titration with silver nitrate.

Protein determination. Protein was determined by the Folin-Ciocalteu method (Lowry, Rosebrough, Farr & Randall, 1951) in the crude and partially purified extracts. For the eluted fractions from the column a spectrophotometric method was employed (Warburg & Christian, 1941).

Fig 1. Elution of aldehyde dehydrogenase from the DEAE-cellulose column. The conditions are given in the text. The continuous line represents the protein concentration; ●, enzyme activity; ▲, gradient of chloride concentration.

Table 1. Purification of aldehyde dehydrogenase from *Pseudomonas AM 1*

Experimental details are given in the Materials and Methods section.

Step	Fraction	Vol. (ml.)	Activity (units/ml.)	Protein (mg./ml.)	Total units	Total protein (mg.)	Sp. activity (units/mg. of protein)	Purification	Yield (%)
1	Supernatant obtained from crude extract after centrifuging	33 (36)*	55 (59)	22.4 (16.4)	1820 (2060)	739 (590)	2.46 (3.5)	1.0 (1.0)	100 (100)
2	Supernatant after removal of protamine sulphate precipitate	35 (37)	51 (46)	18.8 (10.7)	1750 (1700)	650 (395)	2.7 (4.3)	1.1 (1.23)	96 (83)
3	Fraction precipitated by ammonium sulphate (60-70%) after dialysis	2.9 (6.2)	305 (270)	7.9 (7.0)	920 (1620)	23.7 (42.4)	38.6 (38.6)	16.0 (11.0)	52 (78)
4	Combined batches before column chromatography	9.1	283	7.3	2540	66.4	38.6	13.7†	69†
5	Selected combined fractions after column chromatography	7.0	148	0.373	1014	2.6	390	138†	26†

* Values in parentheses refer to the fractionation of a second batch of extract. After step 3 the two batches were combined for column chromatography.

† Average purification values for the two batches.

‡ Net yield for combined batches.

Assay of enzyme activity. The activity was assayed by measuring the rate of reduction of DCPIP* at 600 m μ in the Cary recording spectrophotometer. The complete system, in 1.5 ml. silica cuvettes (light-path 1 cm.), contained 10 μ moles of phosphate buffer, pH 7.0, 0.2 μ mole of DCPIP, 0.05 ml. of enzyme solution, 10 μ moles of formaldehyde and water to 1 ml. The reaction was initiated by addition of the formaldehyde, and the decrease in extinction at 600 m μ was measured by using a reference cell containing all components of the system except formaldehyde. One unit was defined as the amount of enzyme required to reduce 1 μ m-mole of DCPIP/min. under these conditions. In the crude and partially purified fractions the reaction rate was linear for about 3 min.; from step 3 onwards the rate was linear for at least 10 min. In all cases the initial rate of reaction was proportional to the amount of enzyme solution used.

Estimation of formaldehyde. Formaldehyde was determined by the acetylacetone method (Nash, 1953). A calibration curve was prepared by using a solution of formaldehyde previously standardized with dimedone (Yoe & Reid, 1941).

Estimation of formate. A purified formate dehydrogenase from formate-grown *Pseudomonas oxalaticus* was used for the determination of formate (Johnson, Jones-Mortimer & Quayle, 1964), by measuring the reduction of NAD⁺ in the presence of formate. The complete system contained 20 μ moles of phosphate buffer, pH 7.0, 1 μ mole of NAD⁺, 0.05 ml. of formate dehydrogenase solution and the formate solution to be estimated containing approx. 0.05–0.15 μ mole of formate. The reaction was initiated by the addition of formate and the total increase in extinction at 340 m μ measured in the Cary recording spectrophotometer.

Purification of formate dehydrogenase from *Pseudomonas* AM1. Step 1: preparation of crude extract. Freshly harvested methanol-grown cells (5 g.) were suspended in 10 ml. of 10 mM-tris-HCl buffer, pH 7.5, containing 0.01 % (v/v) of mercaptoethanol, and ruptured in the French pressure cell. The crude extract was centrifuged at 25000g for 10 min. at 2°.

Step 2: protamine sulphate treatment. This was carried out as described above for the aldehyde dehydrogenase.

Step 3: ammonium sulphate fractionation and dialysis. The supernatant from step 2 was brought to 50 % saturation with ammonium sulphate as described above. After centrifuging, the precipitate was redissolved in 2 ml. of 10 mM-tris-HCl buffer, pH 7.5, and dialysed for 1.5 hr. against 2 l. of the same buffer containing 0.01 % (v/v) of mercaptoethanol.

Step 4: heat treatment. The dialysed enzyme solution was rapidly brought to 50° by heating in a water bath at 60°, and maintained at 50° in a second bath for exactly 7 min. The solution was cooled in ice and centrifuged to remove a small amount of denatured protein.

Assay of enzyme activity. The activity was assayed in a Cary recording spectrophotometer by measuring the rate of reduction of NAD⁺ (increase in extinction at 340 m μ) in the following reaction mixture at 22°. The complete system consisted of 20 μ moles of tris-HCl buffer, pH 8.4, 0.5 μ mole of NAD⁺, 0.01 ml. of enzyme solution, 25 μ moles of sodium formate and water to 1 ml. The reaction was started by the addition of formate. The blank cell lacked formate. It was

essential to add the NAD⁺ before the formate; reversal of this order resulted in considerable inhibition. One unit was defined as the amount of enzyme required to reduce 1 μ m-mole of NAD⁺/min. under these conditions. In all fractions the measured activity was found to be proportional to the amount of enzyme used. This assay system was also used for the determination of *K_m* values and pH optimum, with the appropriate modifications.

RESULTS

Oxidation of C₁ substrates by crude extracts

Crude cell-free extracts of methanol-grown *Pseudomonas* AM1 catalysed the complete oxidation of methanol to carbon dioxide by oxygen with a specific activity of 0.576 μ mole of methanol oxidized/hr./mg. of protein. Formate and formaldehyde were also oxidized, the initial rate of oxygen uptake with these substrates being almost twice that obtained with methanol. The crude extract catalysed the reduction of DCPIP immediately in the presence of formate and formaldehyde and after a 10 min. lag period in the presence of methanol. On the addition of phenazine methosulphate the rate of oxidation of methanol by the crude extract was markedly stimulated. No indication of NAD⁺ or NADP⁺-linked methanol dehydrogenase or formaldehyde dehydrogenase was observed in crude or fractionated extracts although NAD⁺ was rapidly reduced on the addition of formate.

Comparison of formate dehydrogenase and formaldehyde dehydrogenase in other bacteria grown on C₁ substrates

With the Iowa strain of *Pseudomonas methanica*, Harrington & Kallio (1960) showed the presence of a highly active formaldehyde dehydrogenase, which was specific for formaldehyde and required NAD⁺ and GSH. In view of our failure to observe a similar enzyme in extracts of *Pseudomonas* AM1, extracts of other bacteria grown on C₁ compounds were examined. Table 2 lists the activities of formaldehyde dehydrogenase and formate dehydrogenase in identically prepared ultrasonic extracts of four types of bacteria, grown on methanol. It is evident that under these conditions a high concentration of formaldehyde dehydrogenase may be demonstrated in *Pseudomonas methanica* but not in *Pseudomonas* AM1, whereas all four organisms contain an aldehyde dehydrogenase that can be linked to DCPIP. Methanol-grown cells of *Pseudomonas* AM1 were also disintegrated by ultrasonics, alumina grinding and in the Hughes press, but in no case could an NAD⁺-linked formaldehyde dehydrogenase be detected in the resultant extracts.

* Abbreviation: DCPIP, 2,6-dichlorophenol-indophenol.

The formaldehyde dehydrogenase from *Pseudomonas methanica* (Texas) was specific for formaldehyde and NAD^+ and showed a requirement for GSH that was not satisfied by other thiol compounds. The properties are very similar to those found by Harrington & Kallio (1960) for the enzyme from the Iowa strain of *Pseudomonas methanica*.

It can be seen from Table 2 that all four organisms contain similar concentrations of NAD^+ -linked formate-dehydrogenase activity.

The two enzyme systems capable of oxidizing C_1 substrates observed in *Pseudomonas* AM1 extracts were an aldehyde dehydrogenase that could be linked to DCPIP and an NAD^+ -linked formate dehydrogenase. A fractionation of these two enzyme systems was carried out (see the Materials and Methods section) and the properties of the purified enzymes were studied.

Properties of the purified aldehyde dehydrogenase

Products and stoichiometry. The reaction product resulting from formaldehyde oxidation was identified as formate enzymically by using a purified NAD^+ -linked formate-dehydrogenase preparation (Johnson *et al.* 1964), which was specific for formate and free from other dehydrogenase activities.

To determine the stoichiometry of the formaldehyde oxidation, formaldehyde and DCPIP were incubated with the enzyme solution for 2 hr. at 30° . The change in extinction of the dye at $600\text{ m}\mu$ was noted and then the reaction was stopped by adding an excess of GSH, which reduced any remaining oxidized dye. The complete system contained, in a volume of 1 ml.; $0.18\text{ }\mu\text{mole}$ of DCPIP, $8.5\text{ }\mu\text{moles}$ of phosphate buffer, pH 7.0, $0.5\text{ }\mu\text{mole}$ of formaldehyde and 0.05 ml. of the enzyme solution. In the control, 0.05 ml. of boiled enzyme solution was substituted for the untreated enzyme. After stopping the reaction, 0.4 ml. of each incubation mixture was assayed for residual formaldehyde, and 0.5 ml. assayed for formate (see the Materials and Methods section). Under these conditions the oxidation of $0.175\text{ }\mu\text{mole}$ of formaldehyde was

accompanied by the reduction of $0.18\text{ }\mu\text{mole}$ of DCPIP, calculated from $\epsilon 19 \times 10^3$ (Basford & Huennkens, 1955), and the appearance of $0.166\text{ }\mu\text{mole}$ of formate. With the boiled enzyme solution, $0.045\text{ }\mu\text{mole}$ of formaldehyde was consumed and $0.026\text{ }\mu\text{mole}$ of formate produced. These results show that the enzyme is an aldehyde dehydrogenase, capable of catalysing the reaction:

Specificity for electron acceptors. The following compounds were tested for ability to act as electron acceptor in the assay system described in the Materials and Methods section: DCPIP, phenazine methosulphate, methylene blue, brilliant cresyl blue, benzyl viologen, cytochrome *c*, NAD^+ , NADP^+ , FAD and FMN. Of these, only DCPIP was reduced in the presence of formaldehyde, and the rate of this reduction was stimulated by the presence of phenazine methosulphate. When a manometric assay was employed, coupling of formaldehyde oxidation to phenazine methosulphate reduction could be demonstrated but the rate of reaction soon became non-linear, presumably owing to inhibition of the enzyme by the hydrogen peroxide produced by autooxidation of the dye.

Substrate specificity. The enzyme was found to have a broad substrate specificity and oxidized a wide range of aliphatic aldehydes, including formaldehyde, acetaldehyde, glyoxal, glyoxylate, glyceraldehyde, 3-phosphoglyceraldehyde, butyraldehyde and propionaldehyde at similar rates under the assay conditions described in the Materials and Methods section. The velocities obtained at saturating substrate concentration with formaldehyde, glyoxal and 3-phosphoglyceraldehyde were in the proportions 1.0:1.6:0.5. The K_m values for formaldehyde and glyoxal were measured in the reaction system used for the enzyme assay (see the Materials and Methods section). The values obtained were respectively 3.85 and 0.8 mM. The pH optimum, measured in the same reaction mixture, was 7.0.

Table 2. *Specific activities of formaldehyde dehydrogenase and formate dehydrogenase in ultrasonic extracts of methanol-grown bacteria*

Units of enzyme activity ... Assay system used	NADH produced ($\mu\text{moles/hr.}/\text{mg. of protein}$)		DCPIP reduced ($\mu\text{mole/hr.}/\text{mg. of protein}$) Formaldehyde-dehydrogenase assay system (see the Materials and Methods section)
	Phosphate buffer, pH 8.0, 20 μmoles ; NAD^+ , 0.5 μmole ; GSH, 2.0 μmoles ; extract, 0.05 ml.; formaldehyde, 25 μmoles ; total vol., 1 ml.	Tris-HCl buffer, pH 8.4, 20 μmoles ; NAD^+ , 0.5 μmole ; ultrasonic extract, 0.05 ml.; formate, 25 μmoles ; total vol., 1 ml.	
<i>Protaminobacter ruber</i>	0.36	6.8	0.8
<i>Pseudomonas extorquens</i>	0.828	4.8	0.92
<i>Pseudomonas methanica</i>	9.0	5.5	0.6
<i>Pseudomonas</i> AM1	0	8.2	0.18

Stability of the enzyme. The enzyme was stable to freezing and thawing and to storage for up to 2 months at -14° . Activity was slowly lost at 4° and totally abolished by heating for 2 min. at 50° .

Activators and inhibitors. The purified enzyme was 50 % inhibited by dialysis overnight against 10 mM-EDTA. This inhibition could not be reversed by the addition of a wide range of metal ions, but disappeared when the EDTA was removed by dialysis against 10 mM-tris-hydrochloric acid buffer, pH 7.5, for 2 hr. The addition of 12.5 mM-*o*-phenanthroline caused a lag in DCPIP reduction of 5 min. Iodoacetate (1 mM) produced 50 % inhibition after 60 min. preincubation, and *N*-ethylmaleimide (1 mM) produced 42 % inhibition after 30 min. preincubation. The presence of formaldehyde during incubation with the latter inhibitor did not significantly affect the degree of inhibition. Dialysis for 2 hr. in the presence of 10 mM-mercaptoethanol or -cysteine abolished the activity completely.

Contaminating enzymes. Under the standard assay conditions no reduction of DCPIP was observed when formaldehyde was replaced by methanol, ethanol, formate or malate.

Comparison of enzyme activity in methanol- and succinate-grown cells. The specific activity of the aldehyde dehydrogenase in extracts of methanol-grown cells was found to be twice that in similar extracts of succinate-grown cells.

Properties of the formate dehydrogenase

This enzyme was more labile than the aldehyde dehydrogenase and the presence of mercapto-

ethanol throughout the purification procedure was essential for activity to be retained. The overall purification obtained was only threefold, the specific activity of the crude extract being $0.136 \mu\text{-mole}$ of NADH produced/min./mg. of protein (14800 units) and that of the supernatant from step 4 being 0.42 (10050 units). Further attempts to purify the enzyme by salt fractionation, alumina C_γ gel treatment or column chromatography on DEAE-cellulose (Cl⁻ form) resulted in loss of activity. However, as the heat-treated enzyme solution was free from many interfering activities it was used for the experiments described below.

Products and stoicheiometry. Under the standard assay conditions the production of NADH by the enzyme corresponded to 80 % of the formate supplied (Table 3). By using the manometric assay, it was found that the enzyme catalysed the evolution of stoicheiometric amounts of carbon dioxide from formate, but only 30 % of the expected yield of NADH could be found or possibly accounted for by oxygen uptake. When formate and NAD⁺ were replaced by $1.25 \mu\text{moles}$ of NADH under the conditions shown in Table 3, 95 % of the NADH had been removed after 55 min. incubation. This suggests that the lack of stoicheiometry was due to the presence of enzyme(s) that removed the NADH produced. It was thus not possible to account completely for the electron change in the enzymic reaction, although it seems most likely that the enzyme is a formate dehydrogenase catalysing the reaction:

Table 3. *Stoicheiometry of the formate-dehydrogenase reaction*

For the spectrophotometric method, standard assay conditions were used, the incubation time being 10 min. (see the Materials and Methods section). For the manometric method, the main compartment of each double-side-arm Warburg flask contained: $20 \mu\text{moles}$ of phosphate buffer, pH 7.5; $5 \mu\text{moles}$ of NAD⁺; enzyme solution (containing 3.5 mg. of protein); water to give a final volume of 2.0 ml. Additions of sodium formate ($5 \mu\text{moles}$), KOH (0.2 ml. of 20 %) and H₂SO₄ (0.2 ml. of 3N) were made as indicated. Incubation was at 30° for 55 min.; the gas phase was air. NADH production was measured spectrophotometrically in flasks 1 and 2.

Spectrophotometric method						
Formate supplied 0.1 μ mole						
NADH produced 0.08 μ mole						
Manometric method						
Flask no.	Additions			CO ₂ evolved (μ l.)	O ₂ consumed (μ l.)	NADH produced (μ mole)
	Side arm 1	Side arm 2	Centre well			
1	Formate	None	Alkali	—	29	1.04
2	None	None	Alkali	—	5	0
3	Formate	Acid	None	154	—	—
4	None	Acid	None	44	—	—
5	None	Acid (tipped at zero time)	None	40	—	—
Net CO ₂ evolution (μ mole)				4.93		
Net O ₂ consumption (μ mole)				1.0		
Net NADH production (μ mole)				1.04		

Substrate specificity. The enzyme did not oxidize methanol, formaldehyde, acetate, oxalate, succinate or malate under the conditions of assay. NAD^+ was required as electron acceptor; NADP^+ was inactive.

K_m values and pH optimum. The K_m value for formate was 0.25 mM at 22° and pH 8.4, and that for NAD^+ was 0.09 mM under the same conditions. The pH optimum was 8.4.

Interfering activities. Under the standard assay conditions no reduction of NAD^+ was observed when formate was replaced by formaldehyde, methanol, ethanol, malate or acetate. The preparation still contained some NADH-removing activity. At pH 8.4 and 22°, with 0.08 μmole of NADH in the place of formate and NAD^+ in the standard assay system (see the Materials and Methods section), the rate of disappearance of NADH observed was 5.7 $\mu\text{m-moles/min./mg.}$ of protein.

Inhibition of the reaction. The effect of various inhibitors on the formate dehydrogenase is shown in Table 4. The enzyme is protected from inhibition by *N*-ethylmaleimide by the presence of NAD^+ . It is extremely sensitive to cyanide and to ferrous and cupric salts.

After dialysis to remove mercaptoethanol from the enzyme solution no stimulation of activity by 1 mM- or 5 mM-GSH was observed.

Nature of the methanol-oxidizing enzyme

Anthony & Zatman (1963) found a methanol dehydrogenase in ultrasonic extracts of *Pseudomonas* sp. M27 that readily reduced phenazine metho-

sulphate in the presence of methanol. Partially purified extracts lost all activity on dialysis but the addition of 5 mM-ammonium salts completely restored the activity.

In *Pseudomonas* AM1 a very similar enzyme appears to be present. As mentioned above, crude extracts prepared in the French pressure cell oxidized methanol completely to carbon dioxide and the initial rate of oxygen uptake was stimulated by the addition of phenazine methosulphate. To investigate the cofactor requirements of this reaction, low-molecular-weight compounds were removed from a sample (1.6 ml.) of such an extract (containing 20 mg. of protein/ml.) by passage through a small column (10 cm. $\times$ 1.5 cm.) of Sephadex G-50 (medium grade) previously equilibrated with 10 mM-phosphate buffer, pH 7.0. The enzyme system was recovered in a single fraction (volume, 2.0 ml.) and assayed for methanol-oxidizing activity in the presence of phenazine methosulphate. As shown in Fig. 2, the final oxygen uptake attained was diminished from approx. 1.3 μmoles to 0.95 $\mu\text{mole}/\mu\text{mole}$ of methanol supplied (i.e. yielding a product at the oxidation level of formate). This is consistent with the removal of NAD^+ and thus the failure of formate dehydrogenase to catalyse formate oxidation.

On fractionation of this crude extract with ammonium sulphate all the methanol-oxidizing activity was found in the protein fraction precipitating between 50 and 80 % saturation (50–80 % fraction). Passage of this protein fraction through Sephadex G-50 abolished its methanol-oxidizing activity (Fig. 3). The activity was

Table 4. *Inhibitors of formate dehydrogenase*

Enzyme activity was estimated in the standard assay system (see the Materials and Methods section). The absolute value for the uninhibited reaction was 0.42 μmole of NADH oxidized/min./mg. of protein.

Inhibitor	Concentration (mM)	Conditions	Inhibition (%)
Iodoacetate	4.0	Preincubation for 1 hr.	80
<i>N</i> -Ethylmaleimide	2.0	Preincubation for 30 min.	30
		Preincubation for 30 min. in the presence of 1 mM- NAD^+	0
EDTA	30	—	37
	50	—	50
	50	EDTA removed by 2 hr. dialysis against 10 mM-tris	50
Potassium cyanide	0.1	—	43
	0.5	—	95
Sodium azide	0.01	—	13
	0.08	—	21
8-Hydroxyquinoline	1.0	—	0
			(10% stimulation)
Pyridine-3-sulphonate	20	Preincubation for 5 min.	60
Copper sulphate	0.02	—	92.3
Ferrous sulphate	0.005	—	50

restored by the addition of 5 mM-ammonium sulphate. Such behaviour is very similar to that found by Anthony & Zatman in *Pseudomonas* sp. M27. *Protaminobacter ruber*, *Pseudomonas extorquens* and *Pseudomonas methanica* were all found to contain similar enzyme(s) that exhibited a requirement for NH_4^+ ions when aged or after treatment with Sephadex G-50 as described above.

Role of catalase in the oxidation of methanol

Harrington & Kallio (1960) showed that oxidation of methanol could proceed in extracts of *Pseudomonas methanica* in the presence of hydrogen peroxide generated by an added glucose-oxidase system. They suggested that the oxidation of methanol in *Pseudomonas methanica* took place via a 'catalase-linked peroxidase'. It is well known that methanol can be oxidized by hydrogen peroxide in the presence of catalase. By adding an artificial source of hydrogen peroxide to the bacterial extracts it is to be expected that the oxidation of methanol by any bacterial catalase present would be observed although this may have little relevance

to the oxidation *in vivo*. Since 3-amino-1,2,4-triazole can produce an immediate reversible inhibition of catalase (Heim, Appleman & Pyfrom, 1955) and a slowly developing (1–2 hr.) irreversible inhibition in the presence of a low and constant concentration of hydrogen peroxide (Margoliash & Novogrodsky, 1958; Margoliash, Novogrodsky & Schejter, 1960), this inhibitor was used in an attempt to define the role of catalase in methanol oxidation by extracts of *Pseudomonas* AM1.

Extracts were prepared by rupturing bacteria in the French pressure cell. The presence of catalase in such extracts was demonstrated by the perborate method of Feinstein (1949). For quantitative measurements of catalase activity the crude extract was diluted 100-fold, giving a protein concentration of 0.2–0.3 mg./ml. Preincubation of the crude extract for 5 min. with 20 mM-3-amino-1,2,4-triazole in the presence of 1 mM-ascorbate gave 50 % inhibition of catalatic activity; this was quantitatively similar to that observed with crystalline ox-liver catalase. The inhibitor was found to have no effect on the oxidation of methanol and formaldehyde by the same extract under these conditions (oxygen uptake measured in the absence of artificial electron acceptors). This result indicates that the enzyme system responsible for the complete oxidation of methanol in extracts of *Pseudomonas* AM1 is not catalase. After 60 min. preincubation with the inhibitor in the same experi-

Fig. 2. Effect of Sephadex G-50 treatment on methanol oxidation in the crude extract. The main compartment of each Warburg flask contained: 0.1 ml. of phenazine methosulphate (0.1 %, w/v), 1.55 μ moles of methanol, 20 μ moles of phosphate buffer, pH 7.5; the centre wells contained 0.2 ml. of 20 % (w/v) KOH; 0.2 ml. of the crude extract (containing 4 mg. of protein) was added to the side arm. Methanol was omitted from the control flasks. The final volume was 2 ml. Incubation was at 30°. ○, Untreated extract + methanol; △, untreated extract alone; ●, Sephadex-treated extract + methanol; ▲, Sephadex-treated extract alone.

Fig. 3. Effect of Sephadex G-50 treatment and NH_4^+ ions on the 50–80 % ammonium sulphate fraction. Flask contents and conditions were as in Fig. 2, except that the side arm contained a sample of the 50–80 % fraction containing 5 mg. of protein. Endogenous respiration was negligible and is not shown. ○, Untreated 50–80 % fraction; ▲, Sephadex-treated 50–80 % fraction; ●, Sephadex-treated 50–80 % fraction + 5 mM-ammonium sulphate.

ment the oxidation of methanol was inhibited 50 % and after 75 min. that of formaldehyde by 20 %. However, since the purified aldehyde dehydrogenase is itself 50 % inhibited after incubation for 50 min. with 3-amino-1,2,4-triazole, involvement of catalase need not be invoked to explain the slowly developing inhibition of methanol oxidation by crude extracts in the presence of this inhibitor.

DISCUSSION

Oxidation of methanol. As described above, Anthony & Zatman (1963) found that extracts of *Pseudomonas* sp. M27 contained a methanol dehydrogenase that could be coupled to phenazine methosulphate and required the presence of NH_4^+ ions. Inhibitor studies indicated that methanol oxidation was independent of catalase. In *Pseudomonas* AM1 the present work has shown the independence of methanol oxidation from catalase action and also the presence of a methanol-oxidizing enzyme that appears closely similar to that found by Anthony & Zatman (1963). This enzyme has also been demonstrated in extracts of methanol-grown cells of three other types of bacteria. No evidence has been found for the presence of an NAD^+ -linked methanol dehydrogenase similar to that reported by Kaneda & Roxburgh (1959) in *Pseudomonas* PRL-W 4.

Oxidation of formaldehyde. Three specific formaldehyde dehydrogenases have been described. Two have been purified from mammalian liver (Strittmatter & Ball, 1955) and yeast (Rose & Racker, 1962) respectively. These purified enzymes require NAD^+ and GSH. Other thiol compounds are ineffective, and these authors suggest that the thiohemiacetal of formaldehyde and glutathione may be the actual substrate of the enzyme. Harrington & Kallio (1960) have shown the presence of a similar enzyme in extracts of methanol-grown *Pseudomonas methanica*. No enzyme of this type could be demonstrated in crude or fractionated extracts of methanol-grown *Pseudomonas* AM1 prepared by any of the methods described above, but extracts of *Pseudomonas methanica* (Texas) contained high concentrations of NAD^+ -linked formaldehyde dehydrogenase when prepared by ultrasonic disintegration. Lower concentrations of this enzyme were observed also in the other two organisms tested. It is therefore concluded that if such an enzyme is present in *Pseudomonas* AM1 it must be much more labile than the corresponding enzyme in *Pseudomonas methanica*.

The aldehyde dehydrogenases of broader specificity that have been described are either flavoproteins (e.g. Mahler, Mackler & Green, 1954) or use nicotinamide-adenine dinucleotides as electron acceptors. Such enzymes have been purified from

mammalian liver by Racker (1949) and Deitrich, Hellerman & Wein (1962), from yeast by Black (1951) and Seegmiller (1953), from *Acetobacter suboxydans* by King & Cheldelin (1956) and from *Pseudomonas fluorescens* by Jakoby (1958). Although the enzyme from *Pseudomonas* AM1 is similar to these enzymes in some respects (such as its broad specificity and sensitivity to thiol reagents) it differs in its requirement for a primary electron acceptor.

In view of its broad substrate specificity the significance of this enzyme in the oxidation of C_1 substrates is uncertain. However, the fact that its activity is twofold higher in methanol- than in succinate-grown cells, and its presence in methanol-grown *Pseudomonas methanica*, *Pseudomonas extorquens* and *Protaminobacter ruber*, suggest a role for the enzyme in C_1 metabolism.

An alternative mechanism for formaldehyde oxidation might be by coupling of reactions (3)–(6):

Reaction (3) occurs non-enzymically (Kisliuk, 1957), and the presence of enzymes catalysing reactions (4)–(6) in *Pseudomonas* AM1 has been demonstrated by Large & Quayle (1963). The overall reaction would result in the synthesis of 1 mol. each of NADPH and ATP/mol. of formaldehyde oxidized. However, the extent to which such a scheme may be involved in the oxidation of formaldehyde *in vivo* is not known at present.

Oxidation of formate. Formate dehydrogenase has been described as a component of the formate-hydrogen-lyase system of *Escherichia coli* (Gest & Peck, 1955; Peck & Gest, 1957). Soluble formate dehydrogenase preparations have recently been obtained by deoxycholate treatment of particulate fractions of aerobically and anaerobically grown *E. coli* (Iida & Taniguchi, 1959; Itagaki, Fujita & Sato, 1961; Wrigley & Linnane, 1961). In all these preparations the enzyme was found to be closely associated with cytochrome b_1 .

An NAD^+ -linked formate dehydrogenase has been purified from peas (Mathews & Vennesland, 1950; Davison, 1951; Nason & Little, 1955). Particulate preparations from the leaves of higher plants have been shown to contain a similar enzyme that can be extracted by digitonin treatment (Mazelis, 1960).

Among bacteria utilizing C₁ compounds the only report of the presence of formate dehydrogenase of which we are aware is that of Kaneda & Roxburgh (1959). These authors showed that extracts of methanol-grown *Pseudomonas* PRL-W 4 catalysed the reduction of NAD⁺ in the presence of formate. The present work demonstrates the presence of such an enzyme in methanol-grown *Pseudomonas* AM1. It is similar to the plant enzyme with respect to inhibition by cyanide and metal ions but is much less sensitive to inhibition by azide and 8-hydroxyquinoline. The demonstration and accurate assay of the enzyme in crude extracts is complicated by its instability and by the high NADH-oxidase activity present.

Comparison of enzymic activities with rates of oxidation in whole cells. In whole cells of *Pseudomonas* AM1 grown on methanol the rates of oxidation of methanol, formaldehyde and formate are about 20, 20 and 10 μ moles of oxygen utilized/hr./mg. of protein respectively. In crude extracts the formate dehydrogenase had an activity of 8.2 μ moles of NADH produced/hr./mg. of protein (uncorrected for NADH oxidase). This minimum value for the activity is thus of the order required to account for the level observed in whole cells. The respective values obtained for the activities of the methanol and formaldehyde dehydrogenases in crude extracts were 3.24 μ moles of phenazine methosulphate and 0.18 μ mole of DCPIP reduced/hr./mg. of protein. These values are clearly well below those required to account for the observed rate of oxidation of methanol and formaldehyde in whole cells. Whether this is due to the coupling of the enzymes with artificial electron acceptors in the assay systems or to their playing only a minor physiological role cannot be decided at this stage.

SUMMARY

1. The complete oxidation of methanol to carbon dioxide in cell-free extracts of methanol-grown *Pseudomonas* AM1 has been investigated.

2. By using 3-amino-1,2,4-triazole, a known inhibitor of catalase, the independence of methanol oxidation from catalatic activity has been shown.

3. The only enzyme capable of oxidizing methanol that could be demonstrated was a dehydrogenase that can be linked to phenazine methosulphate and required the presence of NH₄⁺ ions.

4. An aldehyde dehydrogenase that reduced 2,6-dichlorophenol-indophenol or phenazine methosulphate in the presence of formaldehyde was found in cell-free extracts and was purified by ammonium sulphate precipitation and ion-exchange chromatography. It possesses a broad specificity towards aldehyde oxidation, and a pH optimum of 7.0. The enzyme is inhibited by thiol-binding reagents and

ethylenediaminetetra-acetate. Its *K_m* value with respect to formaldehyde is 3.85 mM.

5. A nicotinamide-adenine nucleotide-linked formate dehydrogenase was found in cell-free extracts and purified by ammonium sulphate precipitation. The enzyme is specific for the oxidation of formate, with a *K_m* value for formate of 0.25 mM and a pH optimum of 8.4. It is inhibited by iodoacetate, cyanide and ferrous or cupric salts.

6. The possible significance of these enzymes in the oxidation of C₁ substrates by intact cells is discussed.

7. Cell-free extracts of methanol-grown *Protaminobacter ruber*, *Pseudomonas extorquens* and *Pseudomonas methanica* have been found to possess a methanol dehydrogenase that can be linked to phenazine methosulphate, an aldehyde dehydrogenase that may be linked to 2,6-dichlorophenol-indophenol, and a nicotinamide-adenine dinucleotide-linked formate dehydrogenase, similar to those found in *Pseudomonas* AM1.

8. A specific nicotinamide-adenine dinucleotide-linked formaldehyde dehydrogenase, dependent on the presence of glutathione, was found in high activity in extracts of methanol-grown *Pseudomonas methanica*, in lower activity in extracts of methanol-grown *Pseudomonas extorquens* and *Protaminobacter ruber*, but was not found in extracts of methanol-grown *Pseudomonas* AM1.

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REFERENCES

- Anthony, C. & Zatman, L. J. (1963). *J. gen. Microbiol.* **31**, xxi.
- Basford, R. E. & Huennekens, F. M. (1955). *J. Amer. chem. Soc.* **77**, 3873.
- Bassalik, K. (1913). *Jb. Wiss. Bot.* **53**, 255.
- Black, S. (1951). *Arch. Biochem. Biophys.* **34**, 86.
- Davison, D. C. (1951). *Biochem. J.* **49**, 520.
- Deitrich, R. A., Hellerman, L. & Wein, J. (1962). *J. biol. Chem.* **237**, 560.
- Dworkin, M. & Foster, J. W. (1956). *J. Bact.* **72**, 646.
- Feinstein, R. N. (1949). *J. biol. Chem.* **180**, 1197.
- Gest, H. & Peck, H. D. (1955). *J. Bact.* **70**, 326.
- Harrington, A. A. & Kallio, R. E. (1960). *Canad. J. Microbiol.* **6**, 1.
- Heim, W. G., Appleman, D. & Pyfrom, H. T. (1955). *Science*, **122**, 693.
- Hughes, D. E. (1951). *Brit. J. exp. Path.* **32**, 97.
- Iida, K. & Taniguchi, S. (1959). *J. Biochem., Tokyo*, **46**, 1041.
- Itagaki, E., Fujita, T. & Sato, R. (1961). *Biochem. biophys. Res. Commun.* **5**, 30.

- Jakoby, W. B. (1958). *J. biol. Chem.* **232**, 75.
- Johnson, P. A., Jones-Mortimer, M. C. & Quayle, J. R. (1964). *Biochim. biophys. Acta*, **89**, 351.
- Kaneda, T. & Roxburgh, J. M. (1959). *Canad. J. Microbiol.* **5**, 187.
- King, T. E. & Cheldelin, V. H. (1956). *J. biol. Chem.* **220**, 177.
- Kisliuk, R. L. (1957). *J. biol. Chem.* **227**, 805.
- Large, P. J., Peel, D. & Quayle, J. R. (1962). *Biochem. J.* **82**, 483.
- Large, P. J. & Quayle, J. R. (1963). *Biochem. J.* **87**, 386.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
- Mellwain, H. (1948). *J. gen. Microbiol.* **2**, 228.
- Mahler, H. R., Mackler, B. & Green, D. E. (1954). *J. biol. Chem.* **210**, 465.
- Margoliash, E. & Novogrodsky, A. (1958). *Biochem. J.* **68**, 468.
- Margoliash, E., Novogrodsky, A. & Schejter, A. (1960). *Biochem. J.* **74**, 339.
- Mathews, M. B. & Vennesland, B. (1950). *J. biol. Chem.* **186**, 667.
- Mazelis, M. (1960). *Plant Physiol.* **35**, 386.
- Nash, T. (1953). *Biochem. J.* **55**, 416.
- Nason, A. & Little, H. N. (1955). In *Methods in Enzymology*, vol. 1, p. 536. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Peck, H. D. & Gest, H. (1957). *J. Bact.* **73**, 706.
- Peel, D. & Quayle, J. R. (1961). *Biochem. J.* **81**, 465.
- Racker, E. (1949). *J. biol. Chem.* **201**, 629.
- Rose, Z. B. & Racker, E. (1962). *J. biol. Chem.* **237**, 3279.
- Seegmiller, J. E. (1953). *J. biol. Chem.* **201**, 629.
- Strittmater, P. & Ball, E. G. (1955). *J. biol. Chem.* **213**, 445.
- Warburg, O. & Christian, W. (1941). *Biochem. Z.* **310**, 384.
- Weaver, R. H., Samuels, T. C. & Sherago, M. (1938). *J. Bact.* **35**, 59.
- Wrigley, C. W. & Linnane, A. W. (1961). *Biochem. biophys. Res. Commun.* **4**, 66.
- Yoe, J. H. & Reid, L. C. (1941). *Industr. Engng Chem. (Anal. Ed.)*, **13**, 238.

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Fatty Acids and Intestinal Metabolism

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Long-chain fatty acids have inhibitory effects on many metabolic processes *in vitro*. Munoz & Leloir (1943) observed that decanoic acid, lauric acid, palmitic acid, stearic acid or oleic acid at a concentration of 3 mM inhibited the respiration of a particle preparation from guinea-pig liver. Fluck & Pritham (1961) found that, at a concentration of 1.5 mM, decanoic acid, lauric acid or myristic acid inhibited the conversion of mevalonic acid into cholesterol by rat-liver homogenates by over 90%. Ahmed & Scholefield (1961*a, b*) observed that decanoic acid at concentrations above 1.34 mM inhibited the respiration of rat-liver, -kidney and -brain slices. The incorporation of inorganic phosphate into ATP and ADP was also inhibited.

Since the intestinal mucosa probably absorbs 60% of the dietary fatty acids in the form of free fatty acids the effect of a series of fatty acids on rat intestine has been studied. An inhibition of respiration was observed that depended on the chain length of the fatty acid and its concentration. A study was also carried out on the effect of fatty acids on the uptake and metabolism of glucose by intestine *in vitro* to elucidate the nature of the respiratory inhibition.

EXPERIMENTAL

Materials

Animals. Wistar strain rats, approx. 200 g. each, fed *ad lib.* on a diet of rat cubes (Oxo Ltd., London) were starved for 24 hr. before use.

Chemicals and enzymes. All solvents were of analytical grade. Fatty acids were obtained from L. Light and Co. Ltd., Colnbrook, Bucks., and the California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A. [^{14}C]-Glucose and [^{14}C]-glutamic acid were obtained from The Radiochemical Centre, Amersham, Bucks. 2,5-Diphenyl-oxazole was obtained from L. Light and Co. Ltd. and from Nash and Thompson, Tolworth, Surrey. Lactate dehydrogenase and purified peroxidase were obtained from C. F. Boehringer and Soehne G.m.b.H., Mannheim, West Germany, and glucose oxidase was obtained from George T. Gurr Ltd., London.

Fatty acid solutions were prepared by dissolving the requisite amount of fatty acid in ethanol-ether (1:1, v/v). The calculated equivalent of aqueous NaOH was added and the mixture evaporated to dryness at 60° on a rotary evaporator. The solid was then taken up in the appropriate saline medium, pH 6.8, with heating on a water bath if necessary to disperse the material. The fatty acids are referred to below as undissociated acids since these form the major component of the mixture at this pH for fatty acids with 12 or more carbon atoms (Schmidt-Nielsen, 1946).