THE MICROBIAL DEGRADATION OF POLYETHYLENE GLYCOLS

by

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Jenkins, L.D.J.

ABSTRACT

The work described in this thesis was undertaken to elucidate the metabolic pathways by which oligo- and polymeric glycols are degraded by micro organisms.

From a variety of microorganisms isolated by elective culture on minimal medium containing either ethylene glycol (EG), diethylene glycol (DEG) or polyethylene glycol 400 (PEG 400), three pure cultures, designated Z, R and O, were selected for further study. Strain R showed a marked preference for utilising oligomeric glycols (2-4 EO groups) while strain O grew best on the polymeric materials (6-25 EO groups); strain Z utilised EG exclusively.

The partial degradation of PEG 200 by strains R and O was studied in some detail and the specificity of these isolates for growth within a particular molecular weight range confirmed. Those components of the mixture that were not utilised were converted into acidic derivatives which accumulated in the medium.

Cell-extracts of EG-grown strains Z and R contained a glycol dehydrogenase which resembled in several characteristics the methanol dehydrogenase of methylotrophic bacteria in that it had a high pH optimum, an *in vitro* requirement for phenazine methosulphate (PMS) as an artificial carrier, was activated by NH_4^+ and had a molecular weight in the region of 120000. This enzyme which catalysed the conversion of ethylene glycol to glycolaldehyde also attacked DEG, triethylene glycol (TEG) and a number of primary and secondary alcohols. A NAD^+-linked aldehyde dehydrogenase, glycollate oxidase (located in the particulate fraction) and enzymes involved in the metabolism of glycollate to pyruvate, in particular glyoxylate carboligase, tartronate semialdehyde reductase and glycerate kinase, were demonstrated in the soluble fraction of extracts of strain Z grown on EG and of strain R grown on EG, DEG or TEG. These enzymes were significantly absent (with the exception of aldehyde dehydrogenase) in succinate-grown cells. This is consistent with oxidation of EG and possibly DEG and TEG by way of the glycerate pathway.

The metabolic control of EG-metabolism in strains Z and R was examined but because the appropriately blocked mutants were not isolated, identification of the specific inducers was not possible. Preliminary results suggested that successive inductive events regulate the synthesis of enzymes which convert EG into 2-phosphoglycerate.

Evidence was obtained to implicate an active transport system for ethylene glycol uptake in strains Z and R, which recognised oligomeric but not polymeric glycols. The high Ks value calculated suggested that EG is an unnatural substrate of a glycerol or alkanol transport system.

Oxidation of polymeric glycols but not EG or the oligomeric glycols was effected by PMS-supplemented extracts of strain O grown on PEG 400. The immediate product(s) of PEG oxidation were not identified but are probably carboxylated derivatives.

CHAPTER ONE

INTRODUCTION

1.1 GENERAL INTRODUCTION

Coincident with the widespread domestic and industrial use of synthetic chemical products, there has in recent years developed a concern for the disposal and ultimate fate of these chemicals once they have been used. The introduction of synthetic surfactants into commerce in the late 1940's for example, led to the appearance of thick layers of foam on rivers and in the decade 1958-1968 considerable effort was expended in solving the problem.

Bacteria and fungi are the principle agents which accomplish the biodegradation of organic compounds, an important process in the carbon cycle. Whatever carbon compound is used it is usually broken down to amphibolic intermediates of low molecular weight that enter one or the other of a relatively small number of biosynthetic pathways. For utilisation of compounds such as sugars, fatty acids and amino acids, relatively short pathways of degradation are involved since these compounds bear close structural similarity to intermediate metabolites and are readily converted to such products by enzyme-catalysed reactions. The structures of many other types of compounds, such as the natural (secondary) products of plant biosynthesis and many synthetic polymers, often require extensive structural modification before they can enter central metabolic schemes.

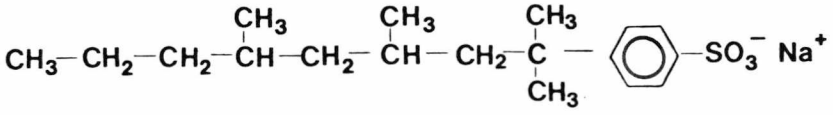
Microorganisms that have acquired the ability to degrade natural products have had a considerable period of time to evolve the enzymes necessary for their biodegradation. With certain commercially important synthetic carbon compounds, however, such as dyestuffs, some herbicides and detergents that have in recent years been manufactured and introduced into the environment, microorganisms have had much shorter periods of time to acquire the necessary catabolic potential. Enzymes possessing appropriate

catalytic ability may be recruited from pre-existing degradation pathways, either previously encoded by the genes of the evolved strain or acquired by an exchange of genetic material (Hegeman, 1972; Chapman, 1979). Their effective functioning in the new setting frequently requires alterations in their specificity and in the regulatory mechanisms which control their synthesis (Ornston, 1971; Clarke, 1978; Chapman, 1979). Other factors which may present special problems to microorganisms for the degradation of structurally useful polymers include their chemical structure, insolubility and size. Until about 1965, for example, the commonest detergents in use were tetrapropylene-derived alkylbenzene sulphonates (Fig. 1). These detergents suffered from the disadvantage that they were incompletely degraded by bacteria in sewage treatment plants, which meant that many such plants and the rivers into which they discharged became filled with foam. Because the failure of bacteria to attack these materials was due to the presence of a large number of branched alkyl groups in the molecule, the use of such detergents has now been abandoned in the United Kingdom and other Western nations. Reduction in the number of branched chains increased the capacity for biodegradation so that most detergents, today, contain linear or only singly branched alkyl chains; they include the linear alkylbenzene sulphonate class of anionic surfactants and the non-ionic linear alcohol ethoxylates (Fig. 1).

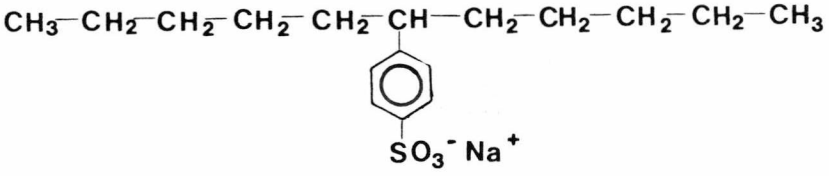
Primary biodegradation of non-ionic surfactants of the alcohol ethoxylate type is a rapid process so that the appearance of polyethylene glycols (the subject chemicals of this thesis) or closely related compounds during biodegradation has been widely reported (Patterson et al., 1967; Nooi et al., 1970 Wickbold, 1972; Cook, 1979). Current knowledge of the environmental impact of these compounds is nevertheless limited although their presence in aircraft de-icing fluids, and consequently in airfield drainage has prompted some investigations (Evans and David, 1974). Little work

a) Examples of anionic surfactants

Tetrapropylene-derived alkylbenzene sulphonate



Linear alkylbenzene sulphonate



b) Example of an alcohol ethoxylate non-ionic surfactant

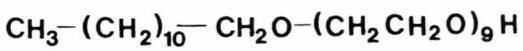


Fig. 1 Synthetic surfactants

has been done on the effect of polyethylene glycols (PEGs) on aquatic biota, partially because they have not been expected to persist in water. PEGs with high molecular weights have, however, been detected in river water and sewage effluents (Patterson et al., 1967) where they enhance the foaming potential of residual anionic and non-ionic surfactants (Patterson et al., 1966).

The biodegradation of such synthetic compounds in natural habitats is thus of considerable importance because of the large volume commercial production of mono-, di-, tri and polyethylene glycols in many countries. With the exception of the monomer, all these compounds have an ether bond which is generally quite resistant to chemical and microbial attack. Biodegradation of mono- and PEGs has nevertheless been observed by several investigators (Patterson et al., 1967; Pitter, 1968; Evans and David, 1974; Ogata et al., 1975; Cox and Conway, 1976). Use of these man-made chemicals results in their eventual discharge to the environment where they are attacked by the microorganisms of sewage, river water and soil. While there is considerable evidence that both ethylene glycol and its oligomers* are degraded in the laboratory by pure bacterial cultures (Fincher and Payne, 1962; Haines and Alexander 1975; Harada and Nagashima, 1975; Watson and Jones, 1977; Child and Willets, 1978; Kawai et al., 1978; Hosoya et al., 1978; Thélu et al., 1980; Wiegant and deBont, 1980) it has been suggested that the higher molecular weight compounds may, under some conditions persist in the natural environment (Tobin et al., 1976a; Tobin et al., 1976b).

Footnote

* Oligomeric - refers to compounds containing 2-4 ethylene oxide residues.
  Polymeric - refers to compounds containing 5 or more ethylene oxide residues.

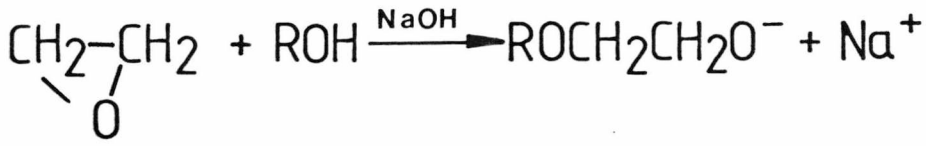
The work described in this thesis is concerned with the microbial degradation of commercially available PEGs with particular reference to the uptake and degradation of mono-, di- and triethylene glycols. Unlike the polymers which are mixtures (see section 3.1.3), the low molecular weight glycols are pure compounds for which biodegradation intermediates, end-products and metabolic pathways can be more readily established. It was believed, however, that such studies could give some insight into the nature of degradation of the higher molecular weight compounds and thus provide a basis of information from which to predict the fate of these compounds in specific environments.

1.2 THE POLYETHYLENE GLYCOLS

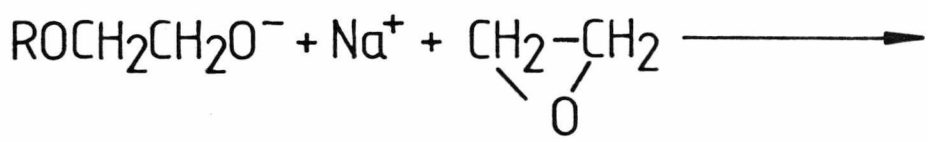
1.2.1 Synthesis

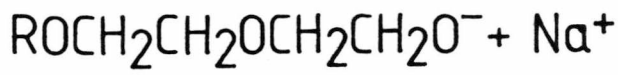
PEGs are polycondensation products of ethylene oxide and ethylene glycol. In the presence of sodium hydroxide at temperatures of the order of 120-135°C and under a total pressure of about 4 atmospheres, a reaction occurs which allows the initiator (ethylene glycol) to react with ethylene oxide to form an ether linkage between the two. The latter, in turn, under alkaline conditions adds onto another molecule of the open chain form of ethylene oxide giving diethylene glycol and so on. To terminate polymer formation, acid is added to neutralise the reaction mixture (Scheme 1). Due to the random nature of growth of polymers in this reaction, a mixture of polymers of various molecular weights results. Other bases (i.e. water, alcohols, etc.) may function in a similar capacity to ethylene glycol in which case the terminal groups are, of course, a function of the initiator molecule. These are hydroxyls when the latter is water or ethylene glycol.

a. INITIATION

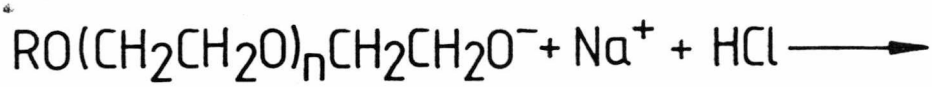


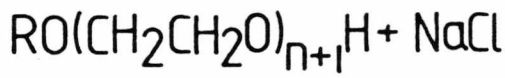
b. PROPAGATION





c. TERMINATION





Scheme 1. The polymerisation of ethylene oxide

1.2.2 Structure and Properties

The oligomeric glycols are low molecular weight materials which include mono-, di- tri- and tetraethylene glycol. They are composed of one to four ethylene oxide units linked by ether bridges and terminated with hydroxyl groups (Table 1). They are colourless, odourless, hygroscopic liquids of high boiling point and low volatility which are completely soluble in water.

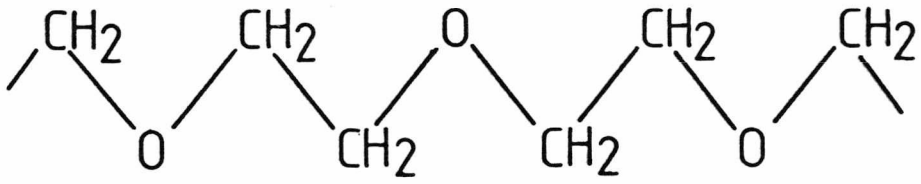
The PEGs are polymeric materials with molecular weights ranging from 200-20,000 and the general formula: $HO(CH_2CH_2O)_nH$ where n can represent an average of from 4 to about 136 ethylene oxide (EO) units. The numerical suffix (n) used to differentiate between the different grades gives an indication of their average molecular weight. Thus PEG 400 has an average molecular weight of about 400 (measured values for commercial samples range between 380 and 420) corresponding to a value of n for this particular polymer of n = 8. At room temperature, PEGs 200, 300 and 400 are colourless, relatively non-volatile liquids; 600 is a soft white material and PEGs 1000, 1500, 4000, 6000 and 20,000 are white waxy solids. Those with molecular weights ranging from 200 to approximately 600 are completely soluble in water while the higher molecular weight polymers exhibit a progressive decrease in water solubility.

The low molecular weight materials with 10 or less EO units are thought to be characterised by a zig-zag structure while the higher molecular weight materials are more convoluted due to EO units bending and folding back on themselves (Cox, 1978), (see Fig 2).

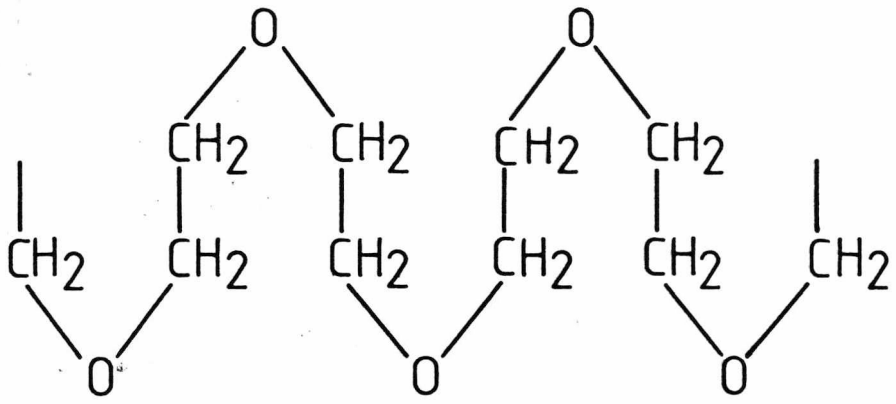
The tertiary structure of these materials has been reported as helical in nature, but their structures in dilute aqueous suspension are only vaguely known. They are, however, thought to at least partially maintain their helical nature (Bailey and Koleske, 1976).

Table 1 The glycols

COMPOUND	STRUCTURE
Ethylene glycol	$HO-CH_2-CH_2-OH$
Diethylene glycol	$HO+CH_2CH_2O+_2H$
Triethylene glycol	$HO+CH_2CH_2O+_3H$
Tetraethylene glycol	$HO+CH_2CH_2O+_4H$



Zig-zag structure of low-molecular weight glycols



'Bent' structure of PEGs containing greater than 10 ethoxylates

Fig.2 Structure of the glycols

1.2.3 Uses

The major uses of ethylene glycol are in the production of automobile antifreeze, liquid refrigerants and anti-icing compounds.

PEGs, together with ethylene glycol, are used in the manufacture of a wide range of pharmaceutical products, where they are frequently employed as ingredients in modern ointment bases and in a variety of cosmetic preparations including skin creams and sun-tan lotions. In addition the solid, waxy, water-soluble substances are used to increase the viscosity of liquid propylene glycols and to stiffen ointment and suppository bases.

PEGs are components of synthetic lubricants and cutting oils in the engineering industry and find considerable use as plasticisers, lubricants, conditioners and finishing agents for processing textiles and rubber. A substantial fraction of PEG goes into the production of non-ionic detergents, such as the alcohol ethoxylates (see Fig. 1). These compounds do not contain an ionized group as their hydrophilic component, but the hydrophilic properties are conferred on them by the presence of the number of oxygen atoms in the 'glycol' part of the molecule which are capable of forming H-bonds with molecules of water. Such non-ionic detergents are being used increasingly for the solubilisation and purification of membrane proteins in biochemistry (Helenius and Simons, 1975).

PEGs also find use in the biomedical field, where they are used for the purification of proteins by fractional precipitation (Polson *et al.*, 1964; Chun *et al.*, 1967; Honig and Kula, 1976) and when mixed with dextran or other water soluble polymers, form aqueous two-phase systems which can be used to purify cells and macro-molecules by counter-current distribution (Albertson, 1971).

1.2.4 Biodegradation of Polyethylene Glycols

Studies with unacclimated and acclimated activated sludge

Early studies on the degradation of PEGs, utilised systems which simulated biological sewage treatment and incorporated the use of commercial products and conventional biodegradation test methods (Lamb and Jenkins, 1952; Mills and Stack, 1954; Bogan and Sawyer, 1954, 1955; Sawyer, Bogan and Simpson, 1956).

20-day Biological Oxygen Demand (BOD) tests and studies using activated sludge were adopted to determine the ability of such systems to degrade ether glycol compounds. The glycol monomer, ethylene glycol, was shown to be readily degraded in these systems but di- and triethylene glycol, which possess one and two ether linkages respectively were less susceptible to microbial oxidation (Lamb and Jenkins, 1952). A series of 10-day activated sludge cultures using several different concentrations of test substances revealed that ethylene glycol was degraded but that di- and triethylene glycol was not (Mills and Stack, 1953). Likewise these authors concluded that the polyethylene glycols were not readily degradable by sewage microflora; when tri- and tetraethylene glycols or PEG 400 (approximately 8 EO units) were added to sludge, only about a third of the original material was degraded within 10 days. In further experiments to determine the biodegradability of a number of organic compounds, these authors observed that adaptation or acclimation of the seed culture to the various chemicals prior to use in BOD studies, generally resulted in a more active biomass (Mills and Stack, 1954). The procedure simply involved the incubation of river water samples in bottles with low levels of the test compound for 1-2 weeks in a mixed, aerobic system. BOD tests performed with adapted cultures on PEGs still did not reveal substantially greater biodegradation.

Gerhold and Maloney (1966) evaluated degradation with respirometric measurements and observed that whereas ethylene glycol was readily degraded in three different sludge mixtures, di- and triethylene glycol were utilised much more slowly or not at all.

One assumption was that the ethoxy ether linkage did not of itself resist biochemical oxidation but that the nature of the groups attached to the PEG chain and the length of the chain itself could exert a considerable influence of the rate of degradation (Bogan and Sawyer, 1954). Using t.l.c. to assess the amount of degradation occurring during laboratory die-away tests on a large number of the commercial ethoxylated non-ionics of the types used in detergent formulations Patterson et al. (1967) confirmed that the structure of the alkyl moiety and the length of the ethoxy chain influence the rate of degradation although none of the materials examined were found to be completely undegradable. In general, the longer the ether glycol chain of the original non-ionic material the more slowly it was degraded. Similar conclusions were made by Nooi et al. (1970) through studies in which removal of polyethoxylate material was estimated by radioactive tracer techniques applied in functional activated sludge plants.

Failure to demonstrate degradation of such long chain ethoxylates was partially explained by Pitter (1968) who found by using combinations of longer incubation periods, adapted sludge and higher concentrations of PEG than those typically employed in BOD studies, that alkyl ethoxylate detergents with higher molecular weight polyglycol moieties could in fact be biodegraded. In subsequent work Pitter (1973) restricted his studies to PEGs and found that sludge, acclimated to a medium containing 1000 mg/l PEG 1000, produced a biomass which when incubated with either PEG 600, 700 or 800 could completely degrade them in less than 30 days. Similarly

acclimated sludge samples degraded PEG 1000 in about 40 days and PEG 1500 in about 70 days. PEG 3000 was only slowly and incompletely degraded. More recently an enrichment culture technique was used to obtain cultures which were capable of degrading high molecular weight PEG polymers (Cox and Conway, 1976). Growth of the adapted biomass was examined turbidimetrically and the degree of PEG removal was calculated from total organic carbon (TOC) data and loss of reactivity of culture samples to Burgers reagent. PEGs with molecular weights up to 4000 were degraded by the biomass enriched on PEG 1540.

Watson and Jones (1977) were unable to demonstrate utilisation of PEG 4000 but did demonstrate the partial degradation of PEG 1500 when bacteria were grown in the presence of artificial sewage, containing other nutrients, namely peptone, yeast extract and urea. Inorganic salts media supplemented with PEG 1500 did not, however, support growth of these bacteria.

Using t.l.c. and n.m.r. analysis to monitor degradation, Watson and Jones (1977) also provided evidence to suggest the production of carboxylated PEG derivatives confirming earlier results of Patterson et al. (1967, 1970) who found similar intermediates from the biodegradation of alcohol ethoxylates. Although these intermediates were shown to be further degraded in sewage at temperate climates, it has been suggested that large quantities of polyethoxylates resulting from the incomplete degradation of non-ionic surfactants may persist in the natural environment (Tobin et al., 1976a). These authors applied a gas chromatographic method to demonstrate Dobanol 25-9 ($C_{12-15}H_{25-31}(C_2H_4O)_9H$) biodegradation in batch cultures. When inorganic salts media containing 20 mg/l Dobanol 25-9 was inoculated with activated sludge and incubated at 20°C on a rotary shaker, samples taken at appropriate times and analysed by g.l.c. showed the persistence of large fractions of the original polyethoxylate long after

removal of the surfactant alkyl moieties. Subsequent experiments performed to examine the degradation of Dobanol 25-9 in a simulated lake environment and in a laboratory-scale activated sludge plant produced essentially similar results (Tobin et al., 1976b). Studies by Cook (1979) on the contrary have indicated that the detergent Dobanol 45-7 ($C_{14-15}H_{29-31}(C_2H_4O)_7H$) could be degraded by activated sludge even at high initial concentrations (500 mg/l) and intermediates formed were not persistent. Material extracted from the supernatants was examined by both t.l.c. and g.l.c. analysis and the results suggested that the original surfactant material had disappeared within 3 days to be replaced by a polyglycol-like compound(s), which was subsequently degraded via acidic intermediates. At 14 days neither parent material nor products were detectable by either t.l.c. or g.l.c. analysis.

Pure culture studies

Although the degradation of PEGs by acclimated or unacclimated activated sludge as a source of biomass indicates their susceptibility to biological attack knowledge of the possible mechanisms employed by microorganisms for the dissimilation of PEGs derives largely from studies of pure cultures able to grow at the expense of these compounds. Such investigations have revealed the existence of microorganisms with wide-ranging glycol-degrading activities.

The ability of microorganisms to utilise ethylene glycol as the sole carbon and energy source has been demonstrated in species of several different genera of bacteria including Acetobacter aceti (Brown, 1887; Kersters and DeLey, 1963); Pseudomonas fluorescens (Jakoby, 1957); Micrococcus (now Paracoccus) denitrificans (Kornberg and Morris, 1965); Alcaligenes faecalis var myxogenes (Harada, 1965); Mycobacterium E_{44} (Wiegant and deBont, 1980) and two halophilic microorganisms; a fungus

Fusarium sp. No.83, isolated from algal bloom (Zeitoun et al., 1971) and a bacterium tentatively identified as a pseudomonad (ATCC 27042) isolated from a brine pond (Gonzalez et al., 1972).

44 strains of bacteria capable of degrading ethylene glycol (and other primary alcohols) were isolated from activated sludge treating the industrial wastes of polyester fibres (Grabinska-Loniewska, 1974). This author observed that the predominant bacteria were pseudomonads although she also isolated bacteria of the genera Achromobacter, Flavobacteria, Mycobacterium and Xanthomonas.

The metabolism of ethylene glycol by species of Mycobacterium is sufficiently well described to suggest its use as a means of species differentiation (Tsukamura, 1966). Of 132 strains tested 5 strains of M. smegmatis, 5 strains of M. fortuitum and 1 unidentified strain were able to grow in medium containing ethylene glycol as the sole carbon and energy source.

Growth of bacteria on oligomeric and/or polymeric glycols has also been described by several investigators. Several strains of soil bacteria have been induced to utilise oligomeric glycols and PEGs with molecular weights up to 400 (Fincher and Payne, 1962; Ohmata et al., 1974; Hosoya et al., 1978; Thélu et al., 1980). Each exhibits slight differences in their substrate specificities as shown in Table 2.

Species of Alcaligenes also grow at the expense of glycols (Harada and Nagashima, 1975). Two species Alcaligenes MC11 and TE8 grew well on ethylene glycol monoethylether, tri- and tetraethylene glycol and PEGs of molecular weight up to 400. In addition strain MC11 also grew well on ethylene glycol monomethylether. A third species Alcaligenes PE18, which could not grow in glucose medium, only exhibited good growth with tri-, tetra- and PEGs.

Table 2. Growth of various soil isolates on polethylene glycols

Carbon source	Soil isolate Fincher & Payne 1962	Strain PG1 Hosoya et al. 1978	Soil isolate Ohmata et al. 1974	Ps. 400 Thelú et al. 1980
Ethylene glycol	+	-	+	-
Diethylene glycol	+	+	-	+
Triethylene glycol	+	+	+	+
Tetraethylene glycol	+	n.d.	n.d.	+
PEG 200	+	+	+	+
PEG 300	+	+	+	n.d.
PEG 400	+	+	+	+

n.d. not determined.

Ogata et al., (1975) isolated cultures by enrichment from soil, waste water and acclimated sludge samples and classified them into five groups according to their PEG-assimilating limits. These groups were characterised by their ability to utilise PEG 400, 600, 1000, 4000, 6000 and 20,000. Representative strains from some of these groups were then tested for their ability to grow on PEGs in a wide range of molecular weights. One strain was reported to metabolise PEG 600 to the exclusion of all others while paradoxically, strains selected for metabolising PEGs optimally between 300 and 1000 were the only strains that could also actively metabolise ethylene glycol. In another study pure cultures of 20 strains of bacteria were obtained which grew using ethylene glycol, diethylene glycol or PEGs with molecular weights up to 1500 (Pearce and Heydeman, 1980), though no single strain used the whole range. One organism designated S8 could grow on several alternative carbon sources related to di-, tri- or tetraethylene glycol, including 2-ethoxyethanol, 2-butoxyethanol and 2'-(2-butoxyethoxy)ethanol. 2-(2'-Methoxyethoxy) ethanol was only partially utilised to yield 2-methoxyethanol which was not further degraded.

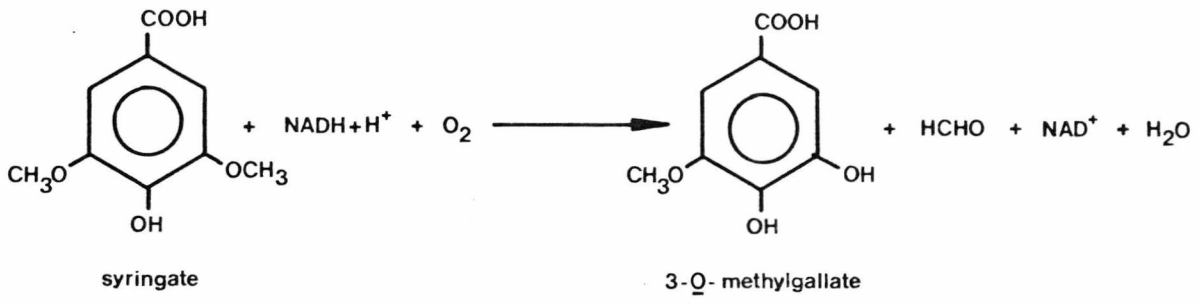
Degradation of PEGs with molecular weights greater than 1500 have been reported on only a few occasions. A strain of Pseudomonas aeruginosa was isolated which was capable of growth with polyethylene glycols ranging from ethylene glycol to PEG 20,000 (Haines and Alexander, 1975) and a species of Nocardia has been obtained from enrichment culture capable of growth on PEG 4000 (Baggi et al., 1978). Kawai et al. (1977) were unable to isolate a single organism with this property but they did obtain a synergistic mixed culture or 'consortium' of 2 strains, a Pseudomonas sp. and a Flavobacterium neither of which alone assimilated PEG for growth but when grown together were able to utilise PEGs of molecular weight between 300 and 20,000.

1.2.5 The Degradation of Ether compounds

Enzymic mechanisms available to microorganisms for the cleavage of ether bonds are now known for both natural and synthetic ethers.

The ether linkage is uncommonly distributed in natural compounds although because it occurs profusely in lignin it is very significant in terms of total biomass. The most commonly encountered form of the ether linkage is the methoxyl substituent of aromatic rings. Despite its chemical inertness, this link is amenable to enzymic cleavage, and most of the research performed has been on aromatic ethers.

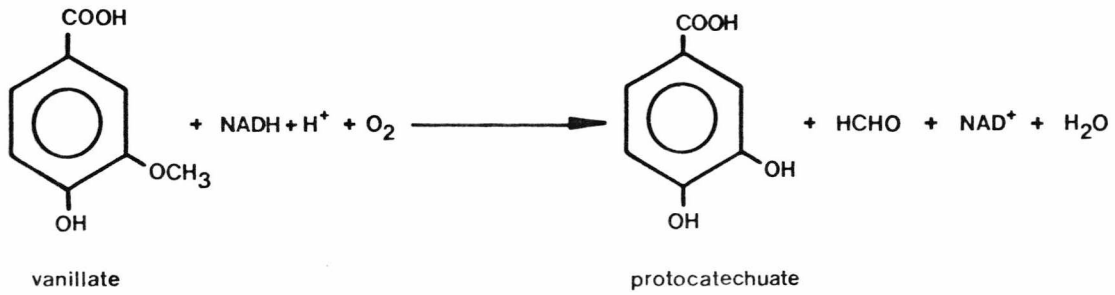
The presence of an O-alkoxyl group on an aromatic nucleus represents a site of potential microbial action. From studies with a number of O-methyl ethers of aromatic compounds, many having structures related to lignin monomers, aerobic microorganisms can be shown to effect demethylation by monooxygenation. With cell free extracts of Pseudomonas putida, Tack et al. (1972) demonstrated that oxidation of syringate was not initiated until NADH was added. This requirement for NADH indicated that ring fission was proceeded by O-demethylation according to the following equation:



syringate

3-O-methylgallate

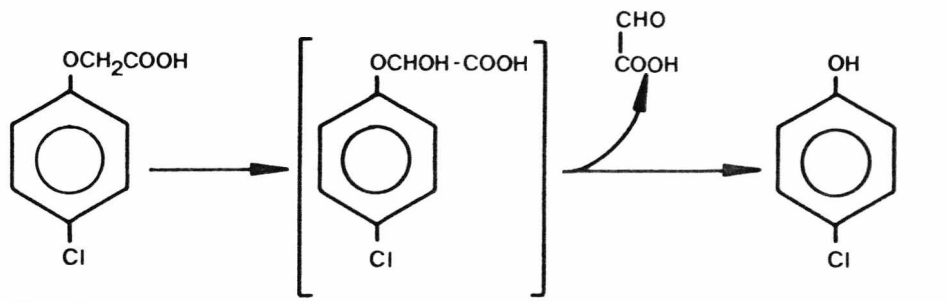
Ribbons (1971) showed that an analogous equation described O-demethylation of vanillate by extracts of Ps. aeruginosa. The enzyme, a monooxygenase was shown to catalyse the conversion of vanillate to protocatechuate and formaldehyde:



vanillate protocatechuate

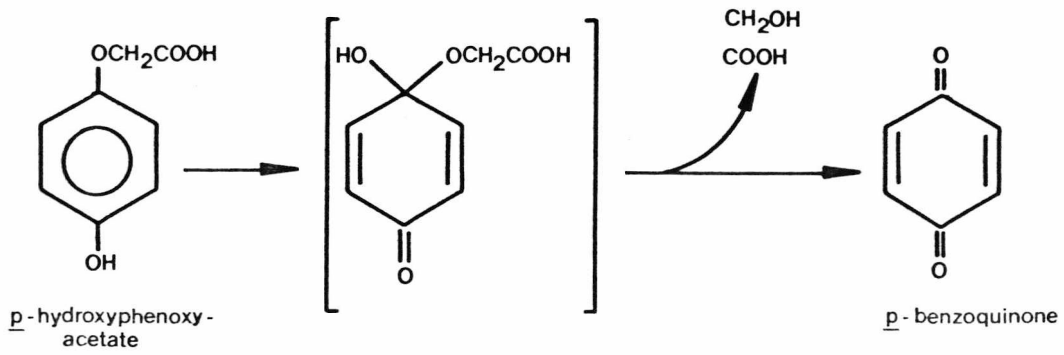
In the latter case the product of the monooxygenase reaction is probably the unstable hemiacetal of the parent phenol which then dismutates to give the phenolic and formaldehyde products observed.

Such reactions also explain the cleavage of the ether sidechain of phenoxyacetic acids such as 4-chlorophenoxyacetic (Evans et al., 1971) and 4-chloro-2-methylphenoxyacetic acids (Gramar and Graunt, 1971). In this instance, glyoxylate is formed together with the parent phenol:



p-chlorophenoxy-acetate p-chlorophenol

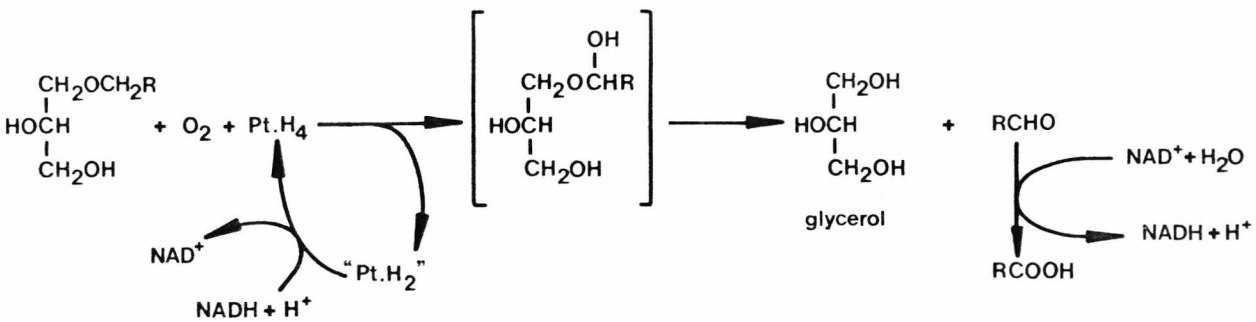
Fortuitous monooxygenation of 4-hydroxyphenoxyacetate at C-1 by an enzyme from Ps. acidovorans, can accomplish cleavage of the ether bond to yield glycollate and 1,4-benzoquinone (Hareland et al., 1975).



Studies on the degradation of strictly aliphatic ethers have not been so extensive but the ability of methane-oxidising bacteria to oxidise and utilise dimethylether as a sole source of carbon and energy has been described (Wilkinson, 1971; Davey, 1971; Hazeu, 1975; Ribbons, 1975; Patel et al., 1976).

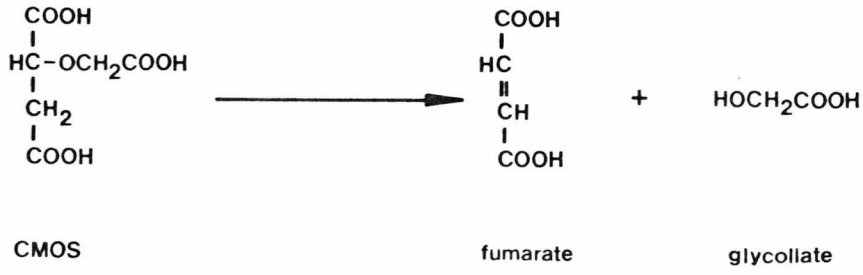
Recently Meyers and Ribbons (1978) and Stirling and Dalton (1980) have shown that although dimethylether can be oxidised (albeit very poorly) by suspensions of Methylococcus capsulatus and readily oxidised by extracts with purified methane monooxygenase preparations, it is a non-growth substrate which is fortuitously oxidised. Observed growth on dimethylether by previous investigators was in fact shown to be due to contamination of the ether with high concentrations of methanol. The reaction catalysed by methane monooxygenase was assumed to proceed via the unstable hemiacetal, methoxymethanol, to yield the observed products methanol and formaldehyde. Oxidation of diethylether occurred at the subterminal carbon atom resulting in the production of ethanol and acetaldehyde, which could be detected in spent reaction mixtures.

A 'mixed-function' oxygenase has been implicated in the cleavage of the ether bond of glyceryl ethers, which occur throughout Nature as components of lipids in which long chain alkyl residues are frequently linked to glycerol by ether bonds rather than by the more common ester linkage. This activity has been demonstrated in rat liver (Tiez et al., 1964) but may conceivably also occur in microorganisms degrading such compounds. The first step in the reaction sequence was shown to be oxidation of the ether by molecular oxygen in the presence of a tetrahydropteridine co-factor. The product of this reaction was assumed to be a hemiacetal which then broke down spontaneously to yield the long chain aldehyde and free glycerol. The aldehyde was subsequently oxidised to the corresponding carboxylic acid in a reaction requiring a nicotinamide nucleotide:



R = alkyl residue ($C_{14} - C_{23}$)

An investigation on the microbial degradation of carboxymethoxysuccinate (CMOS), a potential replacement of phosphate as a detergent builder, has revealed the presence of a non-oxygenative ether-cleaving enzyme. The enzyme CMOS lyase was shown to catalyse a β-elimination type reaction converting CMOS into fumarate and glycollate (Peterson and Llaneza, 1974, Cain, 1981):



While there is presently no precise indication as to how the ether bonds of PEGs are cleaved, intermediates and enzymes which constitute part of the degradation pathway(s) have now been characterised and reported in a number of investigations.

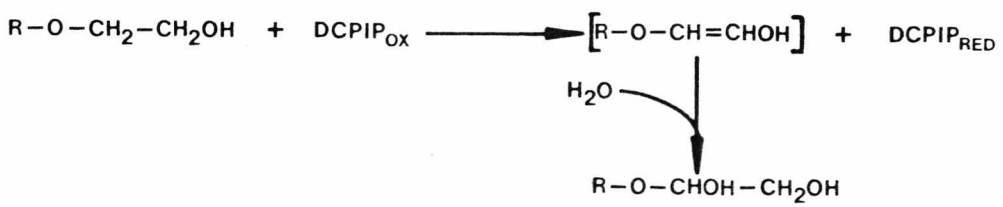
In an extensive review of the acetic acid bacteria, Kersters and DeLey (1963) identified a number of strains of Acetobacter and Gluconobacter which oxidised aliphatic glycols. Resting cells of both A. aceti and G. suboxydans oxidised ethylene glycol to glycollate but di- and triethylene glycol were oxidised progressively less effectively and their products were not identified. A soluble NAD-linked alcohol dehydrogenase and a particulate oxidase system were implicated in these conversions. The primary product, the corresponding aldehyde, was considered to be the same whether the oxidation involved the dehydrogenase or the oxidase system. Further studies showed that di- and triethylene glycol were substrates for oxidation by cell suspensions of G. suboxydans and diglycollate was identified as the product of diethylene glycol metabolism (DeLey and Kersters, 1964).

High NAD-dependent dehydrogenase activities toward ether alcohol compounds including di- and triethylene glycols and PEGs as well as towards ethylene glycol and ethanol were observed by Harada & Sawada (1977) in extracts of Alcaligenes MC11. Enzymes in crude extracts were fractionated by polyacrylamide gel electrophoresis and the NAD-dependent dehydrogenase activity in the gels detected using p-nitroblue tetrazolium chloride. Different bands of dehydrogenase activity were obtained in the crude extracts when the cells were grown with various ether alcohols, ethylene glycol and ethanol. Crude extracts of cells grown with ethylene glycol, ethanol and ethylene glycol monomethyl- and monoethylethers gave 2 bands, b and c. Extracts of cells grown with diethylene glycol gave 4 bands, a, b, c and d whereas extracts of cells grown with triethylene glycol and PEGs gave 2 bands a and c. When succinate was used for growth a single band c was observed. Thus the enzyme in band c appeared to be formed constitutively whereas those in the other bands by induction by the glycols. Enzymes in bands a, b and d showed high activities towards di-, tri and PEGs but not ethylene glycol. Enzyme c on the other hand, attacked ethylene glycol and alcohols to the exclusion of all other glycols. The specificity of the latter enzyme resembles the NAD-dependent alcohol dehydrogenase demonstrated in extracts from cells of an Acinetobacter strain (Jones and Watson, 1976), although in this case the enzyme was shown to be inducible.

Two enzymes with different coenzyme requirements were isolated from a soil bacterium (Ohmata et al., 1974); a NAD-dependent dehydrogenase which showed high activity towards triethylene glycol and PEG 200 and a flavin-linked dehydrogenase catalysing the oxidation of triethylene glycol; whether this enzyme could attack other ether substrates was not determined.

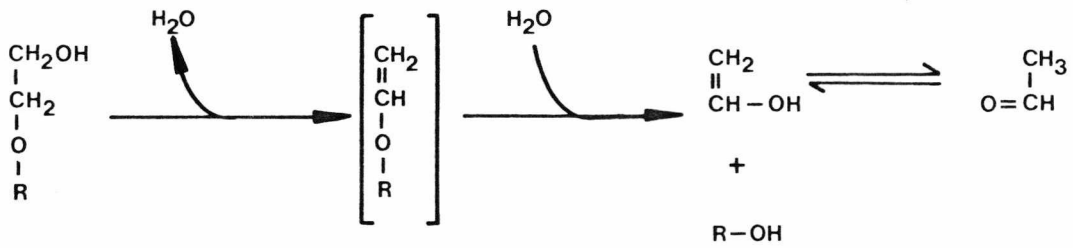
Several other investigators have reported the existence of PEG dehydrogenases which require coenzymes other than nicotinamide nucleotides

concerned solely with the oxidation of the oligomeric glycols. Payne and Todd (1966) showed that extracts of a bacterium, designated TEG.5, dehydrogenated a variety of ether glycols but not ethylene glycol. FAD or ferricyanide were shown to be effective electron acceptors for the reaction. In agreement with this work Caskey and Taber (1981), using a modification of the procedure of Payne and Todd (1966), showed that extracts of bacterium T-52 (ATCC 27042) required the artificial electron acceptor ferricyanide for dehydrogenase activity to be observed. The ability to use other acceptors was not investigated. An active PEG-oxidising enzyme located in crude extracts of PEG-grown Pseudomonas P_{400} gave high dehydrogenase activity with the electron acceptor dichlorophenolindophenol (DCPIP) (Thélu et al., 1980). Both FAD and ferricyanide were required for full activity. Low molecular weight glycols (2-8 EO groups) and PEG oligomers having one methyl or ethyl ether linkage at one end of the chain were shown to be substrates for the enzyme, as were the dimethyl ether derivatives of di- and tetraethylene glycol, albeit less efficiently. This suggested that a free hydroxyl group was not essential for the substrate to be oxidised. As both 2-ethoxyethanol and 2-ethoxyacetate were equally well oxidised, despite the absence of any terminal alcoholic function in the latter, it was concluded that, unless this phenomenon was due to the existence of separate enzymes for these 2 substrates, the enzyme was not a terminal dehydrogenase. Thélu et al. (1980) proposed that the enzyme was a flavoenzyme, acting at some other point of the chain, which resulted in the transient formation of a double bond followed by water addition, according to the following scheme:



The subsequent introduction of a hydroxyl group at the subterminal carbon position followed by either hydrolysis of the hemiacetal produced or by another oxidation to an ester would lead ultimately to C_2 units such as glycolaldehyde or glyoxylate.

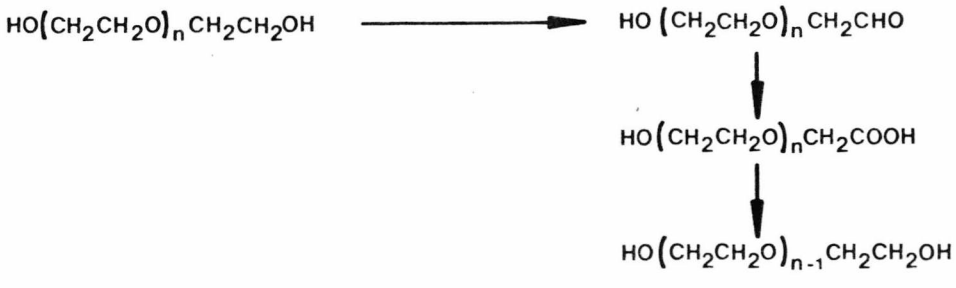
Neither oxygen nor any other cofactor appeared to be needed for the enzyme system responsible for the stepwise degradation of PEGs by a soil isolate, designated Acinetobacter S8 (Pearce and Heydeman, 1980). The protein fraction extracted by deoxycholate from the envelopes of lysed bacteria acted on diethylene glycol and the next few higher homologues, degrading the molecules by removal of successive EO units until ethylene glycol remained. These authors concluded that the conversion of the oligomers to the observed products, ethylene glycol and acetaldehyde was a 2-stage process catalysed concomitantly by a hydrolyase and a hydrolase:



Similarly no cofactors appeared to be required by the particulate enzyme, isolated from ethylene glycol-grown cells of Flavobacterium NCIB 11171 (Child and Willets, 1978). The enzyme, which was inducible, required oxygen and oxidised ethylene glycol but not di-, tri- or PEGs.

A particulate NAD-independent dehydrogenase has also been implicated in the degradation of PEGs (Kawai et al., 1977, 1980) although an artificial electron acceptor (DCPIP) was required to demonstrate enzyme activity. The enzyme was purified 24-fold by precipitation with $(NH_4)_2SO_4$,

solubilisation with laurylbetaine and chromatography with DEAE-cellulose, hydroxylapatite and Sephadex G-200. The enzyme did not require metal ions for activity and oxidised various PEGs among which PEG 6000 was the most active substrate. Primary alcohols (carbon chain length <5) and oligomeric glycols were either not or barely oxidised by the enzyme. These authors suggested that PEG was oxidised via an aldehyde to the monocarboxylic acid followed by cleavage of the ether bond resulting in the release of glycollate and PEG molecules that were reduced by one glycol unit:



An extracellular enzyme from Ps. aeruginosa was reportedly responsible for the depolymerisation of PEG 20,000 to monomers, dimers or possibly several oligomers (Haines and Alexander, 1975). The authors suggested that the conversion of the oligomers to the monomer may require another enzyme not able to function as a depolymerase.

Fermentation of ethylene glycol, but not PEGs, has been described. From mud, Gaston and Stadtman (1963) isolated Clostridium glycolicum which was capable of growing anaerobically on both ethylene and propylene glycols ($HO-\underset{\displaystyle CH_3}{\underset{|}{CH}}-CH_2OH$). Whole cells of glycol-grown but not glucose-grown cells were shown to convert these 1,2-diols to the corresponding acids and alcohols confirming the results of Abeles et al. (1960) who described similar conversions by cell suspensions of Aerobacter aerogenes (Klebsiella pneumoniae) grown anaerobically on glycerol.

In a series of further investigations Abeles and Lee (1961) and Lee and Abeles (1963) isolated a coenzyme B_{12} (adenosylcobalamin)-requiring enzyme responsible for the following dehydrations:

$$R-CH(OH)CH_2OH \longrightarrow R-CH_2CHO + H_2O$$

in which the adenosylcobalamin (5'-deoxy-5'-adenosylcobalamin) acted as an intermediate hydrogen carrier in the reaction. This cobamide coenzyme also showed an absolute requirement for a monovalent ion with maximum activity being obtained in the presence of K^+, NH_4^+ and Ti^+. The enzyme has since been designated as a diol dehydratase (diol dehydrase or DL-1,2-propanediol hydro-lyase EC. 4.2.1.28) and its presence further demonstrated in other genera of the Enterobacteriaceae (Toraya et al., 1979; Ichikawa et al., 1977).

More recently an enzyme isolated from Mycobacterium E_{44}, grown aerobically on ethylene glycol was shown to resemble the dehydratase found in K. pneumoniae grown anaerobically on this substrate (Wiegant and deBont, 1980). The enzyme, catalysing the conversion of ethylene glycol to acetaldehyde, was observed to be coenzyme B_{12}-dependent and required K^+ or NH_4^+ for full activity but unlike that in K. pneumoniae (Abeles et al., 1960; Toraya et al., 1979) was not inhibited by oxygen.

The ease with which ethylene glycol utilising microorganisms can be isolated from natural sources may result from the wide-substrate specificity of the enzymes accomplishing ethylene glycol metabolism. This specificity often encompasses monohydric primary alcohols and, less frequently, secondary alcohols. NAD-linked alcohol dehydrogenases have now been studied in a great number of bacteria including: Acetobacter peroxydans (Tanenbaun 1956; Nakayama 1959, 1960); E. coli (Still, 1940; Dawes and Foster, 1956; Hatanaka et al., 1971a); Clostridium acetobutylicum

(Fogarty and Ward, 1970); Leuconostoc mesenteroides (Hatanaka et al., 1971 a and b) Ps. aeruginosa (Tassin and Vandecasteele, 1972); Streptococcus mutans (Brown and Patterson, 1973); Bacillus stearothermophilus (Kolb and Harris, 1971; Atkinson et al., 1972); Neissseria gonorrhoeae (McDonald et al., 1980) and Mycobacterium tuberculosis var bovis (Bruyn et al., 1981).

Many of these dehydrogenases have very broad substrate specificities and show high affinities for various alcohols in which the length of the carbon chain often determines the rate at which the alcohol(s) are oxidised. For example, the NAD(P)-dependent alcohol dehydrogenases reported in Ps. aeruginosa (Tassin et al., 1973; Neihaus et al., 1978), E coli (Hatanaka et al., 1971a) and Leuconostoc (Hatanaka et al., 1971b) showed high activity towards long chain primary aliphatic alcohols; the ethanol dehydrogenase reported by Racker (1955) in Bakers yeast was active towards ethanol and propan-1-ol and the glycerol dehydrogenase described by McDonald et al. (1980) in Aerobacter aerogenes was particularly active towards propylene glycol and glycerol.

Similarly it may be envisaged that the broad substrate specificity of the NAD-independent alcohol dehydrogenase isolated recently from Acinetobacter calcoaceticus (Duine and Frank, 1981) may also endow this organism with the ability to metabolise ethylene glycol. The alcohol dehydrogenase resembles methanol dehydrogenase (EC 1.1.99.8), an enzyme which is present in methylotrophic bacteria, grown on methane and methanol. These enzymes contain a novel coenzyme form of a nitrogen-containing ortho-quinone referred to as methoxatin (Salisbury et al., 1979) or pyrrolo-quinoline quinone (Duine and Frank, 1980, 1981; Duine et al., 1980). Certainly ethylene glycol is a substrate (albeit a poor one) for methanol dehydrogenases found in species of several different genera of bacteria including: Ps. sp M27 (Anthony and Zatman, 1965); Ps. C (Goldberg, 1976) and a facultative methylotroph PAR (Bellion and Wu, 1978).

1.3 BIOCHEMICAL ROUTES OF DEGRADATION OF GLYCOLS

1.3.1 Further Fate of Glycol Oxidation Products

Ether glycol compounds must ultimately be degraded to yield one or more small aliphatic fragments capable of entering the terminal oxidation pathways.

Growth on C_2 compounds (for example, glycollate, glyoxylate and glycine) by species of pseudomonads led various workers (Kornberg and Gotto, 1961; Dagley *et al.*, 1961; Krakow *et al.*, 1961; Hansen and Hayashi, 1962; Stouthamer *et al.*, 1963: Kornberg and Morris, 1965; Lord, 1972) to formulate several peripheral pathways by which these compounds could generate amphibolic intermediates.

Although there was some evidence to suggest that ethylene glycol might be metabolised in a few bacteria via glycollate and glyoxylate (Kornberg and Morris, 1963; Gonzalez *et al.*, 1972; Jones and Watson, 1976; Caskey and Taber, 1981) and in others via acetaldehyde and acetate (Wiegant and deBont, 1980) this metabolic route has in fact been studied in only a small number of bacteria.

1.3.2 Anaerobic Conversion of Ethylene Glycol

A mechanism for the anaerobic degradation of ethylene glycol was first proposed by Gaston and Stadtman (1963) to account for growth of *Aerobacter aerogenes* (*K. pneumoniae*) on ethylene glycol but has since been found to operate in other genera of the Enterobacteriacae including *Citrobacter freudii* and *C. intermedium* utilising both ethylene and propylene glycols (Toraya *et al.*, 1979) Fig. 3. The pathway involved the dehydration of ethylene glycol to acetaldehyde catalysed by a diol dehydratase (see section 1.2.5); oxidation of acetaldehyde to acetyl-CoA by CoA-dependent

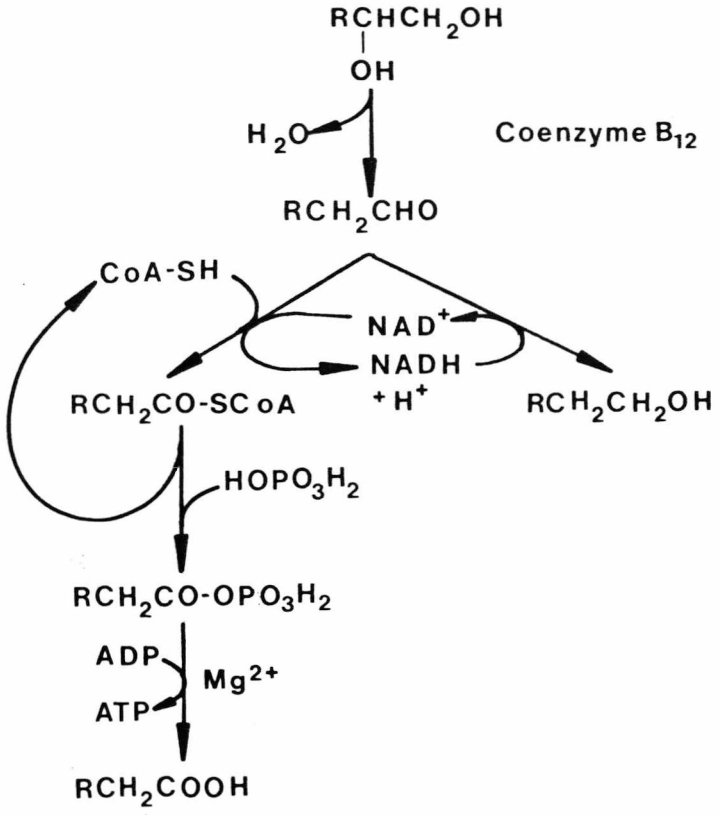


Fig. 3 Schematic representation of the fermentations of propylene glycol and ethylene glycol. R = CH_3 or H

aldehyde dehydrogenase, followed by transacylation from acetyl-CoA to inorganic phosphate, mediated by phosphotransacetylase. Acetyl phosphate was finally hydrolysed to acetate by acetate kinase with concomitant phosphorylation of ADP to ATP. The resulting NADH was used to reduce another molecule of acetaldehyde to ethanol.

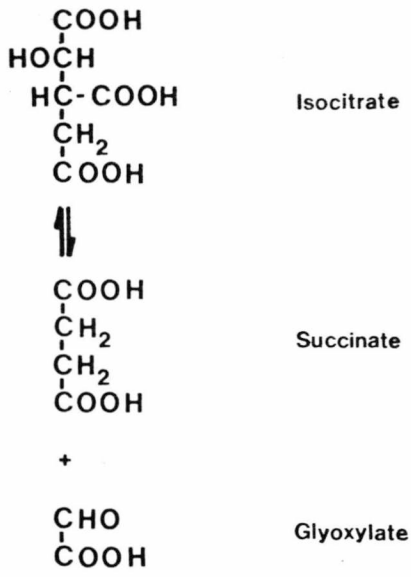
1.3.3 Aerobic pathways of glycol metabolism

A pathway for the aerobic degradation of ethylene glycol was observed in ethylene glycol-grown cells of *Acetobacter aceti* which converted glycollate to glyoxylate and contained fully induced enzymes of the glycerate pathway and glyoxylate bypass (Stouthamer *et al.*, 1963). The energy requirements of the organism were reportedly met by the glyoxylate bypass, the key reactions of which involved the breakdown of isocitrate to succinate and glyoxylate catalysed by isocitrate lyase and the formation of malate from glyoxylate and acetyl-CoA by malate synthase (Fig. 4). These conclusions rested on the observed elevated activities of malate synthase and isocitrate lyase in ethylene glycol-grown cells.

Synthesis of cell constituents from ethylene glycol by *A. aceti* was shown to involve the glycerate pathway, first described by Kornberg and his colleagues in glycollate-grown *E. coli* and a *Pseudomonas* species (see Kornberg, 1966) in which glyoxylate carboligase catalyses the condensation of two glyoxylate molecules, to form a C_3 compound tartronate semialdehyde and CO_2. Reduction of tartronate semialdehyde to glycerate by NADH: tartronate semialdehyde reductase, and conversion of glycerate, after phosphorylation, to pyruvate by the Embden-Meyerhof pathway (Fig. 5) provided a source of glycolytic intermediates.

Contrary to the results of Stouthamer *et al.* (1963) more recent research

The isocitrate lyase reaction



The malate synthase reaction

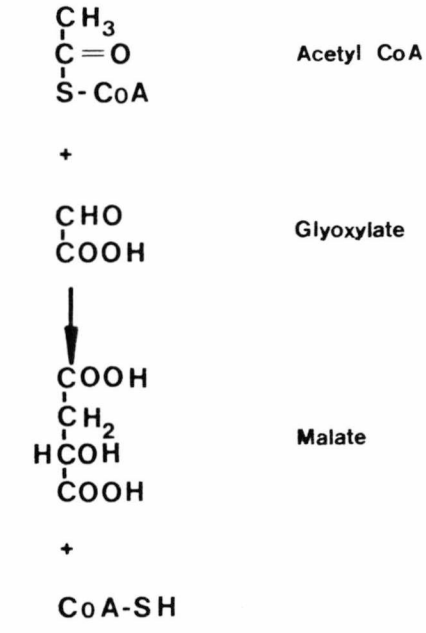


Fig.4 Reactions of the glyoxylate bypass

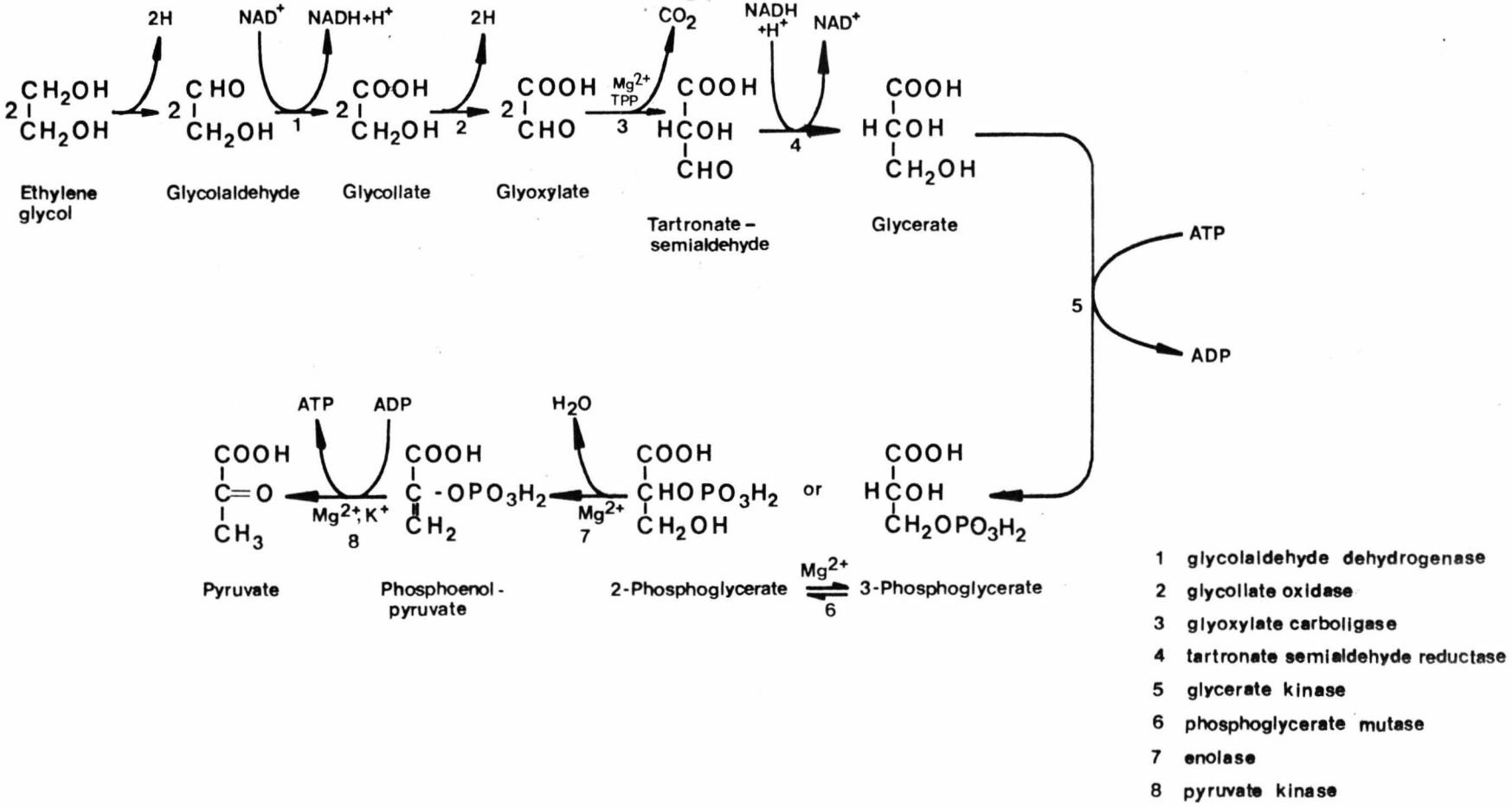


Fig. 5 Postulated pathway for conversion of ethylene glycol to pyruvate via the glycerate pathway

has implicated a major oxidative role for the ethylene glycol-to-glycerate pathway; enzymes catalysing the conversion of ethylene glycol to glycollate and some enzymes of the glycerate cycle have been found in a Flavobacterium sp. (Child and Willets, 1978) and a halophilic bacterium T-52 (Caskey and Taber, 1981). TCA cycle intermediates essential for growth of these bacteria on ethylene glycol were provided by malate synthase.

Evidence implicating glycollate and glyoxylate as intermediates of ethylene glycol metabolism by NCIB 11171 (Child and Willets, 1978) included the simultaneous adaptation of ethylene glycol-grown cells to the oxidation of all three substrates, the identification of [^{14}C]glycollate as a metabolite of [U-^{14}C]ethylene glycol and the identification of glyoxylate as a metabolite of both ethylene glycol and glycollate oxidations. High levels of the glycerate pathway enzymes, glyoxylate carboligase and tartronate semialdehyde reductase were demonstrated.

Oxidation of ethylene glycol sequentially to glycollate and glyoxylate was demonstrated in extracts from cells of ethylene glycol-grown Bacterium T-52 (Caskey and Taber, 1981) but additional evidence for the presence of an ethylene glycol-to-glycerate pathway was not presented.

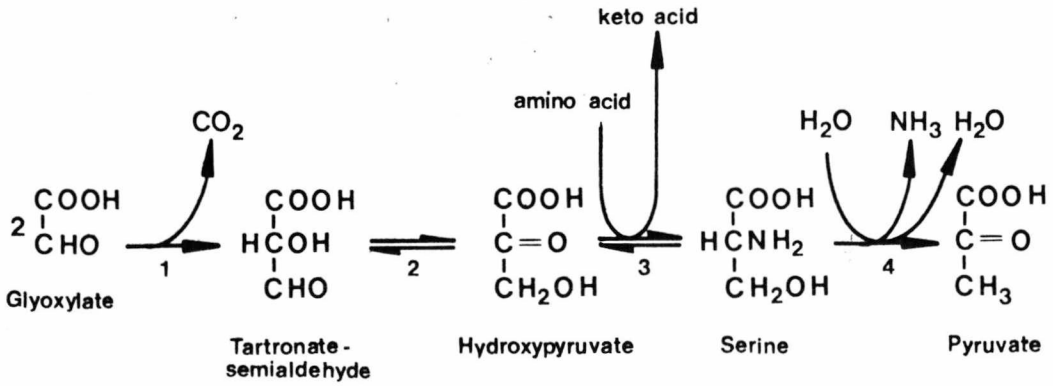
Metabolism of ethylene glycol by a species of Acinetobacter (Jones and Watson, 1976) appeared to involve an alternative route because extracts from cells grown on ethylene glycol metabolised ethylene glycol, glycolaldehyde and glyoxylate but not glycollate or tartronate semialdehyde. Hydroxypyruvate was suggested as an intermediate when hydroxypyruvate reductase activity was demonstrated in these extracts.

Bacillus fastidiosus, which degrades uric acid and allantoin via glyoxylate (Braun and Waltwasser, 1979) possesses the enzyme glyoxylate carboligase when grown on these compounds but a further utilisation of tartronate

semialdehyde via the glycerate pathway appeared to be unlikely as no glycerate kinase could be demonstrated. An enzymatic tautomerisation of tartronate semialdehyde to hydroxypyruvate was observed in the extracts. The subsequent conversion of this latter compound, via serine to pyruvate indicated that the observed enzymic conversion of tartronate semialdehyde to hydroxypyruvate might participate in the formation of C_3 compounds from glyoxylate in this organism (Fig. 6). Such a pathway would account for the route of ethylene glycol oxidation in the Acinetobacter species isolated by Jones and Watson (1976).

The absence of glyoxylate carboligase activity in glycollate-grown Micrococcus (Paracoccus) denitrificans (Kornberg and Morris, 1963) led to the discovery of the β-hydroxyaspartate pathway for the catabolism of glyoxylate in this organism (Fig. 7). The key reactions involve the transamination of glyoxylate to glycine which then condenses with a further molecule of glyoxylate to form erythro-β-hydroxyaspartate which then undergoes a dehydration reaction catalysed by β-hydroxyaspartate dehydratase, to yield oxaloacetate and ammonia.

A further possibility exists for the terminal metabolism of glyoxylate derived from ethylene glycol, namely oxidation by the dicarboxylic acid cycle (DCA). In this case glyoxylate reacts with acetyl-CoA to give malate in a reaction catalysed by malate synthase. Malate is oxidised to oxaloacetate and then via phosphoenolpyruvate to carbon dioxide and pyruvate which undergoes oxidative decarboxylation to acetyl-CoA (Fig. 8). Whereas the operation of the DCA cycle serves to supply metabolic energy to the cell from the catabolism of ethylene glycol, the glycerate pathway serves to replenish those intermediates of the dicarboxylic and tricarboxylic acid cycles that are used for biosynthesis.



1 Glyoxylate carboligase

2 Hydroxypyruvate isomerase

3 Hydroxypyruvate : glycine - amino transferase

4 Serine dehydratase

Fig. 6 Postulated pathway for the conversion of glyoxylate to pyruvate via hydroxypyruvate

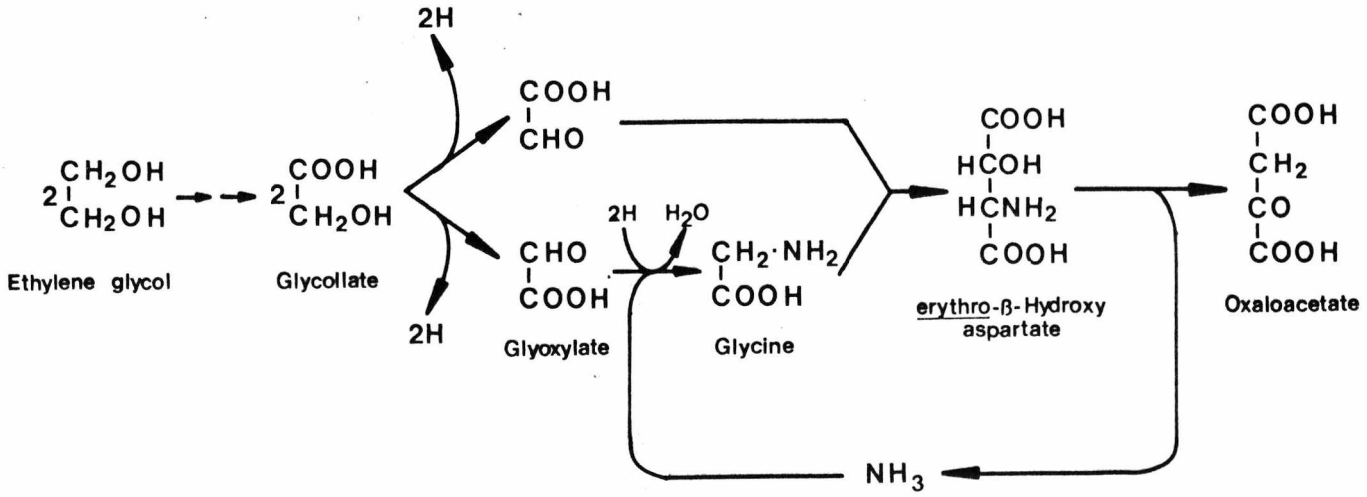


Fig. 7 The ß-hydroxyaspartate pathway.

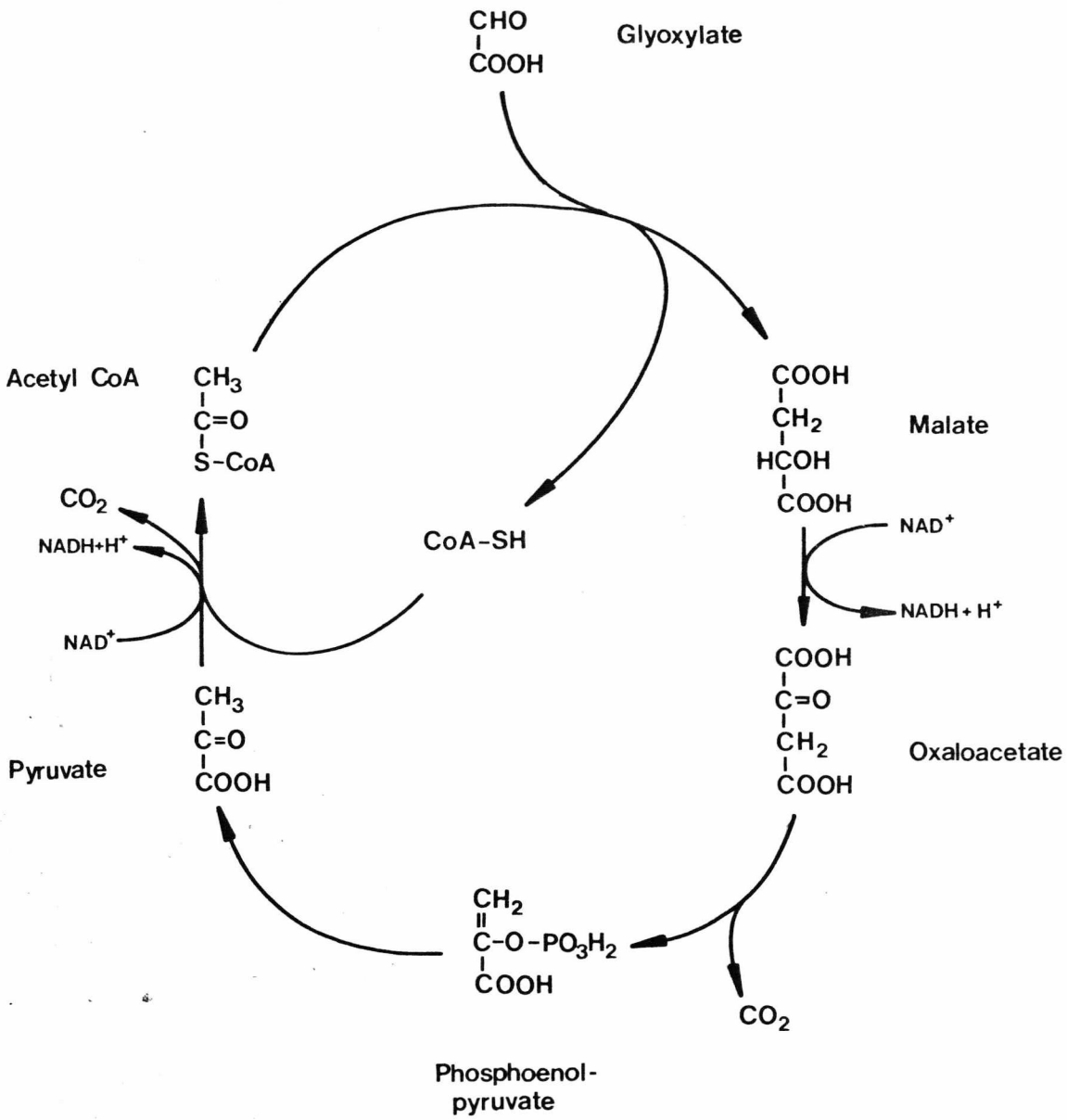


Fig.8 The Dicarboxylic Acid Cycle

Although the DCA cycle has been shown to operate in E. coli W (Kornberg and Sadler, 1960) and in a Pseudomonas strain (Kornberg and Gotto, 1961) during growth on glycollate no evidence has yet been obtained to implicate this cycle in ethylene glycol metabolism.

The inability of Mycobacterium E_{44} to grow on glycollate or glyoxylate and results of oxygen uptake experiments with washed cell suspensions strongly suggested that unlike other ethylene glycol-utilising bacteria, glyoxylate was not an intermediate in ethylene glycol metabolism in this organism (Wiegant and deBont, 1980). Mycobacterium E_{44} was reported to utilise ethylene glycol via acetaldehyde which was subsequently converted to acetyl CoA (see Fig. 3) and further oxidised by the enzymes of the TCA cycle.

The mode of degradation of the higher molecular weight glycols is still unclear although known pathways for C_2 compounds could be available for di-, tri- and possibly PEGs provided that cleavage of the parent molecule yields glyoxylate, acetate or precursors known to be metabolised to these compounds.

Microbial degradation of PEGs to C_2 units has been suggested (Jones and Watson, 1976; Harada and Sawada, 1977; Kawai et al., 1977; Hosoya et al., 1978; Thélu et al., 1980); glycollate, glyoxylate (Kawai et al., 1977; Hosoya et al., 1978) and glycolaldehyde (Thélu et al., 1980) are speculated intermediates.

Recent studies by Pearce and Heydeman (1980) indicated that in Acinetobacter sp S18 assimilation of C_2 units occurred at the oxidation level of acetate.

1.4 REGULATION OF GLYCOL METABOLISM

Studies on the metabolism of PEGs by bacteria have indicated that the enzymes involved are under inductive control. Kawai et al., (1978) showed that PEG was converted to the corresponding aldehyde by an inducible enzyme from a synergistic mixed culture of a Flavobacterium and a Pseudomonas species. Growth with PEG, but not with succinate endowed the organisms with the ability to oxidise PEG. Thèlu et al., (1980) analysing a Pseudomonas sp, designated P_{400} found PEG dehydrogenase activity was not detectable in extracts of succinate or acetate-grown cells but was inducibly synthesised on transferring the organism to a medium in which growth was dependent on utilisation of tetraethylene glycol. Thèlu et al., observed that the enzyme was sensitive to catabolite repression by succinate; when succinate was included in the growth medium with PEG, PEG dehydrogenase activity was not induced until all succinate had been exhausted.

The first evidence indicating that enzymes of glycol metabolism are regulated by catabolite repression emerged from the original studies by Fincher and Payne (1962). Addition of a minimal quantity of yeast extract to a culture of a soil isolate growing on triethylene glycol resulted in the preferential utilisation of this complex substrate followed by a diauxic response to the glycol. Only after the disappearance of yeast extract from the medium (and a transient cessation of growth) was catabolite repression alleviated permitting the synthesis of enzymes necessary for growth at the expense of triethylene glycol.

In an investigation of the metabolism of ethylene glycol, Caskey and Taber (1981) found that resting cells of bacterium T-52 grown on ethylene glycol readily oxidised ethylene glycol, glycollate and glyoxylate. The ability to catabolise ethylene glycol was diminished when the cells were grown on

either glycollate or glyoxylate, but the ability to respire both glycollate and glyoxylate was retained. Glycerol-grown cells oxidised all three compounds at very low rates.

Studies in which $^{14}CO_2$ evolution was measured during catabolism of [^{14}C]ethylene glycol confirmed the low rate of oxidation when cells were grown on glycollate, glyoxylate or glycerol. This data suggested that the enzymes for the metabolism of ethylene glycol, glycollate and glyoxylate were induced during growth with ethylene glycol as the sole carbon and energy source. Similarly in Mycobacterium E_{44} (Wiegant and deBont, 1980) the synthesis of the enzymes of the pathway were shown to be inducible; their formation was elicited by exposure of the bacterium to ethylene glycol but not by growth with succinate.

Although it is now clear that bacteria can catabolise glycol and PEGs via a number of different metabolic routes (see section 1.3) and the enzymes involved have been shown to be inducible, the regulatory mechanisms governing the control of the pathway enzymes remain obscure.

Glyoxylate provides a point of metabolic divergence in that it can participate in either of two condensing reactions; one catabolised by malate synthase and the other by glyoxylate carboligase. If glyoxylate is oxidised via the tricarboxylic acid cycle glyoxylate carboligase plays an oxidative role and malate synthase replenishes those intermediates of the TCA cycle that are used for biosynthesis. If however, the DCA cycle operates the role of malate synthase and glycerate pathway enzymes are reversed so that malate synthase initiates the oxidation of glyoxylate and the enzymes of the glycerate pathway now perform an anaplerotic function.

Ornston and Ornston (1969a) conducted a detailed examination of glyoxylate catabolism in E. coli K-12 and in addition to establishing the relative

contributions of the TCA and DCA cycles to the oxidative catabolism of glyoxylate, determined the regulatory mechanisms governing the control of malate synthase.

Catabolism of glyoxylate in E. coli K-12 was deduced by analysis of mutant strains that were blocked in the formation of glyoxylate carboligase and malate synthase G. Falmagne et al. (1965) had shown previously that E coli K-12 elaborated two chromatographically distinct forms of malate synthase. One enzyme was induced solely during growth with acetate and was designated malate synthase A; the second form (malate synthase G) was induced in the presence of both acetate and glycollate and contributed 40% and 90% of the activity observed in extracts of acetate and glycollate-grown cells respectively (Vanderwinkel and Devlieghere, 1968). In the studies of Ornston and Ornston (1969a) mutant strains unable to form glyoxylate carboligase (and thus unable to oxidise glyoxylate via the glycerate pathway) possessed high levels of malate synthase G and all enzymes necessary for the conversion of malate to acetyl-CoA. Organisms blocked in the synthesis of malate synthase G, however, were unimpaired in their ability to oxidise glyoxylate. Thus the DCA cycle appeared not to play an essential role in the oxidation of glyoxylate because malate synthase, the enzyme that introduces glyoxylate into the cycle, was not catalytically active although it was present at high levels in this organism.

The inability of the glyoxylate carboligase-deficient mutant to oxidise glyoxylate via the DCA cycle was attributed to a low intracellular concentration of acetyl-CoA which prevented glyoxylate from being introduced into the DCA cycle by malate synthase G. The same interpretation was proposed to account for the apparent absence of a contribution by malate synthase G to the oxidative metabolism of cells that contained glyoxylate

carboligase. When cells were exposed to both glyoxylate and an acetogenic substrate, however, the activity of malate synthase increased in order to replenish oxaloacetate, thus increasing the rate of utilisation of acetyl-CoA via the TCA cycle.

The enzyme glycerate kinase, a key enzyme of the glycerate pathway, might also have a regulatory function during growth of microorganisms on ethylene glycol as E. coli K-12 has been shown to elaborate two kinds of glycerate kinase (Ornston and Ornston, 1969b); one form, GK-I was found in cells grown with glycerate, glucarate and glycollate but of these compounds, glycollate was the only carbon source that elicited the synthesis of the second enzymatic form GK-II. These authors (1969b) suggested that the synthesis of two glycerate kinases might enable the cell to exert a refined regulatory control. The two enzymic forms might be the products of distinct structual genes in E. coli: one gene which dictates the synthesis of GK-I during growth with glycerate, glucarate or glycollate and a second gene which directs the synthesis of GK-II only in the presence of glycollate. The expression of the latter but not of the former gene might be postulated to be extremely sensitive to catabolite repression thus enabling the cells to lower the level of glycerate kinase when utilising glyoxylate via a pathway other than the glycerate cycle.

A further regulatory function may be exerted through the activity of the enzyme phosphoglycerate mutase. In ethylene glycol-grown Flavobacterium sp. NCIB 11171 2-phosphoglycerate (rather than 3-phosphoglycerate) was the product of the glycerate kinase reaction. This led Willets (1979a) to suggest that phosphoglycerate mutase may initiate gluconeogenesis during diol-dependent growth of the bacterium. Partially-purified phosphoglycerate mutase from this bacterium was shown to be activated by phosphoenolpyruvate - an intermediate in the catabolism of ethylene glycol to CO_2

by strain NCIB 11171. Willets (1979a) speculated that this activating effect of PEP on PGA mutase could serve a regulatory role by increasing the flow of carbon from the diol into the synthesis of carbohydrate when the potential energy status of the bacterial cells was high, indicated by an accumulation of PEP available for catabolism via the TCA cycle. An equivalent role for phosphoglycerate mutase has been proposed for methanol-grown Hyphomicrobium X (Harder, Attwood and Quayle, 1973) and methylamine-grown Pseudomonas MA (Hersh, 1975) operating the serine pathway.

1.5 BACTERIAL TRANSPORT MECHANISMS

Many microorganisms capable of growth on PEGs are known (see section 1.2.4) and whilst there have been a number of reports to suggest mechanisms by which glycols are degraded (sections 1.2.5; 1.3), very little is known about the transport of these compounds into microbial cells or whether this affects biodegradation.

Bacterial cells are capable of taking up biologically important molecules from the surrounding medium. With a few exceptions such as water and ammonia (Mitchell and Moyle, 1956) and lipophilic solvents passage of metabolites occurs by the agency of specific transport systems that are present in the membrane and are made up of one or more proteins. These transport systems either equilibrate substrates across the membrane (facilitated diffusion) or use energy to concentrate substrates internally above the external concentration (active transport).

Facilitated diffusion has been reported in a wide range of bacterial species and for a variety of metabolites (see Rosen, 1978). Little attention, however, has been paid to systems that facilitate the diffusion of small non-polar molecules with the exception of the glycerol carriers of several genera of bacteria (Lin, 1976) including those for *E. coli* (Richey and Lin, 1972) and *Ps. aeruginosa* (Tsay *et al.*, 1971) or that for propylene glycol in a mutant strain of *E coli* K-12 (Hacking *et al.*, 1978).

At the present time it appears that most bacterial systems carry out active transport and fall into three major groups; those which couple transport to metabolism via the proton circulation; those which utilise ATP or a related compound to drive the transport process, and group translocation, a system which utilises the chemical energy of the modification reaction to accumulate substrates.

Many bacterial transport systems are now thought to be energised by the proton circulation (Mitchell, 1966). The proton motive force (pmf) i.e. the difference in pH and electrical potential across the membrane, does the work and determines the concentration gradient that can be attained. In aerobes (or facultative anaerobes utilising alternative oxidants such as nitrate) this force results from respiration-linked proton translocation, whereas in strict anaerobes it results from the hydrolysis of ATP which is produced by substrate-level phosphorylation. The pmf has both a chemical component (due to protons, the inside of the cell being alkaline with respect to the outside), and an electrical component (the inside being negative due to the transport of H^+ outwards and electrons inwards).

Thus the uptake of amino acids and certain sugars appears to be driven by the pH and/or the membrane components of the pmf. At neutral pH, certain sugars and neutral amino acids enter accompanied by a proton ($X.H^+$) and hence move in response to the total pmf. An example is the lactose permease of E. coli, which mediates electrogenic symport of lactose with protons (see Wilson, 1978). The movement of basic amino acids, for example lysine, in Staph. aureus (Niven and Hamilton, 1974) is also electrogenic, but these are transported as cations (X^+) in response to the membrane potential. Conversely, acidic amino acids combine with a proton ($X^-.H^+$) and enter as electroneutral molecules in response to the pH gradient; an example is the transport system for glutamate in Staph. aureus (Hamilton, 1975). The carriers themselves are substrate-specific and catalyse translocation in either direction in accordance with the electrochemical potential of protons.

Uptake of certain metabolites by Gram-negative bacteria has been shown to occur by an alternative transport system, unrelated to the proton circula-

tion, which involves the participation of small proteins localised in the periplasmic space. According to Robbins and Rotman (1975) binding proteins somehow induce a specific and dramatic increase in affinity that is necessary for active transport to take place, but their precise role is unclear.

ATP-dependent transport systems include those for a range of sugars (galactose, arabinose, ribose and maltose) in E. coli (Rosen, 1978), amino acids (glutamine, arginine, histidine and ornithine) in E. coli (Berger and Heppel, 1974) and ions. At least one transport system in E. coli, that for K^+, requires both ATP and the proton circulation (Rhoades and Epstein, 1977).

Gram-positive bacteria do not synthesise periplasmic binding proteins but ATP is nevertheless implicated in several of their transport systems. In Strep. faecalis the accumulation of phosphate and of glutamate requires ATP and there is some evidence that uptake of K^+ and extrusion of Na^+ are also ATP-dependent (Harold and Spitz, 1975; Harold and Altendorf, 1974; Harold, 1978).

The reasons for the existence in the same organism of transport systems that are proton-coupled and others that are ATP-dependent are not known. The proton-linked systems have the virtue of versatility whilst ATP-driven transport systems can achieve higher concentration gradients (Harold, 1978). A further generalisation that can be made is that ATP-dependent systems appear to have somewhat lower K_s values than proton-linked systems transporting the same or similar substrates (Wilson, 1978).

In many bacteria the uptake of sugars is mediated by a complex of enzymes that transfer a phosphoryl group from PEP to the sugar, concomitant with

its translocation across the membrane (Roseman, 1969). The phosphotransferase system has been found to catalyse the uptake of sugars in many types of anaerobic and facultative bacteria; phosphorylation of glucose by E. coli and by Strep. faecalis and that of lactose by Staph. aureus are well known examples (Roseman, 1969; Hamilton, 1975; Postma and Roseman, 1976). Uptake of glucose by aerobic organisms probably occurs by symport with protons (Harold, 1978).

Several studies have revealed that the cell wall acts as a molecular sieve which restricts the entry of certain molecules such as oligopeptides (Payne and Gilvarg, 1968) and certain antibiotics, dyes and bile salts (Gustafsson et al., 1973; Nakae and Nikaido, 1975) but permits the rapid non-specific diffusion of small hydrophilic molecules. PEG polymers are not taken up uniformly, because of the polydispersity of these molecules; instead only the small molecular size fractions (M.W. less than 1200) have been shown to penetrate the bacterial cell wall (Scherrer and Gerhardt, 1971; Decad and Nikaido, 1976). This passive diffusion of small molecules through the cell does not necessarily apply to the passage of these compounds across the cytoplasmic membrane, even though the passage of simple molecules such as dilute aqueous ethanol and its polyhydroxy homologues, glycol and glycerol, is in many cases very rapid (causing little or no cell plasmolysis) and believed to occur by simple diffusion. The report by Hacking et al., (1978) of the existence of a facilitated diffusion system for propylene glycol uptake in a mutant strain of E. coli K-12, during growth of this organism on low concentrations of this substrate, would support the opposite view and substantiate earlier results of Murooka and Harada (1974) who found an active transport system for the uptake of ethanol in Corynebacterium acetophilum.

In the case of glycerol uptake in Ps. aeruginosa an inducible transport

system associated with a binding protein has been reported (Tsay et al., 1971). Whether this system catalyses only facilitated diffusion remains to be established.

CHAPTER TWO

MATERIALS AND METHODS

2.1 MEDIA

Rl1 mineral salts media described by Cripps (1973) was used routinely. The media contained (g/l): KH_2PO_4, 5.4, adjusted to pH 7.0 with 10M-NaOH; $(NH_4)_2SO_4$, 1.2 and trace elements solution, 2 ml/1. The trace elements solution contained (g/l): $CaCO_3$, 1.25; ZnO, 0.25: $FeCl_3.6H_2O$, 3.38; $MnCl_2.4H_2O$, 0.62; $CuCl_2.2H_2O$, 0.11; $CoCl_2.6H_2O$, 0.18; H_3PO_4, 0.04; $MgCl_2.6H_2O$, 63.5 and conc. HCl, 8.28 ml. All carbon sources were added at a final concentration of 0.1% (w/v) unless otherwise stated. Solutions were sterilised by autoclaving at 121°C for 15 minutes except those containing glucose, glyoxylate and glycerate which were sterilised by filtration (Millipore, 0.22 μm pore-size). Solid medium was prepared by the addition of 1.5% (w/v) Lab M agar (code MC_2, London Analytical and Bacteriological Media Ltd., London, U.K.) to the appropriate liquid medium.

Nutrient agar (Oxoid, Basingstoke, Hampshire, U.K.) was used as a general purpose medium for culture maintenance.

2.2 ISOLATION OF MICROORGANISMS

PEG-utilising microorganisms were isolated from locally collected samples of estuarine muds, activated sludge and soil. Samples were used as inocula into 25 ml volumes of Rl1 mineral salts media contained in 100 ml Erlenmeyer flasks. Media were supplemented with the appropriate glycol (0.5 g/l) and incubated at 25°C on a rotary shaker (Model G-23, New Brunswick Scientific Co. Inc., New Brunswick, N.J., USA), operated at 200 rev/min. After 8 days incubation, 0.5 ml aliquots were removed from the media and used to inoculate fresh media of the same composition. This process was repeated over four transfers. Those cultures showing visible growth within 8 days in the final transfer medium were selected for further

study. Selected cultures were streaked onto nutrient agar and where different colonial types were evident, individual colonies were purified and tested for their ability to grow on PEGs in liquid glycol media.

2.3 GROWTH AND MAINTENANCE OF MICROORGANISMS

All strains were maintained by monthly subculture on slopes of both nutrient agar and solid Rll medium containing the appropriate carbon source. Slopes were incubated at 30°C and stored at 4°C.

Liquid cultures were grown either in a volume of 50 ml in 250 ml capacity Erlenmeyer flasks fitted with side-arms or in a volume of 750 ml in 2 litre capacity Erlenmeyer flasks. All cultures were incubated on a rotary shaker at 30°C unless otherwise stated. Growth was monitored either turbidimetrically with a Klett-Summerson colorimeter (Klett Manufacturing Co. Inc., New York, USA) or spectrophotometrically using a Unicam SP500 spectrophotometer (Pye-Unicam Ltd., Cambridge) at a wavelength of 660 nm and related to dry weights by means of a calibration curve (Fig. 9). Dry weights of organisms were the mean of duplicate 4 ml samples of suspensions dried at 85°C to constant weight on aluminium planchets.

2.4 IDENTIFICATION OF ISOLATES

2.4.1 Colony and cell morphology

Colonial characteristics and pigmentation were studied on plates of nutrient agar, Rll mineral salts medium agar containing 0.1% (w/v) glycol and the media of King *et al*. (1954). In addition isolates designated Z and R were grown on cetrimide agar (nutrient agar to which had been added cetrimide, 0.2 g/l).

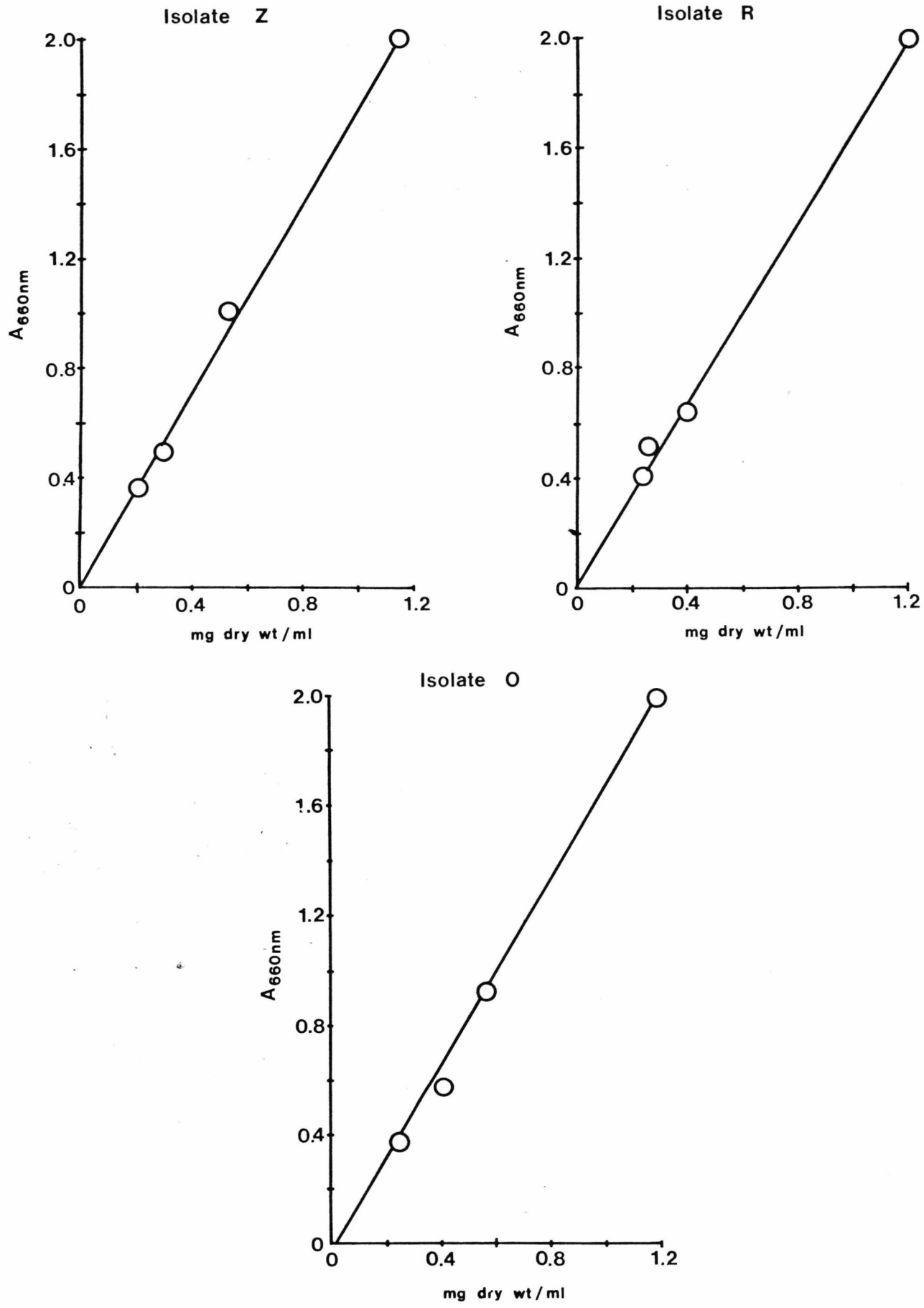


Fig. 9 Calibration curves for the estimation of dry weight

Motility (hanging-drop) and Gram-staining were done on bacteria grown in peptone (1% w/v) water for 24 h.

2.4.2 Physiological tests

Growth at temperatures between 5° and 42°C was tested in liquid Rll mineral salts medium supplemented with the appropriate glycol (0.1% w/v).

The optimum pH for growth was tested in liquid Rll mineral salts medium containing 0.1% (w/v) glycol; the pH was adjusted with 1OM-NaOH to give a pH range of 5.5 to 8.5. Media at each pH without carbon substrate was also inoculated and incubated as a control.

The ability to grow anaerobically was tested either by incubating plates of nutrient agar in anaerobic jars with an atmosphere of hydrogen for 7d or by inoculating 200 ml capacity glass bottles, filled to the neck with glycol (0.1% w/v) medium supplemented with either 10mM-KNO_3 or 10mM-KNO_2. Bottles were incubated without shaking for up to 10d.

2.4.3 Biochemical tests

Strains were tested for catalase and indole production (Kovacs' method); methyl-red and Voges-Proskauer (Barritts' method) reactions; nitrate reduction and gelatin hydrolysis; oxidase (Kovacs' method), urease (Christensens' method) and β-galactosidase activity; citrate utilisation (Kosers' method); degradation of aesculin; arginine dihydrolase production (Thornley, 1960) and decarboxylation of arginine, ornithine and lysine (Møller, 1955). All tests were performed according to Cowan and Steel (1974) and examined after 5 and 10d except where otherwise indicated.

Where more than one procedure was given the chosen method is shown in parentheses.

The O/F test (Cowan and Steel, 1974) was done using the basal medium of Hugh and Leifson (1953) containing 1% (w/v) glucose.

Acid (or acid and gas) production from carbohydrates (Cowan and Steel, 1974) was examined in peptone water cultures containing 1% (w/v) of the appropriate sugar.

Tween 80 was added to nutrient agar plus 0.01% (w/v) $CaCl_2$ to give a final concentration of 1% (w/v). Plates of the media were inoculated and incubated at 30°C for 10d. Hydrolysis of Tween was indicated by a zone of opacity, due to precipitation of calcium salt, around the area of growth.

2.5 GROWTH AND HARVESTING OF CELLS

Cultures were grown in shaken flasks at 30°C from a 1% (v/v) inoculum of cells grown on the same carbon source (2 g/l, unless otherwise stated). Cells were harvested by centrifugation at 10000g for 15 minutes at 5°C in a MSE 'High Speed' 18 centrifuge from mid-exponential phase of growth. Harvested cells were washed in 67mM-potassium phosphate buffer, pH 7.0 and resuspended in the same buffer.

Large scale production of cells were effected in 20 litre glass fermentor pots. Inocula for 18-litre batch cultures were derived from shake cultures grown at 30°C in 2 litre Erlenmeyer flasks containing 1 litre of the appropriate medium, which themselves had been inoculated from 50 ml liquid cultures. The 20 litre fermenter pots were incubated at 30°C with forced aeration (5 litre air/min). The full contents were collected via a sampling tube and harvested by centrifugation.

2.6 ADAPTATION OF SUCCINATE-GROWN BACTERIA TO GROWTH ON ETHYLENE GLYCOL

Microorganisms were taken through five serial subcultures at 30°C on sodium succinate (2g/l) as sole carbon source. When the last culture (11) had grown fully, cells were harvested aseptically and used to inoculate two, 18-litre batch cultures of glycol (1 g/l) mineral salts media contained in 20 litre fermenter pots, and incubated with forced aeration as in section 2.5. Cells were collected at intervals throughout the growth cycle via a sampling tube and harvested by centrifugation.

The sampling protocol was as follows:-

Absorbance (660 nm)	Sample size	Pot Number
0.009	8 litre	1
0.058	8 litre	1
0.160	5 litre	2
0.225	5 litre	2
0.425	2 litre	2
0.670	2 litre	2
0.710	750 ml	2

2.7 MEASUREMENT OF OXYGEN UPTAKE BY WHOLE CELLS

Conventional manometric techniques (Umbreit, Burris and Stauffer, 1957) were used to measure respiration of washed cell suspensions. The main compartments of the Warburg manometer flasks contained in 3 ml: potassium phosphate buffer, pH 7.0, 100 µmol and washed cell suspensions adjusted to give a final cell concentration of approximately 5 mg dry weight/ml (A_{660} = 9.0) on the Unicam SP500 spectrophotometer. The centre wells

contained potassium hydroxide (400 μmol). Substrates (10 μmol) were added from the side-arms after thermal equilibration at 30°C.

2.8 PREPARATION OF CELL FREE EXTRACTS

Microorganisms, 1 g wet weight, were suspended in 5 ml of 67 mM-sodium potassium phosphate buffer, pH 7.0 and sonicated for periods of 15 seconds in a 100W MSE ultrasonic disintegrator at an amplitude of 8 μm peak to peak. The total exposure time was 1 minute and throughout the treatment, the cells were cooled in an ice-bath. Intact cells and large cell debris were removed by centrifugation at 10000 g for 20 minutes at 5°C to yield an opalescent crude extract.

The supernatants were further centrifuged at $180000g_{av}$ for 2 hours at 5°C in a MSE 'Prepspin 50' ultracentrifuge (Measuring and Scientific Equipment Ltd., Crawley, Sussex, U.K.). The particulate fraction was separated from the clear supernatant (HSSF) and washed twice in 0.1M-potassium phosphate buffer and then resuspended in the same buffer to a final absorbance of 1.0 at 660 nm, corresponding to a protein concentration of 6 mg/ml.

2.9 PURIFICATION OF EXTRACTS

2.9.1 Ammonium sulphate fractionation

Ammonium sulphate fractionation was done at 4°C. Solid ammonium sulphate was added slowly with stirring to the supernatant (HSSF). The quantities of $(NH_4)_2SO_4$ to be added were determined from the nomogram of Dixon (1953). The precipitates formed after 20 minutes were dissolved in a suitable volume of 67 mM-sodium potassium phosphate buffer, pH 7.1 and dialysed against several changes of 500 vol. of the same buffer for 2 hours at 4°C, unless otherwise stated.

2.9.2 Column chromatography

Dry Sephadex G-200 gels were swollen in 50 mM tris-HCl buffer, pH 7.2, for 72 hours before the columns (Pharmacia [G.B.] Ltd., London) were packed.

Columns of G-200 (2.5 x 38 cm) were packed under a hydrostatic pressure of 15 cm H_2O and were eluted by downward flow at 4°C, under the same hydrostatic head. Protein samples were applied to the top of the column under a layer of buffer and eluted at a flow rate of 20 ml/h.

Columns were calibrated before use with Blue Dextran (Pharmacia Ltd.) and the following marker proteins; myoglobin (M.W. 17800), bovine serum albumin (M.W. 68000), lactic dehydrogenase (M.W. 135000), fumarase (M.W. 205000) and ferritin (M.W. 500000). Fractions (2 ml) were collected using a LKB UltraRac 2000 fraction collector (LKB Instruments Ltd., LKB House, South Croydon, Surrey) and the enzyme under investigation assayed in the fractions. Marker proteins were determined by the methods given in Table 3.

Columns of LKB Ultrogel AcA34 (2.5 x 42 cm), supplied in the pre-swollen form, were packed under a hydrostatic pressure of 15 cm H_2O and were eluted and calibrated as for Sephadex G-200. Protein samples were applied to the top of the column under a layer of buffer and eluted at a flow rate of 17 ml/h. Fractions (2 ml) were collected and assayed for the enzyme under investigation.

2.10 SDS-POLYARCYLAMIDE-GEL ELECTROPHORESIS

SDS-polyacrylamide-gel electrophoresis was performed according to Best *et*

Table 3. Marker proteins used for determination of Molecular Weights by Gel Chromatography

Marker	Supplier	Source	Determination	Molecular Weight
Blue Dextran 2000	Pharmacia	-	A_{625}	2000000
Ferritin	Sigma	Horse spleen	A_{404}	500000
Fumarase	Sigma	Pig heart	Packer (1950)	205000
Lactic dehydrogenase	Sigma	Rabbit muscle	Kubowitz and Ott (1943)	135000
Albumin	Sigma	Bovine serum	A_{280}	68000
Ovalbumin	Sigma	Egg	A_{280}	45000
Myoglobin	Koch Light	Whale skeletal muscle	A_{405}	17800

al. (1981) in denaturing gels of 10% (w/v) acrylamide containing sodium dodecyl sulphate (0.1% w/v) with a 3% (w/v) acrylamide stacking gel, set in slabs 1 mm thick which facilitated rapid staining and destaining. Samples were electrophoresed for 2 hours at a constant 12 mA. Gels were stained with Coomassie brilliant blue R250 in water:propan-2-ol:glacial acetic acid (65:25:10), destained in the above solvent mixture and stored in 7% (v/v) acetic acid.

Protein standards for calibrating SDS-containing gels were bovine serum albumin monomer (M.W. 68000), catalase (M.W. 63000), glutamate dehydrogenase (M.W. 56000), ovalbumin (M.W. 45000), carboxypeptidase (MW 37000) and myoglobin (M.W. 17800).

2.11 PRODUCTION OF EXTRACTS OF Paracoccus denitrificans NCIB 8944

Extracts of P. denitrificans were prepared as follows: a 10 ml inoculum of bacteria grown to exponential phase in R11 mineral salts media supplemented with the appropriate carbon source was added to 1 litre of media in a 2 litre Erlenmeyer flask containing either ethylene glycol (2 g/l) or succinate (2 g/l) and the culture shaken at 200 rev/min at 30°C until mid-exponential phase, turbidity being monitored at 660 nm. Cells were harvested and extracts prepared as described in sections 2.4 and 2.6 respectively.

2.12 ENZYME ASSAYS

The following enzymes were assayed spectrophotometrically at 30°C in silica cuvettes of 10 mm light path using a Unicam SP1800 spectrophotometer (Pye-Unicam Ltd.) linked to a Unicam AR45 chart recorder and fitted with a constant temperature cuvette holder through which water was circulated at 30°C from a constant temperature bath.

2.12.1 Alcohol(-NAD$^+$) dehydrogenase (EC.1.1.1.-) was assayed by following the increase in A_{340} due to the formation of NADH during the course of the reaction. The reaction mixture contained in a total volume of 1 ml: glycine-NaOH buffer, pH 9.6, 50 µmol; NAD$^+$, 0.67 µmol; and extract. The reaction was initiated by the addition of ethylene glycol (100 µmol) or other appropriate primary alcohols (2 µmol).

2.12.2 Glycolaldehyde reductase was determined by the method of Holzer and Goedde (1971) with slight modifications, by following the oxidation of NADH at 340 nm. The reaction mixture contained in a total of 3 ml; citrate buffer, pH 6.0, 110 µmol; NADH, 0.2 µmol; glycolaldehyde, 1 µmol and an appropriate amount of enzyme. Glycolaldehyde was added to start the reaction.

2.12.3 Glycolaldehyde dehydrogenase (Aldehyde:NAD$^+$ oxidoreductase: EC. 1.2.1.3) was determined by the method of Jakoby (1957), by measuring the increase in A_{340} due to the formation of NADH. The reaction mixture contained in 1 ml: potassium phosphate buffer, pH 7.1, 50 µmol; NAD$^+$, 1.5 µmol; glycolaldehyde, 1 µmol and an appropriate amount of enzyme. Glycolaldehyde was added to start the reaction. At this pH alcohol(-NAD) dehydrogenase (2.12.1) activity was not observed.

2.12.4 Tartronate semialdehyde reductase (D-glycerate:NAD$^+$oxidoreductase: EC. 1.1.1.60) was measured according to the method of Stouthamer et al., (1963) by following the oxidation of NADH at 340 nm in a system containing: phosphate buffer, pH 6.8, 75 µmol; thiamine pyrophosphate (TPP), 100 µg; $MgSO_4$, 10 µmol; glyoxylate, 5 µmol and extract in a total volume of 3 ml. The mixture was pre-incubated at 30°C for 30 minutes in the absence of NADH to obtain formation of tartronate semialdehyde; thereafter the reaction was started by the addition of NADH (0.4 µmol).

2.12.5 Glycerate kinase (ATP:D-glycerate 2-phosphotransferase; EC. 2.7.1.-) was measured according to the method of Heptinstall and Quayle (1970). The enzyme was assayed at 340 nm by following the formation of ADP consequent upon the phosphorylation by ATP of glycerate; the ADP produced was coupled to the oxidation of NADH by adding excess phosphoenolpyruvate and purified pyruvate kinase and lactate dehydrogenase.

The equilibria and enzymes involved are:-

$$\text{D-glycerate} + \text{ATP} \longrightarrow \text{2-phosphoglycerate} + \text{ADP}$$

$$\text{Phosphoenolpyruvate} + \text{ADP} \underset{\text{kinase}}{\overset{\text{pyruvate}}{\rightleftharpoons}} \text{pyruvate} + \text{ATP}$$

$$\text{Pyruvate} + \text{NADH} + \text{H}^+ \underset{\text{dehydrogenase}}{\overset{\text{lactate}}{\rightleftharpoons}} \text{L-lactate} + \text{NAD}^+$$

Quantitative conversion was assured because of the positions of the equilibria of the reactions catalysed by pyruvate kinase and lactate dehydrogenase at pH 7.5. The complete system contained in a total of 1 ml: tris-HCl buffer, pH 7.5, 50 µmol; EDTA, 1 µmol; phosphoenolpyruvate, 2.5 µmol; NADH, 0.1 µmol; ATP, 0.5 µmol; $MgCl_2$, 10 µmol; sodium DL-glycerate, 1 µmol; pyruvate kinase, 4 units; lactate dehydrogenase, 10 units and extract.

2.12.6 Pyruvate kinase (ATP:pyruvate phosphotransferase; EC. 2.7.1.40) was determined by the method of Bücher and Pfleiderer (1955). The reaction mixture contained in a total of 3 ml: tris-HCl buffer, pH 7.5, 150 µmol; $MgCl_2$, 10 µmol; KCl, 100 µmol; ADP, 1 µmol; phosphoenolpyruvate, 5 µmol; NADH, 0.45 µmol; lactate dehydrogenase, 10 units and extract. The reaction was started by the addition of phosphoenolpyruvate and followed by measuring the decrease in A_{340}.

2.12.7 Phosphopyruvate hydratase (2-phospho-D-glycerate hydrolyase; EC. 4.2.1.11) was assayed by the method described by Harder, Attwood and Quayle (1973). The reaction mixture contained: tris-HCl buffer, pH 7.5, 50 μmol; $MgCl_2$, 10 μmol; 2-phosphoglycerate, 5.6 μmol; NADH, 0.15 μmol; ADP, 0.5 μmol; lactate dehydrogenase, 5 units; pyruvate kinase, 2 units and extract in a total of 1 ml. The reaction was followed by measuring the decrease in A_{340}.

2.12.8 Glycerate phosphomutase (2,3-diphospho-D-glycerate:2-phospho-D-glycerate phosphotransferase; EC. 2.7.5.3) was assayed in a similar reaction mixture to that described for phosphopyruvate hydratase except that 2,3-diphosphoglycerate, 0.1 μmol was additionally required and 3-phosphoglycerate, 5 μmol, was substituted for 2-phosphoglycerate.

2.12.9 Citrate synthase (citrate oxaloacetate lyase [CoA acetylating]; EC. 4.1.3.7) was assayed by the method of Reeves et al. (1971) by measuring the appearance of thiol groups arising from acetylcoenzyme A hydrolysis, using 5,5'-bisthiol(2-nitrobenzoate)(DTNB) to measure the free thiol compound. The reaction mixture contained in a total of 1 ml: tris-HCl buffer, pH 8.1, 200 μmol; DTNB, 0.2 μmol; acetyl-S-CoA, 0.1 μmol; fresh potassium oxaloacetate, 0.5 μmol and extract. Oxaloacetate was added to start the reaction which was followed by measuring the increase in A_{412} due to the formation of the p-nitrobenzoate anion.

2.12.10 Isocitrate dehydrogenase (L_s-isocitrate:$NADP^+$ oxidoreductase [decarboxylating]; EC. 1.1.1.42) was determined by the method of Reeves et al.(1971) by following the increase in A_{340} due to the formation of NADPH. The reaction mixture contained in 1 ml: tris-HCl buffer, pH 7.5, 20 μmol; $MnCl_2$, 2 μmol; $NADP^+$, 0.5 μmol; sodium DL-isocitrate, 0.5 μmol and extract. The reaction was started by the addition of isocitrate.

2.12.11 Malate dehydrogenase (L-malate: NAD^+ oxidoreductase; EC. 1.1.1.37) was assayed by the method of Reeves et al. (1971), with slight modifications, by following the decrease in A_{340}. The reaction mixture contained in 1 ml: phosphate buffer, pH 7.4, 25 µmol; NADH, 0.1 µmol; oxaloacetate, 0.5 µmol; and extract. The reaction was started by the addition of oxaloacetate.

2.12.12 Isocitrate lyase (threo-D_s-Isocitrate glyoxylate-lyase; EC. 4.1.3.1) was determined by the method of Dixon and Kornberg (1959) with slight modifications. The enzyme was assayed by following the increase in A_{324}, consequent upon the formation of glyoxylate phenylhydrazone from glyoxylate. The assay system contained in a total of 3 ml: potassium phosphate buffer, pH 7.0, 200 µmol; $MgCl_2$, 15 µmol; phenylhydrazine-HCl, 10 µmol; cysteine-HCl, 6 µmol; sodium DL-isocitrate, 12.5 µmol and enzyme source. The reaction was started by the addition of glyoxylate, 2 µmol.

2.12.13 Malate Synthase (L-malate glyoxylate-lyase [CoA acetylating]; EC. 4.1.3.2) was determined by the method of Dixon and Kornberg (1959) by following the decrease in A_{252} consequent upon the breakage of the thioester bond of acetyl-S-CoA in the presence of glyoxylate. The reaction system contained in a total of 3 ml: tris-HCl buffer, pH 8.0, 50 µmol; $MgCl_2$, 10 µmol; acetyl-S-CoA, 0.2 µmol; sodium glyoxylate, 2 µmol and enzyme.

The following two enzymes were determined manometrically in a Braun (Melsungen, FRG) Warburg-type respirometer at 30°C.

2.12.14 Glycollate oxidase (Glycollate:oxygen oxidoreductase; EC. 1.1.3.1) was determined by the method of Kornberg and Gotto (1961). The main compartment of the Warburg vessel contained: potassium phosphate buffer, pH 7.0, 100 µmol; $MgSO_4$, 10 µmol; phenazine methosulphate, 0.33 µmol and

extract (10000 g crude extract or particulate preparation prepared as described in section 2.8) in a total of 3 ml. The centre well contained 0.2 ml of KOH solution (10% w/v). The reaction was started by the addition of glycollate (10 μmol) from the side-arm.

2.12.15 Glyoxylate carboligase (Glyoxylate carboxy-lyase [dimerising and reducing]; EC. 4.1.1.6) was measured according to the method of Kornberg and Gotto (1961) by following the rate of CO_2 evolution from glyoxylate. The assay was conducted in a manometer cup with two side-arms. The main compartment of the Warburg flask contained: potassium phosphate buffer, pH 6.4, 80 μmol; $MgCl_2$, 2.5 μmol; thiamine pyrophosphate, 0.6 μmol; and extract in a total of 2 ml. One of the two side-arms contained glyoxylate (20 μmol), the other 0.75 ml 5M-H_2SO_4. Control flasks lacked substrate. The flasks were flushed with nitrogen for 15 minutes and after thermal equilibration at 30°C, glyoxylate was added from the appropriate side-arm. The reaction was stopped by the addition of H_2SO_4 which also liberated the dissolved (bound) CO_2.

2.12.16 Ethylene glycol dehydrogenase (PMS-dependent) was assayed polarographically by measuring oxygen consumption. The reaction mixture contained in a final volume of 2 ml: borate-buffer, pH 9.0, 100 μmol; phenazine methosulphate, 0.1 μmol; ammonium chloride, 60 μmol and extract. The reaction was started by the addition of ethylene glycol, 2 μmol. Correction was made for the oxygen uptake that occurs at this pH in the absence of enzyme and for the endogenous uptake by the extracts in the absence of substrate. Oxygen consumption was monitored on a Perkin-Elmer PE 165 chart recorder (Perkin-Elmer Ltd., Beaconsfield, Bucks, U.K.) connected to a Clark Oxygen electrode. At 30°C the oxygen concentration in the air-saturated buffer mixture (stock solution bubbled with air continuously) was assumed to be 0.24 μmol/ml.

2.12.17 Enzyme units

One unit of enzyme activity is defined as the quantity of enzyme that catalyses the formation of 1 μmol of product or the disappearance of 1 μmol of substrate per minute in the assays described.

The molar extinction coefficient (ϵ) of NADH and NADPH at 340 nm was taken to be 6220 litre/mol/cm; of acetyl-S-CoA to be 13,600 litre/mol/cm; of glyoxylate phenylhydrazine to be 17,000 litre/mol/cm. The $\Delta\epsilon_{232}$ for the cleavage of the thioester bond of acetyl-S-CoA was 4500.

2.13 EXTRACTION OF POLYETHYLENE GLYCOLS

Culture samples (100 ml) were centrifuged (17000 g; 10 min) and PEGs and any metabolic products extracted either by direct solvent extraction from culture supernatants (Patterson et al., 1967; Anthony and Tobin, 1977) or by evaporation under reduced pressure at 42°C, followed by sequential extraction of the residual solids with aliquots of chloroform and 'acid' chloroform (acetic acid/chloroform 1:100).

2.13.1 Extraction by the method of Patterson et al. (1967): The sample (25 ml) was mixed with 25 ml of conc. $MgSO_4$ solution (600 g $MgSO_4.7H_2O$/1) in a 100 ml separating funnel and extracted with 4 x 12.5ml of chloroform. A further addition of conc. HCl (5 ml) and a further two extractions with chloroform was used for those compounds more readily extracted under acid conditions.

2.13.2 Extraction by the method of Anthony and Tobin (1977): The extraction was carried out as above except that the sample was first made nearly salt-saturated by adding 9 g of NaCl to the 25 ml sample.

The chloroform fractions were dried by passage through silica-coated phase separating paper (H. Reeve Angel Ltd., London, E.C.4) and taken to dryness under a stream of nitrogen at room temperature. Acetic acid was first removed by bubbling nitrogen through the eluate in a test tube until 5 minutes after no more acetic acid could be smelled. Final traces of solvent were removed by vacuum desiccation over silica gel for a period of 12 hours before i.r., m.s. or n.m.r. analyses were performed.

The efficiencies of the various extraction procedures were determined by removing the solvent by evaporation, resuspending extracted material in distilled water, and measuring the total recovered organic carbon (TOC).

2.14 CHEMICAL ANALYSES

2.14.1 Total organic carbon analysis (TOC)

Total organic carbon in culture supernatants was determined using an aqueous carbon analyser (Tocsin Mark 1, Phase Separations Ltd., Queensferry, Clywd). Samples were first centrifuged (2000 g; 20 min) to remove debris and then acidified to pH 2.0 with analytical grade nitric acid. An automatic sampling device used in conjunction with the carbon analyser incorporated the facility for purging acidified samples with CO_2-free air prior to actual analysis. The machine was calibrated against a standard solution of potassium hydrogen phthalate (BDH Chemicals Ltd., Poole, U.K.).

2.14.2 Trimethyl Silylether (TMS) derivatives

TMS derivatives of glycols were prepared by reacting 0.5-1.0 mg of dried sample with hexamethyldisilazane (25µl) and trimethylchlorosilane (12.5µl)

as described by Suffis et al. (1965) and analysed by gas chromatography using a high efficiency gas chromatograph (Model 7610A, Hewlett Packard Ltd., King Street Lane, Winnersh, Wokingham, Berkshire) equipped with a flame ionisation detector and a Hewlett Packard Integrator (Model 3385a). n-Undecane (10 µl of 3.3% v/v solution in chloroform) was added to the reaction mixture as an internal standard and samples (5 µl) injected onto a glass column (1800 x 3 mm) packed with 5% SE Ultraphase on Chromosorb W (100/120 mesh), with a nitrogen carrier gas flow of 35 ml/min. The temperatures of the injector and detector ports were 280° and 300°C respectively. The column temperature was held at 90°C for 1 minute and then increased to 350° at a rate of 10°C/min. Under these conditions the retention times observed were: ethylene glycol 1.80 min; diethylene glycol 4.53 min; triethylene glycol 7.75 min and tetraethylene glycol 8.42 min, (Fig. 10).

2.14.3 Formation of hydrogen bromide fission products

Polyethylene glycols were converted into dibromoethane by reacting 0.5-1.0 mg of dried material with 0.4 ml of HBr-glacial acetic acid (HBr 40%) in sealed ampoules at a temperature of 150°C for 3 hours (Luke, 1973):

$$HO(CH_2CH_2O)_nH \xrightarrow[150°C,\ 3\ h]{HBr} nC_2H_4Br_2$$

Dibromoethane was subsequently extracted into carbon disulphide (2 ml) to which n-undecane (10 µl) was added as an internal standard. Samples (5 µl) were injected onto a glass column (1800 x 3 mm) packed with 3% OV-17 on Chromosorb WAW-IMCS (80/100 mesh) with a nitrogen carrier gas flow of 45 ml/min. The temperatures of the injector and detector ports were 170° and 250°C respectively. The column temperature was held at 90°C for 1 minute

Fig.10 Gas chromatograms of trimethyl silyl ether derivatives of : a) ethylene glycol ; b) diethylene glycol ; c) triethylene glycol ; d) tetra-ethylene glycol , showing retention times in minutes

then increased to 250°C at a rate of 10°/min. The chromatographic and recording equipment used was described above. Results were quantitated automatically with the integrator set on the internal standard mode and calibrated against an authentic sample of dibromoethane.

2.14.4 Determination of ethylene glycol

Ethylene glycol was estimated by gas chromatography using a Pye-Unicam 204 instrument (Pye-Unicam Ltd., Cambridge) fitted with a flame ionisation detector. Samples, 1 µl (for details see Figs 36,38) were injected onto a glass column (2.1 m x 4 mm i.d.) packed with Poropak Q (60-80 mesh) with a nitrogen carrier gas flow of 60 ml/min. The temperature of the column, injector and detector ports was 225°C.

The ethylene glycol peak was cut out, weighed and the results quantitated by reference to a previously determined calibration curve. A linear correlation was obtained with ethylene glycol concentrations between 0-1 g/l (Fig. 11).

The following three analyses on recovered samples were performed by the Analytical Department, Shell Research Centre, Sittingbourne, Kent.

2.14.5 Mass Spectra were determined using a Finnigan 3200F quadrupole mass spectrometer (Finnigan Corporation, Sunnyvale, California, USA) operated in the chemical ionisation mode using ammonia as the reagent gas.

2.14.6 Nuclear magnetic resonance (n.m.r.) spectra were determined using a JEOL 100 pulsed fourier transform spectrometer (JOEL (U.K.) Ltd., Colindale, London) operating at 100 MHz.

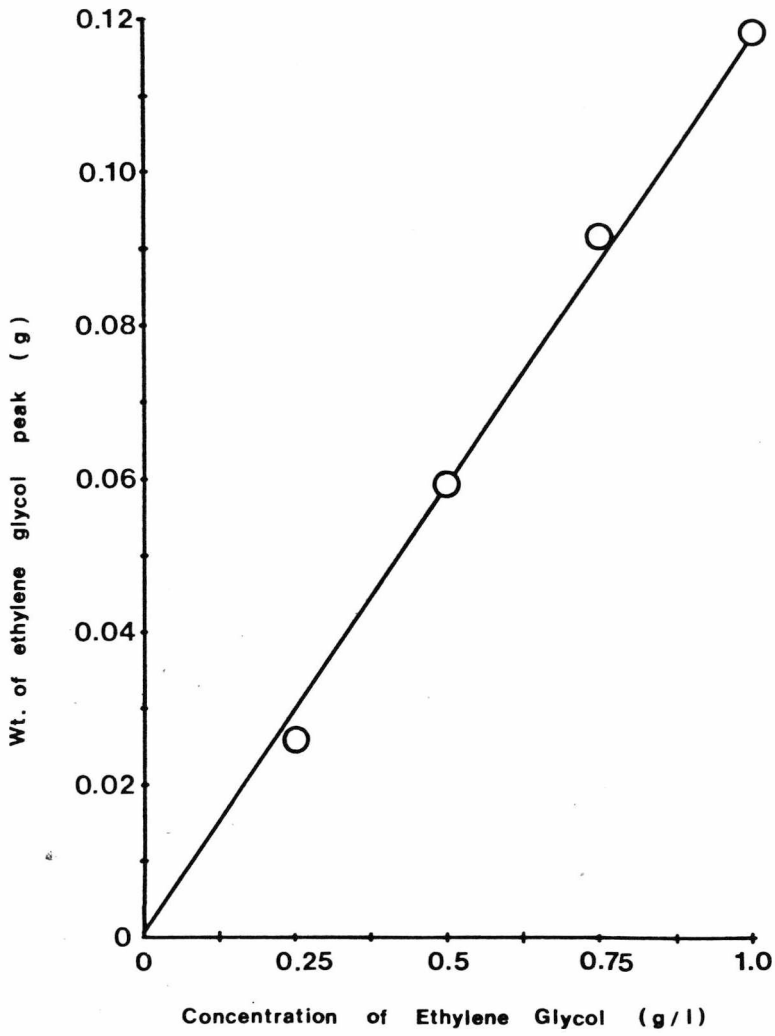


Fig. 11 Calibration curve for the estimation of ethylene glycol

2.14.7 Infrared (i.r.) absorption spectra were recorded (range 2-25 μm) using a Grubb-Parsons grating spectrometer (Grubb-Parsons, Walker Gate, Newcastle-upon-Tyne, U.K.). Samples were taken up in chloroform, deposited on KBr plates and the solvent evaporated.

2.14.8 Silica gel thin-layer (t.l.c.) chromatography and paper chromatography. PEG 200 degradation products and the 2,4-dinitrophenylhydrazone derivatives of glycolaldehyde and glyoxylate (prepared as described in section 2.14.9) were identified by t.l.c. on silica gel plates (Kieselgel 60, 0.25 mm thickness, E. Merck Ltd., Darmstadt, West Germany) which had been activated at 105°C for 30 minutes before use.

Glycollate, glyoxylate and glycerate were identified by ascending paper chromatography on Whatman chromatography paper No. 1. Markers were always run on the same 20 x 20 cm sheets as the unknown. In these cases the reaction mixtures were deproteinised by adding 0.2 ml of 50%w/v trichloroacetic acid, and cations were removed by treating batches with 5 g wet weight of Dowex-50 ion-exchange resin in the hydrogen form. After removal of the precipitated protein and resin by centrifugation, the supernatant fluids were reduced in volume by evaporation.

Details of solvent systems employed are given in the text or tables.

Compounds were detected by the following spray reagents: acidic compounds by 0.04% (w/v) bromocresol purple in 50% aq. ethanol to which was added 1 drop of conc. NH_3; PEGs by modified Dragendorff's reagent (Patterson et al. 1967). This consisted of Dragendorff's reagent (0.17% w/v bismuth oxynitrate and 4% w/v potassium iodide in 0.22M acetic acid), orthophosphoric acid, ethanol and barium chloride (20% w/v) mixed in the ratio 10:1:10:5, respectively.

2.14.9 Isolation and characterisation of 2,4-dinitrophenylhydrazones. Samples (1 ml) of deproteinised reaction mixture suspected to contain glycolaldehyde were treated with 1 ml of 0.2% (w/v) 2,4-dinitrophenylhydrazine in 2M-HCl and after standing for 30 minutes were extracted with ethyl acetate (2 x 3 ml). The ethyl acetate layer was evaporated to a small volume and chromatographed by t.l.c. on silica gel plates.

The 2,4-dinitrophenylhydrazone derivatives of glyoxylate, made by incubating samples (1 ml) of deproteinised reaction mixture with excess 2,4-dinitrophenylhydrazine as above, was first extracted into ethyl acetate (2 x 3 ml) and then isolated by extraction from the ethyl acetate layer into 10% (w/v) Na_2CO_3 solution (2 x 3 ml). The Na_2CO_3 layer was subsequently acidified with conc. HCl and then re-extracted with ethyl acetate, 2 x 3 ml. The organic phase was dried by passage through silica-coated phase separating papers, evaporated to dryness with a stream of nitrogen and the residue dissolved in a small volume of ethanol; samples (10 µl) were applied to silica gel plates.

2.15 ESTIMATIONS

2.15.1 Glucose was determined colorimetrically by the GOD-Perid method using a reagent kit (The Boehringer Corp., London, Ltd.). The calibration curve was linear within the range 0 - 0.3 mg glucose/ml. A typical standard curve is shown in Fig. 12.

2.15.2 Glyoxylate was estimated by the procedure of Friedmann and Haugen (1943). The calibration curve was linear within the range 0-1.0 µmol/ml (Fig. 13).

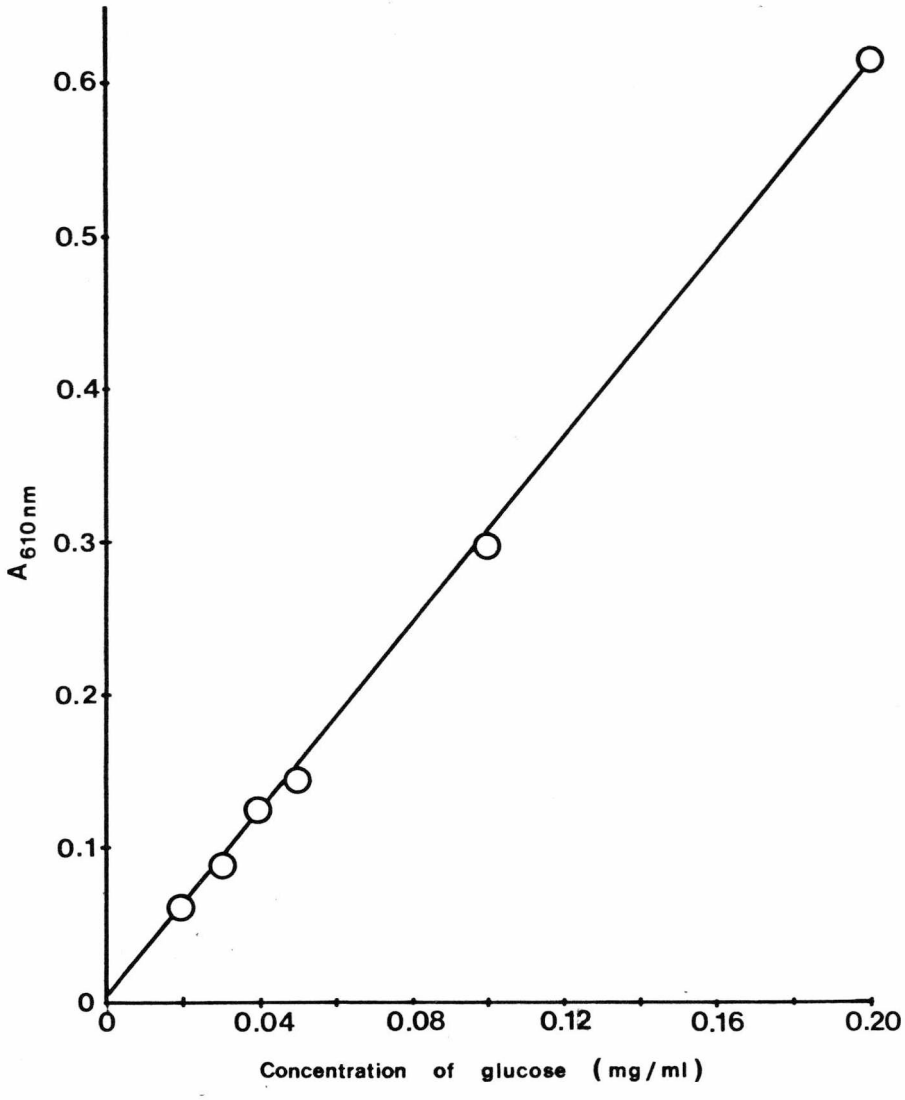


Fig.12 Calibration curve for the estimation of glucose

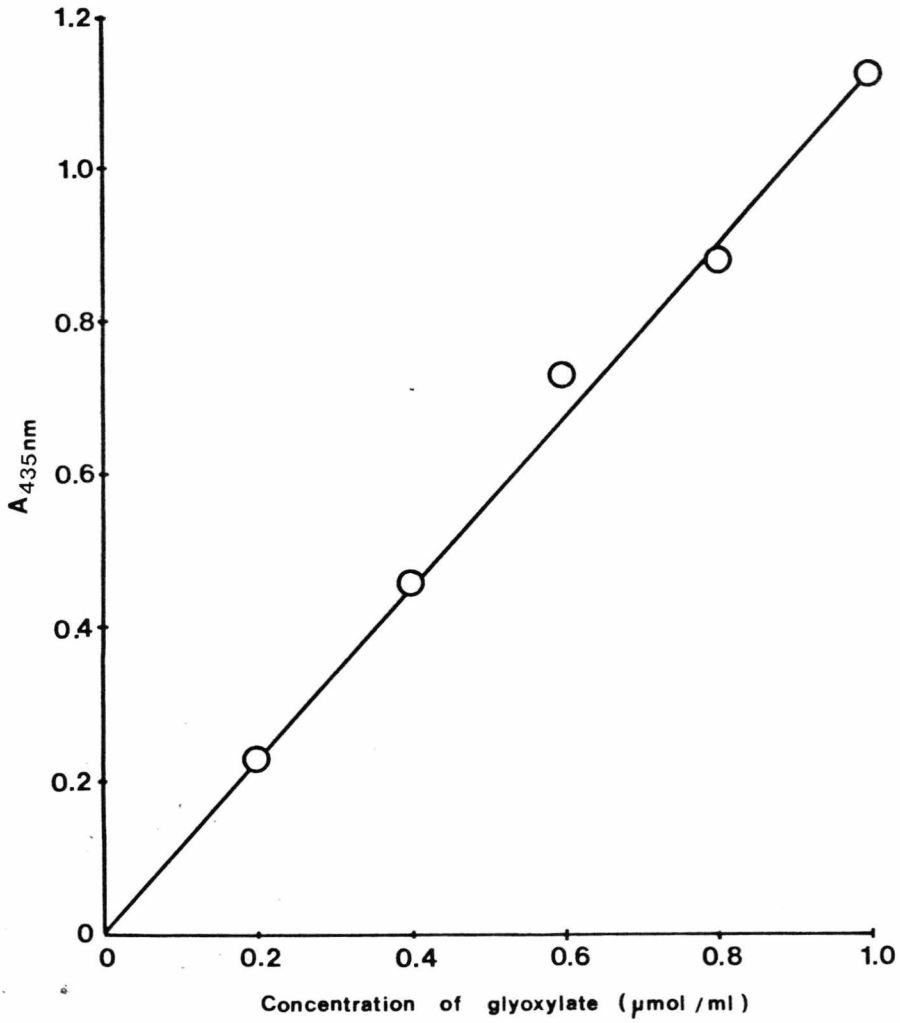


Fig.13 Calibration curve for the estimation of glyoxylate

2.15.3 Protein was determined by the method of Lowry et al. (1951). Bovine serum albumin was used as the standard. A linear correlation was obtained with protein concentrations between 0-0.2 mg/ml.

2.16 CYTOCHROME MEASUREMENTS

Difference spectra were measured at room temperature using a Unicam SP1800 spectrophotometer linked to a Unicam AR45 recorder. Matching silica cuvettes (1 cm path length) were employed in the position closest to the photomultiplier tube and 1 ml of suspension was used in each cuvette. Reduced minus oxidised difference spectra were obtained by reducing the sample in one cuvette with a few grains of sodium dithionite and oxidising the sample in the other cuvette by addition of a few grains of potassium ferricyanide.

Cytochrome concentrations were calculated assuming the following molar absorption coefficients: cytochrome aa_3 (ΔE 605-628nm) = 11 x 10^3 cm^{-1}; cytochrome b (ΔE 559-575) = 17.5 x 10^3 cm^{-1}; cytochrome c (ΔE 551-538) = 17.3 x 10^3 cm^{-1} (Jones and Redfearn, 1967).

Reduced plus CO minus reduced difference spectra were obtained by reducing samples in both cuvettes as described previously and bubbling a slow stream of carbon monoxide for approximately 30 seconds through one cuvette. After a 20 minute incubation with CO to allow time for the peaks to develop, the spectra were recorded.

2.17 EXPERIMENTS ON THE THERMAL INACTIVATION OF ENZYMES

An aliquot of bacterial extract (10 ml, containing 12-15 mg/ml protein) was placed in a test tube and immersed in a water bath maintained at the

required temperature by the use of a Circotherm constant temperature apparatus (Braun Ltd., West Germany). Thermal equilibration of the sample was assumed to be achieved after 30 seconds; after which samples (1 ml) were removed at intervals, immediately transferred to an ice bath and subsequently assayed for enzymes under examination.

2.18 PROCEDURE FOR MEASURING UPTAKE OF ISOTOPICALLY-LABELLED MATERIAL.

Cells for substrate-uptake studies were grown in 750 ml cultures, containing ethylene glycol (2g/l) or glucose (1g/l). Cells were harvested by centrifugation, washed and resuspended in mineral salts media (pH 7.0) to produce a cell density of approximately 0.5 mg dry wt/ml corresponding to an absorbance at 660 nm of 1.0.

Unless noted otherwise cells were incubated at 30°C in 100 ml flasks and stirred with magnetic stir-bars. [U-^{14}C]ethylene glycol was then added to produce final volumes of 10 ml.

To sample the cultures, aliquots (1 ml) were withdrawn at intervals and rapidly applied to preweighed 0.45 μm pore-size Millipore filters and washed twice with 2 ml of distilled water to remove any radioactive medium which may have absorbed to the filter and cells. Filters were dried at 80°C and then reweighed.

Dry filters were suspended in 2 ml volumes of PCS (Amersham, Arlington Heights, Illinois, USA) liquid scintillating fluid and counted in a Beckman (Model LS-200B, Beckman Instruments Inc., Fulerton, California, USA) liquid scintillation spectrometer.

When the inhibition of ethylene glycol uptake was being examined reaction volumes of 2 ml were also used. Details of which appear in the legends to the appropriate tables.

2.19 CHEMICALS

Tetraethylene glycol was obtained from Eastman Kodak Co. (Rochester, N.Y., USA) and PEG 300 and 400 were obtained from Koch-Light Laboratories Ltd., (Colnbrook, Bucks). All other PEGs were obtained from British Drug Houses Ltd., (Poole, Dorset).

Chemicals were obtained from Sigma (Poole, Dorset) with the exceptions of: glyoxylate obtained from Koch-Light Laboratories Ltd.; glucose and thiamine phyrophosphate, British Drug Houses Ltd.; adenosine-diphosphate, adenosine-triphosphate, phosphoenolpyruvate and nicotinamide nucleotide (reduced) from C.F. Boehringer and Soehne G.m.b.h., Manheim Germany.

Radiolabelled ethylene glycol was purchased from the Radiochemical Centre, Amersham, Bucks., U.K.

CHAPTER THREE

RESULTS

3.1 DEGRADATION OF PEGs BY PURE BACTERIAL CULTURES

3.1.1 Isolation and identification of microorganisms from enrichment cultures

Enrichment culture yielded a variety of microorganisms capable of using ethylene glycol and/or polyethylene glycols (PEGs) as sole sources of carbon and energy (Fig. 14). Bacterial cultures designated Z and R, selected by enrichment on mineral salts media containing ethylene glycol and diethylene glycol respectively, were isolated from estuarine mud samples collected near Sittingbourne. Strains designated W, U and O, isolated by enrichment on mineral salts media containing triethylene glycol, PEG 200 and PEG 400 respectively were isolated from activated sludge. All five cultures readily metabolised EGs/PEGswith a molecular weight range close to that of theEG/PEGused in the original enrichment (Fig. 14). Only those isolates derived from activated sludge grew on PEGs.

Three pure cultures Z, R and O provided examples of organisms capable of growing on a very different range of PEGs (Fig. 14) and were thus selected for further study. Colonies of organisms Z and R on nutrient agar were colourless and colonies of organism O, pale yellow. All three organisms grew aerobically in nutrient broth and microscopic examination showed that all three isolates were gram-negative bacteria. Anaerobic growth on nutrient broth or glycol mineral salts media was not detected. The results of various diagnostic tests are given in tables 4, 5 and 6 from which strains Z and R have been tentatively identified as *Pseudomonas fluorescens* and strain O as a *Flavobacterium* sp.

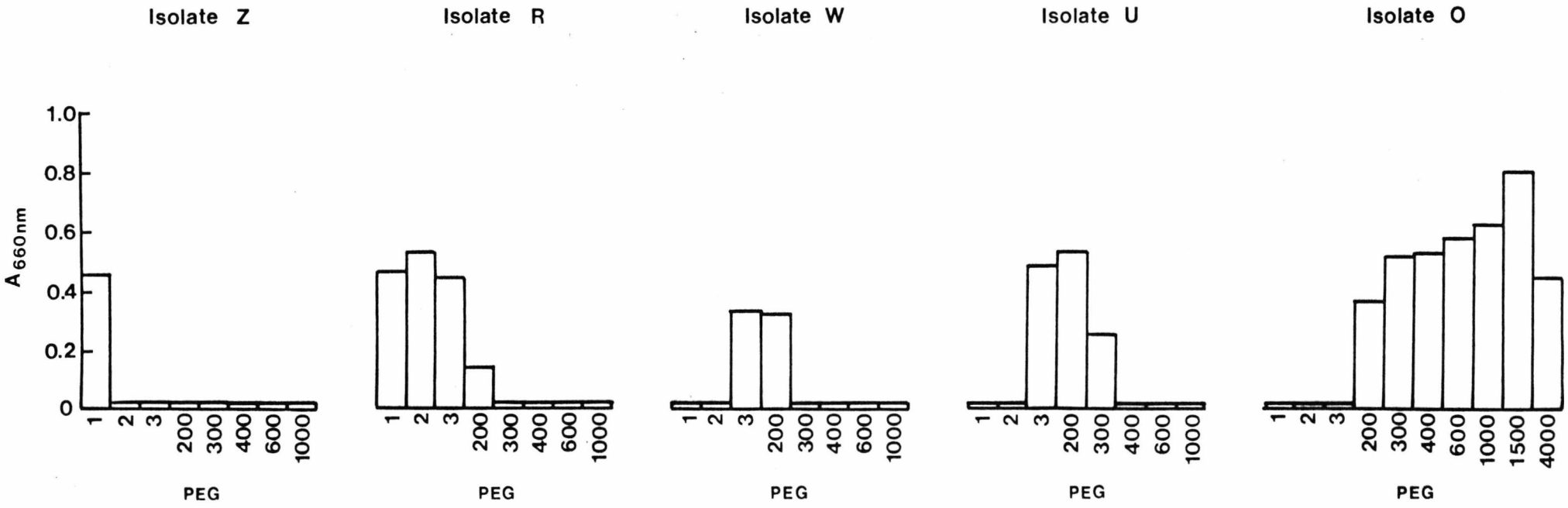


Fig. 14 Growth of various bacterial isolates on ethylene glycol and polyethylene glycols

Bacterial isolates were grown at 25°C in RII mineral salts medium supplemented with the appropriate glycol (1g/l). Growth was measured spectrophotometrically at 660nm when the cultures had entered stationary phase.

Table 4. Diagnostic characteristics of bacterial isolate Z

Test character	Reaction	Test character	Reaction
Shape	Short rods	Acid production in peptone water sugars:	
Gram stain	Gram negative	glucose	+(2)
Motility in peptone water (37°C)	+	arabinose	-(10)
Oxidation-fermentation test	O(2)*	mannitol	-(10)
Indole production	-(1)	inositol	-(10)
Voges-Proskauer	-(1)	sorbitol	-(10)
Methyl red	-(1)	rhamnose	-(10)
Liquefaction of gelatin	-(5)	melibiose	-(10)
Catalase	+		
Oxidase	+		
ONPG	-(1)	Pigment production on:	
Arginine dihydrolase	+(1)	King's A medium	-
Arginine decarboxylase	-(5)	King's B medium	+(yellow)
Ornithine decarboxylase	-(5)		
Lysine decarboxylase	-(5)	Growth on:	
Citrate utilisation	+(2)	Cetrimide agar (0.2g/1)	+(1)
H_2S production	-(7)		
Urease	+(5)	Growth at:	
Nitrate reduction	-(5)	T°: 5 + (12)	pH: 5.5 - (10)
Tween 80 hydrolysis	-(10)	15 + (4)	6.0 + (2)
		25 + (1)	6.5 + (2)
		30 + (1)	7.0 + (1)
		37 - (20)	7.5 + (1)
		42 - (20)	8.0 + (2)
			8.5 + (3)

* Figures in parenthesis indicate days of incubation.

Table 5. Diagnostic characteristics of bacterial isolate R

Test character	Reaction	Test character	Reaction
Shape	Short rods	Acid production in peptone water sugars:	
Gram stain	Gram negative	glucose	+(2)
Motility in peptone water (37°C)	+	arabinose	-(10)
Oxidation-fermentation test	O(2)*	mannitol	-(10)
Indole production	-(1)	inositol	-(10)
Voges-Proskauer	-(1)	sorbitol	-(10)
Methyl red	-(1)	rhamnose	-(10)
Liquefaction of gelatin	-(5)	melibiose	-(10)
Catalase	+		
Oxidase	+		
ONPG	-(1)	Pigment production on:	
Arginine dihydrolase	+(1)	King's A medium	-
Arginine decarboxylase	-(5)	King's B medium	+(yellow)
Ornithine decarboxylase	-(5)		
Lysine decarboxylase	-(5)	Growth on:	
Citrate utilisation	+(2)	Cetrimide agar (0.2g/l)	+(1)
H_2S production	-(7)		
Urease	+(5)	Growth at:	
Nitrate reduction	-(5)	T°: 5 + (12)	pH: 5.5 - (10)
Tween 80 hydrolysis	-(10)	15 + (4)	6.0 + (2)
		25 + (1)	6.5 + (1)
		30 + (1)	7.0 + (1)
		37 - (20)	7.5 + (1)
		42 - (20)	8.0 + (2)
			8.5 + (2)

* Figures in parenthesis indicate days of incubation.

Table 6. Diagnostic characteristics of bacterial isolate O

Test character	Reaction	Test character	Reaction
Shape	Rod	Pigment production on:	
Gram stain	Gram negative	King's A medium	-
Motility in peptone water (37°C)	-	King's B medium	-
Oxidation-fermentation test	O(5)*		
Indole production	-(1)	Growth on:	
Voges-Proskauer	-(1)	MacConkey agar	Weak
Liquefaction of gelatin	-(10)	Horse blood agar	+ (no hydrolysis)
Catalase	+	Nutrient agar	+ (yellow)
Oxidase	+		
ONPG	-(5)	Growth at:	
Arginine dihydrolase	-(5)	T°: 5 - (20)	pH: 5.5 - (10)
Aesculin hydrolysis	-(5)	15 + (4)	6.0 + (3)
Acid production in peptone		25 + (2)	6.5 + (3)
water sugars:		30 + (2)	7.0 + (2)
glucose	-(10)	37 - (20)	7.5 + (2)
arabinose	-(10)	42 - (20)	8.0 + (2)
cellobiose	-(10)		8.5 + (3)
dulcitol	-(10)		
glycerol	-(10)		
lactose	-(10)		
maltose	-(10)		
mannitol	-(10)		
raffinose	-(10)		
salicin	-(10)		
sorbitol	-(10)		
sucrose	-(10)		

* Figures in parenthesis indicate days of incubation.

3.1.2 Growth characteristics

Growth of isolates Z, R and O occurred within the pH range of 6 - 8.5 and within the range 5 to 30°C ± 2°C with the exception of strain O which did not grow at 10°C or below (Table 6). Final growth of each strain was proportional to respective substrate concentration up to 0.4% (w/v) with the exception of strain R for which a linear correlation was obtained with substrate concentrations up to 0.5% (w/v).

In experiments to determine optimum growth conditions, cultures were grown in media containing the EG/PEG that was present in the original enrichment and do not necessarily correspond to the glycol supporting most rapid growth (see table 7).

To examine the range of EGs/PEGs that would act as growth substrates isolates were grown in 50 ml of R11 mineral salts medium containing 1 g/l of the test compound. Flasks were incubated at 25°C and growth estimated turbidimetrically.

Strain R exhibited a marked preference for growth on the lower molecular weight EGs in contrast to strain O, which utilised PEGs of molecular weight 200 and above. Strain Z was unable to use oligomeric glycols or PEGs and grew only with ethylene glycol (Fig. 15). The mean generation times of isolates Z, R and O during exponential growth on PEGs are given in Table 7.

3.1.3 Degradation of PEGs

The degree of substrate utilisation effected by these strains was determined by measurement of total organic carbon (TOC) in the culture super-

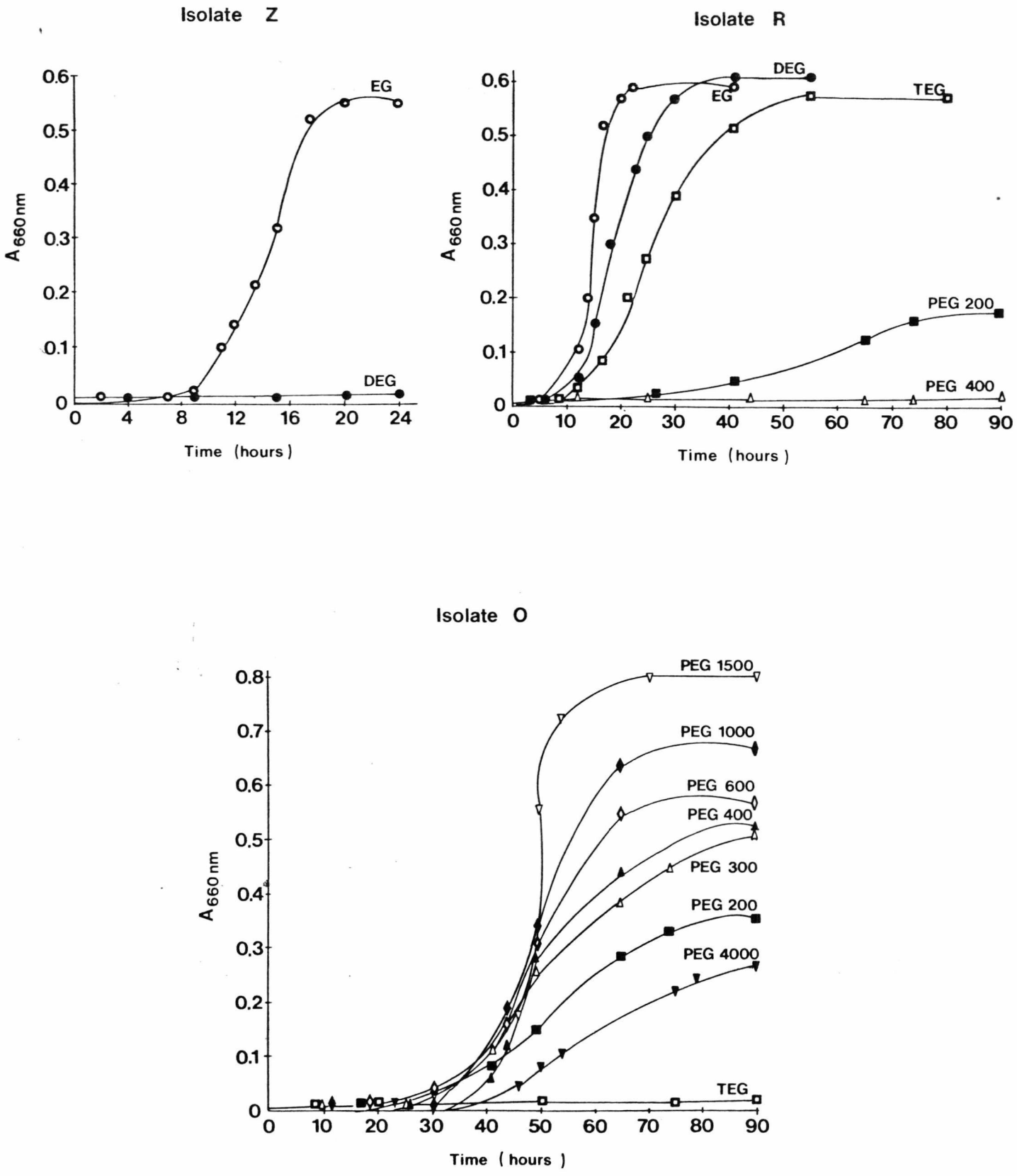


Fig.15 Growth of bacterial isolates Z, R and O on mono- and polyethylene glycols.

Isolates Z, R and O were grown at 25°C in R II mineral salts medium supplemented with the appropriate carbon source (1 g/l). Growth was measured spectrophotometrically at 660nm.

Table 7. Growth of bacterial isolates Z, R and O on ethylene glycol and polyethylene glycols

Isolates were grown on the mineral salts medium described in Materials and Methods supplemented with additions of mono- and polyethylene glycols at 1 g/l. Growth was measured spectrophotometrically at 660 nm.

Substrate	Mean generation time (h)		
	Strain Z	Strain R	Strain O
Ethylene glycol	1.03	1.63	-
Diethylene glycol	-*	2.00	-
Triethylene glycol	-	3.75	-
PEG 200	-	16.60	7.8
PEG 300	-	-	6.6
PEG 400	-	-	4.0
PEG 600	-	-	2.35
PEG 1000	-	-	5.0
PEG 1500	-	-	3.0
PEG 4000	-	-	8.0

* no growth within 10 days

natants at varying stages during growth. At the end of growth almost complete utilisation of substrate was effected by strain Z grown on ethylene glycol, R grown on mono-, di- and triethylene glycol and strain O grown on PEG 1000 and 1500 (Table 8).

Growth yields expressed as g of cell material per g of substrate utilised are given in Table 9. The observed variation probably reflects differences in the efficiency with which the substrate can be used by the organism to generate ATP as well as the proportions of the individual utilisable glycols in the PEG 200 - PEG 400 (mixed) substrates; certainly the pathway(s) for degradation of oligomeric and polymeric glycols are thought to be dissimilar.

Commercially available PEGs with a molecular weight of 200 or greater consist of a mixture of PEGs of different chain lengths. For example, PEG 200 is a mixture of components with molecular weights between 62 and 325 (Fig. 16). Hence the partial degradation of PEG 200 observed with strain R could be the result of degradation of only the mono-, di- and triethylene glycol components. Similarly, the incomplete degradation of PEG 200 by strain O could be attributable to the removal of only the higher molecular weight homologues of the mixture.

The approximate percentage of each component glycol in PEG 200 was determined from the data presented in Fig. 16. Thus if strain R utilised only the mono, di- and triethylene glycol components of the mixture one would expect about 35% degradation. This agrees very closely with the figure of 33% obtained by TOC analysis (Table 8). Similarly if strain O was utilising polymeric glycols with 5, 6 and 7 ethylene oxide groups one would again expect about 35% degradation. In fact TOC analysis revealed as much as 45% degradation, which suggests that strain O was also partially utilising other components in the mixture.

Table 8. Soluble organic carbon from culture filtrates of microorganisms grown with ethylene glycol and polyethylene glycols

Percentage total organic carbon content of culture supernatants was measured at the end of the growth cycle; 23 h for strain Z; $\geqslant$ 23 h for strain R and $\geqslant$ 60h for strain O. Results are expressed as the average of 3 readings obtained from independent experiments which did not differ by more than 5%.

Substrate	TOC removal (percent of that originally present) with designated glycol substrate (1g/1)		
	Strain Z	Strain R	Strain O
Ethylene glycol	93	96	-§
Diethylene glycol	-*	96	-
Triethylene glycol	-	84	-
Tetraethylene glycol	-	<1	<1
PEG 200	-	34	48
PEG 300	-	-†	62
PEG 400	-	-	64
PEG 600	-	-	75
PEG 1000	-	-	86
PEG 1500	-	-	96
PEG 4000	-	-	53

* TOC not measured as no growth occurred within 10d on oligomeric or polymeric glycols
† not tested as no growth occurred within 10d on polymeric glycols
§ not tested as no growth occurred within 10d on oligomeric glycols

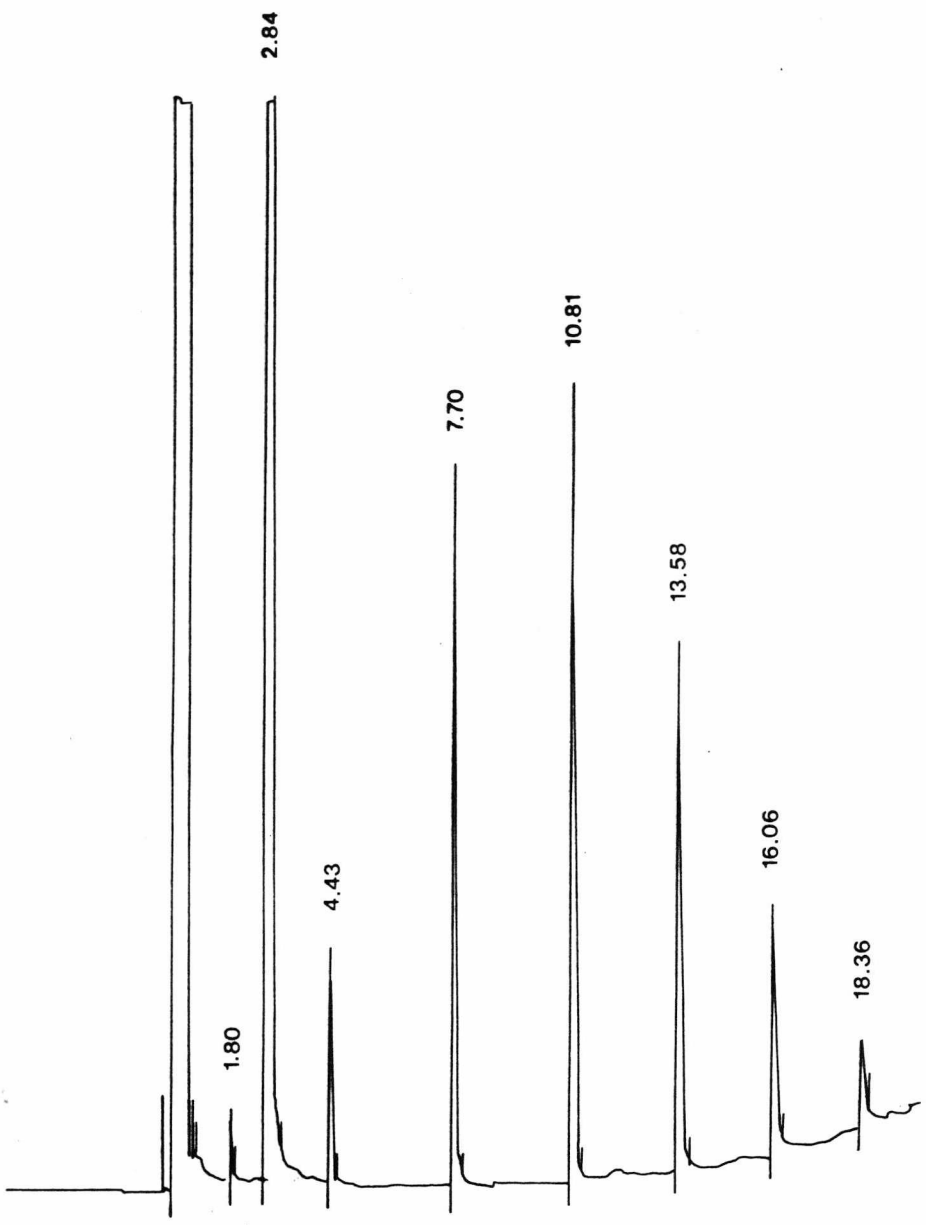
Table 9. Growth yields for isolates Z, R and O grown on ethylene glycol and polyethylene glycols

Cultures were grown at 25°C on R11 mineral salts medium, pH 7.0, supplemented with the appropriate carbon source at concentrations of 0.5, 1, 2, 4 and 5 g/l. Growth was measured spectrophotometrically at 660 nm, and the degree of substrate utilisation effected by these strains determined by measurement of total organic carbon (TOC) level in the culture supernatants at the end of the growth cycle.

The growth yield constant, y, is expressed as grams of cell material per gram of substrate utilised.

Substrate	Growth yield with designated glycol substrate		
	Strain Z	Strain R	Strain O
Ethylene glycol	0.30	0.31	-
Diethylene glycol	-*	0.35	-
Triethylene glycol	-	0.35	-
PEG 200	-	0.13	0.26
PEG 400	-	-	0.31
PEG 1000	-	-	0.35
PEG 1500	-	-	0.42

* no growth within 10d



RT (mins)	Area (units)	% of Total Area
1.80	263	1.72
4.43	1116	7.29
7.70	3936	25.71
10.81	4675	30.54
13.58	3281	21.43
16.06	1581	10.33
18.36	457	2.99

Fig.16 Gas-liquid chromatogram of PEG 200

The numbers represent retention times in minutes. Retention times of: 1.80, 4.43, 7.70, 10.81, 13.58, 16.06 and 18.36 minutes were given by glycols containg 1, 2, 3, 4, 5, 6 and 7 ethylene oxide units, respectively. n-Undecane was used as an internal standard and had a retention time of 2.84 min.

3.1.3.1 Extraction of compounds from culture supernatants

In an attempt to substantiate the hypothesis that strains R and O utilised only certain components of PEG 200, more detailed studies were undertaken and residual substrate components extracted from the growth medium for analysis. A comparison of published procedures used for the extraction of ethylene glycol and PEGs from culture supernatants, however, indicated that both the methods of Patterson et al., (1967) and Anthony and Tobin (1977) gave poor recoveries of the lower molecular weight PEGs. The efficiency of extraction with both methods only approached 50% when PEGs with a molecular weight of 400 or greater was used (Table 10). In contrast, rotary evaporation of culture supernatants under reduced pressure followed by extraction of the residual solids with $CHCl_3$ gave considerably higher recoveries (56% or better) of PEG 200 and the oligomeric glycols with the exception of ethylene glycol. This method was therefore adopted for routine use for the extraction of culture media containing oligomeric and polymeric glycols as growth substrates. Since the amount of ethylene glycol in PEG 200 is very small (~2% of total) analyses for this component were considered relatively unimportant in experiments describing the metabolism of PEG 200. Hence no specific isolations of ethylene glycol were attempted.

3.1.3.2 Growth of strains R and O on PEG 200

TOC analysis indicated that growth of strains R and O on PEG 200 resulted in the removal of 25 and 50%, respectively, of the substrate carbon from culture supernatants within 4 to 7 days (Fig. 17). No significant removal of carbon was observed with either culture after this time.

Metabolites extracted from culture supernatants at intervals throughout

Table 10. Comparison of methods for the extraction of polyethylene glycols from aqueous media

The efficiency of the various extraction procedures was determined by removing solvent by evaporation, resuspending the residue in distilled water and measuring the soluble organic carbon recovered.

Extraction method	Recovery (%) of polyethylene glycols						
	Ethylene glycol	Diethylene glycol	Triethylene glycol	PEG 200	PEG 400	PEG 600	PEG 1000
Extraction from aqueous solution into chloroform in the presence of $MgSO_4$*	<5	<5	<5	<5	48	72	73
As above but in the presence of NaCl †	<5	<5	<5	30	n.d.	56	n.d.
Rotary evaporation §	<5	56	62	68	58	59	54

* Patterson et al., 1967
† Anthony and Tobin, 1977
§ Rotary evaporation followed by chloroform extraction of residual solids
n.d. not determined

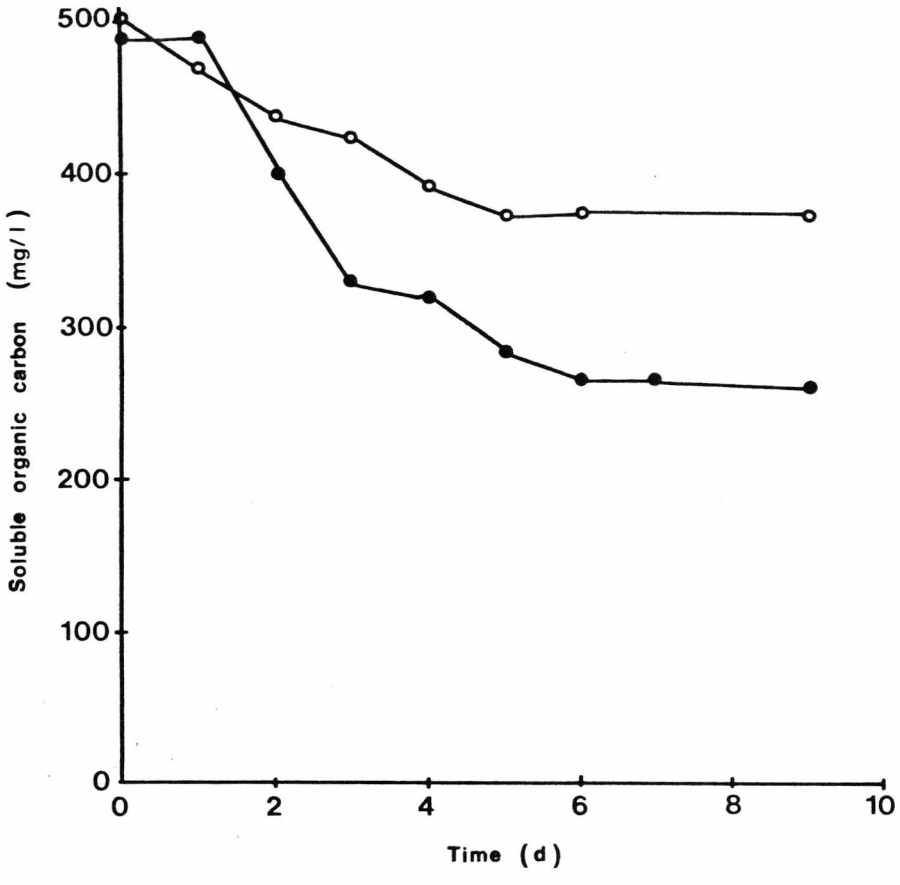


Fig. 17 Degradation of PEG 200 by isolate R and O

Isolates R (o) and O (●) were grown in RII mineral salts medium with PEG 200 (1g/l) as sole carbon source. At intervals culture samples were removed, centrifuged and the soluble organic carbon measured.

growth were converted to their trimethylsilyl ethers and examined by gas liquid chromatography (g.l.c.) (Fig. 18). In samples removed early in exponential growth, the g.l.c. pattern observed with $CHCl_3$ (and acid-$CHCl_3$) extracted samples were consistent with the assumption that strains R and O utilised the low and high molecular weight components of the mixture, respectively, although with strain R, a new peak (retention time, 9.6 min) was detectable after 2 days and was still found in samples taken after this time. The identity of this compound is unknown. With samples taken at 7 days no other significant peaks were observed suggesting that PEGs were no longer present in the culture media at this time. Increased sensitivity was required, however, to detect products by g.l.c. during later stages of growth.

PEGs detected in culture supernatants were determined quantitatively by reaction with HBr and subsequent analysis as dibromoethane by g.l.c. (Table 11). With strain R the results indicated that 155 mg/l of PEGs were in fact present after 7 days incubation, although TOC analysis showed that as much as 375 mg/l carbon (equivalent to 750 mg/l PEG) was present in culture supernatants at this time (Fig. 17). Similarly with strain O, although only 22 mg/l of PEGs could be detected by g.l.c. at 7 days (Table 11) TOC analysis showed that as much as 250 mg/l carbon (500 mg/l PEG) remained in culture supernatants at this time.

Although this apparent anomaly could be partially attributed to a low recovery of PEGs from culture supernatants (only ~50% of PEG 200 was extracted from the initial culture supernatants) a considerable amount of carbon remains unaccounted for.

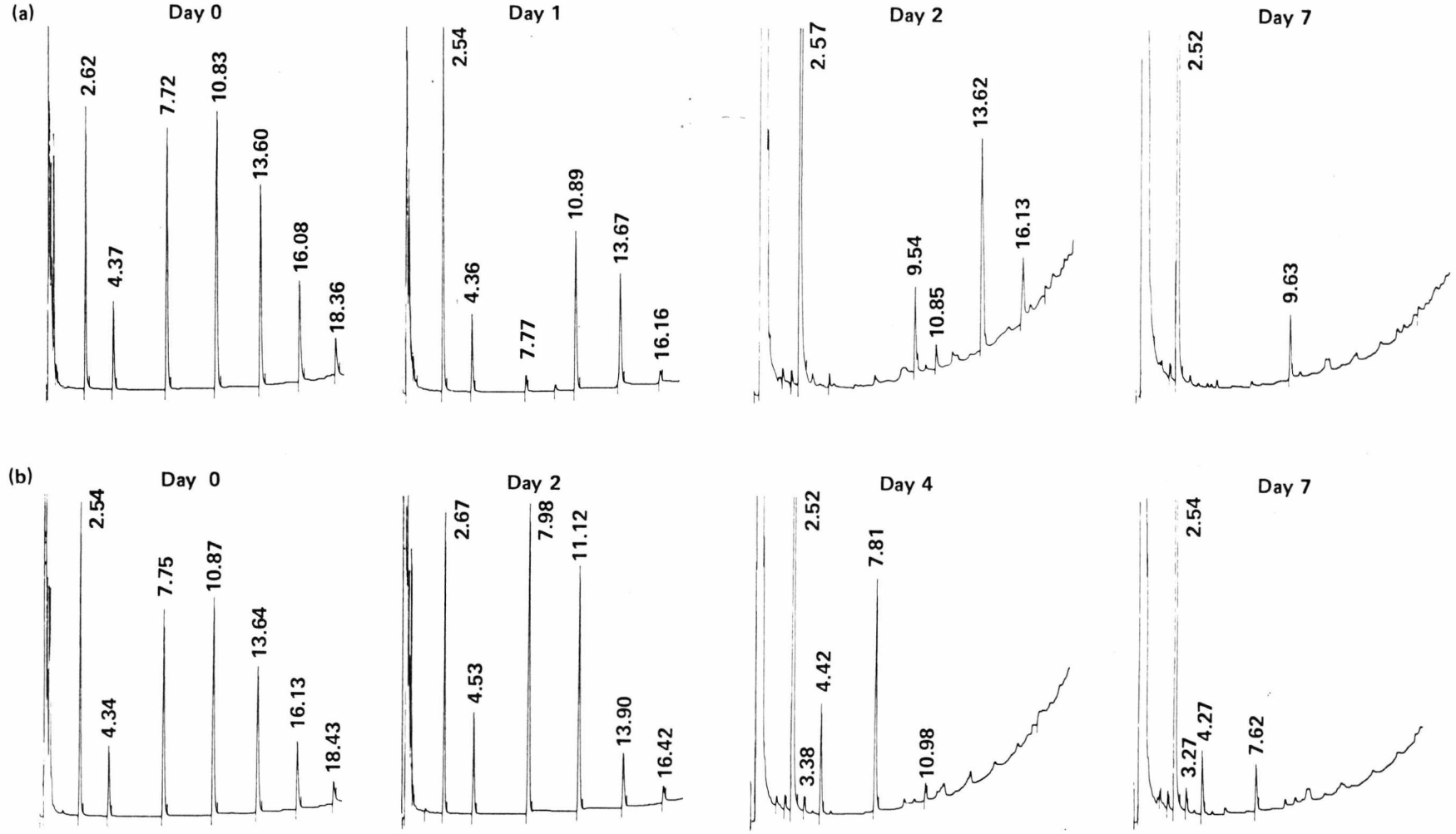


Fig.18 Gas chromatography of trimethyl silyl ether derivatives

Isolates R (a) and O (b) were grown in RII mineral salts medium containing PEG 200 (1 g/ℓ) as sole carbon source. At intervals culture samples (25 mℓ) were removed, centrifuged, evaporated to dryness and the residue extracted in chloroform. After drying the residues from chloroform extraction were converted to their trimethyl silyl ethers and analysed gas chromatographically. The numbers represent retention times in minutes. Retention times of approximately 4.4, 7.7, 10.8, 13.6, 16.1 and 18.3 minutes were given by compounds containing 2, 3, 4, 5, 6 and 7 ethylene oxide units respectively. n—Undecane was used as an internal standard throughout and had an approximate retention time of 2.6 min.

Table 11. Recovery of polyethylene glycols from culture filtrates

Strains R and O grown on PEG 200 (1g/1) were incubated at 25°C with shaking at 200 rev/min and samples (25 ml) taken from these cultures (11) at the times shown. After removing the cells, PEGs were detected by g.l.c. as dibromoethane after reaction with HBr and expressed as concentrations of the unbrominated moieties by relation to a calibrated standard. Figures represent absolute recoveries.

Bacterial Isolate	Fraction	Polyethylene glycol recovered (mg/1) after designated number of days				
		0	1	2	4	7
R	Chloroform extraction of culture supernatant	583	411	199	n.d.	155
	Acid chloroform extraction	33	75	173	n.d.	93
O	Chloroform extraction of culture supernatant	526	n.d.	423	56	22
	Acid chloroform extraction	37	n.d.	21	18	25

n.d. not determined

Reaction of acidic $CHCl_3$-extracted residues with HBr resulted in the formation of dibromoethane (Table 11) suggesting that acidic PEG derivatives were formed during degradation. With strain O the concentration of these compounds detected was very low (25 mg/l) and was fairly constant throughout the course of the experiment. With strain R, however, the concentrations were considerably higher, reaching a maximum of 173 mg/l after 2 days growth and subsequently decreasing to 93 mg/l after 7 days.

Failure to detect these products as their TMS ethers cannot preclude the possibility that these figures may represent the further extraction of undegraded PEG components, which were not removed in the initial $CHCl_3$ extractions, but they were suspected to be metabolites and were examined further.

3.1.3.3 Formation of acidic intermediates

Examination of $CHCl_3$ and acidic $CHCl_3$ extracts by t.l.c. in a solvent system comprising methyl ethyl ketone:methanol:water:conc.ammonia solution (65:20:5:10), followed by staining with bromocresol purple revealed the presence of acidic compounds. In final culture samples, a number of these appeared to be persistent (Fig. 19) and were distinct from others observed on chromatograms sprayed with modified Dragendorff's reagent (Patterson et al., 1966) i.e. they were not PEGs.

Concomitant with the appearance of acidic compounds was the appearance of a series of new spots which reacted with Dragendorff's reagent but which did not correspond to the parent PEG 200. One new spot visible in $CHCl_3$ extracts from culture supernatants of strain R growing on PEG 200 had the characteristic appearance of a PEG-like compound but of apparent increased molecular weight and probably corresponded to an undegraded

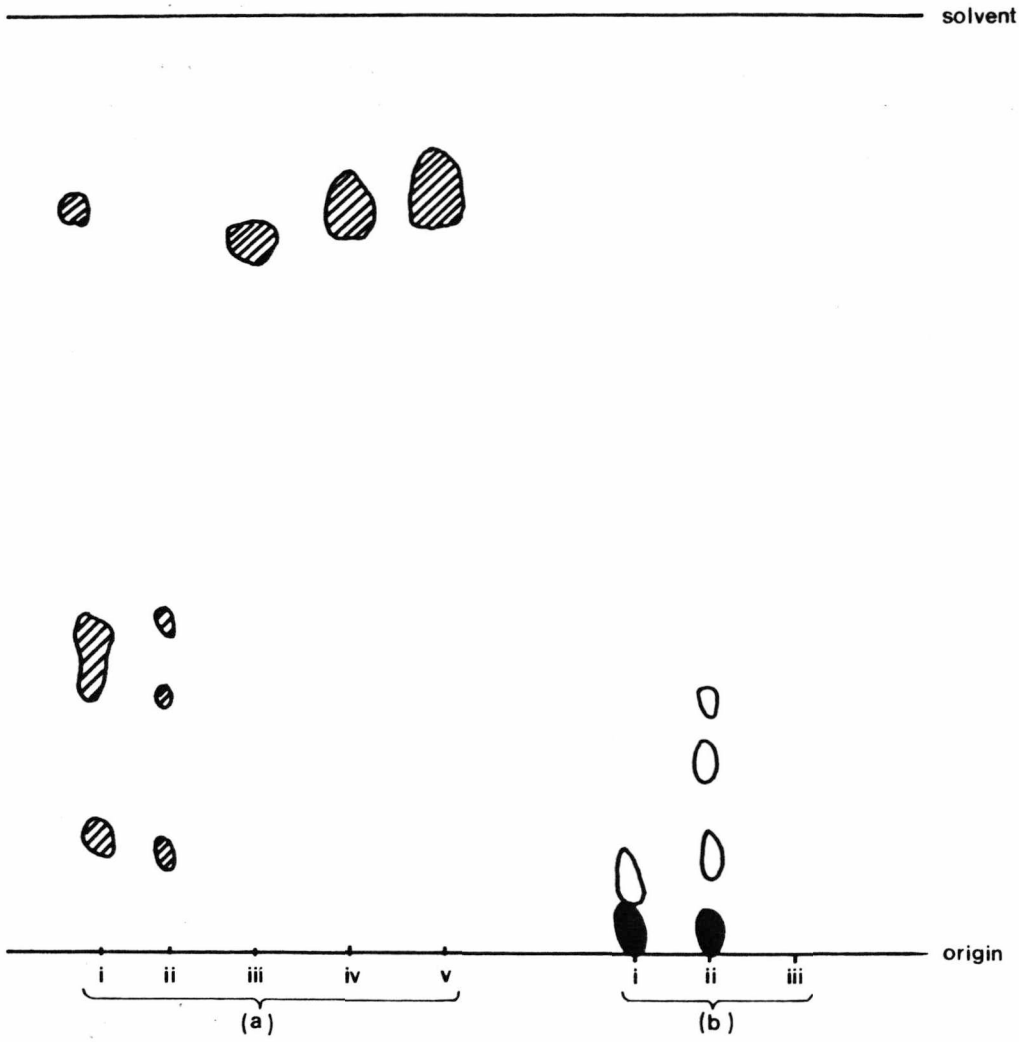


Fig. 19 Analysis of culture supernatants of isolates R and O grown on PEG 200, by thin-layer chromatography

Culture supernatants were evaporated to dryness and the residue extracted with chloroform. After concentration by evaporation, samples (10 µl) of the chloroform extracts were loaded onto silica-gel plates and chromatographed against a mixture of methyl ethyl ketone-methanol-water -conc. ammonia solution (65:20:5:10) and developed by spraying with (a) modified Dragendorffs reagent for demonstrating PEGs, or (b) bromocresol purple [0.04% (w/v) in 50% (v/v) aqueous ethanol] for detecting acidic compounds. Figures in parenthesis represent: the products of PEG 200 degradation by (i) isolate R and (ii) isolate O; (iii) authentic PEG 200; (iv) authentic PEG 300 and (v) authentic PEG 400.

▨ PEG-type material

□ Free acid

■ Salt

high molecular weight component of the PEG 200 mixture.

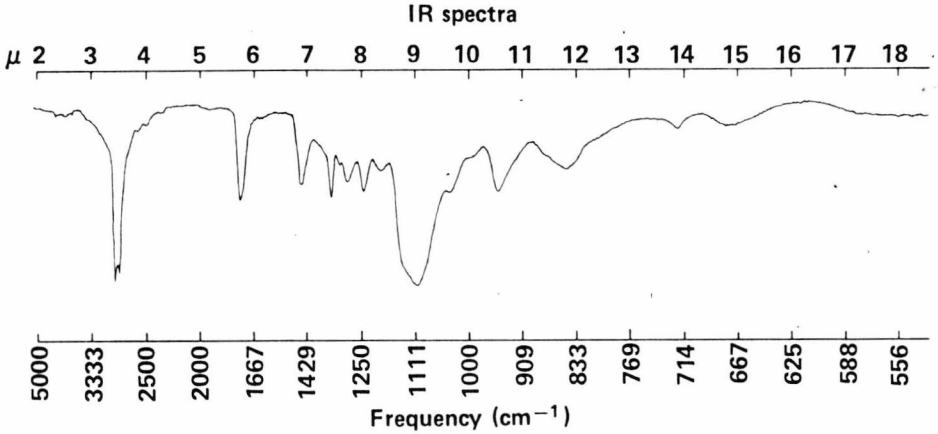
Initial infra-red (i.r.) and nuclear magnetic resonance (n.m.r.) analyses did not substantiate the identity of these products as acidic compounds but they were present as their salts rather than as the free acids. Thus in a further experiment, dried samples were resuspended in 3 ml distilled water and passed through a column (1 x 4.5 cm) of BioRad AG 50-X8 cation-exchange resin and eluted with further additions of distilled water. This procedure converted salts present to the corresponding acids. A total of 20 ml of effluent was collected, rotary evaporated to dryness and the colourless residue analysed by i.r. and n.m.r.

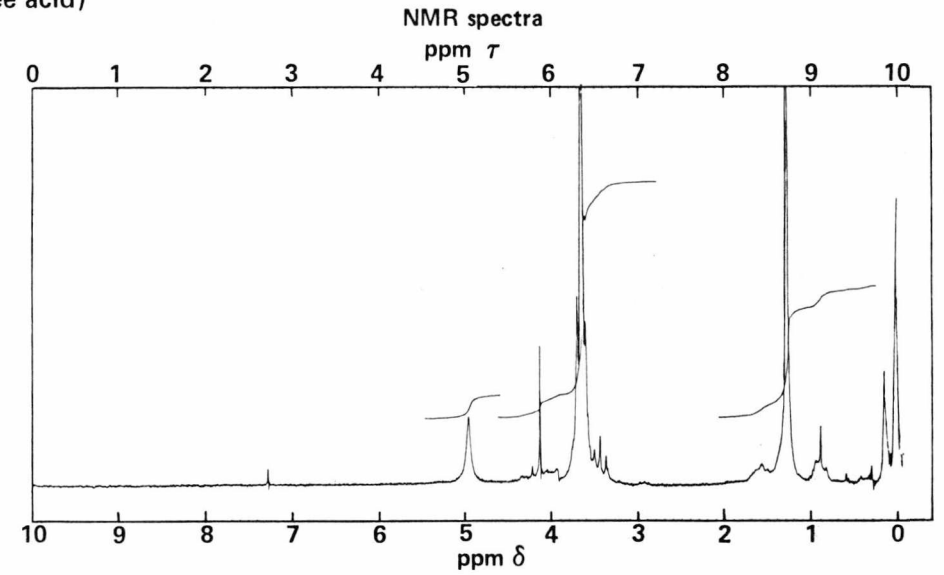
In order to interpret the spectra, an authentic sample of a fatty alcohol polyglycol ether carboxylic acid, Akypo L 100 [$C_{12}H_{25}(OCH_2CH_2)_{10}OCH_2COOH$] was also examined. This compound was analysed both as the free acid, in which the carboxyl band at 1700 cm^{-1} was very much in evidence and as the sodium salt, in which carboxylation was not evident (Fig. 20).

A proton magnetic resonance spectrum of the synthetic carboxylated material showed a pattern of peaks in the region of δ 3.5 - 5.2 ppm (Fig. 20). Peaks at 0.9 and 1.3 correspond respectively to protons of the type $-CH_3$ and $-(CH_2)-$ and peaks at δ 3.7 and 4.2 to the CH_2CH_2O- and $-COCH_2O-$ protons respectively. The peak at ~δ 5.0 has not been identified. The spectrum obtained on analysis of the salt was less complex with peaks at 0.9, 1.3 and 3.7 but not at 4.2 or ~5.0 which suggested that the peak at~δ 5.0 might be indicative of carboxyl group formation. This peak could not, however, be directly assigned to the acidic -OH proton because this generally appears down field between δ 9.0 and δ 13.0 (and is consequently not seen).

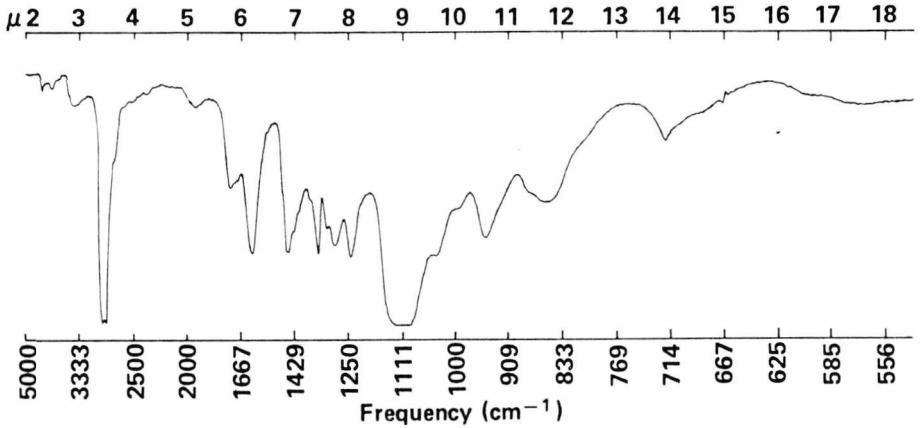
The peak at δ 7.2 can be ascribed to $CHCl_3$.

AK L100 (Free acid)





AK L100 (sodium salt)



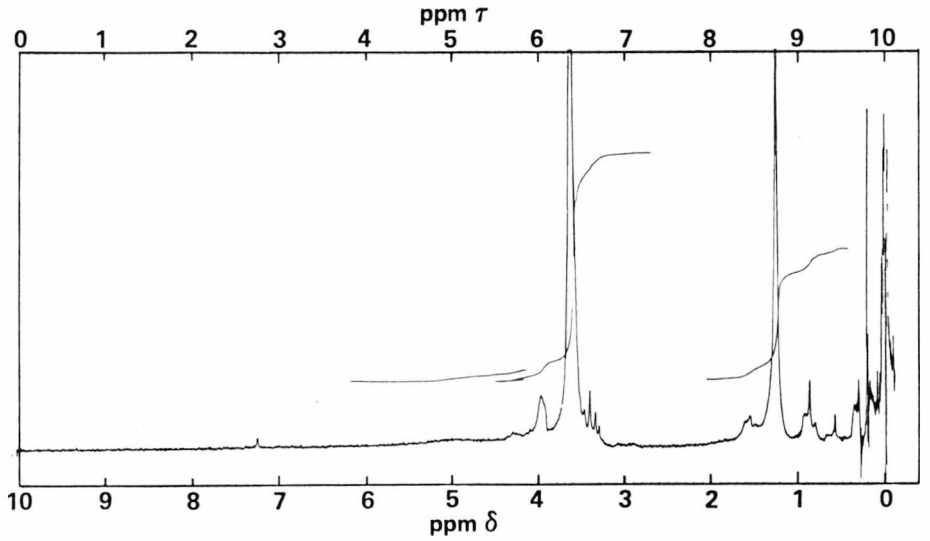


Fig.20 Analysis of Akypo L100 by infrared and nuclear magnetic resonance spectroscopy

I.r. and n.m.r. spectra of extracted residues from PEG 200 degradation by strains R and O closely resembled those of the authentic Akypo L100 compound (Fig. 21). Thus the presence of a peak at 1700 cm^{-1} on i.r. analysis and peaks at δ 3.5, 4.5 (and possibly 5.0) on analysis by n.m.r. spectroscopy were indicative of carboxylated polyethylene glycols.

Further evidence for the carboxylated nature of these compounds was obtained from mass spectroscopy. The mass spectrum of material obtained from PEG 200 degradation by strain R showed peaks at m/e 270, 314 and 358 (Fig. 22) which, because the mass ion was measured in the presence of ammonia, correspond to masses of 252, 296 and 340, respectively; these values are consistent with mono-carboxylated glycols with 5, 6 and 7 ethylene oxide units respectively. The major peak was detected at a m/e ratio of 314 which corresponds to a mono-carboxylated polyethylene glycol with 6 ethylene oxide units.

Similarly with the products obtained from cultures of strain O, masses of 120, 164, 218 & 252, consistent with mono-carboxylated glycols containing 2, 3, 4 and 5 ethylene oxide groups respectively were observed with a peak of highest intensity corresponding to mono-carboxylated tetra-ethylene glycol (Fig. 22).

Thus after selective degradation of either the low or high molecular weight components of the mixture, remaining carboxylated derivatives accumulated in the medium and account for the high residual concentration of organic carbon found in media at the end of growth even though the substrate PEGs had largely disappeared.

Having established the growth substrate specificity of strains Z, R and O on ethylene glycol and PEGs further studies were conducted with whole cells and extracts to determine the metabolic routes by which these isolates degraded such compounds.

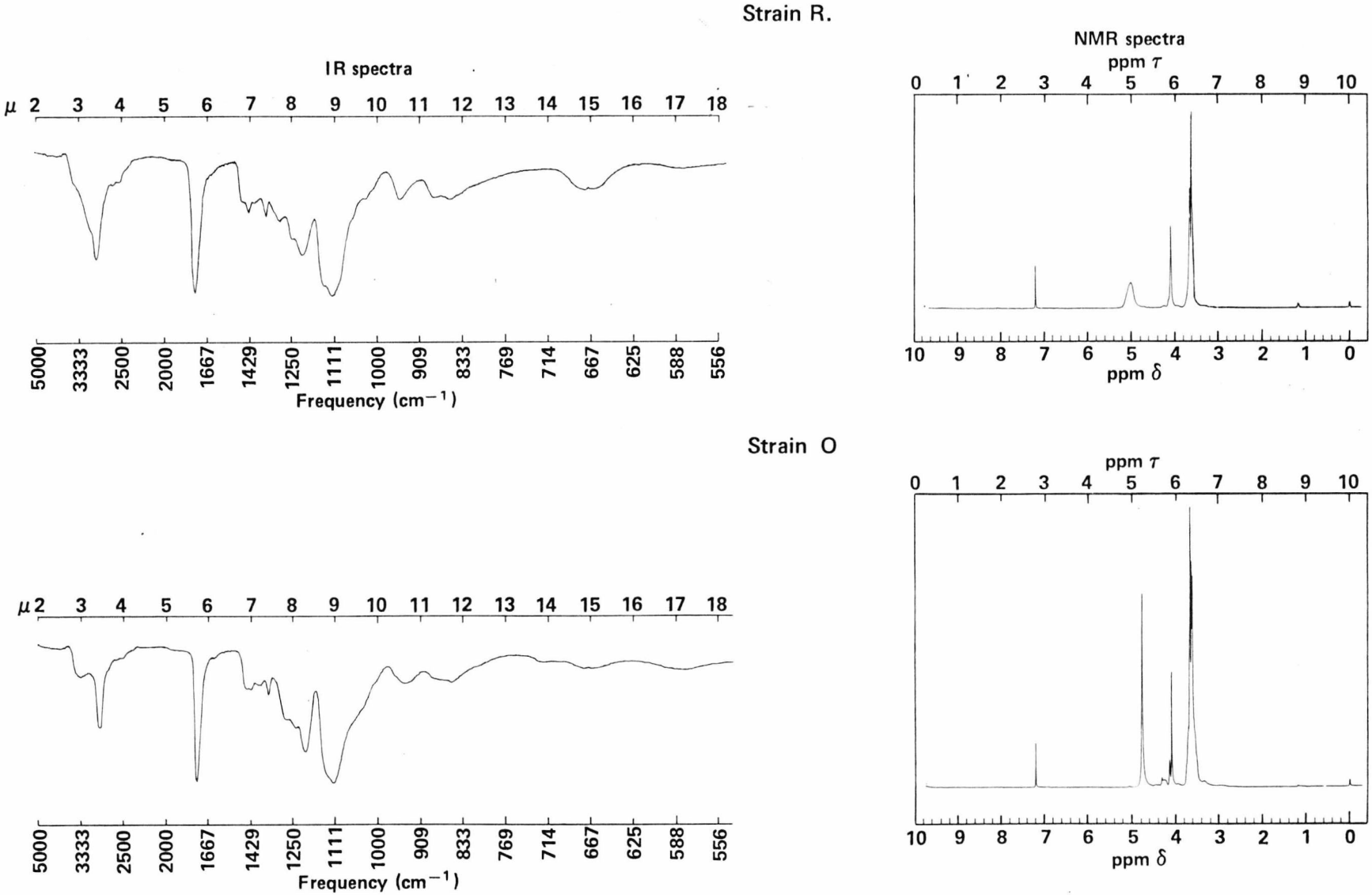


Fig. 21 Analysis of culture extracts by i.r and n.m.r spectroscopy.

Culture samples of R and O were evaporated to dryness and the residual material extracted in chloroform. After concentration by evaporation samples were analysed by i.r and n.m.r spectroscopy.

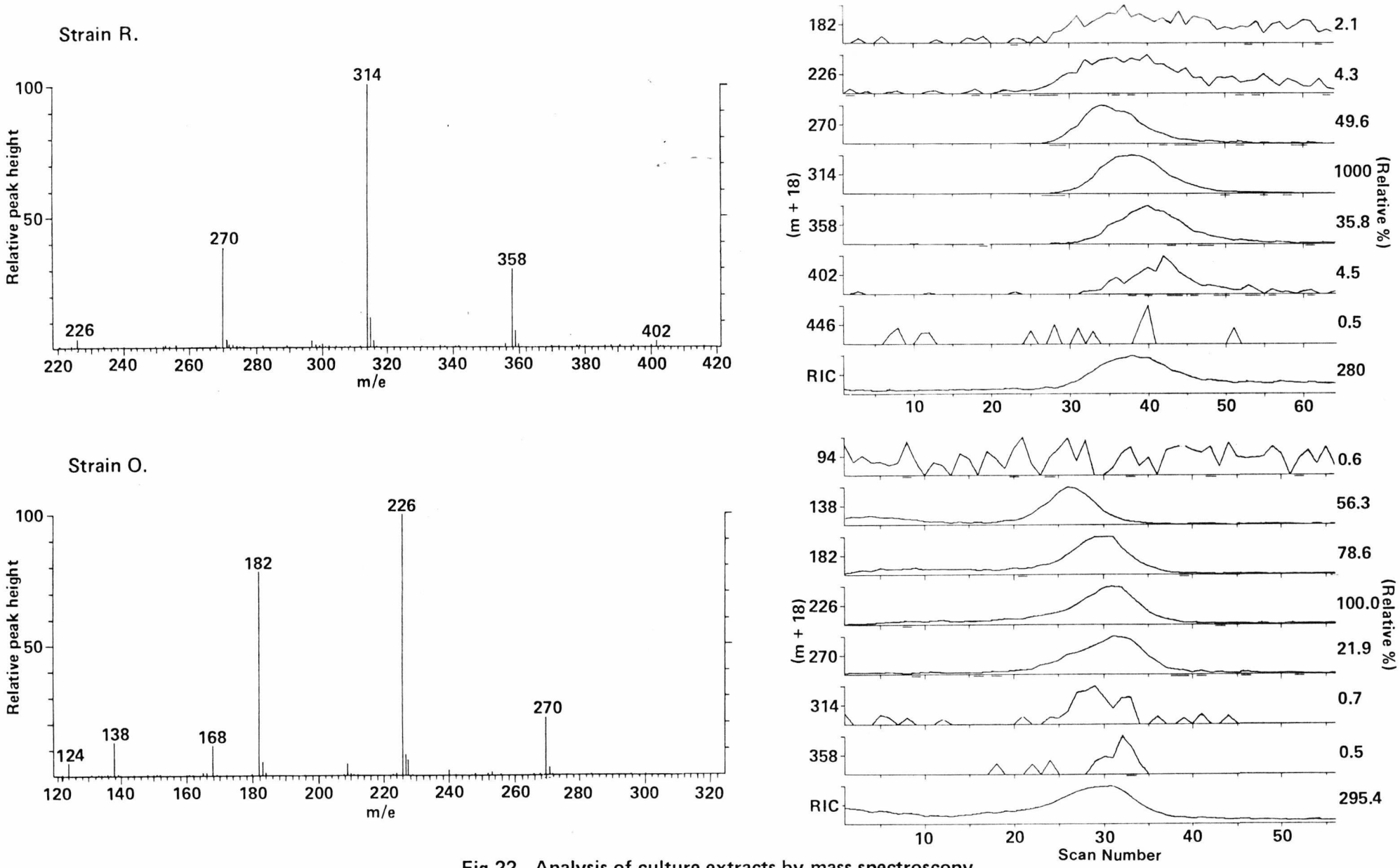


Fig.22 Analysis of culture extracts by mass spectroscopy

Mass spectra of products from the biodegradation of PEG 200 by strains R and O. RIC is the reconstruction ion current and refers to the total ion intensity; other lines show the intensity of ions corresponding to a particular m/e value.

3.2 BACTERIAL UTILISATION OF PEGs

3.2.1 Introduction

Although biodegradation of PEGs has been studied by several investigators (Patterson *et al.*, 1967; Pitter, 1968; Cox and Conway, 1976; Kawai *et al.*, 1977, 1980) the precise metabolic routes involved and the nature of the carbon fragments that enter the central metabolic pathways is unclear.

Identification of degradation intermediates is complicated by the fact that commercially available products are a complex mixture of homologues of different molecular weights. Thus much of the information regarding degradation of PEGs has arisen from work performed on oligomeric glycols. DeLey and Kersters (1964) reported the oxidation of mono-, di- and tri ethylene glycol by *Gluconobacter suboxydans* and identified glycollate and diglycollate as products of the monomer and dimer, respectively. Although Stouthamer *et al.* (1963) produced evidence to show that ethylene glycol-grown *Acetobacter aceti* converted glycollate into glyoxylate which was then further metabolised via the glyoxylate bypass, enzymes for the conversion of ethylene glycol to glycollate were not identified. Subsequent studies by Gonzalez *et al.* (1972); Jones and Watson (1976); and Child and Willets (1978) led to the implication of a functioning glycerate cycle in the pathway of ethylene glycol metabolism although with the exception of the latter investigation, no detailed experimental evidence was presented. An alternative metabolic sequence involving the conversion of ethylene glycol to acetate via acetaldehyde was reported by Wiegant

and deBont (1980) but the most extensive study thus far appears to be that of Pearce and Heydeman (1980). These workers isolated a bacterium that degraded diethylene glycol and low molecular weight PEGs by a route that differed from any reported elsewhere in which assimilation of C_2 units occurred at the oxidation level of acetate.

Bacterial isolate Z grew on glycollate, glyoxylate and glycerate and it seemed reasonable to suggest that the metabolism might involve oxidation of glycollate to glyoxylate and entrance of the latter into the glyoxylate bypass or glycerate cycle. The ability of isolate Z to oxidise such intermediates was thus examined.

3.2.2 Oxidation of substrates by whole organisms of isolate Z

Washed suspensions of ethylene glycol-grown strain Z immediately and rapidly oxidised ethylene glycol, glycolaldehyde, glycollate, glyoxylate and glycerate (Table 12); malate and succinate were oxidised rapidly after a short lag of 15 - 20 minutes; glycine and citrate were not utilised. The rate of oxygen uptake with ethylene glycol was sufficient to account for the oxidation of 2.3 µmol of this compound/mg dry wt./h and the total quantity of oxygen consumed, corrected for the endogenous respiration (1.7 mol O_2/mol substrate) was 68% of the amount required for the complete oxidation of ethylene glycol to carbon dioxide. The deficit is probably accounted for by oxidative assimilation.

Of the substrates tested, malate and succinate were rapidly oxidised by washed suspensions of succinate-grown cells (Table 12) but glycolaldehyde and glycollate were, with this suspension, oxidised only after a lag of between 10 and 15 minutes.

Table 12. Oxidation of substrates by washed suspensions of strain Z grown on ethylene glycol and succinate

Main compartment of Warburg manometer flasks contained in 3 ml: potassium phosphate buffer, pH 7.0, 100 µmol and washed cells (equivalent to 5 mg dry wt.). The centre wells contained KOH, 400 µmol. Substrates as indicated (10 µmol) were added from side-arms after thermal equilibration at 30°C. Results, expressed as µl O_2 consumed/h/mg dry wt., are corrected for the endogenous respiration (28 µl O_2/h/mg dry wt).

Substrate for oxidation	Growth substrate....Ethylene glycol	Succinate
Ethylene glycol	129	<12
Diethylene glycol	47	<12
Triethylene glycol	97	<12
Glycolaldehyde	202	85*
Glycollate	202	92*
Glyoxylate	75	29
Glycerate	129	125
Pyruvate	128	145
L-Malate	119*	183
Succinate	112*	217
Acetate	149	137
Fumarate	130*	173
2-Ketoglutarate	113*	132

* After approximately 15-20 minute lag period

Other glycols, which were unable to support growth of strain Z were incompletely oxidised (Fig. 23). The total quantities of oxygen consumed, corrected for the endogenous respiration were 0.8 mol/mol of diethylene glycol and 0.9 mol/mol of triethylene glycol, only 16 and 12% of the amount required for the complete oxidation of these glycols, respectively.

3.2.3 Enzyme activities in extracts of strain Z

The oxidation of ethylene glycol by crude extracts examined in the presence of various electron acceptors (Table 13) revealed the presence of a PMS-enhanced glycol oxidising enzyme.

Of the single electron acceptors tested no activity was observed with ferricyanide, dichloroindophenol (DCPIP) or cytochrome c.

The reduction of NAD^+ by glycol in these extracts was very slow and $NADP^+$ was not reduced at all. When analysing crude extracts for the occurrence of NAD^+-linked dehydrogenation of ethylene glycol it was found that neither the activity of the extract for, nor its specificity towards, ethylene glycol and primary alcohols was affected by the carbon source used for growth (Table 14). By varying the substrate concentration it was ascertained that the slow reaction with ethylene glycol was not due to too low or high substrate concentration and thus probably reflected a genuine low affinity of this substrate for the NAD^+-linked enzyme; the physiological function of which in glycol catabolism was thus suspect. At pH 6.0 the reverse reaction, the NADH-mediated conversion of glycolaldehyde to ethylene glycol was observed (see Table 26) but this was probably catalysed by the same enzyme as heat denaturation at 65°C caused an equal rate of loss of reductase and dehydrogenase activities (Fig. 24).

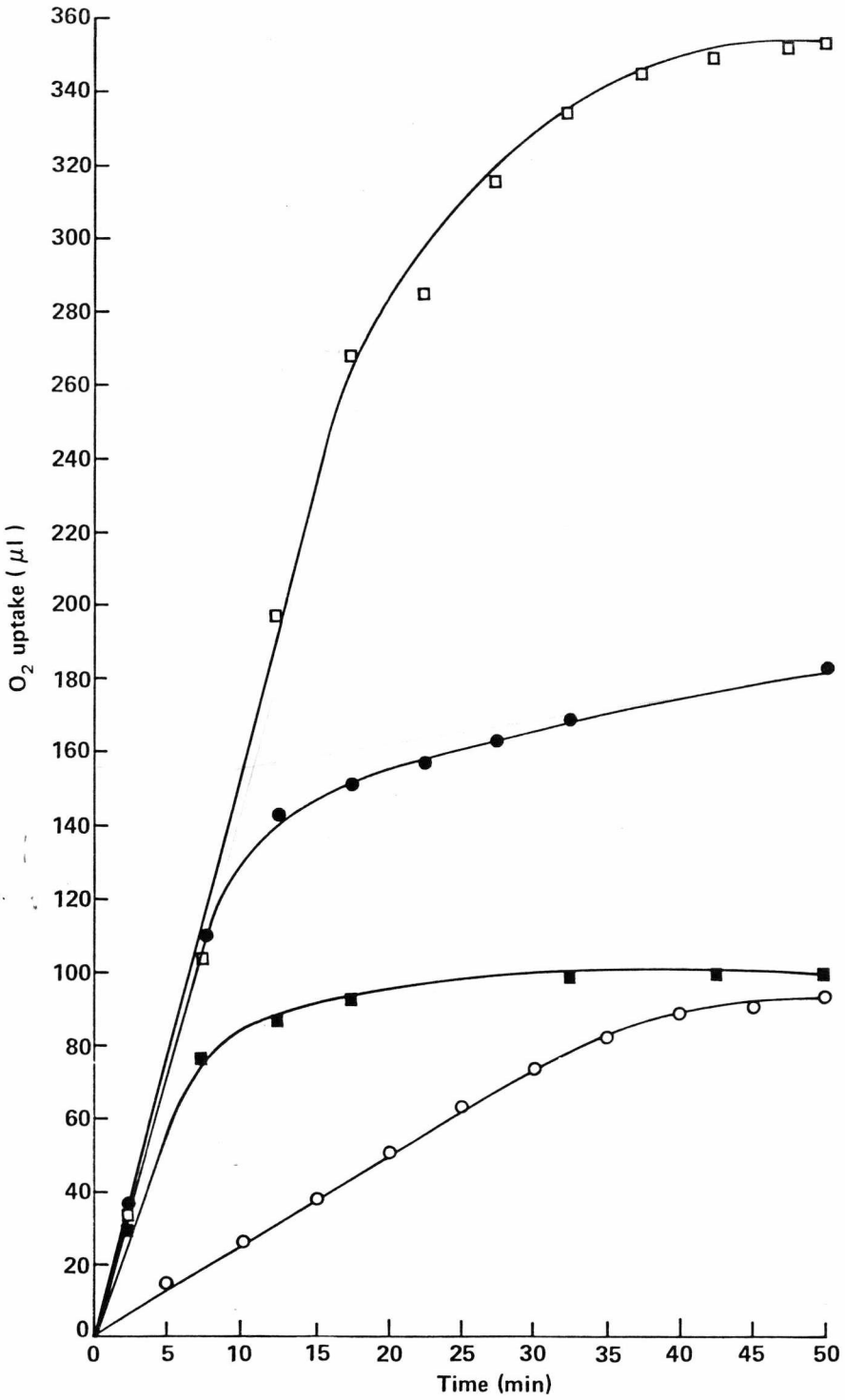


Fig. 23 Oxidation of mono-, di- and triethylene glycol by washed suspensions of isolate Z grown on ethylene glycol

Main compartment of Warburg manometer flasks contained in 3 ml : 100 μmol potassium phosphate buffer, pH 7.0 and washed cells (equivalent to 5 mg dry wt). The centre wells contained 400 μ mol KOH. Substrates as indicated (5 μ mol) were added from side-arms after thermal equilibration at 30°C. Results are corrected for the endogenous respiration of 0.091 μmol O_2/min/mg dry wt :

● , ethylene glycol; ○ , diethylene glycol; ■ , triethylene glycol; □ , oxidation of diethylene glycol by a washed suspension of strain R (equivalent to 5 mg dry wt).

Table 13. Oxidation of ethylene glycol by crude extracts of strain Z with various electron acceptors

Organism Z was grown on ethylene glycol and the cells harvested during exponential growth. Extracts were prepared and activity determined in the presence of various electron acceptors.

Acceptor	Specific activity (nmol/min/mg protein)
NAD	16
NADP	no activity
Ferricyanide	no activity
Cytochrome c	no activity
Dichlorophenolindophenol (DCPIP)	no activity
Oxygen	no activity
Oxygen + phenazine methosulphate	21

Assay systems containing ferricyanide, DCPIP and cytochrome c were measured spectrophotometrically. All reaction mixtures contained in a final volume of 1 ml: tris-HCl buffer at pH 7.5 or 9.0, 100 µmol; KCN (pH 8.0), 6 µmol and crude extract. The standard assay with ferricyanide contained in addition potassium ferricyanide, 0.5 µmol, and readings were taken at 400 nm. The standard assay with DCPIP contained in addition DCPIP, 0.4 µmol and readings were taken at 660 nm. In the experiments with cytochrome c, 0.1 ml of a 1% (w/v) cytochrome c solution was added to each cuvette and readings were taken at 550 nm.

Assay systems containing phenazine methosulphate were measured polarographically as described in Methods, without the addition of ammonium chloride.

Table 14. Influence of growth substrate on the activities of NAD^+-linked alcohol dehydrogenase and ethylene glycol dehydrogenase (PMS-linked) in extracts of strain Z.

Organism Z was grown on the substrates indicated and the cells harvested during exponential growth. Extracts (HSSF) were prepared by sonication and activities of the enzymes determined as described in Methods.

As a control Paracoccus denitrificans, which was found to catabolise ethylene glycol by an NAD^+-linked dehydrogenase (this study), was grown on ethylene glycol, ethanol and succinate and the cells harvested and extracts prepared as described in Methods (2.11). Data for P. denitrificans appears in parentheses.

Growth substrate	Substrate for oxidation:-	Specific activity (nmol/min/mg protein)			
		NAD^+-linked alcohol dehydrogenase		PMS-linked ethylene-glycol dehydrogenase	
		Ethylene glycol	Ethanol	Ethylene glycol	Ethanol
Ethylene glycol		16 (130)	83 (210)	73 (N.D.)	101 (N.D.)
Ethanol		13 (110)	84 (176)	N.D.(N.D.)	N.D.(N.D.)
Succinate		13 (64)	84 (84)	N.D.(N.D.)	N.D.(N.D.)
Glucose		5 (n.d.)	81 (n.d.)	N.D.(n.d.)	N.D.(n.d.)

n.d. not determined
N.D. not detectable

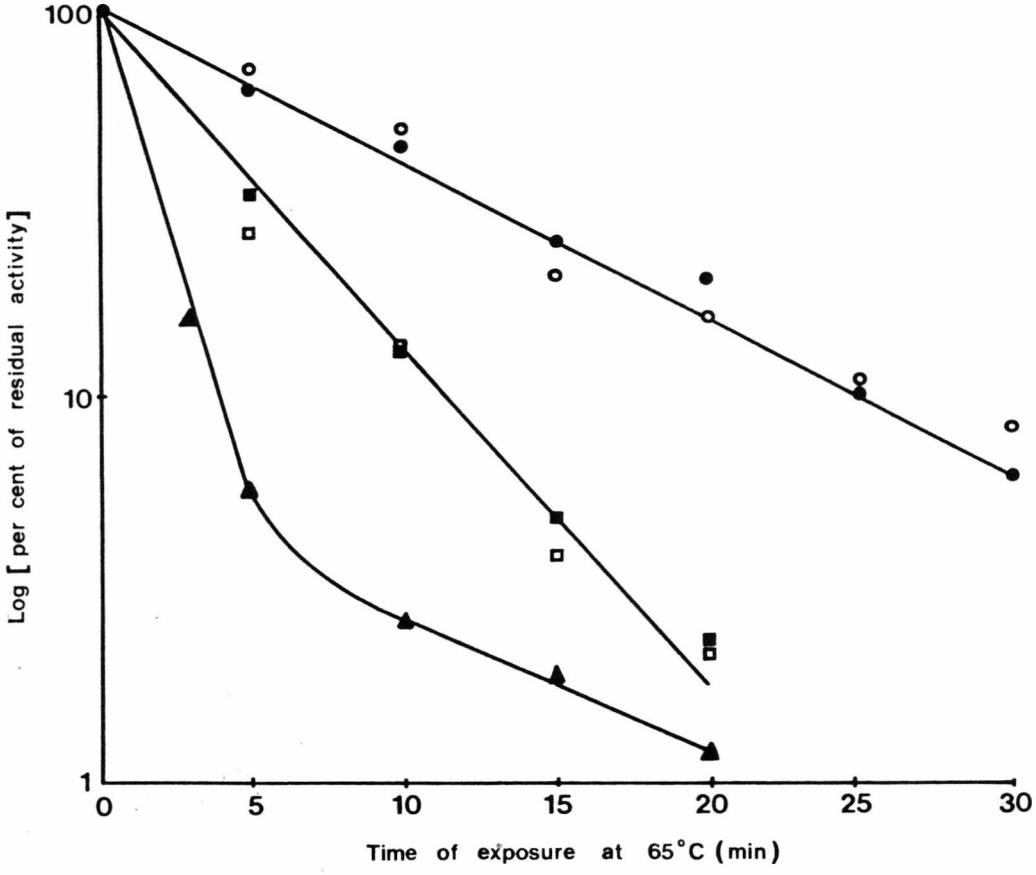


Fig. 24 Comparative thermal inactivation of the enzymes of ethylene glycol metabolism.

An aliquot of crude extract (10ml containing 12-15 mg/ml protein) prepared from isolate Z grown on ethylene glycol at 30°C, was placed in a test tube and immersed in a water bath maintained at 65°C as described in Methods. Samples (1ml) were removed at intervals, immediately transferred to an ice bath and subsequently assayed for residual enzyme activities :

● , ethylene glycol dehydrogenase substrate for oxidation, ethylene glycol ;
o , ethylene glycol dehydrogenase substrate for oxidation, diethylene glycol ;
■ , NAD-alcohol dehydrogenase ; □ , glycolaldehyde reductase ;
▲ , glycolaldehyde dehydrogenase .

In view of the failure to enhance its activity during growth on ethylene glycol, together with the low activities recorded which cannot account for the short generation time of the organism on this substrate, it seems unlikely that the NAD^+-dependent alcohol dehydrogenase has any major physiological role in the metabolism of ethylene glycol.

Detection of PMS-linked activity in crude extracts after growth on ethylene glycol, but not in ethanol- or succinate-grown cells, suggested however, that this enzyme might have an important function in ethylene glycol metabolism. Further characterisation revealed that prolonged dialysis of crude extracts (48 h at 4°C against several changes of 500 vol. 50mM-potassium phosphate buffer, pH 7.2) led to complete loss of PMS-linked activity which was completely restored by including ammonium salts in the assay system, as previously found for methanol dehydrogenase (Anthony and Zatman, 1964). Subsequently it was found that the addition of ammonium salts directly to crude cell free extracts resulted in a pronounced increase of activity (Table 15).

When crude extracts were centrifuged at 180,000 g for 2 h at 5°C almost all the PMS-linked activity (subsequently called ethylene glycol dehydrogenase) was located in the supernatant fraction. Negligible activity was associated with washed (or unwashed) particles (Table 15).

Apparent K_m values for PMS and NH_4Cl were determined as 4.6 μM and 10mM repectively according to the method of Lineweaver and Burke (Fig. 25).

Comparison of the effects of several monovalent cations on ethylene glycol dehydrogenase is given in Table 16. With the exception of the NH_4^+ ion, no increase in activity was observed, and at concentrations of 12.5 mM or higher, inhibition by K^+, Na^+ and Rb^+ became evident. Stimulation of

Table 15. Requirement of ethylene glycol dehydrogenase for NH_4^+

Organism Z was grown on ethylene glycol and the cells harvested during exponential growth. Extracts were prepared by sonication and ethylene glycol dehydrogenase assayed at 30°C by using a Clark oxygen electrode. Reaction mixtures contained: borate buffer, pH 9.0, 100 μmol; PMS, 0.1 μmol; ethylene glycol, 2.0 μmol and extract in a total of 2 ml.

	Specific activity (nmol/min/mg protein)	
Enzyme preparation	$-NH_4Cl$	$+NH_4Cl$*
Crude extract	29.2	83.9
High speed supernatant fraction	28.0	84.0
Particulate fraction	N.D.	5.9

* Final concentration 30 mM

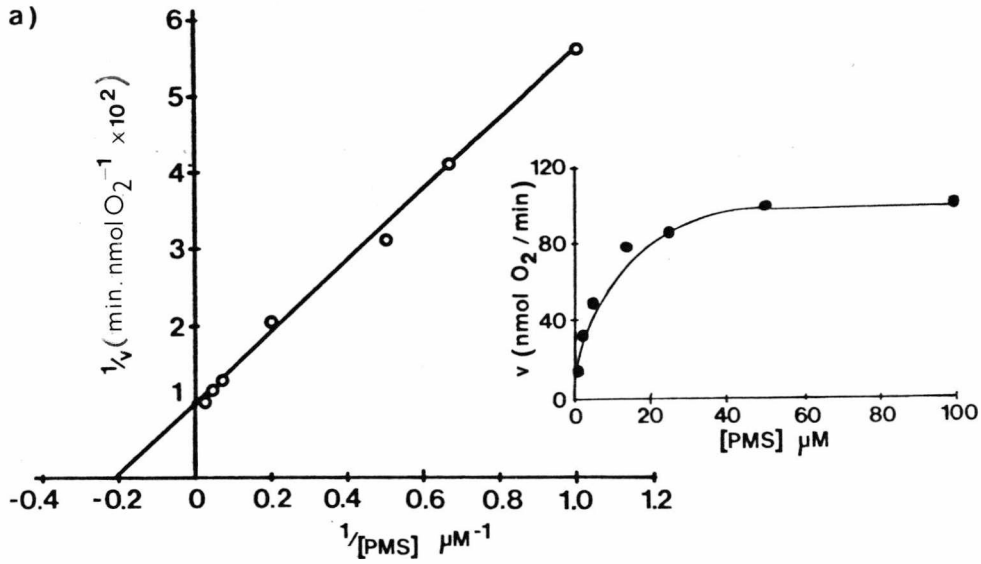
N.D. not detectable

Fig 25 Effect of a) phenazine methosulphate and b) ammonium chloride concentrations on PMS-dependent activity in crude extracts of strain Z.

The polarographic assay was used in each case.

a) Reaction mixtures contained in a total of 2 ml: borate buffer (pH 9.0), 100 µmol; phenazine methosulphate at the concentrations indicated; ammonium chloride, 60 µmol and extract. Reactions were started by the addition of ethylene glycol, 2 µmol.

b) Reaction mixtures contained in a total of 2 ml: borate buffer (pH 9.0), 100 µmol; phenazine methosulphate, 0.1 µmol; ammonium chloride at the concentrations indicated and extract. Reactions were started by the addition of ethylene glycol, 2 µmol.


a)
$^1/_v$ (min. nmol O_2^{-1} x 10^2)
$^1/_{[PMS]}$ μM^{-1}
v (nmol O_2 / min)
[PMS] μM

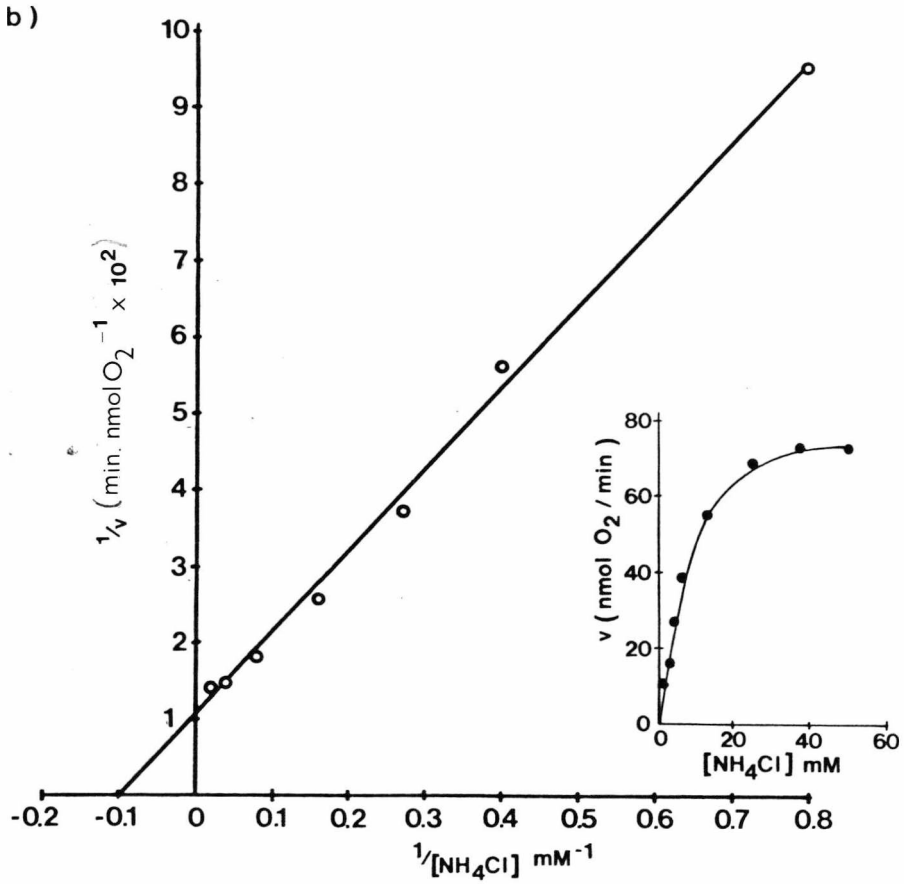

b)
$^1/_v$ (min. nmol O_2^{-1} x 10^2)
$^1/_{[NH_4Cl]}$ mM^{-1}
v (nmol O_2 / min)
[NH_4Cl] mM

Table 16. Effect of various monovalent salts on ethylene glycol dehydrogenase in crude extracts of strain Z

The polarographic assay was used as described in Methods, except NH_4Cl was replaced by various salts at the concentrations indicated. Activity in the absence of salt = 29 nmol O_2/min/mg protein.

		Specific activity (nmol O_2/min/mg protein)		
Salt	Salt conc.(mM).....	5	12.5	30
NH_4Cl		33	55.4	83.9
NaCl		20	12.3	14.8
KCl		28	16.2	9.4
RbCl		29	26.2	18.1

ethylene glycol dehydrogenase was independent of the anion of the ammonium salt; the chloride, nitrate, sulphate and dihydrogen phosphate were equally effective. Multivalent cations (Mg^{2+}, Ca^{2+}, Fe^{2+}, Zn^{2+}) completely inhibited the reaction.

On fractionation of the high speed supernatant with ammonium sulphate, essentially all ethylene glycol dehydrogenase activity was found in the protein fraction precipitated by $(NH_4)_2SO_4$ between 50 and 65% saturation (Table 17), in contrast to the NAD^+-dependent alcohol dehydrogenase activity which precipitated between 20 and 50% saturation.

Dialysis of the 50-65% fraction against 500 vol. of 50mM-potassium phosphate buffer, pH 7.2 for 24h at 4°C resulted in complete loss of activity; as observed previously for the crude extract this was completely restored by inclusion of ammonium salts in the reaction mixture (Fig. 26).

The activity of the enzyme with ethylene glycol as substrate was tested in borate-buffer and the maximum activity found at pH 9 (Fig.27). Only 63% of this activity was observed in glycine-NaOH buffer at the same pH although no inhibition of ethylene glycol dehydrogenase was observed in borate-buffer to which various concentrations of glycine-NaOH at the same pH were added.

A number of other substrates could be oxidised by ethylene glycol dehydrogenase (Table 18). The data indicated that PMS-dependent activity in extracts was not specific for ethylene glycol but shows a broad substrate specificity for several primary and secondary alcohols. 1,1-Dimethyethanol (tert-butyl alcohol) and benzyl alcohol were not oxidised. Other ether glycol compounds that contained a $-CH_2OH$ group, namely 2-methoxyethanol,

Table 17. Separation of enzyme activities by $(NH_4)_2SO_4$ precipitation

Isolate Z was grown at 30°C in R11 mineral salts media supplemented with ethylene glycol to mid-exponential phase. Cells were harvested and extracts prepared as described in Methods. To the high speed supernatant fraction (HSSF) solid ammonium sulphate was added, with constant stirring, to the desired concentration. The mixture was stirred for 20 minutes, the precipitate removed by centrifugation and the activities of ethylene glycol dehydrogenase and NAD-alcohol dehydrogenase determined in the fractions.

	Specific activity (nmol/min/mg protein)			
	$(NH_4)_2SO_4$ satn...20% - 50%		50% - 65 %	
Enzyme substrate	Ethylene glycol dehydrogenase	NAD-alcohol dehydrogenase	Ethylene glycol dehydrogenase	NAD-alcohol dehydrogenase
Ethylene glycol	5	13	267	N.D.
Diethylene glycol	9	N.D.	110	N.D.
Ethanol	11	104	315	N.D.

N.D. not detectable

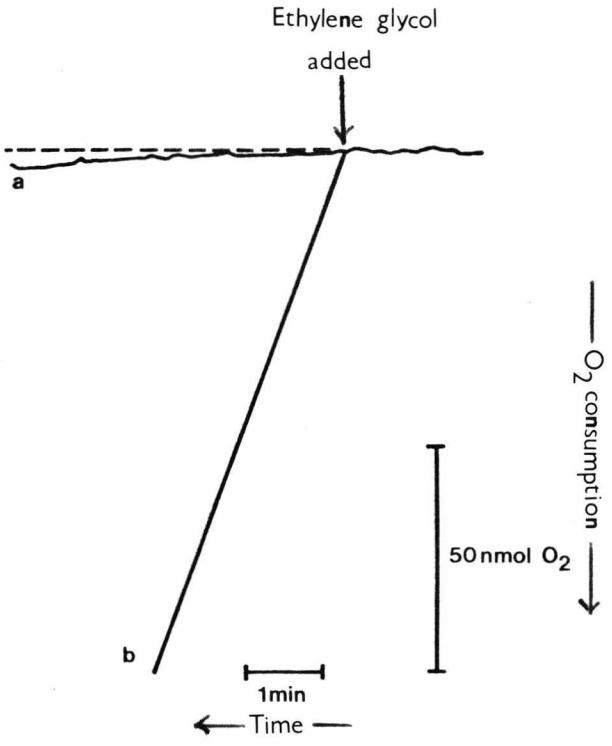


Fig 26 Requirement of ethylene glycol dehydrogenase activity for NH_4^+ in partially-purified extracts of isolate Z grown on ethylene glycol

Enzyme activities were assayed at 30°C using a Clark oxygen electrode. Reaction mixtures contained in 2ml : 100 µmol borate buffer, pH 9.0 ; 0.1 µmol phenazine methosulphate ; 2.5 µmol ethylene glycol ; dialysed fraction precipitating between 50 and 65 % saturation with ammonium sulphate (equivalent to 0.5mg protein) ; Activities were measured (a) without NH_4Cl , and (b) with NH_4Cl , 30 mM.

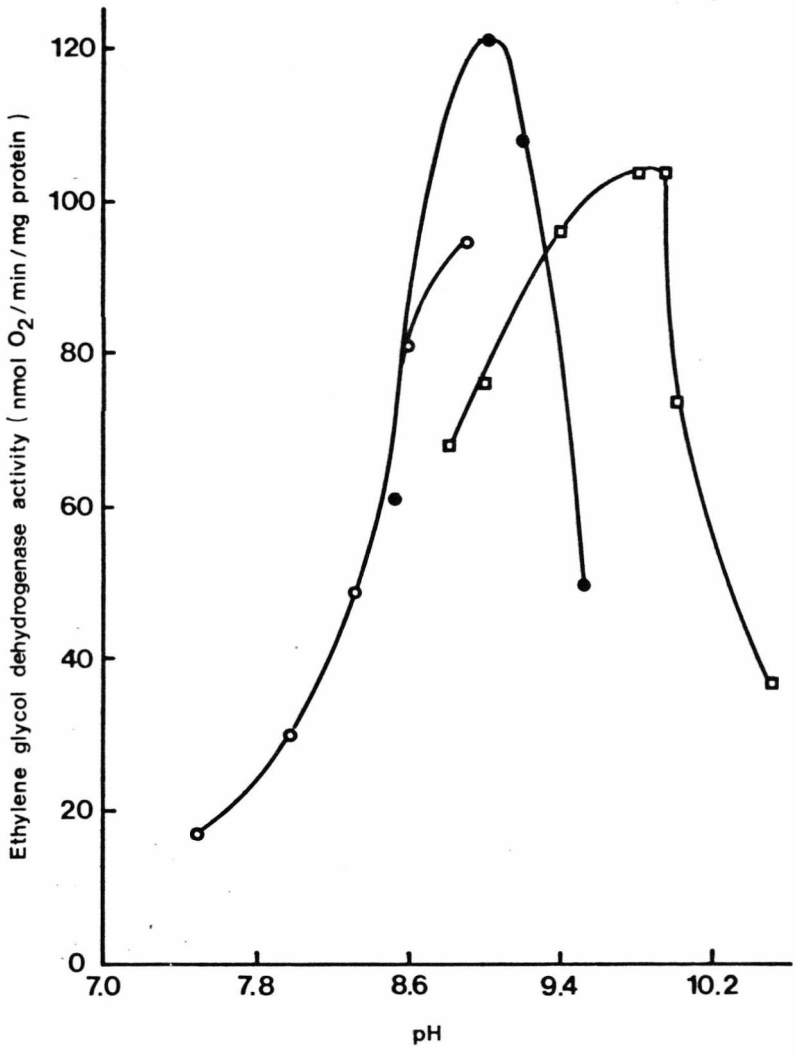


Fig. 27 Effect of pH on ethylene glycol dehydrogenase activity.

Initial activities were assayed at 30°C by using the O_2 electrode. Reaction mixtures contained in 2 ml: 50 µmol buffer; 0.1 µmol phenazine methosulphate, 60 µmol ammonium chloride; 2 µmol ethylene glycol; undialysed fraction precipitating between 50 and 65 % saturation with ammonium sulphate (equivalent to 0.5 mg protein). Activities were measured in : o, tris-HCl buffer; ●, borate buffer and □, glycine-NaOH buffer at the pH values indicated.

Table 18. Relative oxidation rates of various substrates by partially purified ethylene glycol dehydrogenase in the PMS-assay

The polarographic assay was used as described in Methods; the absolute rate of O_2 consumption for ethylene glycol (= 100%) was 250 nmol/O_2/min/mg protein.

Substrate	Oxidation rate (% of that with ethylene glycol
Ethylene glycol	100
Diethylene glycol	41
Triethylene glycol	108
Methanol	0
Ethanol	120
Propan-1-ol	139
Butan-1-ol	120
Propan-2-ol	89
Butan-2-ol	106
1,1-Dimethylethanol (tert-butyl alcohol)	0
Benzyl alcohol	0
2-Chloroethanol	96
Glycolaldehyde	85
2-Methoxyethanol	32
2-(2-Methoxyethoxy)ethanol	88
2-Butoxyethanol	118
2-Phenoxyethanol	105
1,2-Dimethoxyethane	0

2-(2-methoxyethoxy)ethanol, 2-butoxyethanol and 2-phenoxyethanol were also active with ethylene glycol dehydrogenase; even glycolaldehyde was oxidised.

The molecular weight of ethylene glycol dehydrogenase was determined by gel filtation on a 25 x 38 cm column of Sephadex G-200 calibrated with standard proteins (Fig. 28); a molecular weight of 105,000 was estimated.

The results above show that the ethylene glycol dehydrogenase resembles in several characteristics the methanol dehydrogenase (MDH) from methylotrophic bacteria first described by Anthony and Zatman (1964) or the alcohol dehydrogenase from Acinetobacter calcoaceticus (Duine and Frank, 1981) in that it had a high pH optimum; and an NH_4^+ ion activator requirement; it oxidised primary alcohols and a molecular weight in the region of 120,000.

Fig. 29 shows SDS-PAGE analysis of the soluble fractions of succinate- or ethylene glycol-grown isolate Z (tracks 1 and 2 respectively) and the 50-65% saturated ammonium sulphate fraction of ethylene glycol-grown organisms (track 3). Comparison of the soluble fractions from succinate- and ethylene glycol-grown organisms revealed the appearance of several bands in the latter soluble fraction in particular the synthesis of one major polypeptide at about 60,000 Daltons which coincided with the polypeptide band of the MDH marker protein. This band which was particularly distinct in the $(NH_4)_2SO_4$ fraction was significantly absent from the soluble fraction of succinate-grown isolate Z as was the ethylene glycol dehydrogenase activity.

In the absence of further purification, however, it was not possible to identify the natural coenzyme, which has been characterised in MDH

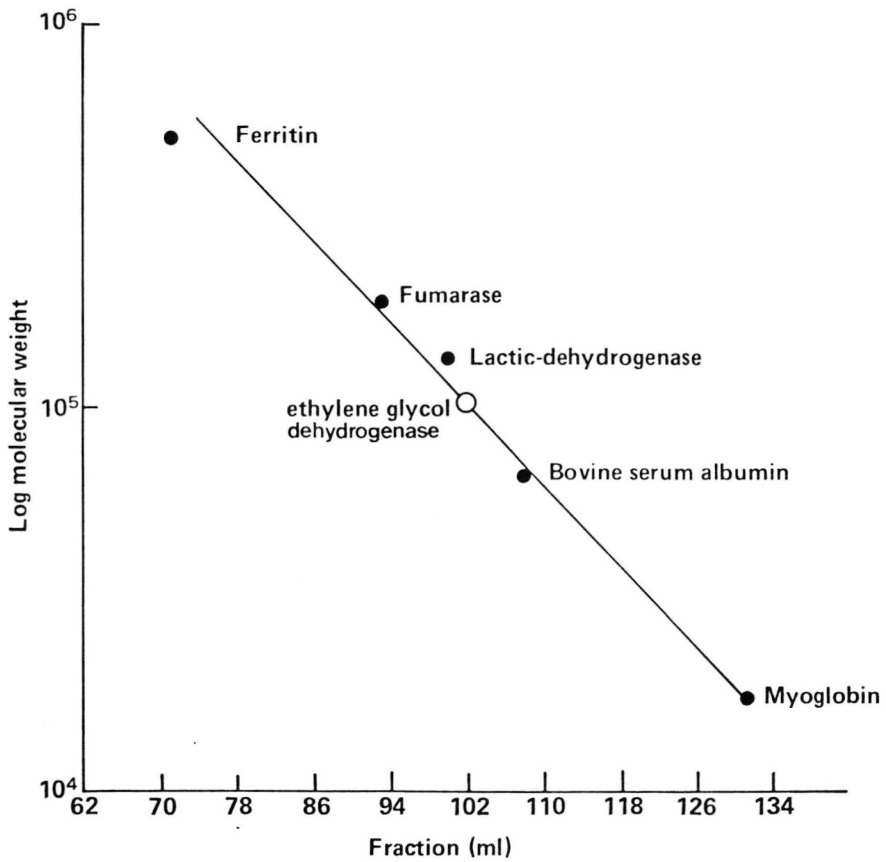


Fig. 28 Estimation of molecular weight of ethylene glycol dehydrogenase on Sephadex G–200

A partially purified enzyme preparation (1.7 ml of a redissolved 50–65% saturated $(NH_4)_2SO_4$ fraction, equivalent to 15 mg protein and containing 3 units of enzyme) was applied to the top of a Sephadex G–200 column prepared as described in Materials and Methods. The column was eluted with 50 mM *tris*–HCl buffer, pH 8.0 and 2 ml fractions collected. Ethylene glycol dehydrogenase was determined in reaction mixtures consisting of 50 μ mol glycine-NaOH buffer, pH 9.6; 0.33 μ mol phenazine methosulphate; 15 μ mol $(NH_4)_2$ SO_4 and 0.5 ml of each fraction. The column was calibrated before use with blue dextran and the following marker proteins; ferritin (750,000); fumarase (205,000); lactic dehydrogenase (135,000); bovine serum albumin (68,000) and myoglobin (17,800).

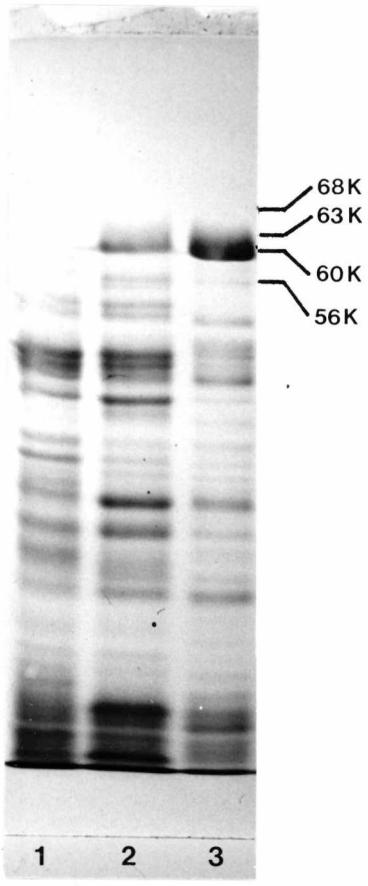


Fig. 29 SDS PAGE Analysis of soluble and 50-65% saturated ammonium sulphate fractions of extracts of isolate Z following growth on ethylene glycol and succinate.

Electrophoresis was performed in 10% (w/v) acrylamide gels. Track 1, soluble fraction (20μg protein), growth substrate succinate; Track 2, soluble fraction (20μg protein), growth substrate ethylene glycol; Track 3, 50-65% saturated ammonium sulphate fraction (10μg protein), growth substrate ethylene glycol. Marker polypeptides were: bovine serum albumin, 68000; catalase, 63 000; methanol dehydrogenase, 60 000; glutamate dehydrogenase, 56 000.

as a pyrroloquinoline quinone (PQQ). The natural cofactor did not seem to be FAD because although the activity could be inhibited some 40% by 0.1mM atabrine, this inhibition was not relieved at all by the further addition of FMN, FAD or riboflavin at 0.1 to 0.2 mM.

3.2.4 Cytochrome complement of isolate Z

The oxidation of growth substrates yields intermediates for carbon assimilation, and reducing equivalents which can be used for energy transduction. Usable energy from the oxidative pathways is obtained by re-oxidising the reduced prosthetic groups and cofactors of dehydrogenases via electron transport chains. Cytochromes are a group of compounds with the ability to undergo reversible oxidation-reduction reactions and because of their haem content, have a characteristic absorption spectrum. This property allows analysis of the oxidation-reduction state, and quantification of individual carriers to be determined spectrophotometrically from their reduced minus oxidised difference spectrum (Smith, 1954). Individual cytochromes are then seen as positive inflections, since the cytochrome absorbs more strongly in its reduced state.

One of the most important conclusions from the work on electron transport in methylotrophs is that electrons from the oxidation of methanol by methanol dehydrogenase, enter the electron transport chain at the level of an unusual CO-binding cytochrome c and after cytochrome b. The evidence for this has been summarised by Anthony (1981). More recently, Duine and co-workers have demonstrated that this cytochrome c is the direct physiological electron acceptor for the quinoprotein, methanol dehydrogenase by isolating a MDH complex from Hyphomicrobium X with a functional coupling to cytochrome c (Duine et al., 1979).

If PQQ is the prosthetic group of ethylene glycol dehydrogenase then

it might be expected that this enzyme, like MDH, donates electrons into the electron transport chain at the level of cytochrome c.

Fig. 30 shows the sodium dithionite-reduced minus potassium ferricyanide-oxidised difference spectrum of the supernatant fraction of isolate Z following growth on ethanol (a), succinate (b) and ethylene glycol (c). Organisms grown on ethylene glycol showed cytochrome c (α -band, 551nm; β -band, 521.5nm) as the predominant species in the soluble fraction. The symmetry of the α -band and the lack of a shoulder at 559nm suggests that there was little or no contribution from a b-type cytochrome. Nevertheless, the extent of the cytochrome c α -band may obscure a low concentration of cytochrome b. The small absorbance peak at 605nm indicates a low concentration of an a-type cytochrome oxidase.

In contrast, supernatant fractions prepared from isolate Z grown on ethanol or succinate exhibited a radically different cytochrome complement (Fig. 30). An alternative cytochrome c (α -band, 552.5nm; β -band, 523nm) was present at about half the concentration of that in ethylene glycol-grown organims (Table 19). Moreover, the significant increase in synthesis of cytochrome b (shoulder at 559nm) following growth on ethanol or succinate (Table 19) indicates the different electron transport requirements for growth on these substrates compared to growth on ethylene glycol. Particulate fractions from ethylene glycol-, succinate- or ethanol-grown Z, sedimented at 180000g for 2 h, contained very low concentrations of cytochromes which could not be estimated accurately from the difference spectra.

Similar gross changes in cytochrome complement have been observed for the facultative methylotroph, Pseudomonas extorquens grown on methanol or succinate (Tonge et al, 1974). Organisms grown on succinate showed about

Fig 30 $Na_2S_2O_4$-reduced <u>minus</u> $K_3Fe(CN)_6$-oxidised difference spectra of the supernatant fraction of strain Z following growth on a) ethanol; b) succinate and c) ethylene glycol.

Cytochrome difference spectra were measured at room temperature as described in Methods.

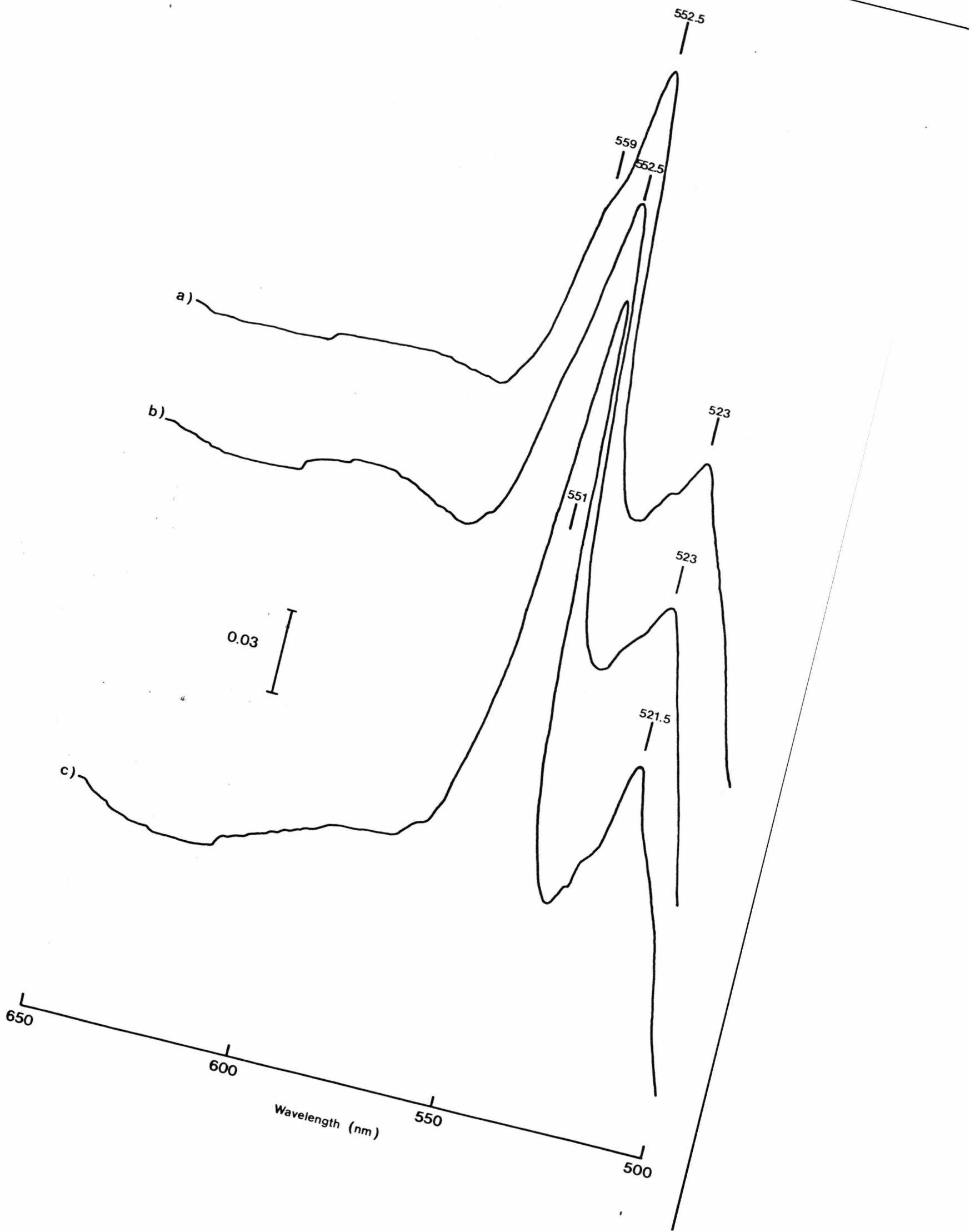

552.5
559
552.5
a)
b)
523
551
523
0.03
521.5
c)
650
600
550
500
Wavelength (nm)

Table 19. Comparison of the cytochrome content of cell extracts of isolate Z following growth on ethylene glycol, ethanol, or succinate.

Sodium dithionite-reduced minus potassium ferricyanade-oxidised difference spectra were recorded at room temperature. The concentrations of the a-, b- and c-type cytochromes were determined from the height of their α -peaks using the extinction coefficients given by Jones and Redfearn (1967).

Growth substrate	Cytochrome concentration (pmol/mg protein)		
	c	b	aa_3
Ethylene glycol	583 (551)*	N.D.	+
Ethanol	334 (552.5)	226	+
Succinate	281 (552.5)	182	+

\+ detectable, but at a concentration too low to measure accurately

N.D. not detectable

* figures in parentheses indicate the λ_{max} of the cytochrome c α-peak.

a four fold increase in cytochrome b and a reduction of 62% of the cytochrome c which exhibited a different λ_{max} (553nm) to the cytochrome c synthesised during growth on methanol (λ_{max}, 551nm).

Fig. 31 shows the reduced plus CO minus reduced difference spectra of the supernatant fraction of isolate Z following growth on ethylene glycol. The presence of a c-type cytochrome able to bind carbon monoxide is indicated by a trough at 554nm and peaks at 539 and 418.5nm. Again this CO-binding spectrum shows a remarkable similarity to the CO-binding c type cytochrome found in supernatant fractions of several methylotrophically grown organisms (Tonge et al.,1974).

Organisms grown on succinate show no such CO-binding cytochrome c in the soluble fraction (Fig. 32).

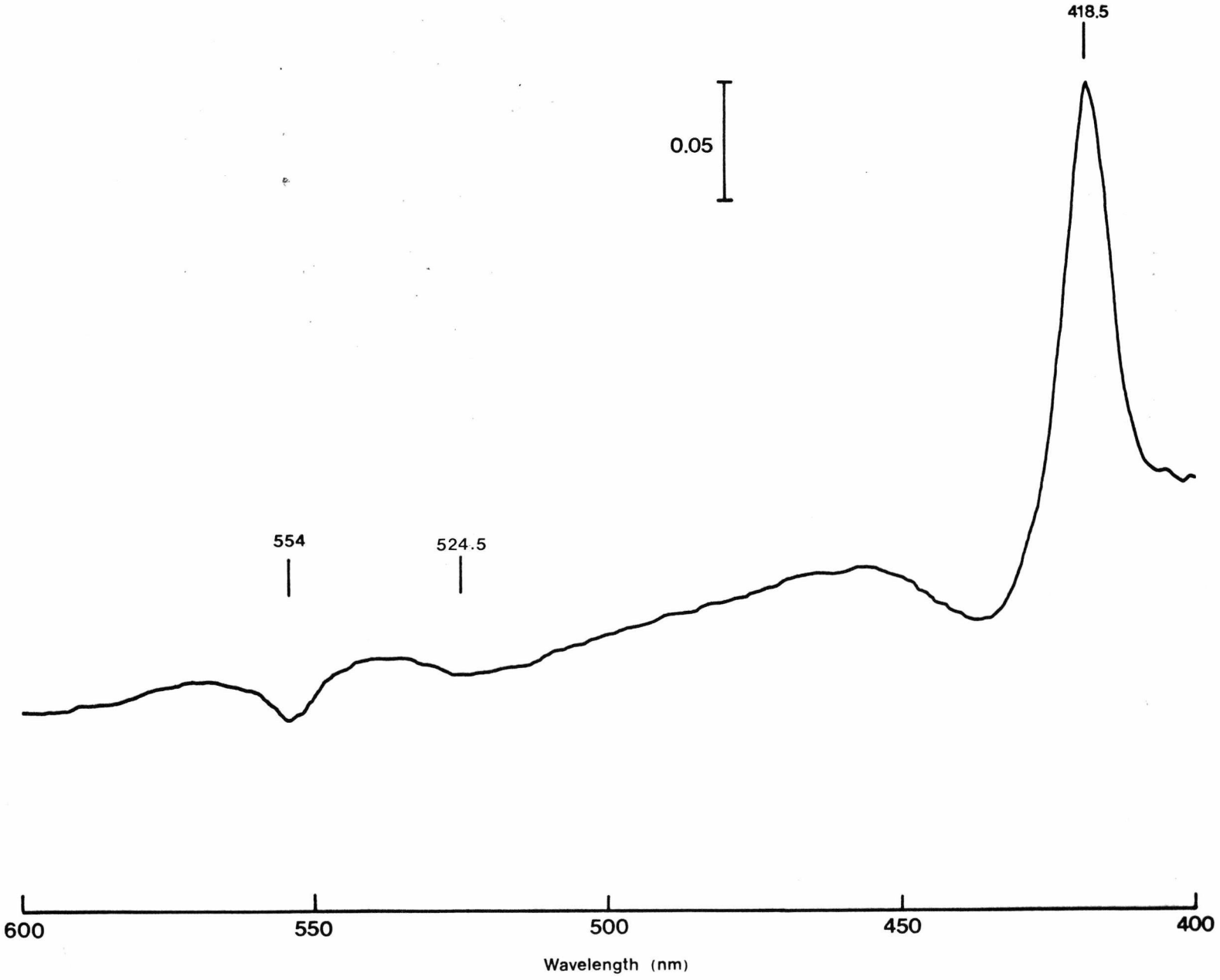
As mentioned earlier, the CO-binding cytochrome c has been shown to be intimately involved in methanol oxidation acting as the direct physiological electron acceptor for the quinoprotein, methanol dehydrogenase (Anthony, 1981; Duine et al, 1979). The presence of this cytochrome at high concentrations in ethylene glycol-, and not succinate- or ethanol-grown isolate Z suggests that this cytochrome has an important function in ethylene glycol oxidation, and provides further evidence to suggest that the ethylene glycol dehydrogenase is a quinoprotein enzyme similar to methanol dehydrogenase.

3.2.5 Conversion of glycolaldehyde to glycollate

An NAD-linked aldehyde dehydrogenase was observed in extracts of organism Z grown on ethylene glycol and ethanol. NAD^+, but not $NADP^+$, was utilised as cofactor and the optimum pH was 7.8 in tris-HCl buffer.

Fig 31 $Na_2S_2O_4$-reduced plus CO minus $Na_2S_2O_4$-reduced difference spectra of the supernatant fraction of strain Z following growth on ethylene glycol.

CO was bubbled for 30 sec through one cuvette followed by a 20 min incubation before recording the spectra.


418.5
0.05
554
524.5
600
550
500
450
400
Wavelength (nm)

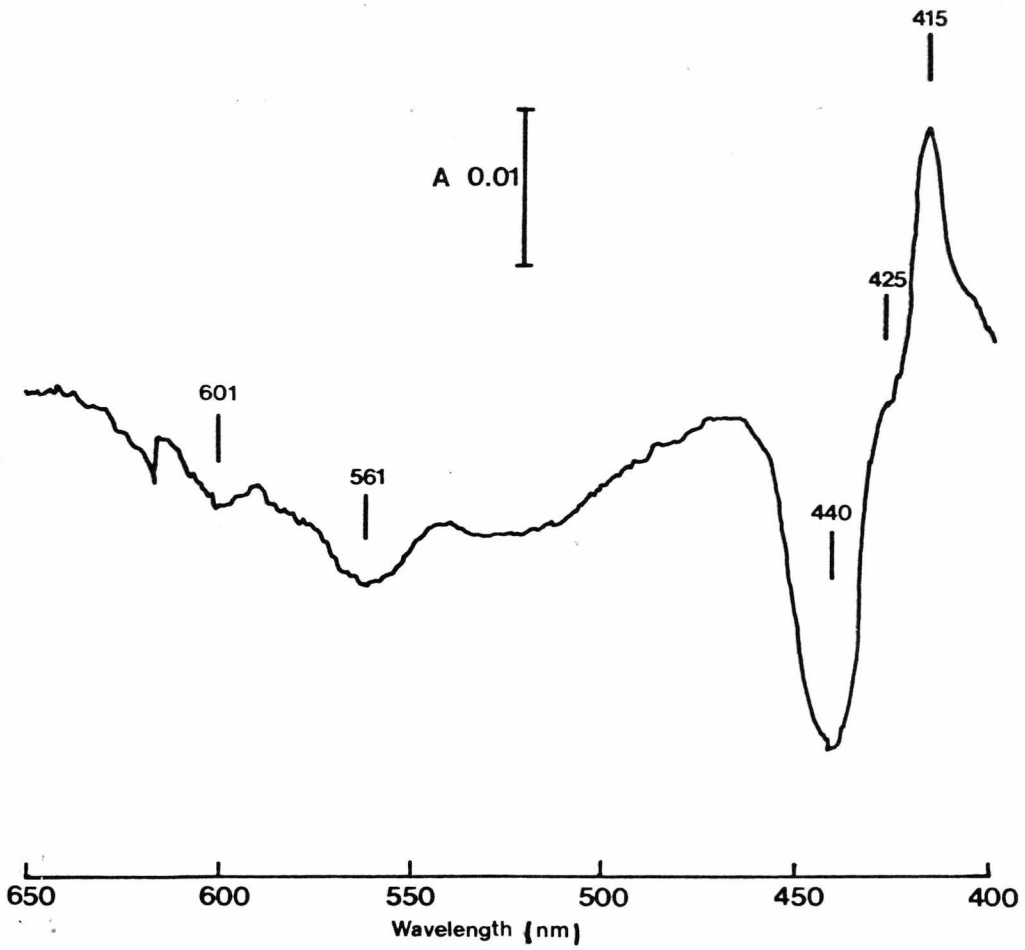


Fig. 32 $Na_2S_2O_4$-reduced plus CO minus $Na_2S_2O_4$-reduced difference spectra of the supernatant of isolate Z following growth on succinate.

The product of the reaction was identified as glycollate by paper chromatography (Table 20). Attempts to find evidence for the reversibility of this reaction were not successful; when glycollate was used as substrate, NADH oxidation was not observed spectrophotometrically at various pH values or at various concentrations of substrates.

Glycolaldehyde dehydrogenase activity was very evident in uninduced cells but specific activity in extracts of ethylene glycol-grown cells was elevated 2- to 4-fold above that in similar extracts of succinate- and glucose-grown organisms (see Table 26).

The aldehyde dehydrogenase activity was not specific for glycolaldehyde because propionaldehyde and particularly acetaldehyde were also good substrates for this enzyme.

The enzyme was unstable to freezing and thawing and complete loss of activity was frequently observed after 5-7 days storage at -14°C. Activity was rapidly lost at 4°C with 50% of the original activity remaining after storage overnight at this temperature. As a result of this instability, all assays for aldehyde dehydrogenase activity were performed on freshly prepared extracts.

Initial attempts to obtain an approximate value for the apparent molecular weight of the aldehyde dehydrogenase were carried out on an Ultrogel AcA34 column (2.5 x 42cm). Preliminary experiments with this column material suggested that the activity was eluted close to the void volume of the column suggesting a high molecular weight. This observation was confirmed when the experiments were repeated with marker proteins and a molecular weight near 295000 indicated (Fig. 33).

Fig. 24 shows inactivation at 65°C of the aldehyde dehydrogenase from

Table 20. Identification of glycollate by paper chromatography

Reaction mixtures contained in a total of 3 ml:potassium phosphate buffer (pH 7.1), 50 μmol; NAD, 5 μmol; glycolaldehyde, 10 μmol and extract (HSSF, 180000g). Flasks were stoppered and incubated for 1 h at 30°C. The reaction was stopped by the addition of 0.2 ml, TCA and protein removed by centrifugation. The supernatant was rotary evaporated to dryness and the residue extracted with ethanol. After concentration the ethanolic samples (10 μl) were spotted onto paper and chromatographed in the 3 solvent systems shown. Chromatograms were developed by spraying with 0.04% (w/v) bromocresol purple in 95% (v/v) aq. ethanol (to which 1 drop of conc. NH_3 was added).

Solvent system	R_f values of			
	Product	Glycollate	Glyoxylate	Glycerate
Butan-1-ol:acetic acid:water (12:3:5 by vol)	0.53	0.54	0.65	0.37
Ethanol:ammonia:water (16:1:3 by vol)	0.64	0.67	0.46	0.48
Ether:acetic acid:water (15:3:1 by vol)	0.52	0.53	0.11	0.18

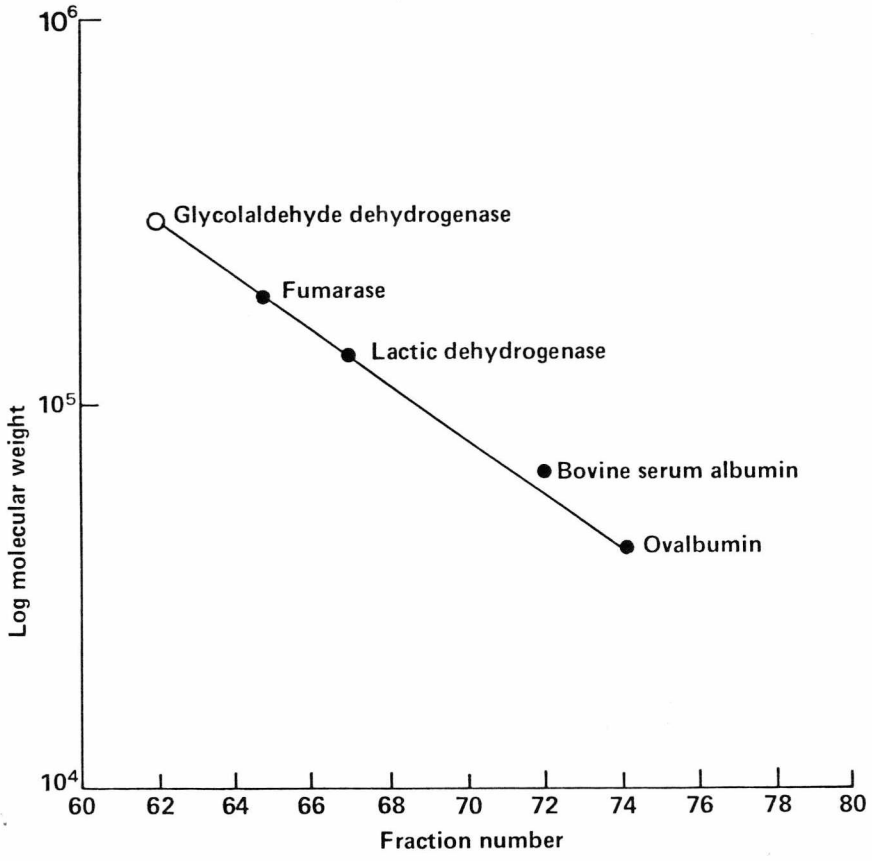


Fig. 33 Estimation of molecular weight of glycolaldehyde dehydrogenase on Ultrogel Ac A34

Crude enzyme (1.5 ml equivalent to approx. 15 units) was applied to the top of an Ultrogel Ac A34 column prepared as described in the Materials and Methods and eluted with 50 mM potassium phosphate buffer, pH 7.2. Fractions (2 ml) were collected and glycolaldehyde dehydrogenase activity determined as described in Methods. The column was calibrated prior to use with blue dextran and the following marker proteins: fumarase; lactic dehydrogenase; bovine serum albumin and ovalbumin.

crude extracts after growth of cells on ethylene glycol. The curved line suggests the presence of at least two aldehyde dehydrogenases which differ in their thermolability. Further work is required to substantiate this theory.

3.2.6 Conversion of glycollate to glyoxylate

The homogenate obtained by ultrasonic disintegration of cell suspensions of Z grown with ethylene glycol as carbon source was centrifuged (180000g for 2 h) and the particulate fraction separated from the clear supernatant (see Methods 2.8).

Rapid oxidation of glycollate was observed in the homogenate and particulate preparation (Table 21) but only a small amount of activity was detected in the supernatant fraction. Although the activity in the supernatant (but not in the particulate fraction) could be stimulated by the addition of phenazine methosulphate (3.25-fold) the activity was still only 33% that of the (unsupplemented) particulate preparation.

These experiments demonstrated a particulate location for glycollate oxidase. The presence of a small amount of activity in the supernatant fraction suggest that the enzyme may be loosely membrane bound and released into the soluble fraction by sonication.

Glyoxylate was identified as the product of glycollate oxidase activity (Table 22). However, the observed stoicheiometry of oxygen utilised: glyoxylate formed (1.6:1) suggests that the conditions of the assay method were not optimal for the measurement of this activity (Table 23).

3.2.7 Metabolism of glyoxylate

When cell extracts were incubated with glyoxylate under anaerobic con-

Table 21. Localisation of glycollate oxidase activity in cell extracts of isolate Z grown on ethylene glycol

Organism Z was grown on ethylene glycol and the cells harvested during exponential growth. Extracts were prepared by sonication and glycollate oxidase determined as described in Methods.

Enzyme preparation	Specific activity (nmol/min/mg activity)
Crude extract	159
High speed supernatant fraction	12
High speed supernatant fraction + phenazine methosulphate (PMS)	39
Particulate fraction (±PMS)	120

Table 22. Identification of glyoxylate by t.l.c. and paper chromatography

Reaction mixtures contained in a total of 2 ml:potassium phosphate buffer (pH 7.1), 180 μmol; sodium glycollate, 10 μmol and particulate preparation (prepared as described in Methods). Flasks were stoppered and incubated for 1 h at 30°C in a shaking water bath. The reaction was stopped by the addition of 0.2ml, TCA and protein removed by centrifugation. The supernatant was subsequently treated in either of 2 ways:
A) 1 ml was incubated with 1 ml of 0.2% (w/v) dinitrophenylhydrazine in 2M-HCl and the 2,4-dinitrophenylhydrazone derivatives isolated and chromatographed as described in the Methods.
B) The supernatant was rotary evaporated to dryness and the residue taken up in ethanol. After concentration the ethanolic samples (10 μl) were spotted onto Whatman No. 1 paper and chromatographed (p.c.) in the 2 solvent systems shown. Chromatograms were developed by spraying with 0.04% (w/v) bromocresol purple in 95% (v/v) aq. ethanol (to which 1 drop of conc. NH_3 was added).

Solvent system	R_f values of				
	Product	Glyoxylate	Glycollate	Glycerate	Pyruvate
A) Butan-1-ol:ethanol:ammonia (7:1:2 by vol) (for t.l.c.)	0.42	0.43	n.d.	n.d.	0.54) 0.74)
B) Butan-1-ol:acetic acid: water (12:3:5 by vol) (for p.c.)	0.67	0.65	0.54	0.37	n.d.
Diethylether:acetic acid: water (15:3:1 by vol) (for p.c.)	0.10	0.11	0.53	0.18	n.d.

n.d. not determined

Table 23. Oxidation of glycollate to glyoxylate by particulate preparations of ethylene glycol-grown strain Z

Bacteria grown on ethylene glycol to exponential phase were harvested and extracts made. Particulate preparations were prepared and glycollate oxidase assayed as described in the Methods except that the main compartment of the Warburg flasks also contained semicarbazide (50 µmol) adjusted to pH 5. At the end of incubation the contents of the Warburg flasks were acidified with 0.5 ml of 6M-HCl and after centrifuging, glyoxylate was estimated in the supernatant solution by the procedure of Friedmann and Haugen (1943).

Incubation mixture	Quantity of glycollate added (µmol)	Quantity of O_2 absorbed (µmol) corrected for endogenous	Quantity of glyoxylate formed (µmol)
Complete system	10	3.2	2
Glycollate omitted	0	0	0
Complete system, extract boiled before incubation	10	0	0

ditions rapid CO_2 evolution was observed; about 0.5 moles of CO_2 were evolved per mole of glyoxylate (Table 24). These findings are similar to those reported for E. coli (Krakow et al., 1959) and Pseudomonas B_2aba (Kornberg and Gotto, 1961) where the anaerobic condensation of two moles of glyoxylate yielded one mol of CO_2 and one mol of a C_3 compound which was assumed to be tartronate semialdehyde or its isomer, hydroxypyruvate. Attempts were made to identify the product of this reaction by converting it to its 2,4-dinitrophenylhydrazone. The absorption spectrum of the bis-2,4-dinitrophenylhydrazone in 1MNaOH showed a broad maximum at 560-570nm and the solution was deep purple. Tartronate semialdehyde is known to decompose (decarboxylate) to glycolaldehyde [whose dinitrophenylhydrazone also gives a purple colour in alkali due to oxidation to glyoxal DNP (bis)] so the sample could have contained some glycolaldehyde, a problem which was discussed by Dagley et al., (1961).

Extracts of ethylene glycol-grown Z oxidised NADH in the presence of glyoxylate (Fig. 34), the cofactors for which were thiamine pyrophosphate and magnesium ions. Dialysis of extracts against 50 mM-potassium phosphate buffer (pH 6.8) for only 2 h led to the loss of 70% of activity. The separate addition of thiamine pyrophosphate and magnesium ions partially restored this activity to 48% and 79% of the activity in the undialysed extract, respectively. Addition of both of these cofactors to the dialysed extract fully restored activity.

When the extract was preincubated with glyoxylate anaerobically, and in the absence of NADH for 10 minutes to from the putative C_3 compound, the subsequent rate of NADH oxidation was at least six-fold greater than that obtained without preincubation. Authentic hydroxypyruvate, in contrast, was very slowly reduced (Fig. 34) and did not give a purple DNP in alkali, indicating that hydroxypyruvate clearly was not the C_3 product of glyoxylate metabolism.

Table 24. Stoicheiometry of the anaerobic decarboxylation of glyoxylate

From strain Z, grown on ethylene glycol, extracts were prepared and glyoxylate carboligase assayed as described in Methods.

Incubation mixture	Quantity of glyoxylate added (µmol)	Quantity of CO_2 evolved) (µmol)	$\frac{\text{Glyoxylate utilised}}{CO_2 \text{ evolved}}$
Complete system	20	8.5	2.4*
Glycollate omitted	0	0	0
Complete system, extract boiled before incubation	20	0	0

* Results for the complete system are expressed as the average of 3 readings ($\pm$ 0.1) obtained from independent experiments.

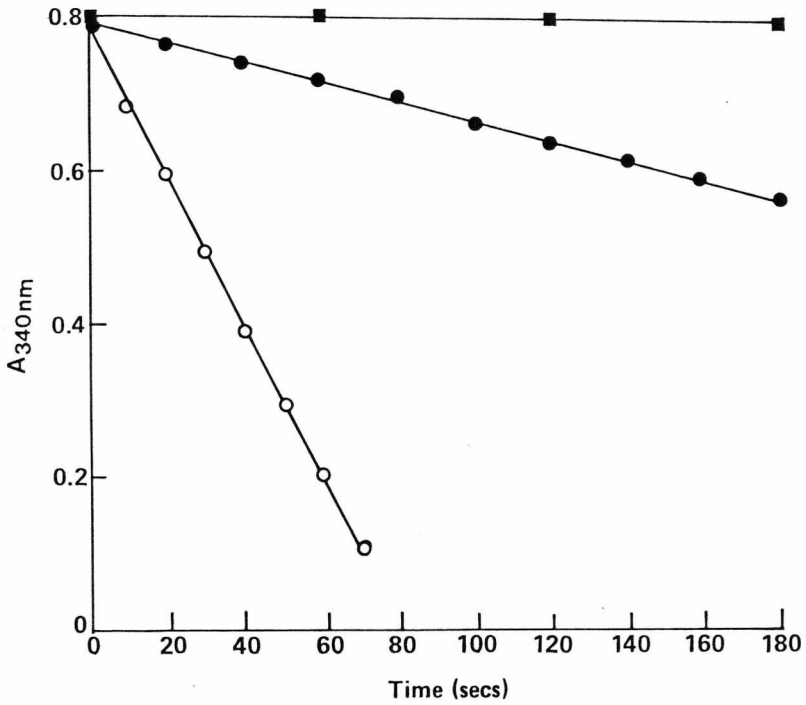


Fig. 34 Oxidation of reduced NAD^+ in the presence of glyoxylate or C_3 compounds

Oxidation of NADH by extracts of strain Z grown on ethylene glycol. The complete system contained in 3 ml: 100 μ mol potassium phosphate buffer pH 7.2; 10μmol $MgCl_2$; 0.5μmol TPP; 0.4μmol NADH; extract (~1.2 mg protein) and 6μmol of sodium glyoxylate (●) or hydroxypyruvate (■). Another cuvette contained the above system but was incubated for 10 min before the addition of NADH (o).

Glycerate was identified as the product of tartronate semialdehyde reductase activity by paper chromatography (Table 25).

These results are consistent with the interpretation that the primary product of the glyoxylate carboligase reaction was probably tartronate semialdehyde which was then reduced by NADH to glycerate.

3.2.8 Metabolism of glycerate

Extracts of ethylene glycol-grown Z catalysed the ATP-dependent conversion of glycerate into phosphoenolpyruvate (PEP), via phosphoglycerate.

The conversion of glycerate to phosphoenolpyruvate can proceed either by direct phosphorylation of glycerate at the 2-position and its subseqeunt dehydration to yield phosphoenolpyruvate, or by phosphorylation of glycerate at the 3-position, requiring isomerisation of the 3-phosphoglycerate product to 2-phosphoglycerate prior to PEP formation. The rate at which glycerate was converted to PEP, measured spectrophotometrically by converting the PEP via pyruvate, to lactate in the presence of excess pyruvate kinase, lactate dehydrogenase and NADH, was 160 nmol/min/mg protein in extracts of ethylene glycol-grown organisms but undetectable in succinate-grown cells. This reaction was dependent on ATP but under identical experimental conditions the same extract of ethylene glycol-grown organisms catalysed the conversion of synthetic 2-phosphoglycerate to PEP in the absence of ATP with a specific activity of 94 nmol/min/mg protein yet could convert 3-phosphoglycerate to PEP with a specific activity of only < 9 nmol/min/mg protein (Table 26). As this latter rate was quite inconsistent with that of the overall conversion of glycerate to phosphoenolpyruvate these results suggested that the phosphorylation of glycerate in Z proceeded via 2-phosphoglycerate and did not

Table 25. Identification of glycerate by paper chromatography

Reaction mixtures contained in a total of 3 ml: potassium phosphate buffer (pH 7.1), 80 µmol; $MgCl_2$, 10 µmol; thiamine pyrophosphate, 0.5 µmol; sodium glyoxylate, 5 µmol; NADH, 5 µmol and extract (crude, 10000g). Flasks were stoppered and incubated for 1 h at 30°C. The reaction was stopped by the addition of 0.2 ml, TCA and protein removed by centrifugation. The supernatant was rotary evaporated to dryness and the residue extracted with ethanol. After concentration the ethanolic samples (10 µl) were spotted onto paper and chromatographed in the 2 solvent systems shown. Chromatograms were developed by spraying with 0.04% (w/v) bromocresol purple in 95% (v/v) aq. ethanol (to which 1 drop of conc. NH_3 was added).

Solvent system	R_f values of				
	Product	Glycollate	Glyoxylate	Glycerate	Pyruvate
Butan-1-ol:acetic acid:water (12:3:5 by vol.; organic layer used)	0.36	0.56	0.66	0.39	0.41
Ethanol:ammonia:water (16:1:3: by vol)	0.48	0.73	0.46	0.48	0.55) 0.63)

Table 26. Enzymic reactions in extracts of isolate Z grown on glycol, succinate and glucose

Organisms of Z were grown on the substrates indicated and the cells harvested during exponential growth. Extracts were prepared by sonication and activities of the enzymes shown determined as described in Methods.

Enzyme	Growth substrate....	Specific activity (nmol/min/mg protein) Ethylene glycol	Succinate	Glucose
Ethylene glycol dehydrogenase (PMS-linked)		73	N.D.	N.D.
NAD-alcohol dehydrogenase		16	13	5
Glycolaldehyde reductase		220	240	240
Glycolaldehyde dehydrogenase		2200	500	540
Glycollate oxidase*		125	N.D.	N.D.
Glyoxylate carboligase		700	N.D.	N.D.
Tartronate semialdehyde reductase		210	N.D.	N.D.
Glycerate kinase		160	N.D.	N.D.
Phosphoglycerate-mutase		N.D.	N.D.	N.D.
Enolase		94	230	130
pyruvate kinase		128	100	93

N.D. not detectable
* crude extract (10000g x 20 min)

involve 3-phosphoglycerate (Fig. 35). This was confirmed by the subsequent addition of crystalline phosphoglyceromutase (5 units) to the reaction mixture containing 3-phosphoglycerate when the rate of PEP formation accelerated to that of experiments using 2-phosphoglycerate directly (Fig. 35).

Identification of the product of glycerate phosphorylation, 2-phosphoglycerate, by paper chromatography, as described by Hansen and Hayashi (1962) was unsuccessful. Authentic standards of 2-phosphoglycerate and 3-phosphoglycerate when applied as a mixture could not be separated by paper chromatography in any of 6 different solvent systems.

The results of the determinations of all the enzyme activities of glycol catabolism in cell free extracts are summarised in Table 26.

3.2.9 Enzyme activities in extracts of strain Z

The activities of key enzymes which are important in the functioning of major oxidation pathways were measured. Citrate synthase and isocitrate dehydrogenase, which are enzymes in the tricarboxylic acid (TCA) cycle were assayed as were enzymes of the glyoxylate bypass; isocitrate lyase and malate synthase.

The specific activities of these enzymes, given in Table 27 indicated that they were present and operating at rates commensuate with the oxidation of ethylene glycol by way of the TCA cycle.

In extracts of ethylene glycol-grown Z, isocitrate dehydrogenase was found at approximately 12 times the activity of isocitrate lyase. It seemed likely therefore that any isocitrate present would be oxidised

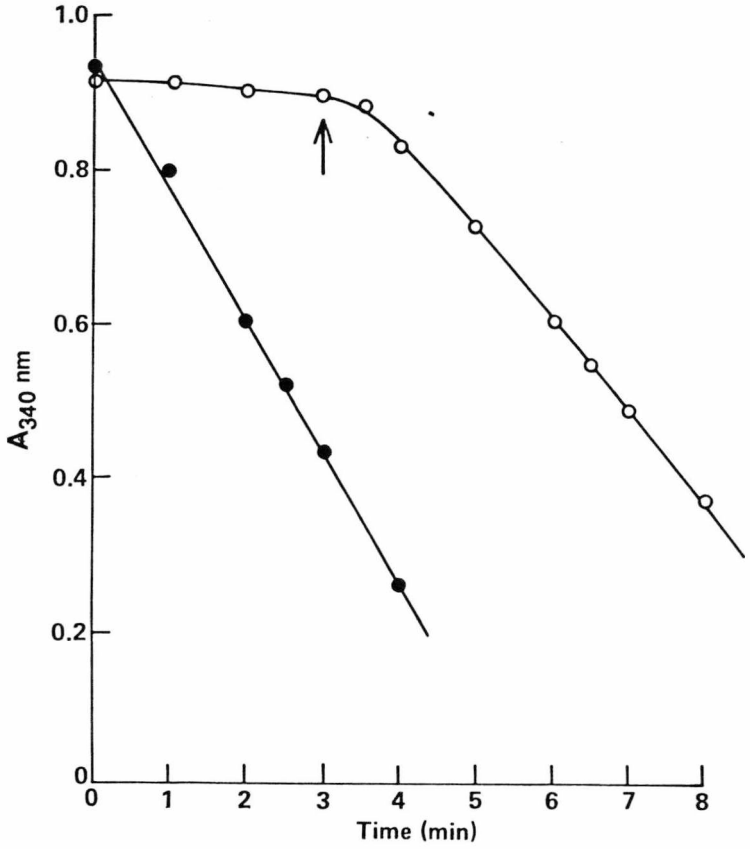


Fig. 35 Enzymatic identification of reaction product of glycerate kinase

Complete system contained in a total volume of 1 ml: 50 μmol *tris*–HCl buffer, pH 7.5; 10 μ mol $MgCl_2$; 0.14 μ mol NADH; 0.5 μ mol ADP; 5 units lactic dehydrogenase; 2 units pyruvate kinase and cell extract (equivalent to 0.35 mg protein). The reaction was started by addition of 5.6 μ mol 2–phosphoglycerate (●) or 3–phosphoglycerate plus 0.1 μmol 2,3–diphosphoglycerate (o). At the arrow (3 min) 5 units of phosphoglycerate mutase was added.

Table 27. Comparison of enzymes characteristic of specific oxidative pathways

Enzyme	Growth substrate....	Enzyme activity (nmol substrate converted/min/mg protein) Ethylene glycol	Ethanol	Succinate
Isocitrate lyase		33	n.d.	11
Malate synthase		56	n.d.	60
Citrate synthase		107	77	371
Isocitrate dehydrogenase (NADP)		393	718	352
Malate dehydrogenase		2500	3000	2620

n.d. not determined

by isocitrate dehydrogenase and continued through the TCA cycle, rather than be diverted to glyoxylate formation by isocitrate lyase. The enzymes of the glyoxylate bypass possibly fulfulling an anaplerotic function.

A further possibility exists for the terminal metabolism of the glyoxylate derived from ethylene glycol, namely oxidation by the dicarboxylic acid (DCA) cycle initiated by malate synthase (Kornberg and Sadler, 1960). In Pseudomonas B_2aba (Kornberg and Gotto, 1961) the operation of the DCA cycle during growth on glycollate was accompanied by increased activities of malate synthase compared with citrate synthase. During growth on succinate, however the physiologically-functioning TCA cycle operated and the activity of citrate synthase was greater than that of malate synthase which fulfilled an anaplerotic role in this case. Little variation in activities of these 2 enzymes was found with Z after growth on ethylene glycol or succinate and although the presence of high levels of citrate synthase in ethylene glycol-grown cells would not exclude a DCA pathway, it appears unlikely that it is the major pathway of terminal oxidation.

3.3 REGULATION

3.3.1 Studies on the regulation of ethylene glycol metabolism in Strain Z

Assay of the ethylene glycol dehydrogenase content of extracts obtained from Z grown on various carbon sources showed that the enzyme was absent after growth on succinate or glucose but was inducibly synthesised on transfer of the organism to a medium in which growth depended on utilisation of ethylene glycol. The inducible character of this enzyme was further suggested by the diauxic growth that was observed when ethylene glycol was supplied together with either succinate or glucose in culture media (Fig. 36).

When mixtures of ethylene glycol and glucose were utilised by Z, ethylene glycol utilisation was delayed until all glucose had been exhausted.

Although succinate disappearance was not followed, it seems likely that initial growth was at the expense of this compound, since only after a transient cessation of growth was ethylene glycol utilisation observed.

3.3.2 Patterns of induction of enzymes of ethylene glycol metabolism

Inductive patterns with respect to enzymes of ethylene glycol metabolism were determined on extracts of strain Z after exponential growth at the expense of all the putative intermediates. Basal uninduced levels of these enzymes were determined on extracts of cells grown with glucose (Table 28).

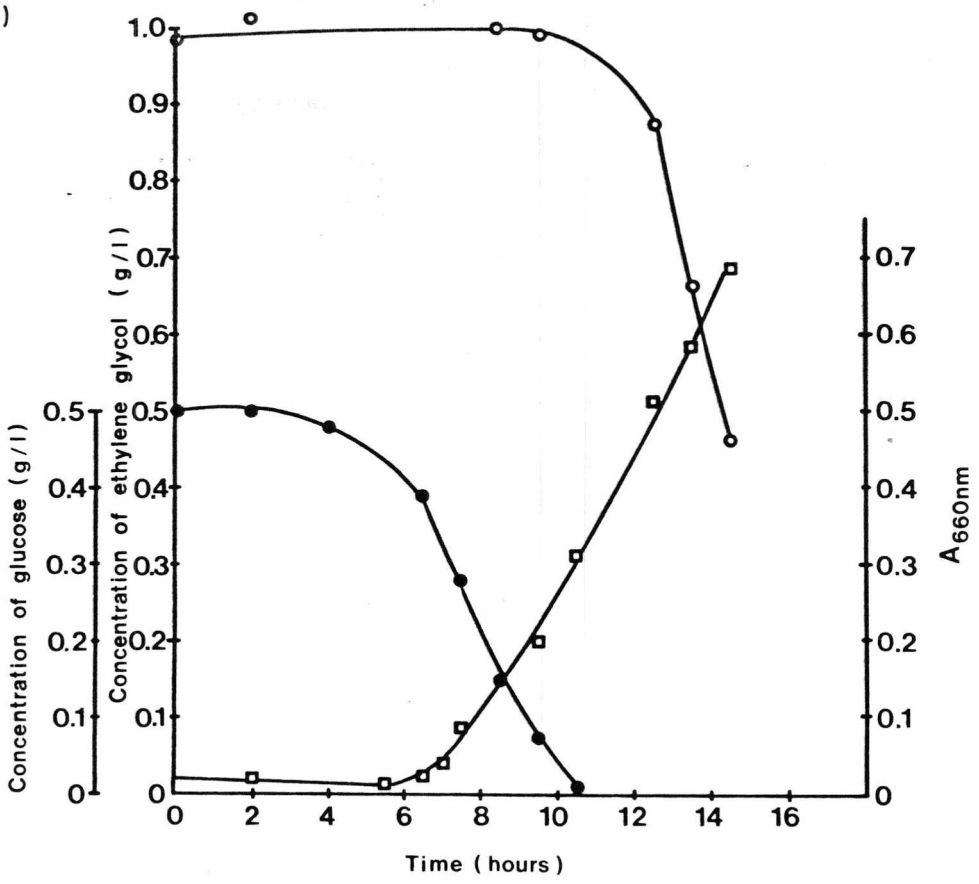
Fig 36 The growth of strain Z on a) ethylene glycol plus glucose and b) ethylene glycol plus succinate.

1 litre flasks containing 750 ml of R11 mineral salts medium (pH 7.0) were sterilised by autoclaving at 121°C for 15 min. The substrates ethylene glycol, glucose and succinate were sterilised by filtration (Millipore, 0.22μm) and added aseptically to give final concentrations of ethylene glycol 1g/l; glucose and succinate, 0.5 g/l.

Cultures of Z were taken through 4 serial subcultures at 30°C on nutrient agar slopes and growth from the last culture suspended in sterile 67mM sodium potassium phosphate buffer (4 ml) diluted to 10 ml and 7.5 ml transfered to flasks containing the following substrates: ethylene glycol, 1 g/l plus a) glucose, 0.5 g/l and b) succinate, 0.5 g/l.

Flasks were subsequently incubated on a rotary shaker at 30 C and samples (2 ml) taken at frequent intervals for determination of (□) A_{660}; (○) ethylene glycol and (●) glucose concentrations as described in Methods.

a)


Concentration of glucose (g/l)
Concentration of ethylene glycol (g/l)
A660nm
Time (hours)

b)

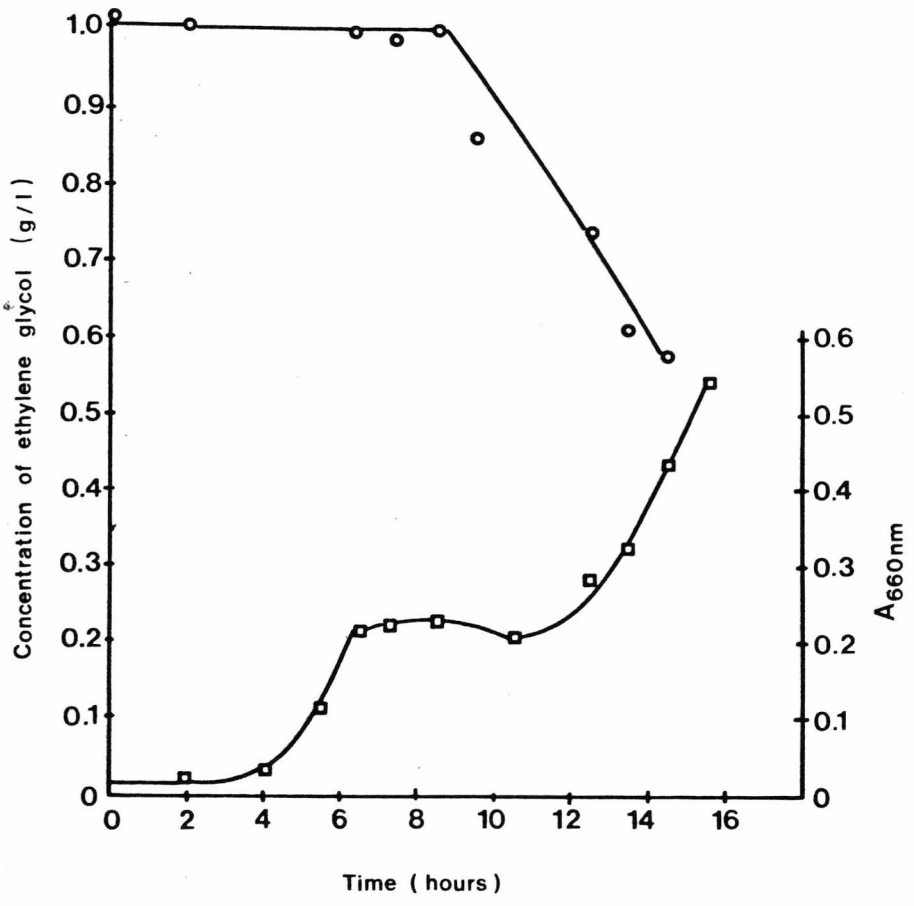

Concentration of ethylene glycol (g/l)
A660nm
Time (hours)

Table 28. Influence of various growth substrates on the activities of enzymes of the glycerate cycle in extracts of isolate Z

Organisms were grown to exponential phase at the expense of the substrate indicated, harvested by centrifugation and extracts prepared by sonication. All substrates were used at a concentration of 20 mM with the exception of glucose which was used at 10 mM.

	Specific activity (nmol/min/mg protein)				
Growth substrate	Ethylene glycol dehydrogenase	Glycolaldehyde dehydrogenase	Glycollate oxidase*	Glyoxylate carboligase	Glycerate kinase
Ethylene glycol	73	2200	125	700	160
Glycollate	N.D.	510	130	710	160
Glyoxylate	N.D.	540	9	690	140
Glycerate	N.D.	400	2	N.D.	140
Glucose	N.D.	540	N.D.	N.D.	N.D.

* crude extract (10000g x 20 min)
N.D. not detectable

After the organism had been grown on glucose, extracts contained negligible ethylene glycol dehydrogenase, glycollate oxidase, glyoxylate carboligase and glycerate kinase, while glycolaldehyde dehydrogenase was present at only 25% of the levels observed in cells fully induced by growth on ethylene glycol.

The enzyme activities observed in extracts of cells grown on different C_2 compounds demonstrated the inducible nature of these enzymes. Growth on glycollate elicited an increased synthesis of all the glycerate cycle enzymes from glycollate oxidase to glycerate kinase whilst growth on glyoxylate induced only glyoxylate carboligase, tartronate semialdehyde reductase and glycerate kinase. After growth with glycerate, enzymes of glycollate and glyoxylate metabolism were absent. These results suggest that each of these individual substrates are the respective inducers of the enzyme that specifically converts them to their subsequent product.

Glycolaldehyde could not be effectively tested since the aldehyde (1 - 10mM) did not support growth of Z and even inhibited growth on glucose; Mr. C. Maslen has found that it will (at 4mM) support growth of strain R, extracts of which then contain all the enzymes except ethylene glycol dehydrogenase.

The absence of induction of ethylene glycol dehydrogenase by any of the other glycerate pathway intermediates confirms that this enzyme is not inductively controlled by any of its degradation products and equally there is no evidence for product induction of the other enzymes in this catabolic sequence.

3.3.3 Examination of di and triethylene glycol as inducers of ethylene glycol metabolism in strain Z

Di- and triethylene glycol did not support growth of strain Z when supplied as a sole carbon source but it was possible that they could be gratuitous inducers of the pathway or partly metabolised in the presence of a readily-utilisable carbon source e.g. glucose. When 0.5 g/l of glucose was added as a supplement to such media the glucose was completely utilised after 8 h but there was no additional increase in growth in flasks containing the glycol supplement, which indicated that neither di- nor triethylene glycol was metabolised. Furthermore enzymes of ethylene glycol degradation and the glycerate cycle were not gratuitously induced by the presence of these substrates.

Such results were surprising in view of the fact that following growth of strain Z on ethylene glycol, whole organisms partially oxidised the dimer and the trimer and equally that an ethylene glycol dehydrogenase was synthesised which showed activity towards di and triethylene glycols (Tables 17 and 18).

These findings could be explained by (a) failure of the organism to cleave the ether bond of the oligomers to produce utilisable metabolites, (b) the inability of di- or triethylene glycol to act as an inducer of the enzymes of ethylene glycol degradation, or (c) oxidation of the oligomers by previously induced cells to products which cannot be further metabolised to utilisable intermediates.

3.4. DEGRADATION OF OLIGOMERIC GLYCOLS

3.4.1 Introduction

The inability of strain Z to utilise di- and triethylene glycol as growth substrates probably reflects the absence of a functional ether-cleaving enzyme(s) in this organism. Since strain R can utilise mono-, di- and triethylene glycol as growth substrates (Fig. 15) it was hoped that enzyme studies with this organism would help substantiate this theory and provide further information to indicate a possible route for the degradation of oligomeric glycols.

3.4.2 Oxidation of substrates by whole cells of strain R

Washed suspensions of strain R grown on mono-, di- and triethylene glycol rapidly oxidised these growth substrates. The rate of O_2 uptake by diethylene glycol-grown cells with mono-, di- and triethylene glycol was sufficient to account for the oxidation of 1.5, 1.1 and 0.56 µmol of the respective substrates/mg dry wt./h.

Theoretically, the complete oxidation of 1 µmol of mono-, di- and triethylene glycol to CO_2 and H_2O requires 2.5, 5 and 7.5 µmol of oxygen, respectively. The total quantities of O_2 consumed by diethylene glycol-grown cells, corrected for the endogenous respiration were 1.43 mol/mol of ethylene glycol; 3.57 mol/mol of diethylene glycol and 5.71 mol/mol of triethylene glycol representing respectively 57.1, 71.4 and 76.2% of the amount required for the complete oxidation of these glycols.

Organisms grown on mono-, di- and triethylene glycol also rapidly oxidised glycolaldehyde, glycollate, glyoxylate and glycerate (Table 29); malate and succinate were oxidised after a short lag of 15 - 20 minutes; glycine and citrate were not utilised.

In contrast to strain Z (Table 12), washed suspensions of succinate-grown organisms rapidly oxidised glycolaldehyde without a lag. Mono-, di- and triethylene glycol were slowly oxidised after a lag of 20 minutes.

3.4.3 Enzyme studies

Extracts of strain R grown on the oligomeric glycols contained an inducible glycol dehydrogenase which showed a high pH optimum (pH 9.0 in borate-buffer) and an *in vitro* requirement for phenazine methosulphate. The effect of various cations on enzyme activity was tested over the range 5 to 50 mM. Of the monovalent ions tested (K^+, Na^+, Rb^+, NH_4^+) only NH_4^+ showed activation; addition of NH_4Cl (30 mM) to crude extracts led to a four-fold increase in enzyme activity. Multivalent cations (Mg^{2+}, Ca^{2+}, Fe^{2+}, Zn^{2+}) completely inhibited glycol dehydrogenase activity.

When the homogenate obtained by ultrasonic disintegration of cell suspensions of R grown with oligomeric glycols was centrifuged (180000g for 2 h) 80% of the activity was retained in the supernatant (Table 30). On fractionation of the supernatant with ammonium sulphate essentially all glycol dehydrogenase activity was found in the protein fraction precipitated by $(NH_4)_2SO_4$ between 50 and 65% saturation.

The Michaelis Constants of the PMS-linked glycol dehydrogenase from strain R for the substrates were:- ethylene glycol, 0.59 mM; diethylene glycol, 1.82 mM and triethylene glycol, 4 mM. The glycol dehydrogenase was also

Table 29. Oxidation of substrates by washed suspensions of strain R grown on mono-, di-, triethylene glycol and succinate

Main compartment of Warburg manometer flasks contained in 3 ml: potassium phosphate buffer, pH 7.0, 100 µmol and washed cells (equivalent to 5 mg dry wt.). The centre wells contained KOH, 400 µmol. Substrates as indicated (10 µmol) were added from side-arms after thermal equilibration at 30°C. Results, expressed as µl O_2 consumed /h/mg dry wt., are corrected for the endogenous respiration (which ranged between 17 and 25 µl O_2 consumed/h/mg dry wt., depending on the growth substrate).

Substrate for oxidation	Growth substrate.... Ethylene glycol	Diethylene glycol	Triethylene glycol	Succinate
Ethylene glycol	130	86	59	26*
Diethylene glycol	128	121	58	19*
Triethylene glycol	130	118	86	19*
Glycolaldehyde	202	202	248	94
Glycollate	151	151	184	102*
Glyoxylate	40	71	85	27
Glycerate	242	110	175	19
Pyruvate	151	161	184	109
L-Malate	95*	117*	145*	133
Succinate	145*	127*	156*	134
Acetate	151	71	78	109
Fumarate	132*	95*	98*	149
2-Ketoglutarate	148*	121*	148*	129

* After approximately 15-20 minute lag period

Table 30. Specific activities of glycol dehydrogenase in extracts of strain R grown on various carbon substrates

Organism R was grown on the substrates indicated and the cells harvested during exponential growth. Extracts were prepared as described in Methods and the glycol dehydrogenase assayed at 30°C by using a Clarke oxygen electrode: Reaction mixtures contained in a total of 2 ml: borate-buffer (pH 9.0), 100 µmol; PMS, 0.1 µmol; NH_4Cl, 60 µmol; glycol, 2 µmol; and extract.

Enzyme preparation	Growth substrate....	Specific activity (nmol/min/mg protein)		
		Ethylene glycol	Diethylene glycol	Succinate or Glucose
High speed supernatant fraction (HSSF)		69	38	N.D.
Particulate		18	9	N.D.
50-65% saturated $(NH_4)_2SO_4$ fraction		159	239	N.D.

N.D. not detectable

active when other substrates were included in the reaction mixture (Table 31).

Polarographic assays were used to estimate the stoicheiometry for the oxidation of mono-, di- and triethylene glycol by partially-purified extracts of strain R. The observed oxygen uptakes per mole of substrate (Table 32) were greater than that required to account for the conversion of mono-, di- and triethylene glycol to the corresponding mono-aldehydes but lower than that required for their conversion to the corresponding mono-carboxylic acids. The identification of the oxidation product of ethylene glycol as glycolaldehyde was supported by t.l.c. analysis (Table 33) and since glycolaldehyde is a substrate for glycol dehydrogenase activity some conversion of the aldehyde to the acid seems likely.

The immediate product of the glycol dehydrogenase with di-and triethylene glycol as substrates has not yet been identified but 2,4-dinitrophenyl-hydrazone derivatives, forming a purple colour in alcoholic KOH were formed from reaction mixtures after precipitation of protein with trichloroacetic acid. These products were not further characterised in the absence of reference compounds. Whether cleavage of the ether bond had occurred with the dimer and trimer at this time is at present unknown.

As a result of the close similarity in properties of the glycol dehydrogenase activity in isolate Z and R, samples of the supernatant, and 50-65% saturated ammonium sulphate fractions of isolate R following growth on ethylene- or diethylene glycol, were examined by SDS-polyacrylamide gel electrophoresis (Fig. 37). Both soluble fractions from ethylene- or diethylene glycol-grown R (tracks 1 and 3, respectively) showed several bands which were either absent or diminished in the soluble fraction of succinate-grown organisms (track 5).

Table 31. Relative oxidation rates of various substrates by partially purified glycol dehydrogenase from strain R in the PMS-assay

The polarographic assay was used as described in Methods; the absolute rate of O_2 consumption for ethylene glycol (= 100%) was 200 nmol/O_2/min/mg protein.

Substrate	Oxidation rate (% of that with ethylene glycol
Ethylene glycol	100
Diethylene glycol	51
Triethylene glycol	112
Methanol	0
Ethanol	138
Propan-1-ol	128
Propan-2-ol	106
Butan-1-ol	134
Butan-2-ol	102
1,1 Dimethylethanol	0
2-Methoxyethanol	54
2-(2-Methoxyethoxy)ethanol	111
2-Butoxyethanol	144
2-Phenoxyethanol	119
1,2-Dimethoxyethane	0

Table 32. Oxidation of mono-, di- and triethylene glycols by extracts of strain R

Stoicheiometry for the oxidation of mono-, di- and triethylene glycols by extracts of strain R, was determined using the polarographic assay. The cuvette contained: borate buffer (pH 9.0), 100 μmol; PMS, 0.1 μmol; NH_4Cl, 60 μmol; substrate, 125 nmol; extract (50-65% $(NH_4)_2SO_4$ fraction) equivalent to 0.5 mg protein and water to 2 ml.

Substrate	Oxygen consumed (nmol)	moles O_2/mole substrate
Ethylene glycol	75	0.60
Diethylene glycol	94	0.75
Triethylene glycol	80	0.64

Table 33. Identification of glycolaldehyde by thin-layer chromatography

Reaction mixtures contained in a total of 2 ml: borate-buffer (pH 9.0), 100 µmol; PMS, 0.1 µmol; NH_4Cl, 60 µmol; ethylene glycol, 5 µmol and extract (50 - 65% $(NH_4)_2SO_4$ fraction equivalent to 2 mg protein).
Flasks were stoppered and incubated in the dark for 5 minutes at 30°C. The reaction was stopped by the addition of 0.2 ml 50% TCA and protein removed by centrifugation. 1 ml of the supernatant was treated with 1 ml 0.2% (w/v) 2,4-dintrophenylhydrazine in 2M-HCl and after standing for 30 minutes was extracted with ethyl acetate. The ester layer containing excess 2,4-dinitrophenylhydrazine and possible neutral 2,4-dinitrophenylhydrazone derivatives was evaporated to a small volume and chromatographed by t.l.c. on silica gel. Neutral derivatives separated from 2,4-dinitrophenylhydrazine which ran with the solvent front.

Solvent system	R_f values of			
	Product	Acetaldehyde	Glycolaldehyde	Propionaldehyde
Hexane:acetone (1:1 by vol)	0.34	0.79	0.35	0.92
Methanol:butan-1-ol (3:1 by vol)	0.75	0.87	0.75	n.d.

n.d. not determined

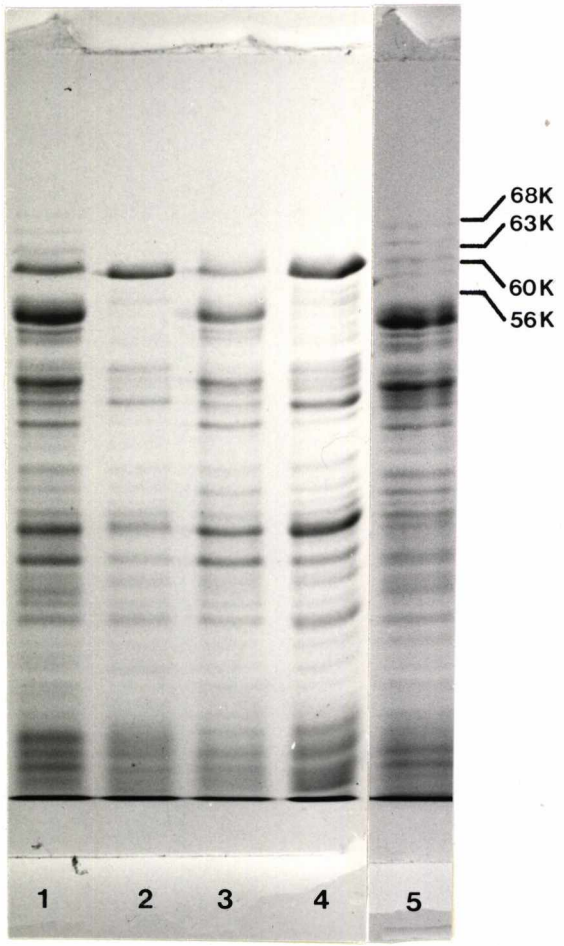


Fig. 37 SDS PAGE Analysis of soluble and 50-65% saturated ammonium sulphate fractions of extracts of isolate R following growth on mono-, diethylene glycol and succinate.

Electrophoresis was performed in 10% (w/v) acrylamide gels. Track 1, soluble fraction (20 µg protein), growth substrate ethylene glycol; Track 2, 50-65 % saturated ammonium sulphate fraction (20 µg protein) growth substrate ethylene glycol; Track 3, soluble fraction (20 µg protein), growth substrate diethylene glycol; Track 4, 50-65% saturated ammonium sulphate fraction (10 µg protein), growth substrate diethylene glycol; Track 5, soluble fraction (20 µg protein), growth substrate succinate. Marker proteins were: bovine serum albumin, 68 000; catalase, 63 000; methanol dehydrogenase, 60 000; glutamate dehydrogenase, 56 000.

The major band in the 50 - 65% saturated ammonium sulphate fraction of ethylene glycol-grown isolate R (track 2) was a 60,000 Dalton (approx.) polypeptide which may be similar to the 60,000 Dalton band from isolate Z grown on ethylene glycol.

The specific activities of enzymes involved in ethylene glycol metabolism by isolate Z were then determined in extracts prepared from cells of strain R grown on mono-, di-, triethylene glycol and succinate (Table 34). Significantly increased activities of glycollate oxidase, glyoxylate carboligase, tartronate semialdehyde reductase and glycerate kinase were noted after growth on glycols, whereas no significant activity of these enzymes could be demonstrated in succinate-grown cells. The specific activity of glycolaldehyde dehydrogenase in extracts of glycol-grown cells was again elevated 2- to 4-fold above that in similar extracts of succinate-grown cells.

It seems probable, therefore, from the enzyme complement that glycol-aldehyde formed by the glycol dehydrogenase is oxidised to glyoxylate, which is subsequently converted to glycerate and then to pyruvate by the glycerate pathway. The very low phosphoglycerate mutase activity in glycol-grown cells (Table 34) also implicates a direct phosphorylation of glycerate to 2-phosphoglycerate in this organism as was shown with Z.

Table 34. Enzymic reactions in extracts of isolate R grown on glycol, succinate and glucose

Organism R was grown on the substrates indicated and the cells harvested during exponential growth. Extracts were prepared by sonication and activities of the enzymes shown determined in high speed supernatant fractions as described in Methods.

Enzyme	Growth substrate....	Specific activity (nmol/min/mg protein)				
		Ethylene glycol	Diethylene glycol	Triethylene glycol	Succinate	Glucose
Glycol dehydrogenase (PMS-linked)		69	38	70	N.D.	N.D.
NAD-alcohol dehydrogenase		14	7	7	15	12
Glycolaldehyde reductase		180	100	96	200	170
Glycolaldehyde dehydrogenase		2200	1400	1500	500	470
Glycollate oxidase*		136	73	79	N.D.	N.D.
Glyoxylate carboligase		650	650	760	N.D.	N.D.
Tartronate semialdehyde-reductase		310	200	430	N.D.	N.D.
Glycerate kinase		220	220	130	N.D.	N.D.
Phosphoglycerate-mutase		9	12	9	N.D.	N.D.
Enolase		114	95	91	590	610
Pyruvate kinase		116	67	61	115	129

N.D. not detectable
* measured in crude extracts (10000g x 20 min)

3.5 REGULATION

3.5.1 Studies on the regulation of glycol metabolism in isolate R

The influence of several growth substrates upon the induced level of glycol dehydrogenase is summarised in Tables 34 & 35. After growth on mono-, di- and triethylene glycol the glycol dehydrogenase activity was several fold higher than that observed in cultures grown with glucose or succinate, indicating its inducible nature.

Since the induction of many microbial enzymes has been shown to be susceptible to catabolite repression by the presence of glucose and other readily utilisable carbon substrates, the sequence of substrate utilisation in cultures of ethylene glycol with either glucose or succinate as an additional carbon substrate was examined in order to identify any diauxic effects in which catabolite repression might be operating. Diauxic growth was evident when mixtures of glucose and ethylene glycol were utilised by strain R (Fig. 38) with ethylene glycol utilisation delayed until all the glucose had been exhausted. Similarly, in mixtures of glycol and succinate, growth at the expense of ethylene glycol was observed only after succinate utilisation (Fig. 38).

The results of this investigation suggested that catabolite repression by glucose (and succinate) was operating on ethylene glycol pathway enzymes. Although the enzymes of ethylene glycol metabolism were apparently subject to varying degrees of catabolite repression by glucose (Table 36), the residual ethylene glycol/glucose concentration and the time of harvesting may have a more significant effect on the enzyme activities than the initial substrate ratios.

Table 35. Influence of various growth substrates on the activities of enzymes of the glycerate cycle in extracts of isolate R

Organisms were grown to exponential phase at the expense of the substrate indicated, harvested by centrifugation and extracts prepared by sonication. All substrates were used at a concentration of 20 mM with the exception of glucose which was used at 10 mM.

	Specific activity (nmol/min/mg protein)					
Growth substrate	Glycol dehydrogenase	Glycolaldehyde dehydrogenase	Glycollate oxidase*	Glyoxylate carboligase	Tartronate semi-aldehyde reductase	Glycerate kinase
Ethylene glycol	70	2200	136	650	310	220
[Glycolaldehyde (4mM)	N.D.	140	40	120	100	40]
Glycollate	N.D.	600	210	800	n.d.	290
Glyoxylate	N.D.	500	18	590	320	140
Glycerate	N.D.	400	N.D.	N.D.	N.D.	190
Glucose	N.D.	530	N.D.	N.D.	N.D.	N.D.

* measured in crude extracts (10000g x 20 min)
N.D. not detectable
n.d. not determined

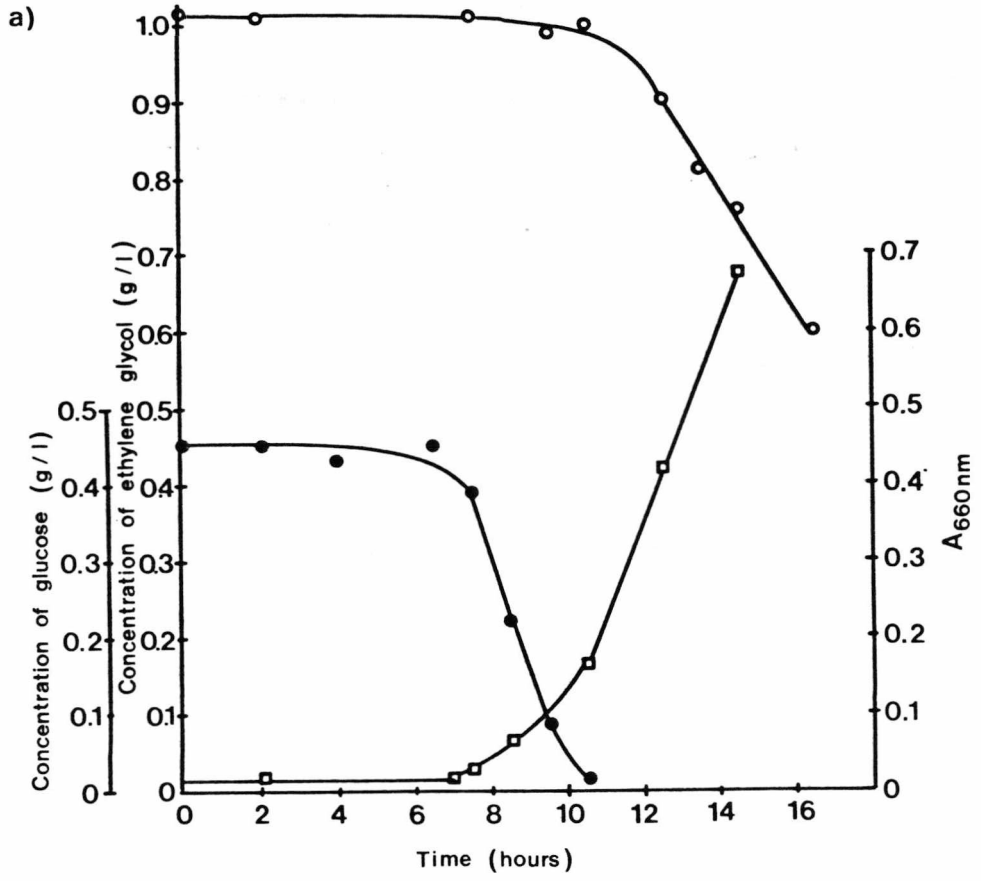
Results in brackets [ ] were obtained by Mr. C. Maslen and are included for comparison. Growth on 4mM glycolaldehyde was poor (t_d ~ 4.5 h, maximum A_{660} 0.22) compared with growth on the other substrates (t_d values between 1.63 and 3.5 h) but concentrations above 4mM were toxic. The glycolaldehyde sample contained approx. 0.2% (w/v) of ethylene glycol by g.l.c. analysis; this amount of glycol does not itself support visible turbidity in growth experiments, so does not account for growth at the expense of glycolaldehyde.

Fig 38 The growth of strain R on ethylene glycol plus a) glucose and b) succinate.

1 litre flasks containing 750 ml of R11 mineral salts medium (pH 7.0) were sterilised by autoclaving at 121°C for 15 min. The substrates ethylene glycol, glucose and succinate were sterilised by filtration (Millipore, 0.22 μm) and added aseptically to give final concentrations of ethylene glycol, 1 g/l; glucose and succinate, 0.5 g/l.

Cultures of R were taken through 4 serial subcultures at 30°C on nutrient agar slopes and growth from the last culture suspended in sterile 67 mM sodium potassium phosphate buffer (4 ml) diluted to 10 ml and 7.5 ml transferred to flasks containing the following substrates: ethylene glycol, 1 g/l plus a) glucose, 0.5 g/l and b) succinate, 0.5 g/l.

Flasks were subsequently incubated on a rotary shaker at 30 C and samples (2 ml) taken at frequent intervals for determination of (□) A_{660}; (○) ethylene glycol and (●) glucose concentrations as described in Methods.


a)
Concentration of glucose (g/l)
Concentration of ethylene glycol (g/l)
A_{660nm}
Time (hours)

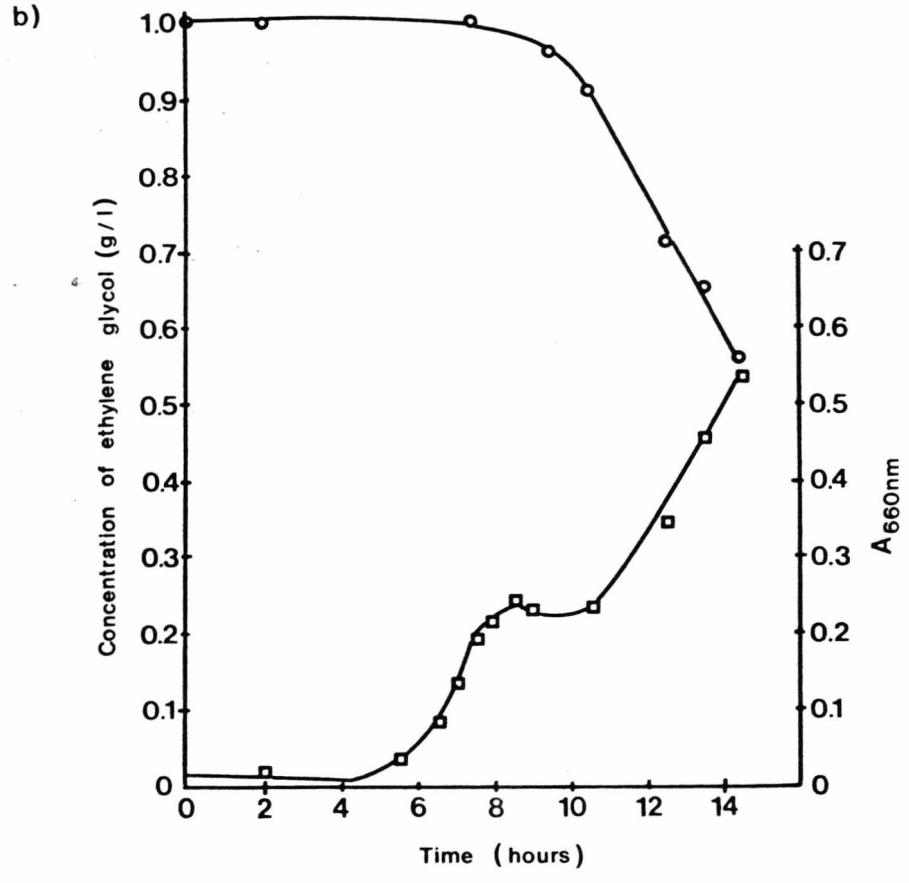

b)
Concentration of ethylene glycol (g/l)
A_{660nm}
Time (hours)

Table 36. Influence of glucose on the activities of enzymes of ethylene glycol metabolism in extracts of strain R

The activities of glycol dehydrogenase, glycolaldehyde dehydrogenase, glycollate oxidase, glyoxylate carboligase and glycerate kinase were determined in high speed supernatant fractions of strain R prepared from cells grown on media containing the carbon sources indicated. Cells were harvested when the A_{660} reached 0.3

Growth substrates (mM)		Specific activity (nmol/min/mg protein)				
		Glycol dehydrogenase	Glycolaldehyde dehydrogenase	Glycollate oxidase*	Glyoxylate carboligase	Glycerate kinase
Ethylene glycol:glucose						
20	0	83	1500	50	460	220
9	1	70	1500	70	600	120
5	5	15	1300	60	70	30
2	8	N.D.	900	50	90	N.D.
0	10	N.D.	500	N.D.	N.D.	N.D.

* measured in crude extracts (10000g x 20 min)
N.D. not detectable

The influence of carbon source on the levels of enzymes directly associated with glycol metabolism is shown by the data presented in Table 35. The glycol dehydrogenase, glycollate oxidase, glyoxylate carboligase and tartronate semialdehyde reductase were not present at significant levels in extracts of R cells after growth with glycerate. Glyoxylate-grown cells contained undetectable levels of the glycol dehydrogenase and glycollate oxidase but high levels of glyoxylate carboligase and glycerate kinase. Ethylene glycol, glycolaldehyde and glycollate were the sole carbon sources that elicited the synthesis of glycollate oxidase.

To obtain further information concerning the role of glycerate cycle enzymes during growth on ethylene glycol, the appearance of enzyme activity during adaptation of the bacteria from growth on succinate to growth on ethylene glycol was followed. When succinate-grown organisms were introduced into a medium containing ethylene glycol, exponential growth commenced 8 h after inoculation (Fig. 39). Utilisation of ethylene glycol required the induced synthesis of several enzymes, including glycolaldehyde dehydrogenase, glycollate oxidase, glyoxylate carboligase and glycerate kinase. Activities (with the exception of glycollate oxidase) were already significantly above basal level before the onset of exponential growth. Glycolaldehyde dehydrogenase activity began to decline rapidly after 12 h whereas the other enzymes remained at approximately the same activity for a further 3 h.

3.5.2 Examination of analogues as inducers of ethylene glycol metabolism

As it seemed likely from the results of the experiments in 3.5.1 that ethylene glycol is the specific inducer of only ethylene glycol dehydrogenase and not the remaining enzymes of the pathway, the likely inductive properties of several analogues of ethylene glycol were examined. A

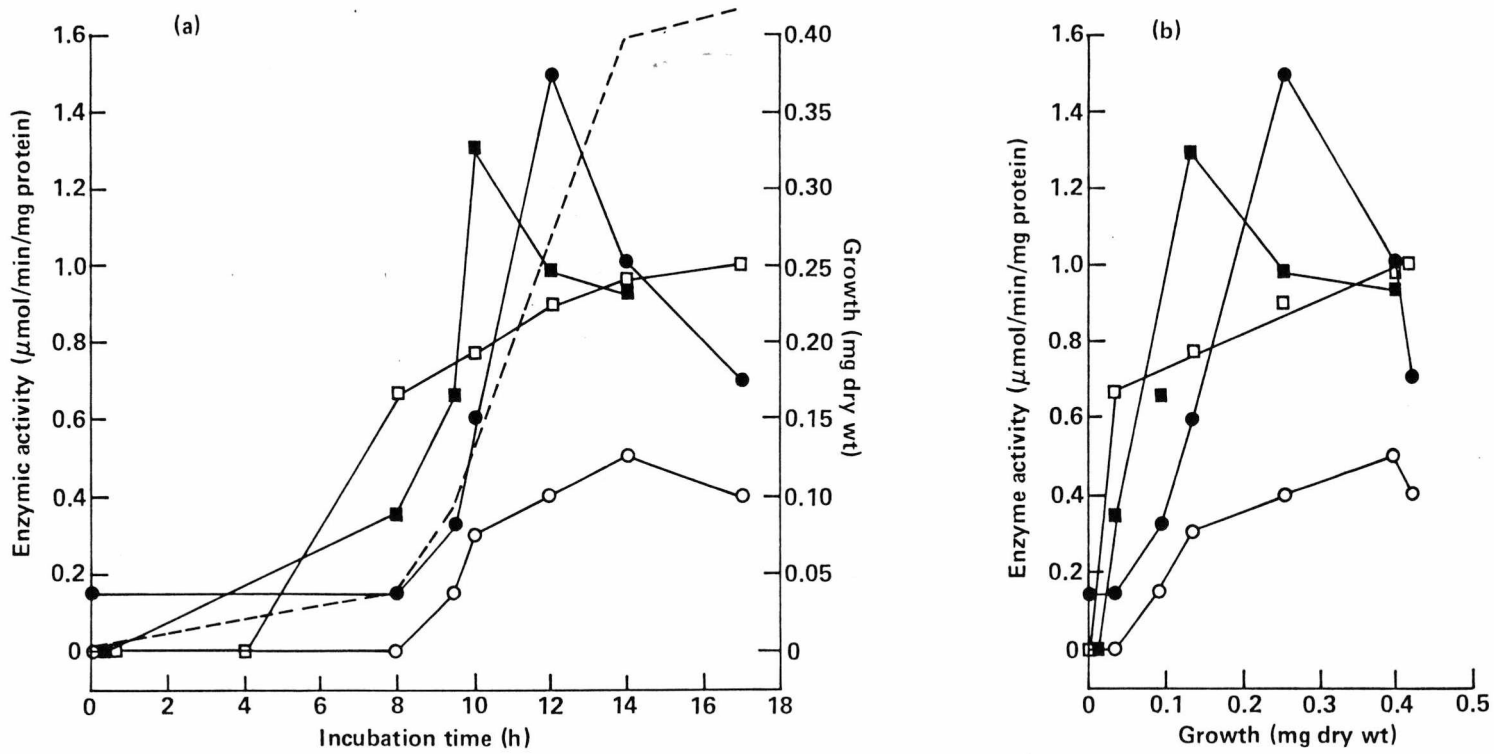


Fig 39 Changes in activities of enzymes of the glycerate cycle during growth of isolate R on ethylene glycol

Strain R was grown in a medium containing succinate (2g/l) as the sole source of carbon. Harvested organisms were transferred to fresh medium containing ethylene glycol (1g/l) and growth followed during aerobic incubation at 30°C. Samples were withdrawn at intervals for enzymic content. Graphs represent (a) activity against incubation time and (b) activity against growth (dry weight).

● , glycolaldehyde dehydrogenase; ○ glycollate oxidase; ■ glyoxylate carboligase and □ , glycerate kinase. Dotted line represents growth. For convenient graphic representation, specific activities of the enzymes glycollate oxidase and glycerate kinase were multiplied by 10.

prerequisite for the use of such analogues as gratuitous inducers was that they should not support or inhibit growth on glucose nor should they be metabolised by the organism. Compliance with these criteria would ensure that any inductive or repressive effects were due solely to the analogue and not to its metabolites. Of the analogues available, 2-butoxyethanol supported growth of strain R and 2-methoxyethanol was utilised in the presence of glucose, though it did not support growth on its own. Other analogues, 2-(2-methoxyethoxy)ethanol and 2-phenoxyethanol were substrates for ethylene glycol dehydrogenase and thus could not be considered as genuine gratuitous inducers, although neither supported growth nor were utilised in the presence of glucose (Fig 40). 1,2-Dimethoxyethane failed to induce any of the enzymes of glycol metabolism above the activities found in extracts of uninduced glucose-grown controls.

3.5.3 Mutant Studies with Isolate R

In the absence of genetically-blocked mutants at the time these observations were made and the failure to find a suitable gratuitous inducer of the glycol dehydrogenase, the identification of the specific inducers of each enzyme in the catabolic pathway depended upon data such as that presented in Table 35 which suggested that the pathway probably consisted of a series of individual sequentially-induced steps, each intermediate acting as inducer for the enzyme which degraded it to the next product. The absence of any co-ordinate induction was confirmed by comparing the relative activities of all the enzymes under a wide range of inducing and catabolite-repressed conditions. Fig. 41 shows no evidence of such a relationship.

Mr. C. Maslen has subsequently obtained two mutants by mutagenic treatment of isolate R with N-methyl, N-nitro-nitrosoguanidine (MNNG). Mutant M-40

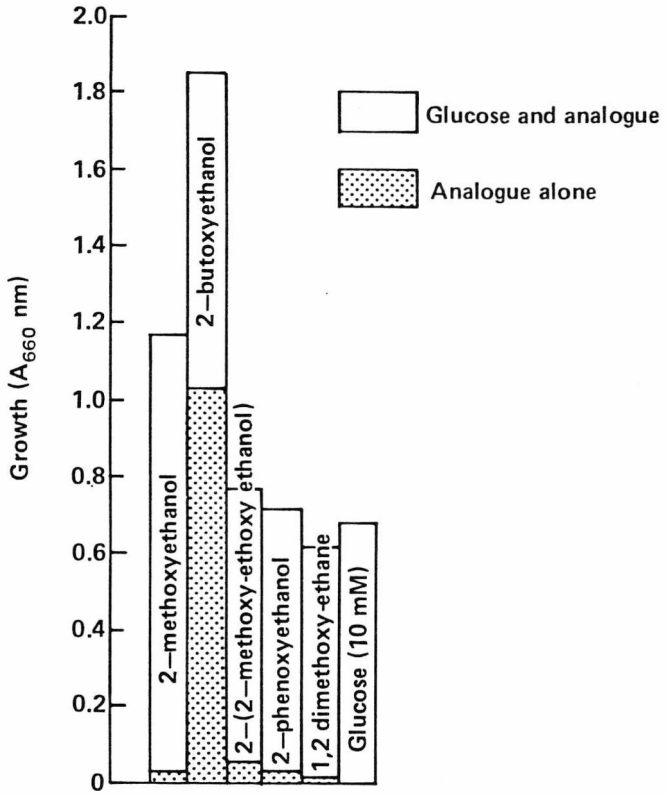


Fig. 40 Growth of isolate R on glucose in the presence of ethylene glycol analogues

Isolate R was grown at 25°C in R11 mineral salts medium supplemented with 10 mM glucose and the appropriate ethylene glycol analogue at 10 mM. Growth was measured spectrophotometrically at 660 nm.

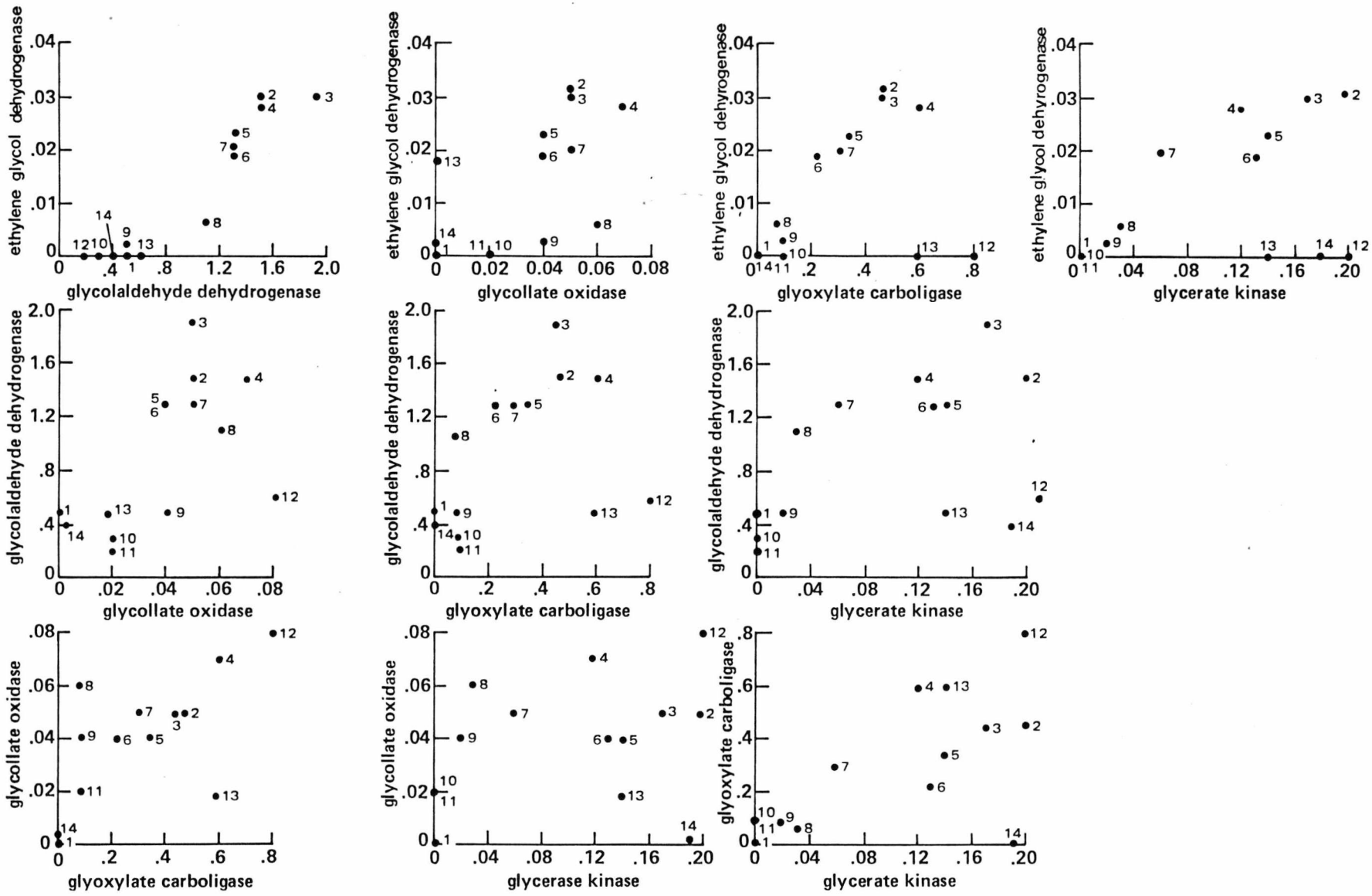


Fig.41 Relative activities of enzymes of ethylene glycol metabolism in extracts of isolate R.

The relative activities of glycol dehydrogenase , glycolaldehyde dehydrogenase, glycollate oxidase, glyoxylate carboligase and glycerate kinase, were determined in extracts of strain R prepared from cells grown on media containing the following carbon sources; (1) 10 mM glucose; (2) 20 mM ethylene glycol; (3) 19 mM ethylene glycol and 1 mM glucose; (4) 9 mM ethylene glycol and 1 mM glucose; (5) 8.6 mM ethylene glycol and 1.4 mM glucose; (6) 7.5 mM ethylene glycol and 2.5 mM glucose; (7) 7.6 mM ethylene glycol and 3.3 mM glucose; (8) 5 mM ethylene glycol and 5 mM glucose; (9) 3.3 mM ethylene glycol and 6.7 mM glucose; (10) 2 mM ethylene glycol and 8 mM glucose; (11) 1 mM ethylene glycol and 9 mM glucose; (12) 30 mM glycollate; (13) 20 mM glyoxylate; and (14) 20 mM glycerate. (units: U/mg protein)

has the phenotype: Ethylene glycol$^-$; glycolaldehyde$^+$, glycollate$^+$, glyoxylate$^+$, glycerate$^+$ and the second, M-1, has the phenotype: Ethylene glycol$^-$; glycollate$^-$; glyoxylate$^-$, glycerate$^+$.

The former, M-40, appeared to be a glycol transport mutant (see section 3.6.2 for experimental evidence) which failed to grow on mono-, di- or triethylene glycols and even when grown on glucose in the presence of any of these three substrates failed to induce any of the subsequent enzymes of the pathway above basal levels whereas in the wild-type strain there is a several-fold induction. Revertants from this mutant (1.4 per 10^6 cells) recovered wild-type phenotype and their sequential inducibility for each step in the pathway simultaneously.

The second mutant, M-1, did take up glycols though more slowly than in the wild-type and its genetic block appeared to be either in the glyoxylate carboligase or tartronate semialdehyde reductase catalysed reactions. Growth of this mutant on glucose plus ethylene glycol, however, gave no increase in the activities of the glycol dehydrogenase or of glycollate oxidase, whereas growth on glucose and glycollate gave rise to an increase (9-fold) in glycollate oxidase. Neither culture showed any elevation of glycerate kinase activity (i.e. beyond the genetic block) confirming that glycol and glycollate were not themselves inducers of the glycerate kinase, but in wild type cells these compounds simply gave rise to the glycerate inducer of this enzyme. Full details of the enzymic complements of these and other mutants will be presented in Mr. Maslens' thesis.

3.6 UPTAKE AND TRANSPORT OF GLYCOLS

3.6.1 Facilitated uptake or diffusible uptake of glycol

The uptake of radioactive ethylene glycol was first examined to determine whether there was an inducible mechanism for its uptake in the organisms isolated in this study.

After growth with glucose, cells of isolate Z accumulated [^{14}C]ethylene glycol for 5 - 7 minutes to approximately 30 nmol/mg dry wt., at which level it remained, for about 30 minutes (Fig. 42). Assuming that (i) all the accumulated activity was actually ethylene glycol and (ii) the intracellular water content was equal to 4 µl/mg dry wt. (Kepes and Cohen, 1962) the internal concentration of ethylene glycol was thus equal to approximately 7mM. Since the external concentration was 5mM this initial uptake to little more than the external concentration was probably due to diffusion.

In contrast ethylene glycol-grown cells rapidly took up labelled substrate at 30°C at an initial rate of 21 nmol/min/mg dry wt., although cells incubated at 0°C showed negligible uptake during the same time interval (Fig. 42). At 30 minutes 660 nmol of ethylene glycol per mg dry wt. were taken up by cells of strain Z (Fig. 42) indicating that induced cells had accumulated approximately 33 times the extracellular ethylene glycol concentration of 5mM at this time. In these experiments, however, both uptake and incorporation of labelled substrate was almost certainly being observed because efflux studies effected by adding 100 µmol of unlabelled ethylene glycol showed no more than 17-20% of this amount of radioactivity.

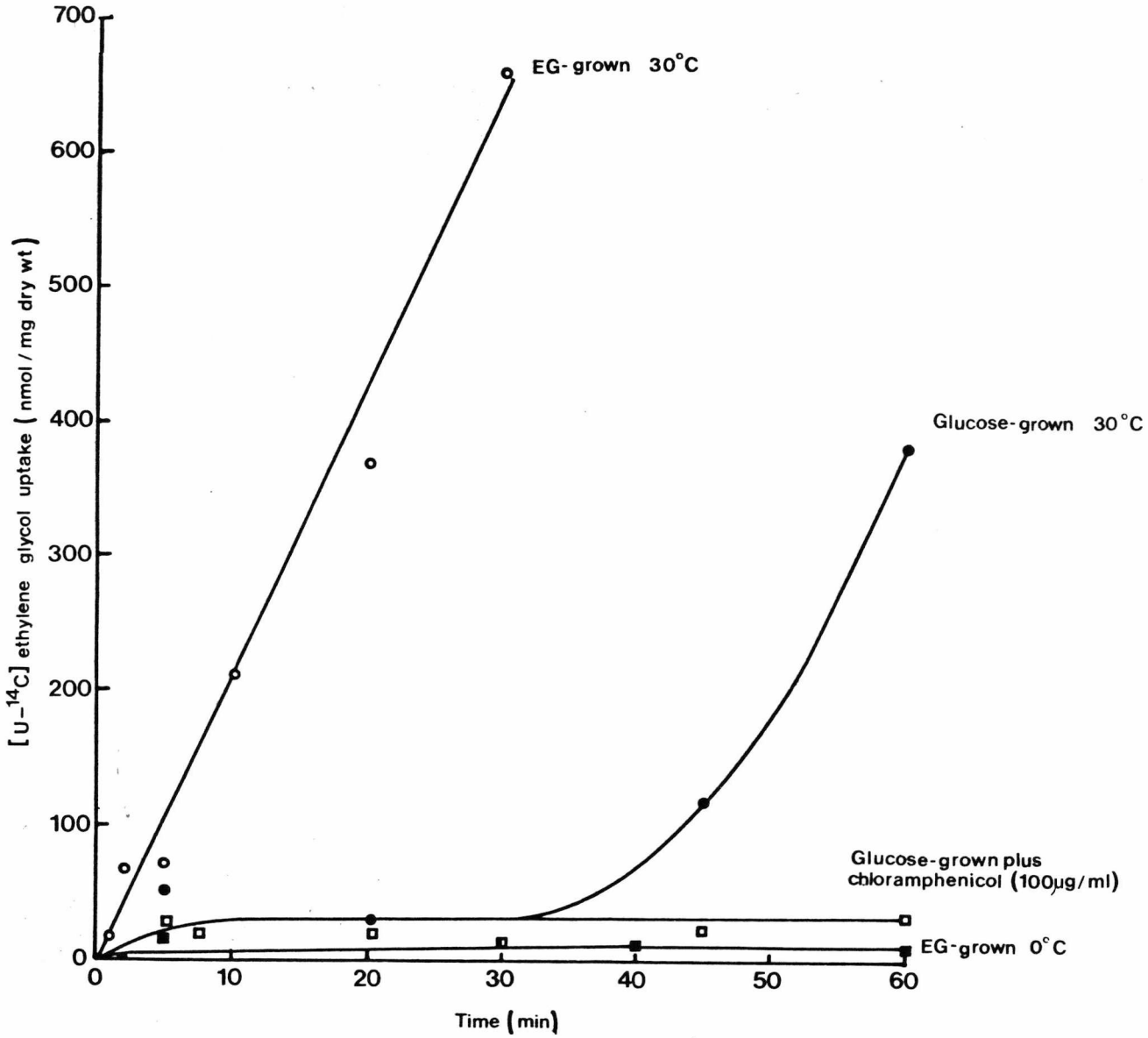


Fig. 42 Uptake of ^{14}C ethylene glycol by suspensions of isolate Z

Organisms grown on ethylene glycol (2g/l) or glucose (2g/l) were harvested by centrifugation, washed and resuspended in 10ml of minimal medium (0.44 mg dry wt/ml) in 100ml conical flasks supplemented with 5mM [^{14}C]ethylene glycol (containing 202246 dpm). Uptake of radioactivity was followed at intervals for 60 min at 30°C as described in Methods

The transport system showed classical saturation kinetics in that the rate of transport ceased to increase appreciably when the concentration of external ethylene glycol was raised above 2mM. An apparent K_s of 0.56mM was calculated by the method of Lineweaver and Burke (Fig 43). This value is rather high for uptake of a natural substrate and suggested that the glycols may be transported by an alkanol uptake system.

When glucose-grown cells were incubated in labelled ethylene glycol media, transport of ethylene glycol began after a period of 35 - 40 minutes (Fig. 42). The rate of uptake increased rapidly and by 55 - 60 minutes eventually paralleled the fully induced rate. In the presence of chloramphenicol (100 μg/ml final concentration), however, this subsequent increase in uptake was completely prevented. These results indicated that some protein synthesis was required before uptake of ethylene glycol reached its maximum rate in uninduced glucose-grown cells. While it was not possible to distinguish unequivocally between uptake due to an inducible transport system and subsequent incorporation of label due to the inducible enzymes of ethylene glycol metabolism, manometric studies with whole cells of succinate-grown isolate Z, showed no significant oxygen uptake with ethylene glycol throughout the 60 minute course of the experiment (Table 12), indicating that enzymes of ethylene glycol metabolism had not been induced within this period. Fig. 42 may therefore represent a genuine inducible transport phenomenon.

Uptake of ethylene glycol was investigated further in strains R and O grown on ethylene glycol and PEG 1500 respectively. Strain O, which was unable to utilise ethylene glycol as a growth substrate, did not accumulate ethylene glycol above the external concentration; the small amount of uptake shown (15 nmol/mg dry wt.) was most likely due to diffusion. In separate experiments glucose (1mM) and PEG 1500 (0.5mM) were added to

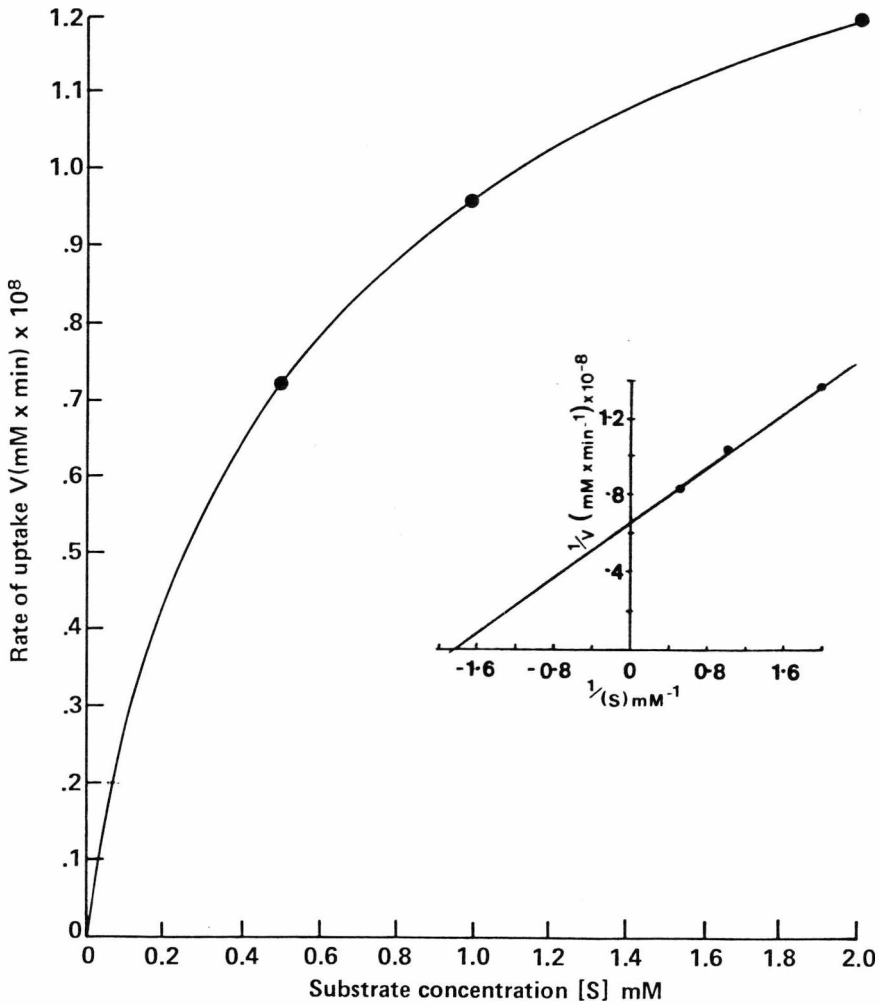


Fig 43 Uptake of [U−^{14}C] ethylene glycol by strain Z

Organisms grown on 20 mM ethylene glycol were harvested, washed and resuspended in 20 ml volumes of minimal media (approx. 0.7 mg dry wt/ml) and incubated at 20°C with a range of [U−^{14}C] ethylene glycol concentrations (0.1–5 mM containing 8×10^5 dpm). Samples (2 ml) were removed at 10 min intervals for 40 min and maximum uptake rates determined.

suspensions of PEG-grown strain O in labelled-ethylene glycol medium to test whether the very low uptake rates observed were due to a requirement by the organism for an available energy or utilisable carbon source. No significant increase in ethylene glycol uptake was observed (Fig. 44).

In contrast, suspensions of ethylene glycol-grown R, which utilised the oligomers but not PEG 1500, readily took up ethylene glycol (0.5mM) [Fig. 44] at an initial rate of 7.6 nmol/min/mg dry wt. at 30°C. While results were probably complicated by the fact that ethylene glycol was being rapidly metabolised upon entry into the cell, efflux experiments, using different batches of cells, nevertheless, showed internal concentrations of 20 - 80 fold above the external concentration when the latter was at 0.5 or 5mM; this further suggested that an active transport system was involved in ethylene glycol uptake.

3.6.2 Facilitated or active transport

Active transport involves the expenditure of energy and allows accumulation against a concentration gradient. Although data in Fig. 42, for example, indicated that cells of strain Z accumulated ethylene glycol 33 times the extracellular concentration (at 30 min), there was no real assurance that active transport was involved. The apparent accumulation may have been due to metabolism of ethylene glycol after entry by facilitated diffusion. Metabolism of ethylene glycol by strain Z and R was aerobic (anaerobic growth on this substrate was never detected, section 3.1.1). If uptake was energetically linked, therefore, no significant uptake should be anticipated under anaerobic conditions. Glycol-grown cells incubated anaerobically accumulated ethylene glycol very slowly (Fig. 45) but on the admission of air the rate of uptake increased from 0.9 nmol/min/mg dry wt. to 11 nmol/min/mg dry wt. implicating an active transport mechanism for uptake of ethylene glycol in this organism.

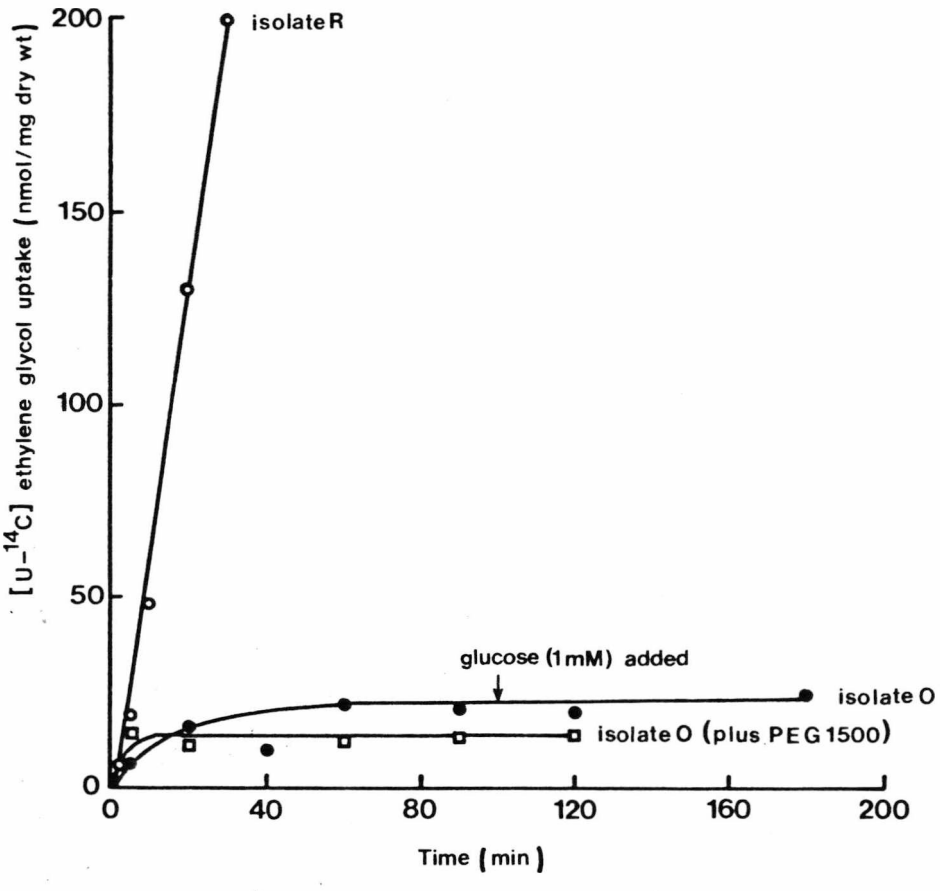


Fig. 44 Uptake of ^{14}C ethylene glycol by suspensions of isolates R and O

Strain R was grown on ethylene glycol (2g/l) and strain O on PEG 1500 (1g/l). Cells were harvested by centrifugation, washed and resuspended in 10 ml of minimal media (final cell concentrations 0.46 and 0.66 mg dry wt/ml in strains R and O respectively) in 100 ml conical flasks supplemented with 0.5 mM [^{14}C]ethylene glycol (containing 202246 dpm). Uptake of radioactivity was followed at intervals as described in Methods.

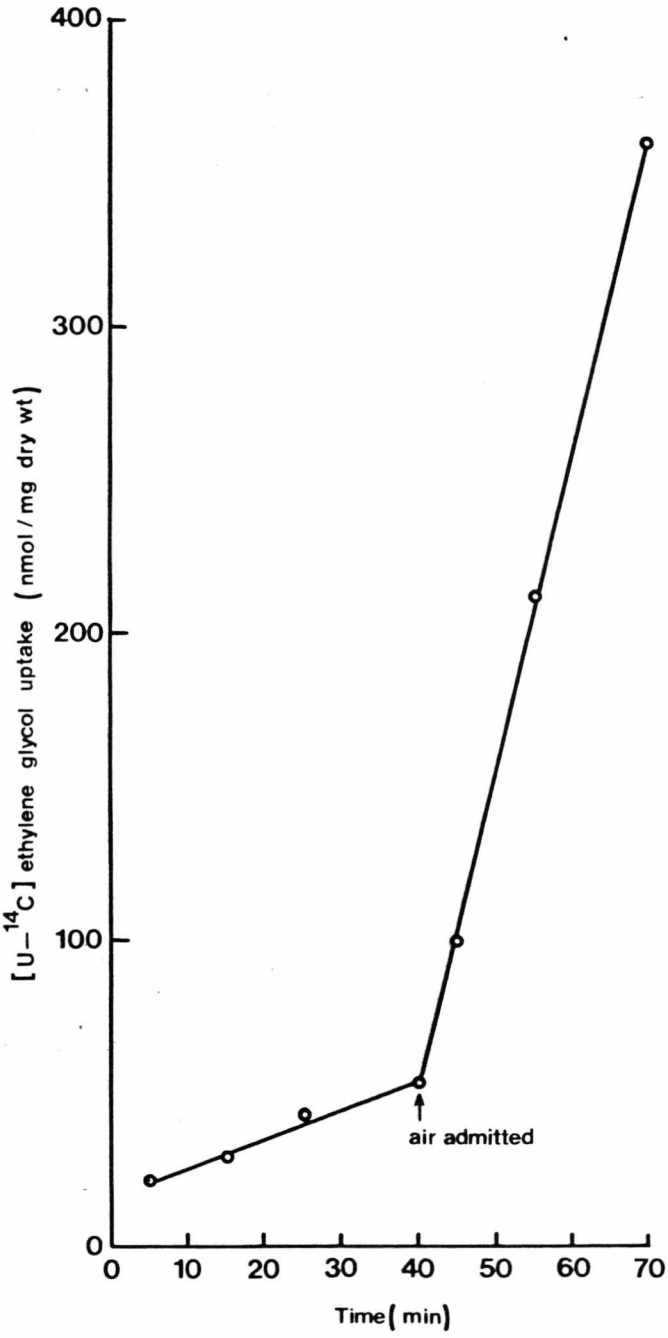


Fig. 45 Anaerobic uptake of ^{14}C ethylene glycol by isolate R

Seven Warburg flasks each contained 0.5 μmol [^{14}C]ethylene glycol (73460 dpm) in the side-arm and 1 ml of cell suspension (0.342 mg dry wt/ml) in the main compartment (cell suspensions gassed for 30 min with N_2). Flasks were agitated and after thermal equilibration at 30°C, were flushed with nitrogen for 1h. Labelled ethylene glycol from the side-arms was subsequently added at 1 min intervals and individual flasks removed at times thereafter. At 40 min air was readmitted and flasks gassed with air for 30 sec. Further incubation and removal of samples was carried out in air. Uptake of radioactivity was determined as described in Methods.

Compounds which inhibit the electron transport pathway were examined because these compounds prevent oxidative phosphorylation and therefore deprive the cell of ATP. If the transport were energy-requiring, i.e. it was an active transport system as opposed to simple or facilitated diffusion systems, then these inhibitors would be expected to inhibit uptake. They have in fact been widely used (Ghei and Kay, 1973; Bellion et al., 1980; Ondrako and Ornston, 1980) to implicate active transport systems for inorganic cations, amino acids, carbohydrates and purines in many bacterial and fungal genera.

Fig. 46 shows that in strain Z, sodium azide and potassium cyanide (added at 0.4mM, 10 minutes after uptake commenced) stopped further uptake of [^{14}C]ethylene glycol and caused the slow leakage of labelled material already accumulated. Substrate uptake was also significantly inhibited by the proton conductors carbonyl cyanide m-chlorophenylhydrazone (CCCP), 4,5,6,7-tetrachloro-2'-trifluoromethylbenzimidazole (TTFB) and valinomycin (Table 37) at the typical very low concentrations (1 to 50 μM). Substantial inhibition of ethylene glycol uptake in the presence of these compounds and 2,4-dinitrophenol was also observed in strain R, examined under similar experimental conditions (Table 37). Such results strongly suggested that transport may be coupled to the provision of metabolic energy.

Di- and triethylene glycol (at 25mM) both caused a marked reduction in the rate of uptake of [^{14}C]ethylene glycol in strains Z and R (Table 38) suggesting that the oligomers were probably taken up by the transport system which recognised ethylene glycol. When PEG 1000 and 1500 (which have no oligomeric components) were tested, they had no significant effect on this uptake rate, commensuate with the belief that the polymeric glycols were not taken up by the glycol transport system. PEG 200, however, (in which the dimer and trimer comprise some 35% of the material)

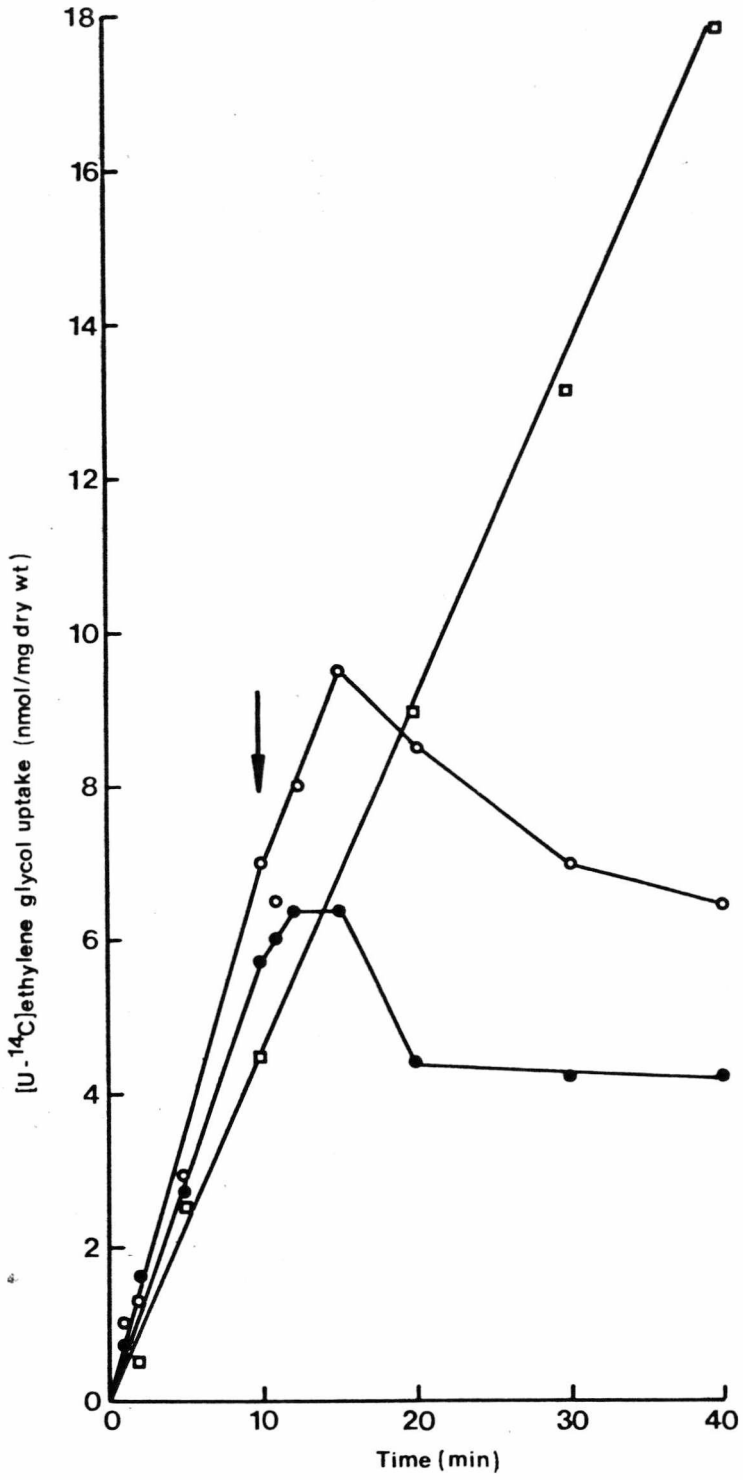


Fig 46. The effect of metabolic inhibitors on the uptake of ^{14}C ethylene glycol by isolate Z

Organisms grown on 20mM ethylene glycol were harvested, washed and resuspended in three separate 20ml volumes of minimal media (approx. 0.7 mg dry wt/ml) and incubated with 5mM ^{14}C ethylene glycol (8×10^5 dpm) for 10min at 20°C and sampled at intervals. 0.4mM sodium azide (●) or 0.4mM fresh potassium cyanide (○) were added to two flasks at the time indicated by the arrow and the third left untreated (□) as the control. Incubation was continued for a further 30 min, sampling at 5 min intervals.

Table 37. Effect of inhibitors on ethylene glycol uptake by strains Z and R

Cells suspended to 0.41 mg dry wt/ml in minimal medium were incubated in test tubes containing radioactive ethylene glycol (0.5mM, 72032 dpm) and inhibitors as shown. The final volume was 2 ml. Tubes were incubated at 30°C for 20 minutes in a reciprocating shaker at 120 oscillations/min. Samples (1 ml) were filtered, washed and counted as in Methods.

Strain Z: The control rate of [^{14}C]ethylene glycol uptake was 1.2 nmol/min/mg dry wt.

Inhibitor	Concentration (μM)	Inhibition (%)
CCCP	0.05	1.5
	5	74.0
TTFB	0.5	7.5
	50	83.9
Valinomycin	0.05	0
	1	64.1

Strain R: The control rate of [^{14}C]ethylene glycol uptake was 6.7 nmol/min/mg dry wt.for Expt. 1 and 7.2 nmol/min/mg dry wt. for Expt. 2.

Inhibitor	Concentration (μM)	Inhibition (%)
Expt. 1.		
CCCP	1	62.7
	5	96.9
TTFB	1	39.9
	5	79.6
Valinomycin	1	57.1
	5	88.7
Expt. 2.	(mM)	
Azide	0.1	2.5
	0.5	33.7
	1.0	65.7
	5.0	72.5
2,4-DNP	0.5	60.6
	1.0	72.9

Table 38. Effect of oligomers and polymers on uptake of ethylene glycol by organisms Z and R

Cells suspended to 0.41 mg dry wt/ml in minimal medium were incubated in test tubes containing radioactive ethylene glycol (0.5mM, 72032 dpm) and analogues as shown. The final volume was 2 ml. Tubes were incubated at 30°C for 20 minutes in a reciprocating shaker at 120 oscillations/min. Samples (1 ml) were filtered, washed and counted as in Methods.

Strain Z: The control rate of [^{14}C]ethylene glycol uptake was 1.7 nmol/min/mg dry wt.

Competitor	Concentration (mM)	Inhibition (%)
Digol	2	21.0
	10	49.6
	25	77.6
Trigol	2	79.8
	10	89.8
	25	93.4
PEG 1000	1	0
Ethanol	1	82.0

Strain R: The control rate of [^{14}C]ethylene glycol uptake was 2.5 nmol/min/mg dry wt.

Competitor	Concentration (mM)	Inhibition (%)
Digol	1	45.8
	5	54.9
	25	70.2
Trigol	0.5	19.1
	1	47.8
	5	47.8
PEG 200	5	35.0
PEG 1500	5	0

caused the anticipated substantial inhibition. The simultaneous presence of ethanol also markedly inhibited accumulation of ethylene glycol in strain Z, when present in an equimolar concentration, which would be expected for a substrate competing for the same transport system; it further suggested that the glycol transport mechanism may actually be a general one for primary alkanols.

Mr.C. Maslen has subsequently obtained two mutants by mutagenic treatment of isolate R with N-methyl-N-nitro-N-nitrosoguanidine (MNNG). Mutant M-40 has the phenotype: Ethylene glycol$^-$, glycolaldehyde$^+$, glycollate$^+$, glyoxylate$^+$, glycerate$^+$ and the second, M-1 has the phenotype: Ethylene glycol$^-$, glycollate$^-$, glyoxylate$^-$, glycerate$^+$. M-40 was suspected to be a glycol transport mutant (see section 3.5.3) from its growth phenotype and from the consistent failure to induce in it any enzymes of ethylene glycol dissimilation by exposure of cells to media containing mixtures of ethylene glycol and glucose.

Cells of the mutant M-40 and a revertant M-40R with the wild type phenotype (obtained from M-40 by counterselection on ethylene glycol plates) were grown on an ethylene glycol:glucose (8:2) mixture, harvested and then incubated with [^{14}C]ethylene glycol. Whereas cells of the M-40 mutant took up the glycol at a rate of only 0.1 nmol/min/mg dry wt. the revertant took up the labelled substrate at a rate of 1.5 nmol/min/mg dry wt. The wild type strain R grown under the same experimental conditions accumulated labelled ethylene glycol at a rate comparable to that of the revertant (Fig 47). This result is consistent with the M-40 lesion being due to a defect in the ethylene glycol uptake system; if it was a metabolic lesion in the glycol dehydrogenase, significant uptake into the cells would be noticed. This result confirmed that ethylene glycol was transported into the cells by a specific uptake system and not by simple diffusion.

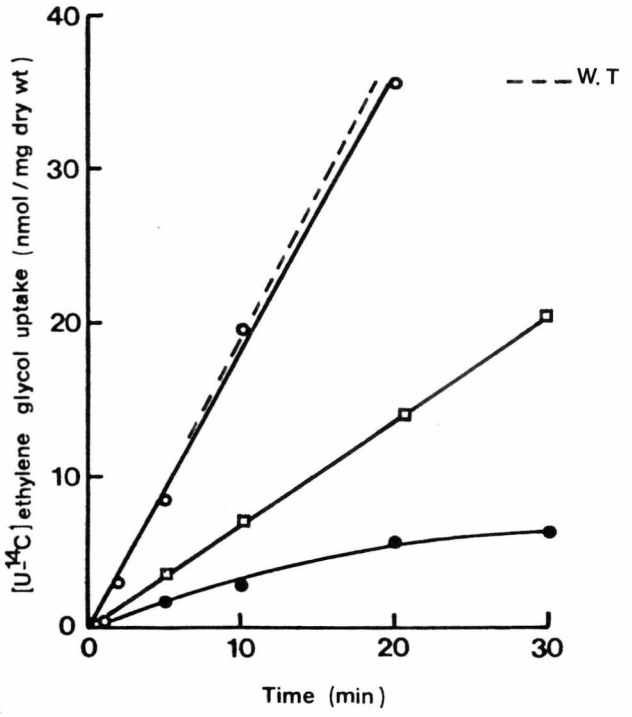


Fig. 47 Uptake of ^{14}C ethylene glycol by mutant strains M-40 & M-1

Wild-type R & mutants M-1 (□); M-40 (●) & the revertant from it M-40R (○) were grown on 8 mM ethylene glycol plus 2 mM glucose. Cells were harvested & resuspended in 10 ml of minimal media containing 0.5 mM [^{14}C] ethylene glycol (11249 dpm) & uptake followed at intervals for 1 h at 30°C as described in methods.

Even though strain M-40 took up ethylene glycol in measurable amounts (6 nmol/mg dry wt.; approx. 1.5mM) this amount was clearly insufficient to support full enzyme induction and measurable growth on this substrate.

The second mutant M-1 was a metabolic mutant, with a genetic block at either glyoxylate carboligase or tartronate semialdehyde reductase; it was thus unable to divert ethylene glycol carbon in significant amounts to many biosynthetic intermediates. It took up [^{14}C]ethylene glycol at about 40% of the wild type rate (Fig. 47) but like the wild-type could still efflux approximately 15% of label with 'cold' ethylene glycol. This result suggested that ethylene glycol must be rapidly converted to early intermediates such as glycolaldehyde, glycollate and glyoxylate even if it is not significantly incorporated into biomass but more importantly confirms that an early metabolic lesion in contrast to a transport mutation does not lead to failure to accumulate label.

3.7 DEGRADATION OF POLYETHYLENE GLYCOLS

3.7.1 Introduction

Because of the inherent difficulties in identifying degradation products of high molecular weight (mixed) PEGs, only preliminary experiments were conducted with this organism. In section 3.1.2 it was reported that strain O did not utilise mono-, di- or triethylene glycol for growth, but grew on PEGs of molecular weight 200 or above. This suggested that strain O degraded PEGs by a route different from that employed by strain R for the degradation of the oligomeric glycols. It was hoped that further studies with resting cells and extracts would substantiate this theory and provide some information to indicate the route of degradation of the polymers.

3.7.2 Oxidation of substrates by whole organisms of strain O

Washed suspensions obtained from cultures grown on PEG 400 rapidly oxidised PEG 400, 600 and 1000. Triethylene glycol and PEG 200 were oxidised much more slowly (Fig. 48). Mono- and diethylene glycol and various glycerate and TCA cycle intermediates were not oxidised at rates significantly above the endogenous respiration level (Table 39).

3.7.3 Oxidation of PEGs by extracts of strain O

The oxidation of PEGs by crude extracts (10000g for 20 minutes) was examined in the presence of $NAD(P)^+$ and electron acceptors such as ferricyanide, dichlorophenolindophenol, phenazine methosulphate, cytochrome c and oxygen.

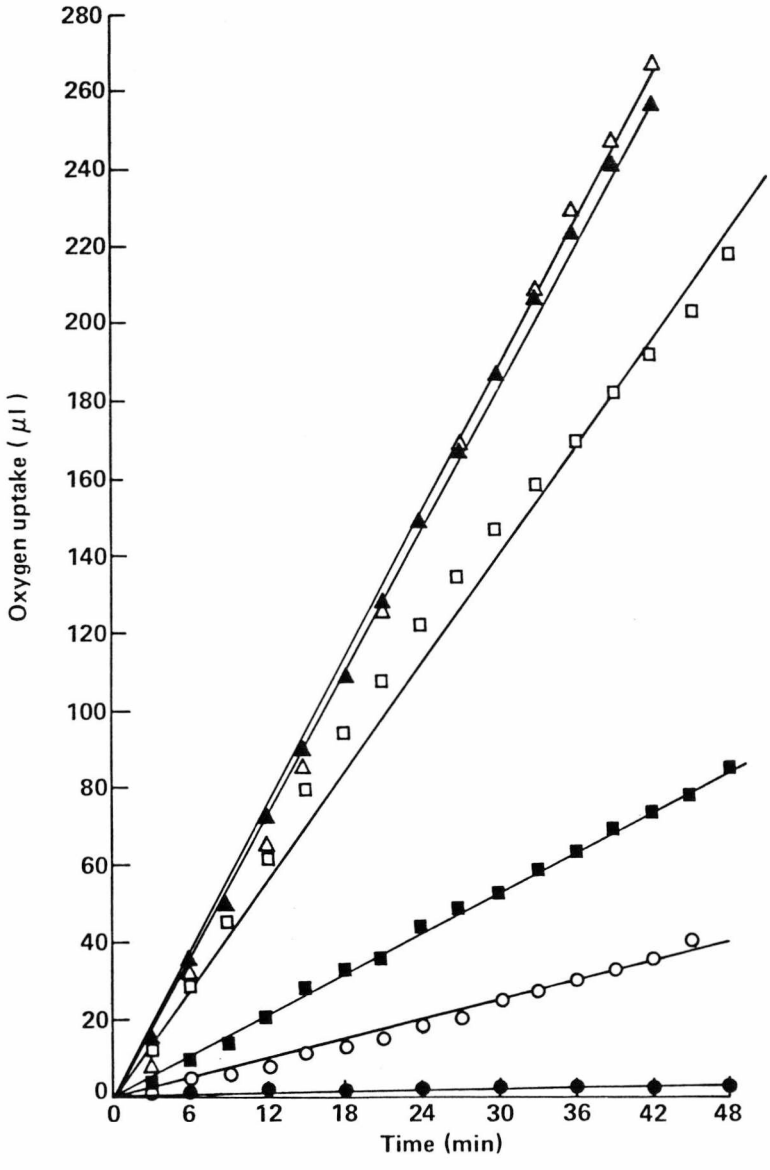


Fig 48 Oxidation of polyethylene glycols by washed suspensions of isolate O grown on PEG 400.

Main compartment of Warburg manometer flasks contained in 3 ml: 150 μmol potassium phosphate buffer, pH 7.0 and washed cells (equivalent to 5 mg dry wt). The centre wells contained 400 μmol KOH. Substrates as indicated (10 μmol) were added from side-arms after thermal equilibration at 30°C. Results are corrected for the endogenous respiration of 0.011 μmol O_2/min/mg dry wt: ● , ethylene glycol; ○ , triethylene glycol; ■ , PEG 200; □ , PEG 400; ▲ , PEG 600 and △ , PEG 1000.

Table 39. Oxidation of substrates by washed suspensions of strain O grown on PEG 400

Main compartment of Warburg manometer flasks contained in 3 ml: potassium phosphate buffer (pH 7.0), 100 µmol; and washed cells (equivalent to 5 mg dry wt.).The centre wells contained KOH, 400 µmol. Sustrates (10 µmol) as indicated were added from side-arms after thermal equilibration at 30°C. Results are corrected for the endogenous respiration (17 µl O_2/h/mg dry wt.).

Substrate for oxidation	Oxygen consumed (µl O_2/h/mg dry wt.)
Ethylene glycol	<12
Diethylene glycol	<12
Triethylene glycol	<12
PEG 200	20
PEG 400	56
PEG 600	73
PEG 1000	75
Glycolaldehyde	<12
Glycollate	<12
Glyoxylate	<12
Glycerate	<12
Pyruvate	<12
L-Malate	<12
Succinate	<12
Acetate	<12
Fumarate	<12
2-Ketoglutarate	<12

Phenazine methosulphate (PMS) was the only electron acceptor tested which enhanced the reaction. The specific activity in its presence was five-fold higher than in its absence (Table 40). The optimum pH was 9.5 in borate-buffer which suggested that the enzyme activity measured may be similar to the glycol dehydrogenase found in extracts of strains Z and R after growth on mono- and oligomeric glycols. The enzyme showed no activity towards low molecular weight glycols and alcohols (Table 41) and activity was not influenced by added ammonium salts, so it is clearly a different enzyme from that found in organisms Z and R.

Although the conversion was not particularly rapid (Table 40) it may, by analogy with the results of page **89** represent the primary step in the pathway of PEG metabolism by this organism. Certainly the formation and accumulation of carboxylated PEGs (see page **68**) from the PEG substrates would require steps analogous to those required for the conversion of ethylene glycol to glycollate via the aldehyde.

The immediate product(s) of PEG oxidation by extracts of O has not yet been identified due to the lack of suitable reference compounds. Application of g.l.c.-mass spectrometry is being used by Mr. C. Maslen (whose study is directed to PEG catabolism) to enable a clearer picture of these early steps to be obtained.

Table 40. Oxidation of PEG by crude extracts of strain O with various electron acceptors

Organism O was grown on PEG 400 and the cells harvested during exponential growth. Extracts were prepared and activity determined in the presence of various electron acceptors.

Acceptor	Specific activity (nmol/min/mg protein)
NAD	no activity
NADP	no activity
Ferricyanide	no activity
Cytochrome c	no activity
Dichlorophenolindophenol (DCPIP)	no activity
DCPIP + phenazine methosulphate	12
Oxygen	3
Oxygen + phenazine methosulphate	15.7

Assay systems containing ferricyanide, DCPIP and cytochrome c were measured spectrophotometrically. See legend to Table 13 for details of assay systems and methods.

Assay systems containing phenazine methosulphate were measured polarographically. The reaction mixture contained in a final volume of 2 ml: borate-buffer (9.5), 100 µmol; phenazine methosulphate, 0.1 µmol; PEG 400, 5 µmol and extract.

Table 41. Oxidation of substrates by crude extracts of strain O grown on PEG 400

Organism O was grown on PEG 400 and the cells harvested during exponential growth. Extracts were prepared as described in Methods and PMS-dependent activity assayed at 30°C by using a Clarke oxygen electrode. Reaction mixtures contained in a total of 2 ml: borate-buffer (pH 9.5), 100 µmol; PMS, 0.1 µmol; PEG, 5 µmol and extract.

Substrate for oxidation	Specific activity (nmol/min/mg protein)
Ethylene glycol	0
Diethylene glycol	0
Triethylene glycol	0
PEG 200	12
PEG 400	16
PEG 1000	11
PEG 1500	7
Ethanol	0
Propan-1-ol	0
Butan-1-ol	0

CHAPTER FOUR

DISCUSSION

4.1 ISOLATION OF MICROORGANISMS

Microorganisms were isolated from various sources which showed dissimilar abilities to degrade ethylene glycol or PEGs. Estuarine mud samples, which may be envisaged as containing glycols as a result of effluent disposal from coastal towns, supported the growth of bacteria capable of readily degrading low molecular weight glycols but not PEGs. Activated sludge, on the otherhand, which contains oligomeric and polymeric glycols as waste products of industrial processes and domestic effluent provided a source of organisms capable of degrading PEGs, though there was no isolate corresponding to that reported by Haines and Alexander (1975) which could grow on PEG 20000. As it is unlikely that free ethylene glycol would occur in soil it is perhaps not surprising that no glycol-utilising organisms were isolated from this source.

Bacteria isolated in this study could be distinguished by their ability to utilise PEGs of different molecular weights as growth substrates (Fig. 15). In general growth of isolates on the higher molecular weight PEGs was characterised by a longer initial lag period than that encountered with the lower molecular weight compounds but in most cases subsequent growth was equally rapid (Fig. 15). In selecting three markedly different organisms it was hoped that during the course of study it might prove possible to demonstrate the existence of different metabolic pathways for the dissimilation of PEGs.

Isolate R, a *Pseudomonas* sp., resembles organisms isolated in a number of different laboratories (Fincher and Payne, 1962; Ohmata *et al.*, 1974; Ogata *et al.*, 1975; Harada and Nagashima, 1975; Watson and Jones, 1977; Pearce and Heydeman, 1980) in its ability to metabolise PEGs with molecular weights of up to 400. Growth on these compounds was rapid and

and degradation of mono-, di- and triethylene glycol was virtually complete within 30 h. Similarly one isolate, O, tentatively identified as a Flavobacterium sp., completely degraded PEG 1000 and 1500 within 70 h after an initial lag period of 30 - 40 h and partially degraded 4000 within the same time period. This contrasts with the growth rates reported for other bacteria with similar substrate specificities where degradation of PEG 1000 required from 10 - 15 d (Watson and Jones, 1977; Ogata et al., 1975).

The third bacterium, a Pseudomonas sp. designated strain Z, while able to degrade ethylene glycol rapidly and completely, was unable to utilise PEGs for growth. In this respect strain Z resembles Flavobacterium NCIB 11171 (Child and Willets, 1978) and Mycobacterium E_{44} (Wiegant and deBont, 1980) both of which utilised ethylene glycol to the exclusion of all other glycols for growth.

Lowering the temperature of incubation considerably affected the ability of the isolated organisms to degrade glycols. Growth of organisms Z on ethylene glycol and R on oligomeric glycols occurred within the temperature range of 5 to 30°C (Tables 4 and 5). At 5°C (which would approximate to European winter river temperatures) results indicated that visible growth on glycols did not occur until after 12 d and at 15°C after 2 d. Organism O did not grow at 10°C or below and showed a lag of 4 d at 15°C (Table 6). This is consistent with the results of Evans and David (1974) who gained evidence to show that at low temperatures (<8°C) mixed bacterial populations could not degrade di- and triethylene glycol within 7 days. Ethylene glycol was, however, partially degraded within the same time interval. These observations could be significant as 10°C is above the minimum temperatures that are reached in North temperate winters and approximates to most European river and lake temperatures at warmer times

of the year. Thus although PEGs have acceptable biodegradability at temperatures above 15°C, which may be applicable to many countries for substantial parts of the year, in winter time the fall in temperature may result in insufficient biodegradation.

The biodegradation of PEGs is of importance because of their widespread production and utilisation which results in their eventual discharge to the environment. In order to further assess their potential impact on the environment it was felt useful to determine what the biodegradation intermediates and end products were and whether these were resistant to further biodegradation.

4.2 BIODEGRADATION OF PEG 200

Commercially available PEG products with average molecular weights greater than 200 are complex mixtures of materials of different molecular weights. An analytical g.l.c. technique was developed, however, which resolved the PEG 200 mixture into its component glycols by forming their trimethylsilyl (TMS) derivatives.

At intervals throughout the growth of strain R on PEG 200 metabolites were extracted from the culture supernatants, converted into their TMS derivatives and examined by g.l.c. Within the first 24 h of incubation the lower polyethylene glycol components were preferentially removed from the culture medium leaving only tetraethylene glycol and the higher PEGs in solution (Fig. 18). In subsequent samples the concentration of these higher PEGs also diminished until they too were undetectable in $CHCl_3$ extracts at 7 d. A new peak (of unknown identity) was detectable, after 2 d however and was still found in samples taken after this time.

PEGs detected in culture supernatants were determined quantitatively by reaction with HBr and subsequent analysis of the resulting dibromoethane by g.l.c. (Table 11). Such analyses indicated that after 7 days incubation only 155 mg/l of PEGs from the original 1000 mg/l were present although TOC analyses showed as much as 375 mg/l carbon (equivalent to 750 mg/l PEG) was still present in culture supernatants at this time (Fig. 17). This anomaly was attributed to the formation of acidic products which no longer reacted as PEGs. In extracts prepared from samples taken at 7 d these intermediates were subsequently identified as carboxylated PEGs by i.r. and n.m.r. spectroscopy (Fig. 21). Mass spectrometry confirmed the presence of mono-carboxylated PEGs with 5, 6 and 7 ethylene oxide (EO) units (Fig. 22).

Hence although certain components of PEG 200 could not be completely degraded, by strain R, they could certainly be converted into intermediate metabolites. The detection of similar carboxylated intermediates during the degradation of PEGs has been reported by other workers (Watson and Jones, 1977; Hosoya et al., 1978).

The inability of strain R to use polymeric glycols as carbon and energy sources may reflect the existence of a permeability barrier for the transport of such compounds into the intact cells. The accumulation of carboxylated PEGs would nevertheless suggest that PEG compounds must have gained access at least to the periplasm of the cell to have undergone partial oxidation. Accumulation of mono-carboxylated PEGs with 5, 6 and 7 EO groups (Fig. 22) from PEG 200, which contains components with that EO range, indicates that depolymerisation of the polymers by cleavage of the ether linkages had not occurred. The limit may lie in the cells' inability to cleave the longer chain compounds; the specificity of the ether-cleaving enzyme(s) allowing recognition of only oligomeric glycols. As a result of the conformation of the PEG molecule, sites on the polymer where the 'etherase' enzyme would bring about cleavage, may simply be inaccessible.

The selective utilisation of high molecular weight components of the PEG 200 mixture by strain O was also demonstrated by g.l.c. Analysis of metabolites of PEG 200 degradation as their TMS derivatives indicated that this time, the polymeric glycols were preferentially utilised leaving only di- and triethylene glycols in the culture media at 4 d. In subsequent samples, however, the concentrations of these low molecular weight glycols decreased and they were only detectable at low concentrations, in $CHCl_3$ extracts after 7 d.

Quantitative analysis demonstrated that only 23 mg/l of PEGs were present after 7 d (Table 11) although TOC analysis indicated that 250 mg/l carbon (equivalent to 500 mg/l PEG) was present in culture supernatants at this time. The reaction of acid-extracted samples with HBr resulted in the formation of some dibromoethane although the concentration detected was very low (Table 11). This observation suggested that carboxylated PEGs were formed and were persisting during growth of strain O on PEG 200. Analyses of samples by i.r. and n.m.r. confirmed the presence of such carboxylated intermediates (Fig. 21) and mono-carboxylated glycols containing 2, 3, 4 and 5 EO groups were subsequently detected, by mass spectrometry, in culture supernatants at the end of growth (Fig. 22).

Accumulation of such products suggested that the inability of strain O to grow on oligomeric glycols and the absence of their oxidation in whole cells, grown on PEGs, did not reflect the lack of a transport mechanism for the oligomeric substrates. The accumulation of mono-carboxylated oligomeric glycols suggested that these compounds must have gained access to the cell and undergone partial oxidation. This conversion of the oligomers to carboxylated intermediates may represent the peripheral activity of an enzyme for which polymeric glycols are the usual substrates. Carboxylated low molecular weight glycols may subsequently accumulate because they do not undergo depolymerisation by the ether-cleaving enzyme-(s), for which a minimum number of EO units may be necessary.

4.3 ROUTE OF ETHYLENE GLYCOL METABOLISM IN STRAIN Z

Previous studies of the metabolism of ethylene glycol (Gonzalez et al., 1972; Child and Willets, 1978; Caskey and Taber, 1981) offered putative schemes to show that bacteria oxidised ethylene glycol with the intermediate formation of glycollate, glyoxylate and tartronate semialdehyde. In the studies of Gonzalez et al. (1972) and Caskey and Taber (1981), however, the experimental evidence was extremely superficial. The data presented in section 3.2 differ in detail from those of Child and Willets (1978) even though both can be interpreted in terms of a reaction scheme (Fig. 5) in which 2 mol of ethylene glycol are converted into 1 mol of glycerate and 1 mol of carbon dioxide. This sequence in strain Z was deduced largely from whole cell studies in which ethylene glycol-grown, but not succinate-grown, cells were able to oxidise the growth substrate and glycerate pathway intermediates at appreciable rates and from the induced enzyme activities found in extracts of cells grown on ethylene glycol.

The first enzyme of the sequence, a glycol dehydrogenase, resembled in several characteristics the methanol dehydrogenase of methylotrophs (Colby et al., 1979)) and the alcohol dehydrogenase of Acinetobacter calcoaceticus (Duine and Frank, 1981) in that it had a high pH optimum, an in vitro requirement for phenazine methosulphate as an artificial carrier, was activated by NH_4^+ and had a molecular weight in the region of 120,000. These features in other bacteria have been found characteristic of quinoprotein dehydrogenases (Duine et al., 1979; Duine and Frank, 1980; DeBeer et al., 1980).

Further, although the dehydrogenase activity of this enzyme was inhibited 40% by 0.1mM atabrine, the inhibition was not reversed by FAD or FMN

as is typical of many atabrine inhibited flavoprotein oxidases.

The similarities of glycol dehydrogenase to methanol dehydrogenase extended to further indirect observations, e.g. (1) the enzyme was readily released into the soluble fraction upon cell breakage (2) the 50-65% saturated ammonium sulphate fraction showed a broad substrate specificity towards primary and secondary alcohols, although unlike methanol dehydrogenase did not oxidise methanol (3) the presence of a 60000 Dalton polypeptide, which in methanol dehydrogenase is a subunit of the native dimer, 120000 and which was the major polypeptide in the 50-65% saturated ammonium sulphate fraction in ethylene glycol-, but not succinate-grown organisms and (4) the presence in extracts of glycol-grown, but not succinate-grown cells, of an unusual CO-binding cytochrome c, which has recently been shown to be the natural electron acceptor for the quinoprotein methanol dehydrogenase (Duine and Frank, 1979).

Low levels of an NAD^+-alcohol dehydrogenase were also demonstrated but the activities of this enzyme were not dependent on growth with ethylene glycol (Table 14). The synthesis of a distinct glycol dehydrogenase during growth on ethylene glycol was anticipated because the NAD^+-alcohol dehydrogenase oxidised ethylene glycol at a rate which was well below that required to account for the short generation time of the organism on this substrate. The physiological function of the NAD-linked enzyme and its significance in the oxidation of ethylene glycol is uncertain but it may be dependent on the degree of aeration. An important factor in natural waters is the relation between dissolved oxygen concentration and oxidation-reduction (redox) potential. In oxygenated waters the measured redox potential or E_h is usually approximately 500 mV, and remains fairly stable as long as the concentration of dissolved oxygen is greater than approximately 1 mg/l; hence the oxygen concentration really has

little effect on the redox potential in aerobic conditions. Reducing conditions (-100 to -400 mV) can be reached however in certain environments, such as estuarine mud from which strains Z and R were isolated and in sediments where oxygen diffusion from overlying waters is extremely slow and mixing may not occur for long periods. Normally microorganisms can tolerate only a specific E_h range but small fluctuations in potential may lead to a change in nutrition or physiology. For example the pathway of propylene glycol metabolism by a species of Flavobacterium able to grow on the diol as the sole source of carbon was influenced by the degree of aeration of the growth medium (Willets, 1979b). Under strongly aerobic conditions the degradation of the diol was exclusively initiated by a diol oxidase. Under microaerophilic conditions, however, some propylene glycol was catabolised by an alternative diol dehydrase-initiated pathway.

The influence of oxygen on the two metabolic routes for propylene glycol metabolism by Flavobacterium sp. NCIB 11171 recalls the ability of Hyphomicrobium X to metabolise dimethylamine by distinct enzymes when grown aerobically (dimethylamine monooxygenase) and anaerobically (dimethylamine dehydrogenase) (Meiberg et al., 1980) and the metabolic versatility of various bacteria grown on glycerol under different conditions (Lin, 1976). The relative contribution of NAD^+-alcohol and ethylene glycol dehydrogenases to ethylene glycol oxidation in strain Z might also be governed by oxygen but only the NAD^+-alcohol dehydrogenase was found in strain Z during growth on ethanol, which suggested that this enzyme may have a specific function only in the oxidation of mono-alcohols.

In this respect isolate Z resembled the facultative methylotroph, organism PAR (Bellion and Wu, 1978) in which the quinoprotein methanol dehydrogenase was replaced by a NAD^+-alcohol dehydrogenase when the organism was grown on ethanol instead of methanol.

The utilisation of a step mediated by a coenzyme with a high redox potential e.g. pyrrolo-quinoline-quinone (which is replaced *in vitro* by the PMS dye), in preference to NAD or flavin would have the physiological value of facilitating growth on ethylene glycol, by displacing the equilibrium of the glycol ⟶ glycolaldehyde reaction towards the formation of the aldehyde; an aspect which may be favourable in the natural environment where glycol is probably present, if at all, at quite low concentrations.

The ethylene glycol dehydrogenase and NAD^+-alcohol dehydrogenase were shown to be two distinct enzymes which differed in thermolability (Fig. 24) and could be separated by ammonium sulphate fractionation.

The observed rapid oxidation of glycolaldehyde by washed cell suspensions and extracts of ethylene glycol-grown cells afforded evidence to support the active participation of glycolaldehyde in the metabolism of ethylene glycol.

The enzyme aldehyde: NAD oxidoreductase was present in highest concentrations in extracts after growth of the organism on ethylene glycol, confirming the earlier observations of Jakoby (1957); von Tigerstrom and Razzell, (1968); Razzell and Blackmore (1969) who all found aldehyde dehydrogenases after growth of various *Pseudomonas* species on ethylene glycol and ethanol. It was however still present in high activities in extracts of glucose-grown cells. Heat treatment resulted in a curved denaturation line which suggested the presence of at least two aldehyde dehydrogenase enzymes which differed in their thermolability. The ability to synthesise more than one enzyme could provide the organism with an important regulatory function.

Although glycolaldehyde was oxidised by whole cells and extracts, growth of strain Z with the aldehyde as the sole carbon and energy source was

not successful presumably because of the toxicity of this compound to the organism (den Dooren deJong, 1926). The product of glycolaldehyde dehydrogenase activity, glycollate (identified by paper chromatography) was shown to be converted to glyoxylate by an inducible glycollate oxidase located in the particulate cellular preparation. Some activity, well below that required to account for the observed rate of oxidation of glycollate in whole cells, was located in the high speed supernatant fraction (HSSF) and then required the addition of phenazine methosulphate to complete the H-transport system. This was probably due to some particulate enzyme being lost to the supernatant during the disruption and extraction procedure.

The enzymic composition of extracts indicated that the glyoxylate formed from ethylene glycol was metabolised via tartronate semialdehyde to glycerate. The following inducible enzymes which lie on the proposed pathway were present at high activities in extracts of ethylene glycol-grown cells: glyoxylate carboligase, tartronate semialdehyde reductase and enzymes converting glycerate into pyruvate.

The product of glyoxylate carboligase was assumed to be tartronate semialdehyde because an acidic bis-2,4-dinitrophenylhydrazone derivative was obtained which could not be extracted into Na_2CO_3 but was extractable into NaOH. Tartronate semialdehyde, however, is an unstable compound which is known to undergo a non-enzymic decomposition to glycolaldehyde (Dagley et al., 1961). Further characterisation of the product of the glyoxylate carboligase reaction will be required to effect positive identification of this compound as tartronate semialdehyde.

Tartronate semialdehyde isomerises to hydroxypyruvate especially in acid solution (Krakow at al., 1961) which was employed for deproteinisation

prior to preparation of the hydrazone derivative. The rate of oxidation of NADH, however, measured spectrophotometrically in the presence of glyoxylate and hydroxypyruvate was 239 and 5 nmol/min/mg protein respectively in ethylene glycol-grown Z (Fig. 34) suggesting that hydroxypyruvate was not the physiologically-important intermediate in this organism.

The conversion of glycerate to phosphoenolpyruvate (PEP) can proceed either by phosphorylation of glycerate at the 2-position and its subsequent dehydration to yield PEP, or by phosphorylation of glycerate at the 3-position, requiring the subsequent conversion of 3-phosphoglycerate to 2-phosphoglycerate prior to PEP formation. The metabolism of 3-PGA, measured spectrophotometrically by converting its phosphoenolpyruvate product into lactate in the presence of excess pyruvate kinase, lactic dehydrogenase and NADH, only occurred rapidly on the addition to the reaction mixture of purified phosphoglycerate mutase which isomerised 3-PGA into 2-PGA. The latter, in contrast, was converted to pyruvate rapidly without such addition, as was a mixture of glycerate and ATP, thus demonstrating the virtual absence of and non-requirement for the mutase in extracts of strain Z and supporting the view that the direct product of the glycerate kinase reaction was 2-phosphoglycerate.

In Hyphomicrobium X the finding that 2-PGA was the product of the glycerate kinase raction led Harder et al. (1973) to suggest that the conversion of 2-PGA to 3-PGA provided a route for gluconeogenesis. Since 2-PGA was also the product of glycerate kinase in strain Z, the formation of 3-PGA from ethylene glycol metabolism would perform a similar role. Willets (1979a) reported that phosphoenolpyruvate stimulated the phosphoglycerate mutase reaction in a Flavobacterium sp. and suggested that this compound could control the fate of 2-phosphoglycerate.

Consideration of the data presented on the oxidation of tricarboxylic acid cycle intermediates by whole cells (Table 12) implied that the TCA cycle could provide a major pathway of ethylene glycol oxidation by strain Z. TCA cycle intermediates (with the exception of citrate) were rapidly oxidised without a lag and at a rate commensurate with the observed rates of ethylene glycol oxidation. Assays of key enzymes of the major metabolic pathways, such as citrate synthase and isocitrate dehydrogenase in the TCA cycle and isocitrate lyase and malate synthase in the glyoxylate bypass, provided further evidence for a functional TCA cycle.

Since isocitrate dehydrogenase was found at approximately 12 times the activity of isocitrate lyase in extracts of ethylene glycol-grown Z, it seemed likely that any citrate formed would be oxidised by isocitrate dehydrogenase and continued through the 2-oxoglutarate stage rather than undergo diversion to glyoxylate formation by isocitrate lyase. The enzymes of the glyoxylate bypass possibly fulfulling an anaplerotic function.

A further possibility exists for the metabolism of glyoxylate derived from ethylene glycol, namely oxidation by the dicarboxylic acid cycle initiated by malate synthase. The small variation in the activities of malate- and citrate synthases found after growth on ethylene glycol or succinate suggested that the possible operation of a DCA cycle was not physiologically significant.

4.4 ROUTE OF GLYCOL METABOLISM BY STRAIN R

The release of ethoxylate (EO) units from PEGs may be effected by a number of mechanisms including: 1) hydrolysis (Haines and Alexander, 1975; Pearce and Heydeman, 1980); 2) oxidative cleavage of the ether bond (Jones and Watson, 1976; Harada and Sawada, 1977; or 3) oxidation of the α-carbon atom and hydrolysis of the resulting ester. These mechanisms would generate from a PEG, ethylene glycol (1), glycolaldehyde (2) or glycollate (3), if they acted on the terminal ether linkage.

Ethylene glycol, glycolaldehyde and glycollate were oxidised by strain Z via the glycerate pathway and thus the appropriate enzymes were also sought in strain R.

An ammonium-ion activated, NAD^+-independent, glycol dehydrogenase in R, with properties similar to the enzyme isolated from extracts of strain Z, oxidised mono-, di- and triethylene glycol. The glycol dehydrogenase showed activity towards mono-, di- and triethylene glycol that was not separable during partial purification, which suggested that the same enzyme acted on all 3 substrates.

The enzyme catalysed the formation from the dimer and trimer of products capable of yielding 2,4-dinitrophenylhydrazone compounds; these were most likely the aldehydes because the product of ethylene glycol metabolism under identical circumstances has been identified as glycolaldehyde (Table 33).

The inability of the glycol dehydrogenase to attack 1,2-dimethoxyethane and the affinity of the enzyme for substrates possessing a terminal $-CH_2OH$ group suggested that a terminal CH_2OH group must be available

for catabolism to be initiated. It is uncertain, however, whether cleavage of the ether bond occurred before or after dehydrogenation of the terminal $-CH_2OH$ group of such ether glycol compounds. It was possible, therefore, that di- and triethylene glycol were converted to their aldehydes and perhaps their acids by the glycol dehydrogenase prior to actual cleavage of the ether bond(s). The dicarboxylates of di- and triethylene glycol, however, were not intermediates (C. Maslen, personal communication).

Glycollate was formed as an intermediate during growth of strain R on di- and triethylene glycol. The observation of elevated activities of glycollate oxidase, glyoxylate carboligase, tartronate semialdehyde reductase and glycerate kinase in di- and triethylene glycol-grown cells but not in succinate or glucose-grown cells was indicative of the involvement of a pathway converting glycollate into glycerate and carbon dioxide. It was conceivable, however, that induction of these enzymes could have been effected by the chemically similar homologues, i.e. by similarity of diglycollate, etc. to the natural (monomeric) substrate. A possible reaction sequence is shown in Fig. 49 with the stoicheiometry of 2 mols of glyoxylate from 1 mol of diethylene glycol.

A man-made compound will be biodegraded if it is susceptible to attack by the enzymatic apparatus acquired by microorganisms during the course of evolution. This, in turn, depends on two factors 1) the ability of microbial enzymes to accept as substrates compounds having chemical structures similar to, if not identical with, those found in Nature and 2) the ability of these substrates, when in the presence of microorganisms, to induce or derepress the synthesis of the necessary degradative enzymes. The first category must also include transport processes that will allow the substrate to gain access to the degradative enzymes.

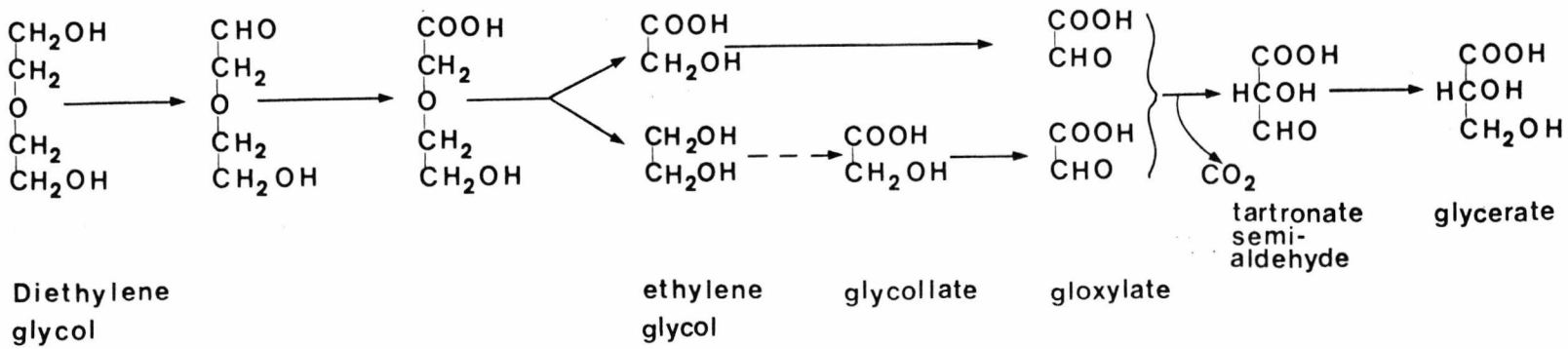


Fig 49 Postulated pathway for the metabolism of diethylene glycol to glycerate

The stages by which mono-, di- and triethylene glycol are degraded (see section 1.2.5 and 1.3) can be related to known enzyme mechanims, which presumably have already evolved for the utilisation of natural product(s). The degradation of keto acids including pyruvate and glyoxylate in the sediments of some lakes (Dovoledo, 1959) and of glycollate, detected in both ocean and lake waters (Fogg, 1964), will require microbial enzyme and transport systems capable of taking up and respiring these substrates. Glycollate is a known excretory product of algae and aquatic higher green plants and represents a potentially important energy source for heterotrophic bacteria in marine, river and lake environments (Wright and Shah, 1975; Fogg, 1964). The fact that so many bacteria have been found to metabolise these substrates in such environments indicates the availability of enzyme systems already capable of degrading products derived from glycol metabolism.

4.5 REGULATION OF ETHYLENE GLYCOL METABOLISM IN STRAINS Z AND R

Successive inductive events appeared to regulate the synthesis of the enzymes which converted ethylene glycol to 2-phosphoglycerate. The first enzyme of the sequence, glycol dehydrogenase, was independently regulated and was induced by growth with its substrate, ethylene glycol, but not by intermediates further down the pathway (Table 35). Each individual intermediate was found to induce the synthesis of its own specific enzyme and those of subsequent steps but not the synthesis of enzymes that catalysed earlier steps in the breakdown i.e. there was no evidence for product induction. Strict sequential inductive control offers an economy of protein synthesis that gives an obvious selective advantage to cells that employ it because this type of control prevents the synthesis of inducible enzymes in the absence of their substrates and permits the utilisation of pathway intermediates should they ever become available exogenously, without the wasteful synthesis of physiologically non-functional enzymes.

Regulatory units that undergo sequential induction are always controlled independently. If, alternatively, several enzymes are induced by the same metabolite, their synthesis may be controlled either independently- coincident induction - or by a more tightly united regulatory control- coordinate induction. Coordinancy is usually, but not always, the consequence of a close association on the chromosome of the structual genes governing the enzymes in question, the phenotypic expression of these genes being subject to a common regulatory control unit or operon. The inductive patterns presented in Tables 28 and 35 indicated that the synthesis of none of the enzymes converting ethylene glycol to phosphoglycerate was coordinately controlled.

Convincing proof that the catabolic sequences suggested were indeed employed by organism Z for the metabolism of ethylene glycol and by organism R for the metabolism of mono- and diethylene glycol will require the isolation of mutant strains that have lost the ability to catalyse component reactions. Although such organisms are unable to utilise the primary growth substrate they may accumulate an identifiable intermediate in its presence. If the mutant strain, unlike the wild type, lacks an enzyme that attacks the accumulated compound, then there is good reason to believe that the enzyme plays an essential role in the sequence.

To identify the specific enzyme inducers involved, it will be necessary to test the inductive properties of each of the intermediates in isolation. Degradation of intermediates under test must be suppressed to prevent the formation of degradation products which may themselves have inductive properties. The most convenient method would again be to use suitably blocked mutants. In the case of the ethylene glycol pathway proposed here such mutants were not available when this thesis was written but have been obtained by a successor investigating the same problem. None of the glycol analogues available acted as genuine gratuitous inducers for strain R so the chemical requirements of the inducing molecule have proved difficult to define more precisely.

4.6 ETHYLENE GLYCOL UPTAKE IN STRAINS Z, R AND O

In a growing culture, heterotrophic organisms must possess the capacity to take up carbon substrate at a rate sufficient to meet the cells biosynthetic and bioenergetic demands.

Glycols, glycerol (and other small non-polar molecules) were believed to penetrate the bacterial cell by passive diffusion, the high intrinsic permeability of phospholipid bilayers to such molecules precluding any necessity for a transport system. Facilitated diffusion systems have been demonstrated however (although only at low external substrate concentrations) for glycerol in species of E. coli (Sanno et al., 1968), B. subtilis (Lin, 1976) and Pseudomonas aeruginosa (Tsay et al., 1971) and for propylene glycol in a mutant strain of E. coli K-12 (Hacking et al., 1978).

Ethylene glycol is likely to be present at low concentrations in the environment and in these cicumstances a facilitated or active transport system would be advantageous for utilisation of this substrate.

The results of uptake studies showed that ethylene glycol-grown cells of strains Z and R rapidly took up [^{14}C]ethylene glycol at 30°C although no such uptake was observed at 0°C (Fig. 42). The accumulated label was not all in ethylene glycol, however, because both uptake and incorporation of labelled material was almost certainly being observed. Nevertheless, the pool material from efflux experiments has been shown to contain [^{14}C]ethylene glycol (and other labelled metabolites) by paper chromatography (C. Maslen personal communication).

The transport system showed classical saturation kinetics for which an apparent K_s of 0.56mM was calculated. This value is several orders of magnitude higher than those in other bacteria for uptake of metabolites such as sugars, amino acids and dicarboxylic acids but is comparable with the K_s of 0.25mM and 0.5mM for glycerol in B. subtilis and Ps. aeruginosa, respectively (Lin, 1976; Tsay et al., 1971) and K_s values of 1 to 3mM for methylamine transport by Pseudomonas sp. MA (Bellion et al., 1980). Such a high K_s, however, could indicate that ethylene glycol is an unnatural substrate of a glycerol or alcohol transport system.

The transport system was probably inducible like that for glycerol in Ps. aeruginosa (Tsay et al., 1971) because cells previously grown on glucose took up ethylene glycol only after a lag of 35 - 40 minutes. The observed initial uptake to approximately 30 nmol/mg within a few minutes was probably attributable to diffusion since the external and internal concentrations were very nearly equal (5mM) and there was no change in this value until induction occurred. In these experiments it was not possible to distinguish unequivocally between uptake due to the transport system and subsequent incorporation of label due to the enzymes of ethylene glycol metabolism, both of which could have been induced by exposure to the glycol of glucose-grown cells. Such induction could be prevented by the addition of chloramphenicol (100 μg/ml) to uninduced cells.

Strain O, which is unable to utilise ethylene glycol as a growth substrate, did not accumulate ethylene glycol above the external concentration which indicated that no glycol transport system existed in this organism.

Internal concentration estimates from the efflux experiments could be obtained in nmol of ethylene glycol/mg dry wt but was more useful for comparison with external ethylene glycol concentrations if it were

expressed in mM concentrations. The nmol/mg to mM conversion factor of 4 µl H_2O/mg dry wt is unlikely to be completely accurate as it was a literature value obtained with E coli (Kepes and Cohen, 1962). Though electron micrographs show that the cellular dimensions of strains Z and R are comparable with those of E. coli, more recent estimates of the intracellular volume of Pseudomonas cultures suggest that this estimate of the ratio may be from 16% (Higgins and Mandelstam, 1972) to 40% (Guymon and Eagon, 1974) too high. This value, however, does enable a minimum estimate of pool concentrations to be made to show whether the transport system was capable of concentrating ethylene glycol to an internal concentration several times higher than the external concentration. From a series of efflux experiments done with separate batches of cells of both Z and R strains, the internal pool concentration of ethylene glycol ranged between 19- to 44-fold higher than the external concentration when the latter was at 0.5 or 5mM and were 6.5-fold higher even when the external concentration was 0.05M.

Cyanide and azide which function at the level of cytochrome oxidase were effective in blocking ethylene glycol uptake (Fig. 46) as were the ATPase inhibitors, 2,4-DNP, CCCP, TTFB and valinomycin(Table 37). These results suggested that transport was probably coupled to the provision of metabolic energy. If this was the case then it was anticipated that there would be a significant difference between uptake in aerobic and in anaerobic conditions, for strain R is an obligate aerobe in its utilisation of the oligomeric glycols. The rate of uptake increased 13-fold on transition from anaerobic to aerobic conditions (Fig. 45) which further points to a likely active transport system.

Competition studies with both strains Z and R indicated that di- and triethylene glycol (at 25mM) competed significantly with ethylene glycol

for uptake (Table 38). PEG 200 caused a 35% decrease in uptake, in strain R, which probably reflects competition due to the mono-, di- and tri-ethylene glycol components of the mixture (which constitute approximately 35% of the glycol). Such results indicate that these oligomers were probably taken up by the transport system which recognised ethylene glycol. PEG 1000, in contrast was without significant effect which suggested either that these metabolites entered the cell by separate transport systems or that they have very low affinities for the system mediating ethylene glycol transport.

Ethanol also caused a marked reduction in the uptake of ethylene glycol transport which suggested that ethanol was taken up by the same system that recognised ethylene glycol.

A mutant, M-40, unable to accumulate ethylene glycol (Fig. 47), obtained by Mr. C. Maslen, was shown to be a glycol transport mutant. The existence of such a mutant implied that uptake was not a result of simple diffusion but due to a specific transport system.

A revertant M-40R concomitantly regained both the ability to grow on ethylene glycol as a sole carbon source and the ability to transport ethylene glycol normally when compared to the wild-type strain (Fig. 47). Furthermore, whereas the M-40 mutant cannot grow with ethylene glycol, di- or triethylene glycol, the revertant M-40R simultaneously regained with the transport mechanism the ability to utilise these oligomers. This further substantiates the belief that the oligomers enter strain R by the glycol uptake system and supports the conclusions of uptake competition data.

A second mutant M-1 (lesion at glyoxylate carboligase or tartronate semialdehyde reductase) took up ethylene glycol at a rate significantly above that of mutant M-40 (Fig. 47) which indicated that such a metabolic lesion did not lead to failure to accumulate label.

4.7 DEGRADATION OF POLYETHYLENE GLYCOLS BY ISOLATE O

Studies on the degradation of polymeric glycols have been hampered by the lack of suitable quantitative methods which can separate the PEG mixtures into their component glycols. The g.l.c. technique used to follow PEG 200 degradation was unsuitable for PEGs with molecular weight 300 and above due to the effect on the g.l.c. columns of the temperatures required to separate the high molecular weight fractions.

In the absence of a suitable analytical method, which could have given some insight into the nature of the degradation intermediates only very preliminary experiments were conducted with this organism.

The inability of strain O to utilise mono-, di- and triethylene glycols for growth and the absence of their oxidation by whole cells suggested either the lack of a transport system for these compounds or absence of enzymes required for their degradation. Since mono-carboxylated derivatives of mono-, di- and triethylene glycol were found in culture media (Fig. 22) after growth of strain O on PEG 200 the latter alternative would seem to be the most likely.

A number of reports have indicated a requirement for various electron acceptors to increase the rate of oxygen uptake of cell-free extracts with PEGs (Ohmata *et al.*, 1974; Kawai *et al.*, 1978; Thélu *et al.*, 1980) and NAD- and flavin-dependent dehydrogenases have been isolated. Polarographic studies have established that the enzyme(s) synthesised by strain O growing on PEG as a sole carbon source was specific for the higher polymers. The addition of phenazine methosulphate stimulated the rate of oxygen uptake by extracts with PEG 200, 400, 1000 and 1500, but not with

mono-, di- or triethylene glycol on which the organism will not grow. Whether this enzyme cleaves the ether-linkage or is merely a dehydrogenase that acts on the terminal $-CH_2OH$ group has not been established. However, like the PEG dehydrogenase enzyme isolated by Kawai et al, (1977), 1980), could effect depolymerisation by catalysing the conversion of PEG, via an aldehyde, to the monocarboxylic acid with the subsequent cleavage of the ether bond.

Further characterisation of this enzyme and its role in the degradation of PEGs is currently being investigated by Mr. C. Maslen.

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