A New Route for Ethylene Glycol Metabolism in *Mycobacterium* E44

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A *Mycobacterium* sp. which grows on ethylene and ethylene glycol was isolated from ditch water. Growth of the organism, respiration of washed organisms, experiments on excretion of metabolic intermediates and enzyme studies were consistent with a new route for the degradation of ethylene glycol via acetaldehyde and acetate. Cell-free extracts of organisms grown on ethylene glycol contained a diol dehydratase which required K^+ or NH_4^+ for activity as well as coenzyme B_{12} (5'-deoxyadenosyl cobalamin). Ethylene glycol was not an intermediate in ethylene metabolism.

INTRODUCTION

The metabolism of ethylene in *Mycobacterium* E20 involves the epoxidation of the double bond by a mono-oxygenase (de Bont *et al.*, 1979). The product, ethylene oxide, is converted to acetyl-CoA in a reaction requiring NAD^+ , CoA and an unknown cofactor (de Bont & Harder, 1978). Ring opening of ethylene oxide with water to form ethylene glycol might be a plausible alternative to this reaction because enzymic diol formation from an epoxide is a common reaction (Jakoby & Fjellstedt, 1972). Growth of an organism on ethylene and ethylene glycol could indicate the existence of this alternative route of degradation, but previously such an organism has not been isolated (de Bont, 1976; S. B. Primrose, personal communication). The recent isolation of an ethylene-utilizing bacterium, *Mycobacterium* E44, able to grow on ethylene glycol, prompted us to investigate the metabolism of ethylene glycol and its possible role in ethylene metabolism.

METHODS

Organism, media and culture conditions. A Gram-positive pleomorphic bacterium classified as *Mycobacterium* E44 (de Bont *et al.*, 1980) was isolated from ditch water using ethylene as the sole carbon and energy source by a method described previously (de Bont, 1976). The organism was maintained on a medium containing ($g\ l^{-1}$ in tap water): glucose, 10; yeast extract, 7; and Oxoid no. 3 agar, 12. The growth medium contained ($g\ l^{-1}$ in deionized water): K_2HPO_4 , 1.55; NaH_2PO_4 , 0.85; NH_4Cl , 2.0; $MgCl_2 \cdot 6H_2O$, 0.075; $(NH_4)_2SO_4$, 0.1; yeast extract, 0.1; and 0.2 ml of a trace element solution as described by Vishniac & Santer (1957). Carbon sources were added to give final concentrations of $1\ g\ l^{-1}$. Cultures (500 ml medium in 5 l Erlenmeyer flasks) were inoculated with 100 ml inoculum cultures grown in the same medium. The cultures were incubated at $30\ ^\circ C$ with shaking at 1 Hz. For growth on ethylene, the flasks were sealed with rubber stoppers fitted with Suba-seals through which 200 ml ethylene was injected.

Chemical estimations. Acetaldehyde and propionaldehyde were determined with a Becker 417 gas chromatograph fitted with a Porapak R column; the column temperature was $180\ ^\circ C$ and the carrier gas was N_2 . Acetic acid and ethylene glycol were measured with a Varian Aerograph series 2400 gas chromatograph fitted with a Chromosorb 101 (80 to 100 mesh) column; the column temperature was $190\ ^\circ C$ and the carrier gas was N_2 saturated with formic acid.

Dissolved O_2 concentrations were measured at 30 °C with a Yellow Springs Instrument Co. model 53 monitor equipped with a polarographic sensor.

Protein was measured by the Lowry method, with bovine serum albumin as the standard.

Respiration measurements and determination of excretion products. Organisms were harvested by centrifugation at 16000 g for 20 min at 4 °C, washed with 50 mM- KH_2PO_4/K_2HPO_4 buffer, pH 7.2 and resuspended in the same buffer.

Endogenous oxygen uptake by 10 ml washed cell suspensions was measured for at least 5 min at 30 °C. Subsequently, 0.1 ml of substrate solutions (5 g l⁻¹), or 0.1 ml of water in equilibrium with an ethylene gas-phase, was added and oxygen uptake was followed for at least 4 min.

Excretion of metabolic intermediates by washed cell suspensions (10 ml final volume) was measured in screw-cap bottles (40 ml) that were incubated at 30 °C in a shaking water bath. Acetaldehyde production was followed by taking samples (0.2 ml) from the gas-phase for analysis. Breakdown of ethylene glycol in incubation mixtures was stopped at different times by injecting 1 ml formic acid, and ethylene glycol and acetic acid were measured in the supernatant fluid after centrifugation at 27000 g for 10 min at 4 °C.

Enzyme assays. Cell-free extracts were obtained by ultrasonic disintegration with a Branson B-12 sonifier (6 × 30 s, 0 °C) of the washed cells in 50 mM- KH_2PO_4/K_2HPO_4 buffer, pH 7.2 containing 1 mM-dithiothreitol and 2 mM-MgCl₂. All subsequent procedures with the extract were carried out in the dark. The resulting suspension was centrifuged at 27000 g for 20 min at 4 °C and the supernatant, containing 5 to 10 mg protein ml⁻¹, was used for the enzyme assays. Gel filtration of the extract used in the experiments shown in Fig. 2(b) was performed by passing 1 ml of the extract down a 25 ml Sephadex G-25 column, eluting with 50 mM-Tris/HCl buffer, pH 7.8 containing 1 mM-dithiothreitol.

The following enzymes were assayed as previously described: NAD⁺-dependent isocitrate dehydrogenase [*threo*-D₂-isocitrate:NAD⁺ oxidoreductase (decarboxylating); EC 1.1.1.41] (Kornberg, 1955b); NADP⁺-dependent isocitrate dehydrogenase [*threo*-D₂-isocitrate:NADP⁺ oxidoreductase (decarboxylating); EC 1.1.1.42] (Kornberg, 1955a); NAD⁺-dependent acetaldehyde dehydrogenase [aldehyde:NAD⁺ oxidoreductase; EC 1.2.1.3] (Racker, 1955); isocitrate lyase [*threo*-D₂-isocitrate glyoxylate-lyase; EC 4.1.3.1] (Dixon & Kornberg, 1959); malate synthase [L-malate glyoxylate-lyase (CoA-acetylating); EC 4.1.3.2] (Dixon & Kornberg, 1962). Citrate synthase [citrate oxaloacetate-lyase (CoA-acetylating); EC 4.1.3.7] was assayed using the malate synthase method and replacing glyoxylate with oxaloacetate. NAD⁺- and CoA-dependent oxidation of ethylene oxide was assayed according to de Bont & Harder (1978).

Acetothio kinase [acetate:CoA ligase (AMP-forming); EC 6.2.1.1] was measured in a coupled assay with citrate synthase [citrate oxaloacetate-lyase (CoA-acetylating); EC 4.1.3.7] and malate dehydrogenase [L-malate:NAD⁺ oxidoreductase; EC 1.1.1.37]. The final mixture contained, in 1.0 ml, 50 μmol Tris/HCl, pH 8.0, 0.1 ml extract, 10 μmol malate, 5 μmol ATP, 0.5 μmol CoA, 20 μmol acetate, 3 μmol NAD⁺, 0.04 mg malate dehydrogenase and 1 mg citrate synthase. The reaction was started by the addition of CoA, ATP or acetate and the change in A_{340} due to NAD⁺ reduction was followed continuously with a Beckman model 25 spectrophotometer. The reaction mixture in the reference cuvette lacked CoA, ATP or acetate. A lag period of several minutes preceded a linear rate of NADH formation. Under these conditions a change of 6.22 absorbance units was taken to correspond to the formation of 1 μmol acetyl-CoA per cuvette.

Diol dehydratase in crude extracts was assayed by following aldehyde production. Vacutainer tubes (13.0 ml; Becton-Dickinson, France) containing 0.8 ml 50 mM- KH_2PO_4/K_2HPO_4 buffer, pH 7.2 and 0.1 ml 100 mM-ethylene glycol or propylene glycol were stoppered and flushed with N₂ for 20 s at a rate of 10 ml s⁻¹. Tubes were wrapped in aluminium foil and incubated at an angle of 45° at 30 °C with shaking at 2.7 Hz. The reaction was started by injecting 0.1 ml of crude extract. When gel-filtered extract was used the assay mixture contained, in 1.0 ml, 30 μmol Tris/HCl, pH 7.8, 40 μmol KCl, 24 nmol 5'-deoxyadenosyl cobalamin (DBC), and 10 μmol ethylene glycol or propylene glycol. The reaction was followed by periodically analysing 0.2 ml samples for acetaldehyde production in the gas-phase by gas chromatography. Aldehyde in the gas-phase was not readily in equilibrium with aldehyde in solution. Under the conditions employed, it took about 5 min to reach complete equilibrium between the two phases.

Chemicals. NAD⁺, NADP⁺, ATP, AMP, malate dehydrogenase and citrate synthase were obtained from Boehringer. 5'-Deoxyadenosyl cobalamin (DBC) and hydroxocobalamin hydrochloride (vitamin B_{12b}) were obtained from BDH.

RESULTS

Growth characteristics of Mycobacterium E44

Mycobacterium E44 grew on ethylene, ethylene glycol and succinate, with culture doubling times of 18, 8.5 and 9.5 h, respectively, and on ethanolamine and glucose as sole carbon and energy sources. Ethane, propylene, ethanol, glycollate and glyoxylate did not support growth.

Table 1. *Rates of substrate-dependent oxygen uptake by washed cell suspensions of Mycobacterium E44*

Values are expressed as the percentage increase in the rate of oxygen uptake in the presence of a substrate compared with the endogenous rate of oxygen uptake [24 to 42 nmol O₂ min⁻¹ (mg protein)⁻¹].

Assay substrate	Rate of oxygen uptake		
	Ethylene glycol	Growth substrate: Ethylene	Succinate
Ethylene	0	450	0
Ethylene oxide	0	175	0
Ethanol	25	50	0
Ethylene glycol	200	25	50
Acetaldehyde	275	150	150
Acetate	500	100	75
Glycolaldehyde	575	50	50
Glycollate	100	25	50
Glyoxylate	75	ND	75
Succinate	25	100	150

ND, Not determined.

Simultaneous adaptation studies

Substrate-dependent oxygen uptake by washed cell suspensions was determined for bacteria grown on ethylene, ethylene glycol or succinate (Table 1). Ethylene glycol did not significantly stimulate oxygen uptake by ethylene-grown cells, indicating that it was not an intermediate in the metabolism of ethylene. Furthermore, acetaldehyde and acetate greatly increased oxygen uptake by cells grown on ethylene glycol. This suggested that acetaldehyde and acetate were intermediates in ethylene glycol metabolism. The pattern of ethylene oxide stimulation of oxygen uptake suggested that this compound was an intermediate in ethylene metabolism.

Excretion of intermediate metabolites during ethylene glycol catabolism

The transient accumulation of acetaldehyde from ethylene glycol was detected using suspensions of washed organism grown on ethylene glycol (Fig. 1*a*). Under these conditions acetate was not detected but it accumulated as a result of ethylene glycol catabolism in the presence of 20 mM-fluoroacetate (Fig. 1*b*).

Activities of enzymes in cell-free extracts

The results described above suggest that acetaldehyde and acetate are intermediates in the metabolism of ethylene glycol by *Mycobacterium* E44. Additional evidence for this suggestion was obtained by estimating activities of the relevant enzymes in extracts of *Mycobacterium* E44 grown on ethylene glycol or succinate. Acetaldehyde dehydrogenase and acetothiokinase activities were higher in extracts of ethylene glycol-grown organisms than in extracts of succinate-grown organisms (Table 2). The aldehyde dehydrogenase was not specific for acetaldehyde because propionaldehyde and, particularly, glycolaldehyde were good substrates for this enzyme as well.

Extracts were also assayed for enzymes of the glyoxylate cycle. The specific activity of isocitrate lyase in ethylene glycol-grown organisms was much higher than in succinate-grown organisms, whereas the specific activities of malate synthase were similar. As expected, the specific activities of citrate synthase and isocitrate dehydrogenase were similar in both extracts.

Oxidation of ethylene oxide occurred in extracts of ethylene-grown organisms when

Fig. 1. Formation of acetaldehyde (a) and acetate (b) from ethylene glycol by washed cell suspensions of *Mycobacterium* E44 grown on ethylene glycol. (a) Suspensions (25 mg protein) were incubated with different initial amounts of ethylene glycol – 30 μmol ($\blacktriangle$), 50 μmol ($\circ$) and 100 μmol ($\bullet$) – and acetaldehyde production was measured as described in Methods. Inset ($\triangle$): the incubation mixture contained 20 μmol acetaldehyde and no ethylene glycol. (b) Suspensions (26 mg protein) were incubated with 100 μmol ethylene glycol and 200 μmol fluoroacetate and the production of acetate ($\circ$) and ethylene glycol remaining ($\bullet$) were determined as described in Methods.

Table 2. *Specific activities of enzymes in cell-free extracts of Mycobacterium* E44

Enzyme	Enzyme activity [nmol substrate converted min^{-1} (mg protein) $^{-1}$]		
	Ethylene glycol	Growth substrate: Ethylene	Succinate
Aldehyde dehydrogenase (NAD^+)			
Acetaldehyde	200	ND	10
Propionaldehyde	160	ND	ND
Glycolaldehyde	310	ND	ND
Acetothio kinase	48	ND	9
Isocitrate lyase	19	ND	1
Malate synthase	75	ND	60
Ethylene oxide dehydrogenase (NAD^+ , CoA)	0	2	ND
Ethylene glycol dehydratase	90	0	0
Citrate synthase	30	ND	50
Isocitrate dehydrogenase (NADP^+)	33	ND	25
Isocitrate dehydrogenase (NAD^+)	0	ND	0

ND, Not determined.

NAD^+ and CoA were included in the reaction mixture, but it did not occur in extracts of ethylene glycol-grown organisms (Table 2).

Diol dehydratase activity

The evidence presented above indicates the possible involvement of a glycol dehydratase (Barker, 1972) in ethylene glycol metabolism. Ethylene glycol-grown organisms contained a diol dehydratase that catalysed the conversion of ethylene glycol to acetaldehyde (Table 2). No diol dehydratase activity was detected in extracts of organisms grown on either ethylene or succinate.

Propylene glycol was also a substrate for the enzyme but ethanolamine was not. The rate of aldehyde production from ethylene glycol or propylene glycol decreased with time

Fig. 2. Aldehyde formation from ethylene glycol and propylene glycol by crude cell-free extract (*a*; 0.75 mg protein) and extract chromatographed on Sephadex G-25 (*b*; 0.31 mg protein). (*a*) Reaction mixtures contained: 10 μ mol ethylene glycol (●); 10 μ mol propylene glycol (○); ethylene glycol and 24 nmol DBC (×). (*b*) Reaction mixtures contained 10 μ mol ethylene glycol together with: 40 μ mol KCl and 24 nmol DBC (●); 40 μ mol NH₄Cl and DBC (○); 40 μ mol NaCl and DBC (□); KCl (▲); DBC (△); KCl and 24 nmol vitamin B_{12b} (■). (For details, see Methods).

of incubation (Fig. 2*a*) at both 30 and 20 °C. Complete loss of activity was observed when the extract was incubated at 30 °C for 15 min in the absence of substrate. Although DBC did not stimulate activity of the diol dehydratase in crude extracts, the enzyme was strictly dependent on DBC. This was shown with an extract which had been passed down a Sephadex G-25 column to remove DBC (Fig. 2*b*). Vitamin B_{12b} could not replace DBC. Activity of the enzyme was also dependent on either K⁺ or NH₄⁺ but Na⁺ could replace these cations to a limited extent. In crude extracts enzyme activity was completely inhibited by 1 mM-glycolaldehyde. N₂O and O₂, other possible inhibitors of diol dehydratase, did not affect the activity of the enzyme from *Mycobacterium* E44 when the atmosphere over the assay mixture contained either of these gases. Storing the crude extract for 3 h on ice in the absence or presence of ethylene glycol under an atmosphere of pure N₂O or O₂ did not affect enzyme activity when compared with an extract that had been stored under N₂. Activity of the enzyme under pure O₂ was established after correcting for the consumption of acetaldehyde by the extract under these conditions.

DISCUSSION

On the basis of the results obtained in the present study, we propose a pathway for the metabolism of ethylene glycol and ethylene in *Mycobacterium* E44, as shown in Fig. 3.

The inability of *Mycobacterium* E44 to grow on glycollate or glyoxylate and the results of oxygen uptake experiments with washed cell suspensions strongly suggested that, unlike other ethylene glycol-utilizing bacteria studied so far (Stouthamer *et al.*, 1963; Kersters & de Ley, 1963; Kornberg & Morris, 1963; Jones & Watson, 1976; Gonzalez *et al.*, 1972; Child & Willetts, 1978), glyoxylate was not an intermediate in ethylene glycol metabolism. Strong stimulation by glycolaldehyde of oxygen uptake by suspensions of ethylene glycol-grown organisms (Table 1) was probably due to the broad substrate specificity of the aldehyde dehydrogenase (Table 2).

Fig. 3. Proposed pathways for the utilization of ethylene glycol and ethylene in *Mycobacterium* E44.

For the following reasons we consider that *Mycobacterium* E44 degrades ethylene glycol via acetaldehyde. Firstly, acetaldehyde was produced from ethylene glycol by suspensions of washed organisms grown on ethylene glycol (Fig. 1a). If it is assumed that acetaldehyde oxidation proceeded at a constant rate during the course of the experiment [40 nmol acetaldehyde oxidized min⁻¹ (mg protein)⁻¹] an almost stoichiometric conversion of the diol into the aldehyde at a rate of approximately 95 nmol min⁻¹ (mg protein)⁻¹ can be calculated. Secondly, ethylene glycol dehydratase was present in extracts of ethylene glycol-grown *Mycobacterium* E44 (Table 2). Furthermore, the acetaldehyde produced was oxidized to acetate (Fig. 1b, Tables 1 and 2), and acetyl-CoA from acetate was oxidized by the enzymes of the tricarboxylic acid cycle (Table 2). During growth on ethylene glycol the glyoxylate bypass operated since isocitrate lyase was induced. The isocitrate lyase assay method has been criticized because any oxoglutarate formed would also be trapped as a phenylhydrazone (Attwood & Harder, 1977). However, since the isocitrate dehydrogenase activities in extracts of ethylene glycol- and succinate-grown organisms were similar it can be concluded that an isocitrate lyase activity was measured.

The diol dehydratase acting on ethylene glycol and propylene glycol in *Mycobacterium* E44 resembles the dehydratases found in *Aerobacter aerogenes* grown anaerobically on glycerol (Abeles & Lee, 1961; Lee & Abeles, 1963; Pawelkiewicz & Zagalak, 1965) since these enzymes were also coenzyme B₁₂-dependent and required K⁺ or NH₄⁺. The kinetic behaviour of the two enzymes from *Aerobacter aerogenes* and that from *Mycobacterium* E44 with respect to ethylene glycol was similar in that a gradual decrease in the reaction rate occurred with time. However, the diol dehydratase from *Aerobacter aerogenes* converted propylene glycol at a constant rate which was higher than the initial rate of ethylene glycol conversion, whereas for the enzyme from *Mycobacterium* E44 propylene glycol was a poor substrate compared with ethylene glycol and the rate decreased with time. Inhibition by N₂O of propionaldehyde formation by diol dehydratase has been observed by Schrauzer *et al.* (1971), but no inhibition by N₂O was found in the present investigation. No inhibition by oxygen, as reported by Wagner *et al.* (1966), was observed, but, as reported by these authors, inhibition by glycolaldehyde was found for the enzyme from *Mycobacterium* E44.

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