

PHYSIOLOGICAL STUDIES OF TRIMETHYLAMINE OXIDE

REDUCTION IN ALTEROMONAS PUTREFACTENS

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بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

قُلْ لَوْ كَانَ الْخَضِرُ مِدَادًا لَكَلَّمْتُ رَبِّي لَنَفِدَ الْخَضِرُ قَبْلَ أَنْ نَنْفَدَ كَلِمَتُ رَبِّي وَلَوْ جُنَّا بِمِثْلِهِ مَدَدًا ۖ قُلْ إِنَّمَا أَنَا بَشَرٌ مِثْلُكُمْ يُوحَىٰ إِلَيَّ أَنَّمَا إِلَهُكُمْ إِلَهُ وَاحِدٌ ۖ فَمَنْ كَانَ مِنْكُمْ رِبِيًّا فَلْيَعْمَلْ عَمَلَهُ صَالِحًا وَلَا يُشْرِكْ بِعِبَادَةِ رَبِّهِ ۚ أَحْكَا ۝

In the name of God, Most Gracious, Most Merciful

"Say: "If the ocean were ink (wherewith to write out) the words of my Lord, sooner would the ocean be exhausted than would the words of my Lord, even if we added another ocean like it, for its aid."

Say: "I am but a man like yourselves, (but) the inspiration has come to me, that your God is one God: whoever expects to meet his Lord, let him work righteousness, and, in the worship of his Lord, admit no one as partner."

To the memory of my father and to the people
who affectionately support me:

my mother
and Bashir

I declare that this thesis has been composed by
myself and that the work herein presented is
my own.

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Physiological Studies of Trimethylamine Oxide

Reduction in Alteromonas putrefaciens

The reduction of trimethylamine oxide to trimethylamine was studied in the marine bacterium Alteromonas putrefaciens. Neither reduced methyl viologen : trimethylamine oxide reductase nor the formate : trimethylamine oxide respiratory chain were repressed in cells grown in the presence of nitrate or fumarate, and cells grown on a combination of trimethylamine oxide with either nitrate or fumarate had slightly enhanced activities of trimethylamine oxide reductase. However, nitrate apparently repressed fumarate reductase and vice versa, possibly indicating that they are under the same regulatory system.

Location of the respiratory enzymes was investigated: trimethylamine oxide reductase, nitrate reductase, and fumarate reductase was predominantly periplasmic, while formate dehydrogenase was membrane bound. Formate dehydrogenase could be completely solubilised from the membrane by the detergent cetyltrimethylammonium bromide; other reagents were inferior. Formate dehydrogenase was protected partially against inactivation by preparation of membranes in Tris-HCl buffer containing EDTA.

Molybdate always enhanced the activities of the molybdoenzymes nitrate reductase and trimethylamine oxide reductase, but not the activity of the complete electron transport chain (Formate : terminal electron acceptor). In contrast, formate dehydrogenase activity was decreased in the presence of molybdate but enhanced by tungstate, as is the case for obligate anaerobes like Clostridium spp and Methanococcus vannielii.

The optimum conditions for complementation of NADPH : nitrate reductase in extract of Neurospora crassa nit-1 by Alteromonas putrefaciens molybdenum cofactor were determined. The time required for complementation was 20 min at room temperature. Molybdenum cofactor of Alteromonas putrefaciens complemented the nit-1 nitrate reductase without adding exogenous molybdate to the complementation mixture. Tungstate was co-ordinated preferentially to molybdate in the molybdenum cofactor and led to loss of complemented nit-1

nitrate reductase activity.

Molybdenum cofactor of Alteromonas putrefaciens behaved similarly to molybdenum cofactors from other sources in being inactivated upon exposure to oxygen. Dithiothreitol was able to afford some protection from oxygen inactivation.

Molybdenum cofactor distribution between the cellular fractions depended on the growth conditions; the periplasm where the molybdo-enzymes were located was found to contain a considerable proportion of the free molybdenum cofactor which was mainly concentrated in the cytoplasmic fraction. Molybdenum cofactor synthesis was induced by partial anaerobiosis and was not affected by the addition of molybdate or tungstate to the growth medium.

Different classes of mutants defective in trimethylamine oxide respiration were isolated and characterised. A high percentage of these mutants were pleiotropic and were unable to grow on nitrate anaerobically. The frequency of mutants defective only in the synthesis of trimethylamine oxide reductase was very low, probably because of the complexity of the trimethylamine oxide respiratory chain which shares a common molybdenum cofactor with other anaerobic electron transport components.

Loss of molybdenum cofactor led to the disappearance of trimethylamine oxide reductase, nitrate reductase, and formate dehydrogenase activities but not fumarate reductase activity. Mutants lacking molybdenum cofactor were affected in the regulation of anaerobic respiration and showed derepressed activities for nitrite reductase.

ABBREVIATIONS

A	Absorbance
ADP	Adenosine 5'-pyrophosphate
ATP	Adenosine 5'-triphosphate
BV ^{•+}	Reduced benzyl viologen
BV ⁺⁺	Oxidised benzyl viologen
cmc	Critical micellar concentration
CoA	Coenzyme A
CTAB	Cetyltrimethylammonium bromide
DCPIP	2,6-dichlorophenolindophenol
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EPR	Electron paramagnetic resonance
EXAFS	Extended X-ray Absorption Fine Structure
FAD	Flavin adenine dinucleotide
FADH ₂	Reduced flavin adenine dinucleotide
FDH	Formate dehydrogenase
GTP	Guanosine 5'-triphosphate
HPLC	High performance liquid chromatography
INT	Iodonitrotetrazolium
MV ^{•+}	Reduced methyl viologen
MV ⁺⁺	Oxidised methyl viologen
MAD ⁺	Nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
NANA	Nutrient agar containing 2% NaCl
NADP ⁺	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NTG	N-methyl-N'-nitro-N-nitrosoguanidine
O-F test	Oxidation fermentation test
PMS	Phenazine methosulphate

Abbreviations (contd.)

PMSF	Phenylmethanesulfonyl fluoride
QAE	Quaternary amino ethyl
TLC	Thin layer chromatography
TMA	Trimethylamine
TMAO	Trimethylamine oxide
Tris	Tris (hydroxymethyl)-aminomethane

Abbreviations of units are defined according to The Symbols Committee of The Royal Society (1975).

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CHAPTER 1

INTRODUCTION

Alteromonas putrefaciens is a Gram negative rod-shaped bacterium (Lee, 1979). All strains of A. putrefaciens are polarly flagellated and have a non-fermentative metabolism. Although biochemically similar to pseudomonads, alteromonads have been placed in a separate genus because the base composition of their DNA (mol % GC 43-54) differs markedly from those of pseudomonads (mol % GC 58-72) (Baumann et al., 1972). Lee et al. (1977) in a taxonomic study on pseudomonas-like bacteria postulated three phenons C, D, and E including bacteria of mol % GC 43-48. The isolate used in this work A. putrefaciens NCMB400 was grouped in phenon D. Strains of A. putrefaciens isolated by Gillespie (1981) showed less metabolic versatility than Pseudomonas spp. and were characterised by their production of sulphhydryl type odours, inability to produce H_2S during growth in peptone iron agar and their ability to reduce trimethylamine oxide. Most of the alteromonads were able to utilise maltose and sucrose but not lactose or mannitol.

A. putrefaciens has been isolated from a wide range of environments especially from proteinaceous foods (Lee, 1979; Parker & Levin, 1983). Easter (1982) showed that all members of phenon D are able to reduce TMAO, nitrate and fumarate. These bacteria can synthesise TMAO reductase and its related components on oxygen depletion to continue growth via anaerobic respiration at the expense of TMAO which is available in marine fish tissues. Alteromonads may therefore have a competitive advantage over other non-TMAO reducing bacteria during fish spoilage and thus may assert their dominance in the spoilage flora.

1. Trimethylamine Oxide

TMAO which can serve as an electron acceptor for anaerobic respiration of bacteria during fish spoilage is a nitrogenous compound having the formula $(CH_3)_3NO$. The oxide is a solid compound and is soluble in water acting as a weak base. It is present in fluids and tissues of marine animals but not in

marine plants. The distribution of the oxide is widely varied in animal tissues but it is more concentrated in the muscles. TMAO content differs from one organism to another and depends on seasonal changes, geographical areas, and type of environment (Groninger, 1959; Yamada, 1967).

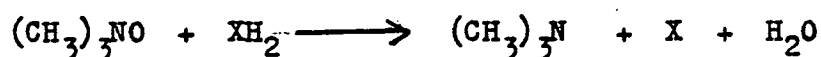
The origin of TMAO is unknown, but it is thought to be derived from protein metabolism; most probably zooplankton are the major producers of TMAO for the marine food chain. Strøm (1979) showed that exposing marine Calanus spp to ^{14}C -labelled TMA in seawater resulted in the oxidation of TMA to TMAO and the latter was accumulated in the animals; the enzymes involved appear to be monooxygenases (Neilands, 1945). The oxidation catalysed by monooxygenases is thought to occur in humans, since oral administration of TMA resulted in the appearance of TMAO as the major product in the urine, while TMAO administered orally is excreted unchanged (Tarr, 1941).

The actual function of TMAO in marine animals is not fully known, but it could have a role either in osmotic pressure regulation or in lowering the freezing point of animal fluids in cold waters (Groninger, 1959).

TMAO when reduced to TMA gives rise to the characteristic off-flavour of spoiled marine fish and TMA has been used as a convenient index of spoilage (Shewan, 1962). The ability to reduce TMAO to TMA has also been used in bacterial classification (Wood & Baird, 1943). TMAO is not toxic to bacteria up to 90 mM concentration (Castell, 1946) and Salmonella typhimurium can grow in the presence of 50 mM TMAO without any apparent adverse effects (Kim & Chang, 1974).

Reduction of TMAO under anaerobic conditions has been reported in different bacteria: Escherichia coli, Salmonella typhimurium, Rhodopseudomonas capsulata, Proteus vulgaris, Vibrio parahaemolyticus, Achromobacter spp and Alteromonas spp (Sakaguchi group; Kim & Chang, 1974; Davidson et al., 1979; Madigan & Gest, 1978; Strøm et al., 1979; Unemoto et al., 1964, 1965; Watson, 1939b; Easter, 1982).

The induction of TMAO reductase by TMAO and the fact that the product TMA is not further metabolised implies that its physiological role is as an alternative electron acceptor which operates under anaerobic conditions. Many reports have shown that bacteria can use a variety of hydrogen donors for TMAO reduction according to the equation :



2. Trimethylamine Oxide Reductase

Earlier studies about TMAO reduction were done on whole cell systems, since methods for cell fractionation and enzyme assays were not well developed. The conclusions from these studies were that the TMAO reducing system in resting cells of different bacteria showed different responses to pH, temperature, and NaCl concentration. Nitrite inhibited TMAO reducing activity in all organisms tested but at varying degrees depending on the bacterial species, while nitrate inhibited TMAO reduction in some strains but not others (Castell & Snow, 1951). Oxygen was found to retard the evolution of TMA, i.e. oxygen prevented the microbial reduction of TMAO to TMA (Watson, 1939a). Tarr (1940) showed that different bacterial cultures reduced TMAO anaerobically and found that the product of reduction was TMA. As induced cells were able to reduce trimethylamine oxide and tri-n-propylamine oxide, Tarr suggested that the enzyme be named "Triamineoxidase" rather than TMAO reductase. Triamineoxidase was appreciably inhibited by cyanide and azide, accordingly it was concluded that the enzyme contains a heavy metal.

TMAO reduction appears to require a dehydrogenase for oxidisable substrate and also component(s) or carrier(s) between the dehydrogenase and the terminal reductase (Neilands, 1945). Further evidence for intermediate carriers was provided by the work of Davidson et al. (1979) who showed that some mutants unable to grow on TMAO anaerobically, retained TMAO reductase activity as assayed using an artificial electron donor.

The induction of TMAO reductase in whole cells of E. coli and Proteus vulgaris was studied by Tomizawa (1951). The system was of an adaptive nature with a pH optimum of 7.5 and required unknown factors as reflected by the requirement for yeast extract. These factors could be trace elements like molybdenum.

TMAO reductase was induced when the dissolved oxygen in the medium was completely consumed, addition of TMAO under these conditions led to a change in the redox state of the medium and further induction of TMAO reductase (Sakaguchi & Kawai, 1977b). Under these conditions the increase in activity was attributed to induced synthesis of TMAO reductase as evidenced by the prevention of de novo synthesis of the enzyme protein by actinomycin D and chloramphenicol (Sakaguchi & Kawai, 1975a). Among the factors affecting the synthesis of TMAO reductase in E. coli is the presence of nitrate which inhibited the synthesis of TMAO reductase but not the activity of the already synthesised enzyme (Sakaguchi & Kawai, 1975c), while oxygen inhibited both the synthesis and the activity of the enzyme (Sakaguchi & Kawai, 1976). In A. putrefaciens, TMAO reductase was induced by TMAO and lack of oxygen and showed no inhibition by TMA the product of reduction; nitrate did not affect TMAO reductase synthesis (Easter, 1982).

In Vibrio parahaemolyticus, TMAO reductase had an optimum pH for activity of 7.0-7.5, and the enzyme lost about 70% of activity upon storage at 2°C or -20°C for 24h; inactivation was prevented in the presence of mercaptoethanol during enzyme preparation. However, this reducing agent failed to stabilise the enzyme during storage. TMAO reductase in Vibrio parahaemolyticus contains essential thiols since it was reversibly inhibited by p-chloromercuribenzoate. The enzyme showed varying degrees of inhibition towards metal-chelating agents (Unemoto et al., 1964, 1965).

In E. coli, the inducible form of TMAO reductase PII, showed different properties from those mentioned above with an optimum pH of 5.0-5.5, substrate inhibition at low concentration (0.3 mM) and inhibition by the ferrous chelating agents α , α' -

dipyridyl and *o*-phenanthroline. Enzyme activity was stimulated by Fe^{++} and Mn^{++} (Sagai & Ishimoto, 1973).

A. putrefaciens TMAO reductase showed high affinity for TMAO, an optimum pH of 6.7-7.0 for activity and was inhibited by azide and cyanide (Easter, 1982).

The broad substrate specificity of TMAO reductase led Tarr to name it "Triamineoxidase". Shimokawa & Ishimoto (1979) found the constitutive and inducible forms of E. coli had a wide specificity including chlorate, bromate and some N-oxides. In another study using a different strain of E. coli, the inducible form was also found to have a similar substrate specificity, which led the authors to emphasise that the enzyme should be given the systematic name tertiary amine N-oxide (acceptor) oxido-reductase (EC 1.7.99) or amine N-oxide reductase (Sagai & Ishimoto, 1973). In A. putrefaciens, the enzyme also has wide substrate specificity (Easter, 1982).

The purified enzyme from E. coli grown anaerobically with TMAO showed three bands of activity on polyacrylamide gel after electrophoresis. On a gel filtration column three peaks of activity were obtained corresponding to three different molecular weights. These results were interpreted as being due either to formation of oligomers or to formation of complexes with other proteins (Sagai & Ishimoto, 1973). In more detailed studies by Shimokawa & Ishimoto (1979), only one band of TMAO reductase activity was detected on polyacrylamide gel with extracts of E. coli cells grown in the absence of TMAO. However, four bands were detected using extracts from TMAO grown cells. One band was due to the constitutive enzyme and the most active band (NoRI) has been named the TMAO induced enzyme; the other two bands were probably due to other inducible enzymes or different forms of the induced enzyme. The NoRI enzyme had a molecular weight of 220,000 daltons; under denaturation conditions this fraction gave two polypeptides with molecular weights of 180,000 and 60,000 daltons on sodium dodecylsulphate-polyacrylamide gel. NoRI catalysed the reduction of various amine N-oxides, both aromatic and aliphatic, and reduced hydroxylamine, chlorate, bromate, but not nitrate at rates lower than with TMAO. The

optimum pH for activity was determined using viologen dyes and was found to be 5.3. The enzyme had an isoelectric point of 5.2 and an iron content of 1.8 mol/mol of enzyme.

Unemoto et al. (1965) found that TMAO reductase in Vibrio parahaemolyticus was firmly bound to the membrane since washing the membrane increased the specific activity. In E. coli strain IFO 3301 the enzyme is also membrane bound (Sakaguchi & Kawai, 1975b), while Shimokawa & Ishimoto (1979) using E. coli K-10 Hfr suggested that the inducible enzyme NoRI was loosely bound to the membrane. By contrast in A. putrefaciens Easter (1982) found that TMAO reductase is predominantly periplasmic and may be attached to the outer aspect of the cytoplasmic membrane.

Kim & Chang (1974) isolated chl D mutants following NTG mutagenesis; upon addition of high concentrations of molybdate to the growth medium the mutants regained both nitrate reductase and TMAO reductase activities. Inhibition of TMAO reductase activity by tungstate and the recovery in the presence of molybdate suggests that amine N-oxide reductase is a molybdoenzyme like nitrate reductase (Shimokawa & Ishimoto, 1979). Recent studies on the complete TMAO reduction system using wild type and chlorate resistant mutants strongly confirmed that TMAO reductase is a molybdoenzyme and shares with nitrate reductase only molybdenum cofactor but not the polypeptides (Cox & Knight, 1981; Takagi et al., 1981; Takagi & Ishimoto, 1983).

3. Anaerobic Respiration

ATP can be generated either by substrate level phosphorylation or through reactions in which phosphorylation is coupled to electron transport. The former occurs mainly in obligate anaerobes and involves soluble enzymes; the latter can occur either aerobically with oxygen as the electron acceptor or anaerobically using an alternative electron acceptor.

Anaerobic respiration can be defined as biological oxidation using inorganic or organic compounds as the ultimate hydrogen acceptor in place of oxygen. Generally anaerobic respiration is associated with oxidative phosphorylation and

in turn with active transport of amino acids and sugars against a concentration gradient. The molar growth yield of bacteria is usually higher in the presence of a terminal electron acceptor than in its absence. This higher yield can be due to more complete oxidation of substrate and consequently less formation of reduced products or because of oxidative phosphorylation occurring during the reduction of the hydrogen acceptor (Stouthamer, 1969).

Several compounds other than oxygen can act as terminal electron acceptor: nitrate, fumarate, thiosulphate, elemental sulphur, tetrathionate, carbonate, TMAO, nitrite and dimethylsulphoxide.

3.1 Anaerobic respiration with TMAO

TMAO reduction system is one of a number of anaerobic electron transport chains and its study may provide more evidence for understanding the metabolic controls governing aerobic and anaerobic metabolism. The system may be different in different bacteria. In an early report mentioned by Neilands (1945) it was suggested that TMAO reduction might involve more than one carrier. Biochemical studies in E. coli have shown that formate, NADH and NADPH were very effective electron donors for TMAO reduction and both c-type and b-type cytochromes have been implicated as electron carriers (Sakaguchi & Kawai, 1977a, 1978a, b; Sakaguchi et al., 1979). The proposed electron transport chain is shown in Figure 1.

The Japanese workers concluded that ubiquinone or menaquinone might be involved in the chain somewhere between cytochrome b and cytochrome c. Confirmation on this point came from the finding that a quinone-deficient double mutant (ubi A, men A) was unable to grow on TMAO unless the necessary precursors were added to the growth medium (Cox & Knight, 1981).

In a study of oxidative phosphorylation coupled to TMAO in Salmonella typhimurium, Kim & Chang (1974) calculated the amount of ATP produced with limiting amounts of glucose and various levels of TMAO and nitrate; they found that TMAO supported about one-half of the growth of nitrate. In Proteus

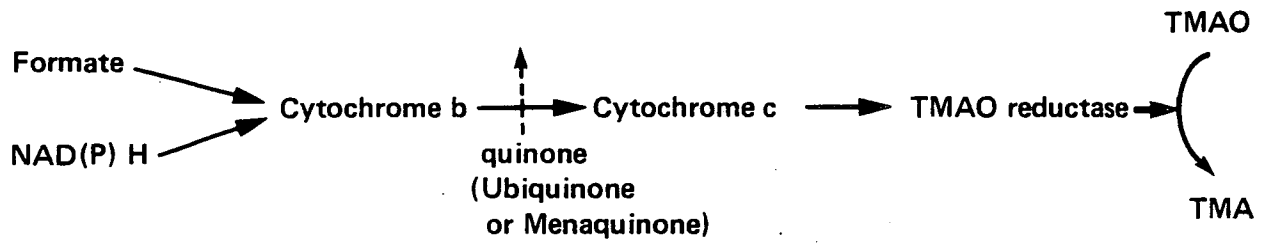


Figure 1. The proposed TMAO electron transport chain in E. coli (Sakaguchi & Kawai, 1978b)

spp, Ström et al. (1979) suggested from free energy considerations that it is thermodynamically feasible that the reduction of TMAO can be coupled to oxidative phosphorylation. In E. coli, similar molar growth yield values were obtained with nitrate, fumarate, and TMAO; each gave 1 mol of ATP per mol formate oxidised, although the theoretical value for TMAO should be 2-3 mol. From these results it was suggested that the phosphorylation site may be located between FDH and a cytochrome (Yamamoto & Ishimoto, 1977).

Proton translocation coupled to TMAO reduction was observed in intact cells of E. coli and a membrane potential was generated by formate-linked TMAO reduction as evidenced by changes in fluorescence quenching of the cyanide dye 3,3'-dipropylthia-carbocyanine. It was concluded that anaerobic TMAO reduction caused generation of a protonmotive force which could then be used for ATP synthesis by the reversible proton-translocating ATPase (Takagi et al., 1981). It has been also demonstrated that TMAO reduction can be coupled to active transport of amino acids like L-serine (Stenberg et al., 1982).

3.2 Anaerobic respiration with nitrate

Microorganisms can utilise nitrate for two distinct processes: firstly nitrate can be used as a sole source of nitrogen for synthesis of cellular materials (assimilation), a process which can occur both aerobically and anaerobically; secondly nitrate can be used anaerobically as a terminal electron acceptor (dissimilation), a process also called nitrate respiration. The immediate product of nitrate respiration is nitrite which can be also used as terminal electron acceptor. Some bacteria reduce nitrite to gaseous products, while others reduced it to ammonia.

In E. coli the respiratory chain to nitrate is membrane bound and formate is the preferential electron donor for nitrate reduction. The first step in nitrate reduction is mediated by formate dehydrogenase which is a complicated enzyme in E. coli since it plays a versatile role in the oxidative metabolism of E. coli. Depending upon the growth conditions formate can

provide electrons for the reduction of oxygen to water (formate oxidase), nitrate to nitrite (formate : nitrate reductase) and H^+ to hydrogen (formate hydrogenlyase). The activity of formate dehydrogenase(s) of each of these membrane-associated electron transport systems requires the presence of trace amounts of selenium, molybdenum, and iron in the culture medium (Pinsent, 1954; Fukuyama & Ordal, 1965; Lester & De Moss, 1971).

Purified FDH is composed of three subunits α , β and γ in a molar ratio of 1 : 1.2 : 0.55 with respective molecular weights of 110,000, 32,000, and 20,000 daltons. The enzyme contains 1.0 haem 0.95 molybdenum, 0.96 selenium, 14 non-haem iron, and 13 acid-labile sulphide per mol protein (Enoch & Lester, 1975) the γ subunit corresponds to associated cytochrome b.

There are two b_1 -type cytochromes involved in nitrate reduction in E. coli, one associated with FDH and the other with nitrate reductase (Ruiz-Herrera & De Moss, 1969; Ruiz-Herrera et al., 1969; Enoch & Lester, 1974). Using both the I^{125} /lactoperoxidase and the transglutaminase methods for labelling proteins, it was found that the cytochrome b_1 associated to the nitrate reductase is facing or on the periplasmic aspect of the membrane (Boxer & Clegg, 1975; MacGregor & Christopher, 1978).

In addition to FDH and cytochromes, quinones are necessary redox carriers for electron transport to nitrate as the acceptor. This was shown for the formate : nitrate respiratory chain of E. coli (Itagaki, 1964) by extraction of quinones and subsequent restoration of the inhibited activity with quinones. The separated components of the nitrate reduction system of E. coli, FDH and nitrate reductase, have been shown to restore an overall formate : nitrate reductase activity when either ubiquinone or menaquinone was present as mediators (Enoch & Lester, 1974). As ubiquinone is more efficient, both in reconstitution and the reactivation after extraction, it was concluded that ubiquinone rather than menaquinone is involved in the nitrate reduction pathway, moreover, the NADH : nitrate reductase of Klebsiella aerogenes could only be reactivated by quinone after lipid extraction (Knoch & Planta, 1973). However, in some Gram-

positive bacteria, which contain only menaquinone, electron transport to nitrate is mediated by this quinone. Thus a mutant of Staphylococcus aureus deficient in menaquinone was found to lack electron-transport-dependent nitrate reduction (S  sarman et al., 1974).

Nitrate reductase, the last component of the nitrate reduction chain serves as acceptor of electrons from the quinone via the associated cytochrome b_1 . In most cases the enzyme is membrane bound (Stouthamer et al., 1980). The isolated enzyme is composed of three distinct subunits (Enoch & Lester, 1975). Two of these subunits, α with a molecular weight 155,000 daltons and β , 60,000 daltons, are present in all active preparations as assayed with artificial electron donors (MacGregor et al., 1974; Lund & De Moss, 1976; Clegg, 1976). The third γ subunit (19,000 daltons) is a cytochrome b_1 which tends to dissociate from the enzyme during purification, but appears to be required for association with the cell membrane (MacGregor, 1975a, b).

The formation of nitrate reductase is affected by mutation at several distinct genetic loci designated chl (see Section 5). Chl C, has been directly implicated as a structural gene for the α catalytic subunit of the nitrate reductase (De Moss, 1978; MacGregor, 1975c). Chl C and chl I (coding for cytochrome b_1) were found to be closely linked genes and they are part of a multicistronic operon (Bonnefoy et al., 1981). Recent studies of transposon insertions in the chl C region of the chromosome of E. coli have shown that such mutations affect the formation of both the α and β subunits leading to the conclusion that the genes coding for the α and β subunits were part of a chl C (nar) operon that is transcribed in the direction $\alpha \rightarrow \beta$ (Edwards et al., 1983).

The α subunit of nitrate reductase contains the catalytic site of the enzyme which includes the molybdenum cofactor (Chaudhry & MacGregor, 1983a), while the β subunit has been postulated to be involved in membrane attachment of nitrate reductase (De Moss, 1977; Graham & Boxer, 1980). In E. coli nitrate reductase contains one atom of molybdenum (Forget, 1974;

MacGregor et al., 1974; Lund & De Moss, 1976). Bacillus licheniformis, Paracoccus denitrificans and Klebsiella aerogenes nitrate reductases also contain one molybdenum atom (Van't Riet et al., 1979; Forget, 1971; Bosma et al., 1978). Nitrate reductase from different bacteria also contains different amounts of acid-labile sulphide and iron-sulphur clusters (Stouthamer et al., 1980).

Ota et al. (1964) were the first to show that oxidative phosphorylation can be coupled to nitrate reduction using cell-free extracts of E. coli. From indirect studies in E. coli oxidative phosphorylation was shown to be coupled to formate oxidation by nitrate (Yamamoto & Ishimoto, 1977), and also to glucose oxidation (Ishimoto & Yamamoto, 1977). It was found that 2-3 mol of ATP were formed per mol nitrate reduced and this reduction provided energy for active transport of lactose and amino acids. Recent developments in the genetics and biochemistry have been covered in recent reviews (Stouthamer, 1976; Haddock & Jones, 1977; Stouthamer et al., 1980).

In general the pathways for TMAO reduction and nitrate reduction were assumed to have some similarity (Kim & Chang, 1974). Recently it has been proved that the similarity is only at the molybdenum cofactor level and that the gene(s) coding for TMAO reductase is distinct from that of chl genes (Takagi et al., 1981; Takagi & Ishimoto, 1983).

3.3 Anaerobic respiration with fumarate

Fumarate is an anaerobic electron acceptor which can be used by several bacteria (Kröger, 1977, 1980). Electron transport to fumarate has been extensively studied in Vibrio succinogenes (Wolinella succinogenes, Tanner et al., 1981) and in E. coli. In both organisms the fumarate reduction system is localised in the cytoplasmic membrane.

The system is essentially composed of FDH which is similar in E. coli and Vibrio succinogenes with respect to its content of molybdenum and cytochrome b, but different in that the former organism has a high iron-sulphur protein content, uses selenium

as an integral component and has a different acceptor specificity. FDH of Vibrio succinogenes has been purified and shown to contain identical subunits of molecular weight 110,000 daltons. Each subunit contains one atom of molybdenum and iron-sulphur groups, the latter represented 10% of the total iron-sulphur present in the membrane, whereas 90% was associated with fumarate reductase. The enzyme was found to be associated with a low potential (-200 mV) cytochrome b as an obligatory component of the electron transport chain and this cytochrome is thought to be the direct acceptor of electrons from FDH and only one form of FDH was observed during purification. These observations led the authors to suggest that molybdenum is the primary acceptor of reducing equivalents from formate and that the iron-sulphur groups may serve as mediators between molybdenum and the electron acceptor of FDH. This would be analogous to the case in xanthine dehydrogenase and other aldehyde dehydrogenases which contain molybdenum and iron-sulphur (Kröger et al., 1979).

FDH in Vibrio succinogenes occupies the outer aspect of the cytoplasmic membrane and the oxidation of formate to CO₂ takes place at the periplasmic aspect, so that formate does not cross the membrane (Kröger, 1980; Kröger et al., 1980).

The other components of this chain following FDH are cytochromes and menaquinone. Bacteria capable of reducing fumarate were found to contain b-type cytochromes and these were thought to be involved in fumarate reduction because fumarate could oxidise them. The concomitant occurrence of either menaquinone or desmethylmenaquinone with the ability to reduce fumarate in various bacteria suggests that these quinones seem to be essential mediators of electron transport to fumarate. Cytochrome b (-200 mV) interacts between FDH and menaquinone, and cytochrome b (-20 mV) between menaquinone and the iron-sulphur protein of the fumarate reductase complex (Kröger & Innerhofer, 1976a, b).

The final component of this chain is fumarate reductase; this enzyme is membrane bound, can use BV⁺⁺ as an electron donor for the reduction of fumarate and is similar to the mitochondrial

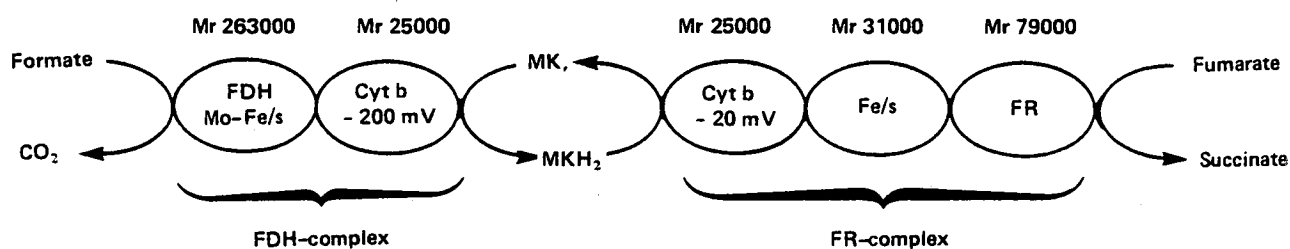
succinate dehydrogenase in that FAD is covalently bound to the protein. This enzyme is located at the inner aspect of the cytoplasmic membrane of Vibrio succinogenes (Kröger et al., 1980).

Fumarate reductase has been purified and the purified complex shown to consist of three subunits: cytochrome b (-20 mV) and two non-identical subunits. The larger subunit contained FAD as a prosthetic group as shown by fluorescence absorption spectra. The two subunits of molecular weight 79,000 and 31,000 daltons respectively were found in equal molar ratio. The larger subunit catalyses the reduction of fumarate by FADH_2 , while the smaller one mediates electron transport between the large subunit and cytochrome b (-20 mV). Therefore fumarate reductase has two different reactive sites which are essential for its function in electron transport. Iron-sulphur protein is the physiological donor for fumarate reductase as it reacts on the fumarate side of menaquinone confirming that menaquinone is an obligatory redox mediator in the formate : fumarate chain (Kröger & Innerhofer, 1976a; Uden et al., 1980; Uden & Kröger, 1981).

In most cases so far investigated, the reduction of fumarate proved to be coupled to electron transport phosphorylation. The cell yields from various bacteria indicate that about 1 ATP molecule is formed from each molecule of fumarate when cells were permitted to grow with hydrogen and fumarate as the only energy substrates (Kröger, 1978). Confirmation of the coupling of phosphorylation to fumarate reduction was investigated also by using strong uncoupling agents such as carbonylcyanide phenylhydrazone derivatives (Kröger & Winkler, 1981).

Using the reconstitution of the separated components of the fumarate reductase system it has been shown that the reconstituted complex is more or less similar to that found in the native cellular membrane (Uden & Kröger, 1982). From the above discussion the electron transport from formate to fumarate is as shown in Figure 2.

In addition to the function of fumarate mentioned above, fumarate can be used as the sole carbon source for Vibrio



Mr, molecular weight; Mo, molybdenum; Fe/s, non-haem iron sulphur protein; FDH, formate dehydrogenase; MK, menaquinone; Cyt, cytochrome; FR, fumarate reductase

Figure 2. Electron transport system for fumarate.
(See Kröger et al., 1979; Unden & Kröger, 1982)

succinogenes grown anaerobically in a mineral medium, i.e. fumarate can be used both as catabolic and anabolic substrate (Bronder et al., 1982; Mell et al., 1982).

In E. coli, the electron transport chain to fumarate is similar to that in Vibrio succinogenes, but most information has come from genetic rather than biochemical studies. Spencer & Guest (1973) isolated frd mutants unable to grow on fumarate as terminal electron acceptor. Involvement of menaquinone in fumarate reduction was indicated by the inability of men mutants to grow on fumarate (Lambden & Guest, 1976b). Purified fumarate reductase contained α and β subunits, with FAD covalently bound to the α subunit. The enzyme was extremely sensitive to thiol modifying reagents and the amino acid sequence was found to be quite similar to the succinate dehydrogenase of Rhodospirillum rubrum (Robinson & Winer, 1982). Recently it has been found that fumarate reductase is associated with two hydrophobic polypeptides and the suggestion is that these polypeptides function to anchor the enzyme in the membrane and maintain the structure of the two original subunits. The hydrophobic subunits are coded for by frd C and frd D genes respectively, while the β subunit (iron-sulphur) is coded for by the frd B gene and the α subunit (FAD) by the frd A gene; all these genes are in the same operon (Cole et al., 1982; Lemire et al., 1982; Plas et al., 1983). However, when the cellular levels of fumarate reductase were amplified using multicopy plasmids the associated b-type cytochrome was not, although the amplified fumarate reductase was found in the cytoplasmic membrane. It was concluded that cytochrome b is essential for electron flow but not for incorporation of fumarate reductase in the membrane (Robinson & Winer, 1982), in contrast to nitrate reductase as mentioned previously.

3.4 Anaerobic respiration with other electron acceptors

As mentioned above a variety of metabolites can be used as terminal electron acceptors for anaerobic respiration. Recently experimental work has shown that sulphate reduction is associated with proton translocation in the sulphate-reducing

bacterium Desulfovibrio vulgaris (Kobayashi et al., 1982).

Anaerobic respiration with sulphate has been reviewed by Thauer & Badziong (1980). Nitrite reduction can also provide a means of anaerobic respiration as proton translocation is associated with nitrate and nitrite reduction in some denitrifying bacteria e.g. Paracoccus denitrificans and Pseudomonas denitrificans (Hollocher, 1981); and similarly in Desulfovibrio desulfuricans (Steenkamp & Peck, 1981). In E. coli energy can be conserved during formate — dependent reduction of nitrite (Motteram et al., 1981).

Most of the electron transport chain components are membrane bound, but in Pseudomonas aeruginosa, nitrite reductase was found in the periplasmic fraction with associated cytochrome C₅₅₁ (Wood, 1978); this shows some similarity with the situation in A. putrefaciens (Easter, 1982).

A fourth mode of energy production, distinct from aerobic respiration, anaerobic respiration and fermentation is thought to be associated with nitrite and TMAO reduction under certain conditions. This is termed "electron acceptor limited growth" and is characterised by energy production by substrate level phosphorylation and regeneration of oxidised pyridine nucleotides through anaerobic electron transport. In E. coli grown on formate as electron donor and nitrite as terminal electron acceptor, ATP was produced by oxidative phosphorylation, but when NADH replaced formate there was no oxidative phosphorylation. This was explained as the reducing components including fermentation products being metabolised anaerobically at the expense of the exogenous electron acceptor nitrite (Cole, 1981; Pope & Cole, 1982). A similar observation was found in Proteus spp: TMAO dependent oxidation of lactate yielded no ATP (Strøm et al., 1979). The purple photosynthetic bacterium Rhodospseudomonas capsulata when grown anaerobically on fructose and TMAO or dimethylsulphoxide as an accessory oxidant, produced ATP by substrate level phosphorylation only. Thus TMAO and dimethylsulphoxide can function as electron acceptors for the reoxidation

of NADH providing NAD^+ required for fermentation as shown in Figure 3. TMAO and dimethylsulphoxide reductase are soluble enzymes found in the cytoplasm (Madigan et al., 1980; Cox, 1983).

4. Molybdoenzymes

Molybdoenzymes are a major group of redox enzymes that catalyse many reactions including the oxidation of purines, pyrimidines and aldehydes and the reduction of molecular nitrogen and nitrate. Steinberg (1937) was the first to show a requirement of molybdenum in the utilisation of ammonium and nitrate by Aspergillus niger, while Nicholas & Nason (1954a) were the first people to demonstrate that molybdenum is an integral part of the nitrate reductase of Neurospora crassa. The best studied molybdoenzyme is nitrate reductase which is distributed in a wide range of organisms from lower microbes to the higher plants and animals, the majority are known to contain molybdenum in the prosthetic group. There are differences among the molybdoenzymes in their cellular location, function, and structure (Scazzocchio, 1980). Hydroxylases (aldehyde oxidase, xanthine oxidase and xanthine dehydrogenase) containing molybdenum have been discussed by Coughlan (1980).

The general chemical groups that can be attacked by molybdoenzymes are depicted in Figure 4. In most cases it has been proved that the electron transfer reactions in these enzymes which have the common property of transferring 2e^- and 2H^+ occur at the molybdenum centre (Branzoli & Massey, 1974a, b; Olson et al., 1974a, b). Table 1 lists some of the known molybdoenzymes and their derivation.

It is known that tungsten competes with the molybdenum in molybdoenzymes due to their similarity. Molybdenum, tungsten, and vanadium belong to Group VI B of the periodic table forming a group of chemically similar elements. The atomic radii of molybdenum, tungsten, and vanadium are 1.29, 1.3, and 1.22 Å^o respectively, while the ionic radii are 0.68, 0.58, and 0.64 Å^o.

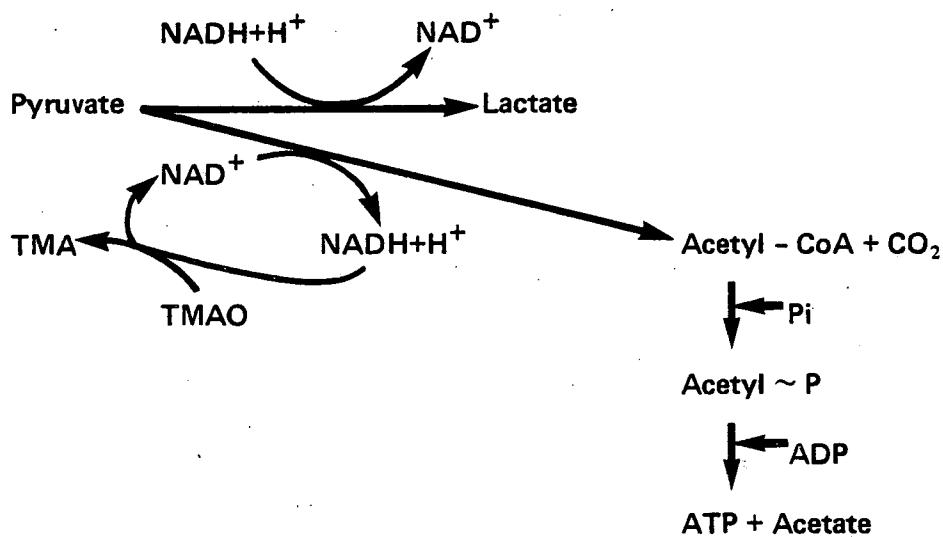


Figure 3. Scheme for TMAO re-oxidation of NADH_2 in *Rhodopseudomonas capsulata* (Cox, 1983)

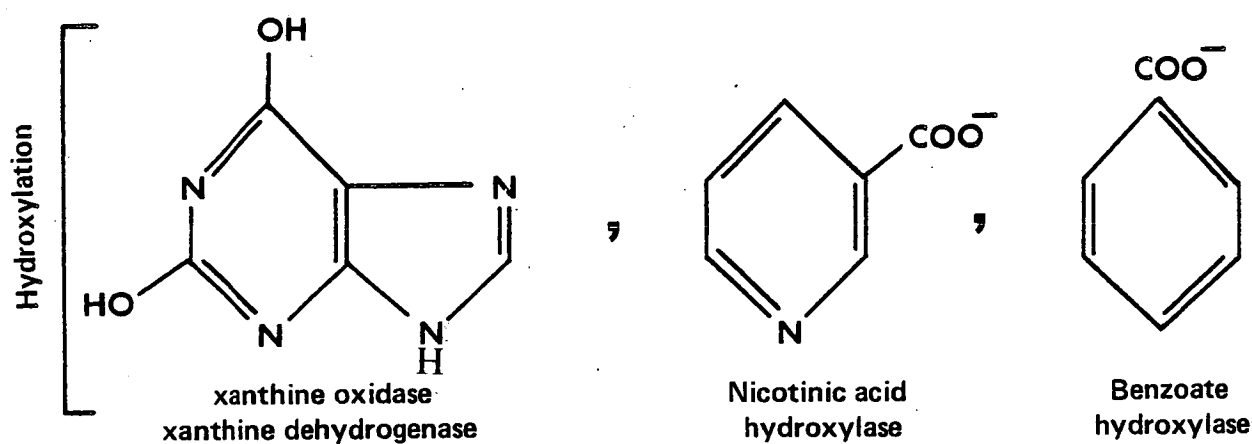
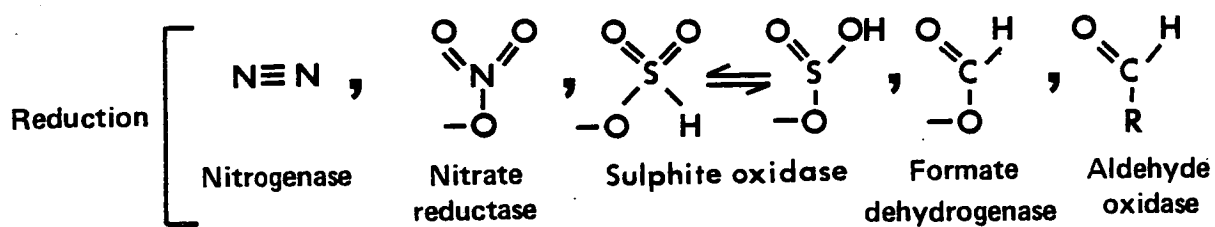


Figure 4. Chemical structures which are attacked by molybdoenzymes

Table 1. Molybdoenzymes

Enzyme	Organism	Evidences for molybdenum centre	References
Nitrate reductase	<u>Escherichia coli</u>	Enzyme purification	Taniguchi & Itagaki, 1960; Enoch & Lester, 1975.
		EPR spectra	Der vartanian & Forget, 1975.
		Genetic and nutritional studies	Lester & De Moss, 1971; Hackett & MacGregor, 1981; Blum & Poole, 1982.
"	<u>Scenedesmus obliquus</u>	Vanadium competition	Arnon & Wessel, 1953.
"	<u>Anabaena</u> spp	Vanadium competition	Arnon, 1958.
"	<u>Thiobacillus denitrificans</u>	Enzyme purification	Adams <u>et al.</u> , 1971.
"	<u>Aspergillus nidulans</u>	Nutritional and genetic studies	Arst <u>et al.</u> , 1970, 1982.
"	<u>Achromobacter fischeri</u>	Biochemical studies	Sadana & McElroy, 1957.
"	<u>Bacillus licheniformis</u>	Biochemical studies and enzyme purification	Van't Riet <u>et al.</u> , 1979.
"	<u>Nicotiana tabacum</u>	Biochemical and genetic studies	Mendel <u>et al.</u> , 1981.
"	<u>Spinacea oleracea</u>	Nutritional studies and tungsten competition	Notton & Hewitt, 1971, 1974.
"	<u>Chlorella vulgaris</u>	Biochemical studies and tungsten competition	Gewitz <u>et al.</u> , 1981.
"	Soyabean (<u>Glycine max</u>)	Biochemical studies and tungsten competition	Aslam, 1982.
"	<u>Azotobacter vinelandii</u> , <u>Azotobacter chroococcum</u>	Radioactive studies	Beneman <u>et al.</u> , 1973.

Table 1. (contd.)

Enzyme		Organism	Evidences for molybdenum centre	References
Nitrate reductase		<u>Pseudomonas denitrificans</u>	Biochemical studies	Radcliffe & Nicholas, 1970.
"	"	<u>Staphylococcus aureus</u>	Biochemical studies	Burke & Lascelles, 1975.
"	"	<u>Micrococcus denitrificans</u>	Biochemical studies and EPR spectra	Lam & Nicholas, 1969; Forget & Der vartanian, 1972.
"	"	<u>Paracoccus denitrificans</u>	Genetic studies and tungsten competition	Calder <u>et al.</u> , 1980; Burke <u>et al.</u> , 1980.
"	"	<u>Klebsiella aerogenes</u>	Inhibition studies and EPR spectra	Van't Riet <u>et al.</u> , 1975; Bosma <u>et al.</u> , 1978.
"	"	<u>Pseudomonas aeruginosa</u>	Enzyme purification	Carlson <u>et al.</u> , 1982.
"	" II	<u>Salmonella typhimurium</u>	Chlorate resistance	Barrett & Riggs, 1982b.
"	" A	<u>Proteus mirabilis</u>	Genetic studies and chlorate resistance	Oltmannet <u>et al.</u> , 1976a; Claassen <u>et al.</u> , 1982a.
"	" (W)*	<u>Nostoc muscorum</u>	Nutritional studies, tungsten competition, and chlorate resistance	Singh <u>et al.</u> , 1978.
Nitrogenase component	I	<u>Anabaena cylindrica</u>	Nutritional studies and tungsten competition	Fay & De Vasconcelos, 1974.
"		<u>Rhodopseudomonas capsulata</u>	Genetic studies and tungsten competition	Hallenbeck <u>et al.</u> , 1982.
"		<u>Azotobacter vinelandii</u>	Biochemical and genetic studies	Nagatani & Brill, 1974; Page & Collinson, 1982; Bishop <u>et al.</u> , 1982.
"		<u>Lobaria pulmonaria</u> , <u>Lobaria oregana</u>	Biochemical studies	Horstmann <u>et al.</u> , 1982.

Table 1. (contd.)

Enzyme		Organism	Evidences for molybdenum centre	References
Nitrogenase component I	(W)	<u>Nostoc muscorum</u>	Nutritional studies, tungsten competition, and chlorate resistance	Singh <u>et al.</u> , 1978.
"	(W)	<u>Rhodospirillum rubrum</u>	Nutritional studies	Paschinger, 1974.
FDH		<u>Escherichia coli</u>	Genetic, biochemical studies, and tungsten competition	Lester & De Moss, 1971; Enoch & Lester, 1972; Glaser & De Moss, 1971.
"			Enzyme purification	Enoch & Lester, 1975.
"		<u>Proteus mirabilis</u>	Genetic studies, chlorate resistance, and tungsten competition	Oltmann <u>et al.</u> , 1976b, 1979.
"		<u>Vibrio succinogenes</u>	Enzyme purification	Kröger <u>et al.</u> , 1979.
"	(W)	<u>Methanococcus vanniellii</u>	Nutritional studies	Jones, 1978; Jones & Stadtman, 1977.
"		<u>Paracoccus denitrificans</u>	Genetic studies and tungsten competition	Calder <u>et al.</u> , 1980; Burke <u>et al.</u> , 1980.
FDH (PMS) & FDH (BV)		<u>Escherichia coli</u>	Biochemical studies	Cox <u>et al.</u> , 1981.
"	(W)	<u>Clostridium thermoaceticum</u>	Biochemical and nutritional studies	Andreesen & Ljungdahl, 1973; Ljungdahl & Andreesen, 1976.
"		<u>Clostridium pasteurianum</u>	Enzyme purification	Scherer & Thauer, 1978.
"	(W)	<u>Clostridium acidurici</u> , <u>Clostridium cylindrosporum</u>	Nutritional studies	Wagner & Andreesen, 1977.

Table 1. (contd.)

Enzyme	Organism	Evidences for molybdenum centre	References
FDH (W)	<u>Clostridium formicoaceticum</u>	Radioactive labelling and nutritional studies	Leonhardt & Andreesen, 1977; Ljungdahl & Andreesen, 1976.
Biotin-d-sulphoxide reductase	<u>Escherichia coli</u>	Chlorate resistance and tungsten competition	Cleary & Dykhuizen, 1974; Del Campillo-Campbell & Campbell, 1982.
TMAO reductase	<u>Escherichia coli</u>	Tungsten competition, genetic studies, and chlorate resistance	Shimokawa & Ishimoto, 1979; Stewart & MacGregor, 1982; Takagi <i>et al.</i> , 1981.
" "	<u>Salmonella typhimurium</u>	Genetic studies and chlorate resistance	Kim & Chang, 1974; Davidson <i>et al.</i> , 1979; Cox & Knight, 1981.
Chlorate reductase	<u>Proteus mirabilis</u>	Enzyme purification and chlorate resistance	Oltmann <i>et al.</i> , 1976b; Oltmann <i>et al.</i> , 1979.
Tetrathionate reductase	<u>Proteus mirabilis</u>	Enzyme purification and chlorate resistance	Oltmann <i>et al.</i> , 1976b; Oltmann <i>et al.</i> , 1979.
NADPH : nitrate reductase	Plants, Chlorella, <u>Candida utilis</u>	Biochemical and nutritional studies	Losada, 1975/1976.
Nicotinic acid hydroxylase (W)	<u>Clostridium barkeri</u>	Biochemical studies and nutritional studies	Dilworth, 1983; Imhoff & Andreesen, 1979.
NADH dehydrogenase	Mammals	Biochemical studies and EPR spectra	Albracht & Slater, 1970.
NADH dehydrogenase	<u>Azotobacter vinelandii</u>	Radioactive labelling and nutritional studies	Der vartanian & Barmlett, 1970.

Table 1. (contd.)

Enzyme	Organism	Evidences for molybdenum centre	References
Molybdenum hydroxylases	Mammals, Birds, Insects, and Bacteria	Enzyme purification	Coughlan, 1980.
NADPH : nitrate reductase	<u>Neurospora crassa</u>	Enzyme purification and genetic studies	Garrett & Nason, 1969; Tomsett & Garrett, 1980.

* Tungsten (W) can replace molybdenum without negative effect (see text).

Molybdenum and tungsten are similar in being able to form complexes with strong ligands in the four and five oxidation states. In some organisms molybdate and tungstate enter the cell via a common transport system (Elliott & Mortenson, 1975). Although tungsten is a competitive inhibitor of molybdenum in some molybdoenzymes, interactions between molybdenum and tungsten are not always negative. Tungstate can replace molybdate in nitrate reductase of Chlorella fusca (Vega et al., 1971). The same is true for the nitrate reductase of spinach leaves (Notton & Hewitt, 1971, 1974) as judged by incorporation of ^{185}W into the enzyme. A similar observation has been made for nitrogenase component I in Azotobacter vinelandii and Azotobacter chroococcum, while vanadium cannot replace molybdenum (Benemann et al., 1973). Replacement of molybdenum by tungsten in nitrogenase reduce the amounts of component I protein (56% with respect to the normal component I protein), this might reflect replacement of molybdenum by tungsten causing other abnormalities beside losing the activity (Nagatani & Brill, 1974).

In E. coli tungsten can replace molybdenum in nitrate reductase and the resultant inactive form migrates faster than the normal form on sodium dodecylsulphate-polyacrylamide gel (Scott et al., 1979), the effect can be reversed by incubation with molybdate in the absence of protein synthesis. The same observations have been reported in Proteus mirabilis (Oltmann et al., 1979). Tungstate can compete with molybdate by inhibiting the uptake or utilisation of molybdate in Aspergillus niger (Higgins et al., 1956) and has been found to inactivate the chl A gene product in E. coli (Giordano et al., 1982). In addition tungsten can cause production of a rat liver sulphite oxidase apoprotein devoid of molybdenum cofactor but activity could be reconstituted by any source of molybdenum cofactor (Johnson et al., 1977).

In addition to consequences caused by replacement of molybdenum in molybdoenzymes, tungsten can exert several other effects such as the modification of processing of E. coli nitrate reductase and superinduction of NADPH : cytochrome c reductase in barley which can be suppressed by addition of high concentration

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of molybdate (Wray & Filner, 1970). Hewitt & Notton (1980) have reviewed this subject in detail.

Tungstate can have different effects towards different enzymes, Pinsent (1957) found that FDH of Coli-aerogenes bacteria grown on nitrate anaerobically was inactivated by tungstate while FDH produced under other conditions was not effected. Tungstate was found to inactivate only the inducible nitrate reductase in soyabean leaves while the constitutive nitrate reductase showed no effect (Aslam, 1982).

Tungstate can also play a positive role in the catalytic centres of enzymes from many sources. For example the FDH's of some obligate anaerobes, that catalyse oxidation of CO_2 to formate have higher activities in the presence of tungstate than molybdate (Ljundahl, 1976, 1980). Other enzymes such as nitrate reductase and nitrogenase are surprisingly active with tungstate. In Nostoc muscorum some mutants showed an absolute dependence on tungsten and chromium for both nitrate assimilation and nitrogen fixation; the authors suggested that whether molybdenum, tungsten, or chromium is the preferred element for the cofactor of either nitrate reductase or nitrogenase may depend on genetic factors (Singh et al., 1978). Rhodospirillum rubrum is able to fix nitrogen in medium supplemented with tungstate and cells are able to accumulate higher concentrations of tungstate than molybdate (Paschinger, 1974). H_2 -evolution can also occur in nitrogenase with tungsten in the prosthetic group. Recently, some tungstate resistant mutants of Azotobacter vinelandii have been isolated and found to have an alternative pathway for nitrogen fixation (Bishop et al., 1980, 1982; Riddle et al., 1982). In model chemical reactions both tungsten and molybdenum are able to catalyse nitrogen reduction to ammonia in acidic solution in a similar manner (Haight & Scott, 1964).

It is now known that molybdenum is present in the molybdo-enzymes as a part of a prosthetic group (molybdenum cofactor), whose structure is considered in sections 4.8 and 4.9.

4.1 Discovery of molybdenum cofactor

The existence of a molybdenum cofactor common to several enzymes was first suggested by Pateman *et al.* (1964). They isolated several mutants of *Aspergillus nidulans* unable to grow on nitrate or hypoxanthine as sole nitrogen source, but able to grow on other types of media and to synthesise the proteins for both nitrate reductase and xanthine dehydrogenase. They suggested that both enzymes shared a common cofactor and designated the mutated genes as cnx.

4.2 Genetics of molybdenum cofactor

In *Aspergillus nidulans* five unlinked loci designated as cnx AB, cnx E, cnx F, and cnx H are involved in the synthesis of molybdenum cofactor (Scazzocchio, 1980; Brill, 1980). However, recently another gene has been reported and designated as cnx J, the product might be involved in the regulation of cofactor level (Arst *et al.*, 1982).

In *N. crassa*, it has been found that at least four or possibly more genes control the synthesis and expression of molybdenum cofactor and consequently the activity of nitrate reductase (Sorger & Giles, 1965; Sorger, 1966). The nit-3 locus codes for the nitrate reductase apoprotein, while the nit-1 gene product is the molybdenum cofactor itself. However, production of complete active molybdenum cofactor also requires the function of three other genes, nit-7, nit-8, and nit-9 (Tomsett & Garrett, 1980, 1981; Tachiki & Nason, 1983).

4.3 Systems for molybdenum cofactor assay

Many systems have been used to detect the molybdenum cofactor from different sources, these are summarised in Table 2.

There are two main types of assay systems: those using acidification and those without acidification. Acid treatment of molybdoenzymes followed by neutralisation leads to loss of activity but acidified extracts when deproteinised are able to restore activity of nitrate reductase in the molybdenum cofactor mutant nit-1 of *N. crassa*. The most commonly used

Table 2. Molybdenum cofactor assay systems

A. Assays without acidification

Cofactor Source	Acceptor Molecule	Source and Form of Acceptor Molecule	Reference
<u>Neurospora</u> wild type or <u>nit</u> mutant other than <u>nit-1</u>	nitrate reductase	<u>Neurospora nit-1</u> extract	Nason <u>et al.</u> , 1970.
<u>E. coli</u> wild type	nitrate reductase	<u>Neurospora nit-1</u> extract	Nason <u>et al.</u> , 1971; Amy & Rajagopalan, 1979.
N ₂ or nitrate metabolizing bacteria	nitrate reductase	<u>Neurospora nit-1</u> extract	Ketchum & Sevilla, 1973; Ketchum & Swarin, 1973.
<u>Aspergillus</u> wild type or <u>nia D</u> mutant	nitrate reductase	<u>Neurospora nit-1</u> extract	Ketchum & Downey, 1975; Garrett & Cove, 1976.
Rat liver mitochondrial outer membrane	nitrate reductase	<u>Neurospora nit-1</u> extract	Johnson J.L., unpublished
<u>Aspergillus nia D</u> mutant	nitrate reductase	<u>Aspergillus onx</u> extract	Garrett & Cove, 1976.
<u>Nicotiana tabacum nia</u> mutant	nitrate reductase	<u>Nicotiana tabacum onx</u> extract	Mendel & Müller, 1978.
<u>E. coli</u> wild type	nitrate reductase	W-grown <u>Chlorella</u> extract	Amy N.K. & Solomonsen L.P., unpublished.
Rat liver homogenate	nitrate reductase	W-grown <u>Chlorella</u> extract	Amy N.K. & Solomonsen L.P., unpublished.
<u>Neurospora</u> wild type	sulfite oxidase	purified demolybdo sulfite oxidase	Johnson <u>et al.</u> , 1977.
<u>E. coli</u> wild type	sulfite oxidase	purified demolybdo sulfite oxidase	Johnson <u>et al.</u> , 1977.
Rat liver mitochondrial outer membrane	sulfite oxidase	purified demolybdo sulfite oxidase	Johnson <u>et al.</u> , 1977.

Table 2. (contd.)

3. Cofactor released by acidification

Cofactor Source	Acceptor Molecule	Source and Form of Acceptor Molecule	Reference
Acidified molybdoenzymes	nitrate reductase	<u>Neurospora nit-1</u> extract	Ketchum <u>et al.</u> , 1970; Nason <u>et al.</u> , 1971.
<u>E. coli</u>	nitrate reductase	<u>Neurospora nit-1</u> extract	Nason <u>et al.</u> , 1971; MacGregor & Schnaitman, 1972.
Acidified xanthine oxidase	nitrate reductase	<u>Neurospora nit-1</u> extract	Pienkos <u>et al.</u> , 1977
N ₂ metabolizing bacteria	nitrate reductase	<u>Neurospora nit-1</u> extract	Pienkos <u>et al.</u> , 1977
<u>Neurospora</u> wild type	nitrate reductase	W-grown <u>Chlorella</u> extract	Amy N.K. & Solomonsen, L.P., unpublished
Mo-grown spinach	nitrate reductase	W-grown spinach extract	Rucklidge <u>et al.</u> , 1976.
Acidified xanthine oxidase	nitrate reductase	W-grown spinach extract	Hewitt <u>et al.</u> , 1977.
Acidified xanthine oxidase	sulfite oxidase	purified demolybdo sulfite	Johnson <u>et al.</u> , 1977.

* After Johnson, 1980.

assay system is the use of nitrate reductase complementation of N. crassa nit-1 mutant, therefore a brief account about the nitrate reductase complex of N. crassa will be considered.

Wild type N. crassa can assimilate nitrate to ammonia via nitrite. The first step is a two electron transfer reaction whereby nitrate is converted to nitrite by NADPH : nitrate reductase, while the second step involves transfer of six electrons and is catalysed by nitrite reductase.

The assimilatory NADPH : nitrate reductase is a complex sulphhydryl protein containing a FAD, cytochrome b_{557} and molybdenum as components and the holoenzyme has a sedimentation coefficient of 7.9S (Garrett & Nason, 1969). The majority (98%) of this complex is found in the cytoplasm associated with cell wall-plasmalemma region and in the tonoplast membranes surrounding the vacuoles (Garrett & Nason, 1969; Roldan et al., 1982).

Garrett & Nason (1969) purified the enzyme. The native purified enzyme has a molecular weight of 228,000 daltons and under denaturing conditions dissociates into subunits with molecular weights of 130,000 and 115,000 daltons (Pan & Nason, 1978). Extensive studies have led to the formulation of the electron transport pathway shown in Figure 5 (Nason & Evans, 1953; Nicholas & Nason, 1954a, b; Nicholas et al., 1954; Garrett & Nason, 1967). One polypeptide chain appears responsible for the transfer of electrons from NADPH to FAD (and thence to cytochrome c) and the other for electron transfer from reduced FAD via molybdenum to nitrate (Sorger & Giles, 1965; Sorger, 1966). Antonie & Nason (1969) reported that the complex in wild type strain (STAL) contained the following activities: NADPH : nitrate reductase; $FADH_2$: nitrate reductase; MV^{++} : nitrate reductase and FAD dependent NADPH : cytochrome c reductase (cytochrome c, however, is not a component of the enzyme) in the constant ratio of 5:1:2:60. Selective inhibition studies indicated that the active sites for nitrate and cytochrome c reduction were different. The first part of the pathway is nitrate inducible, while the second part is constitutive (Nason et al., 1974).

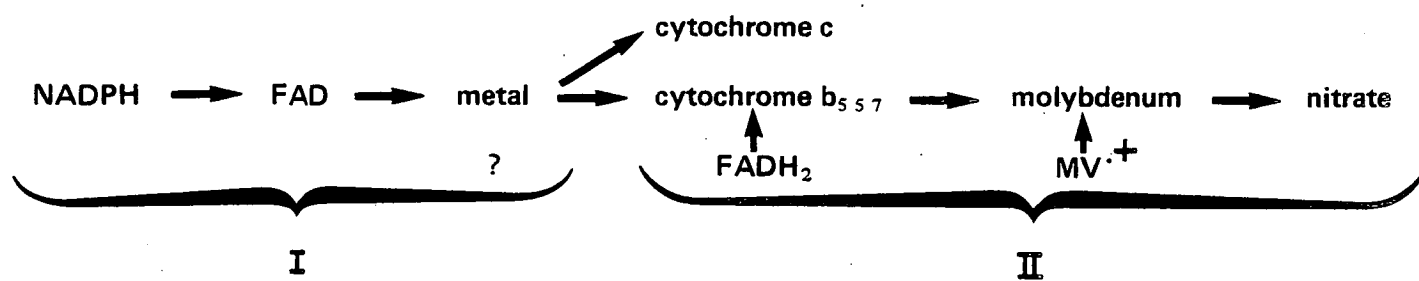


Figure 5. The nitrate reductase of *N. crassa*
(Nason *et al.*, 1974; see also text)

The nit-1 mutant of N. crassa possesses only inducible NADPH : cytochrome c reductase activity (Nason et al., 1970; Nason et al., 1971; Horner, 1983). However, the NADPH : nitrate reductase activity can be restored in extracts of induced nit-1 by mixing with uninduced wild type extract (Nason et al., 1971) or acid treated molybdoenzymes. Other potential sources of molybdenum such as molybdenum-amino acid complexes or molybdate (100 mM) are ineffective (Nason et al., 1974; Lee et al., 1974b). Addition of molybdate at high concentrations (10 mM) is necessary for the complementation process though this concentration has no effect on the activity of the wild type enzyme. The reconstituted enzyme is shown to incorporate ^{99}Mo from $^{99}\text{MoO}_4^-$; therefore the authors conclude that the nit-1 gene product is a structural component of the molybdenum cofactor or more specifically is the cofactor moiety which interacts with molybdate.

Before complementation NADPH : cytochrome c reductase shows a sedimentation coefficient on sucrose gradients of 4.5S but after complementation this is converted to a 7.9S species similar to the native enzyme. Simultaneously the associated activities of the wild type are restored and the reconstituted enzyme is similar to the native enzyme in molecular weight, substrate affinities, sensitivity to inhibitors and temperature. However, the associated activities are at variable ratios, so it has been named as Neurospora like assimilatory NADPH : nitrate reductase (EC 1.6.6.2) (Ketchum et al., 1970; Horner, 1983).

4.4 Function of molybdenum cofactor

The diversity of the reactions catalysed by molybdoenzymes suggests that molybdenum functions in a variety of chemical environments.

Two functions for the molybdenum cofactor have been investigated: a structural one and catalytic one. Molybdenum cofactor has a structural function as shown by the dimerisation of the polypeptides of NADPH : nitrate reductase of N. crassa nit-1 to form a 7.9S species from a 4.5S species. A similar phenomenon occurs in Aspergillus nidulans (Lewis, 1975). The

catalytic function is as an electron carrier (Nason et al., 1971; Alikulov et al., 1980). Only the first function can be performed by an inactive molybdenum cofactor. The structural function of molybdenum cofactor cannot be ruled out in bacterial molybdoenzymes (see section 5).

4.5 Forms of molybdenum cofactor

The classification of molybdenum cofactor forms depends on the criteria used e.g. structure, response to chemical and physical agents, control of biosynthesis etc. In N. crassa only one constitutive form was synthesised independently of nitrate reductase induction and this was absent in the nit-1 mutant (Lee et al., 1974b). Addition of molybdate to the growth medium did not enhance the synthesis of molybdenum cofactor or repair the defect in the nit-1 strain. However, this finding is not general to all systems since Mendel et al. (1982b) showed that in Nicotiana tabacum there were both a constitutive molybdenum cofactor and a nitrate-induced molybdenum cofactor; the increase in inducible molybdenum cofactor was followed by an increase in nitrate reductase activity.

Shah & Brill (1977) and Pienkos et al. (1977), in an investigation on the structural components of molybdenum cofactor, isolated the molybdenum cofactor and iron-molybdenum cofactor from molybdoenzymes and nitrogenase component I respectively with N-methylformamide under anaerobic conditions. They showed that the molybdenum : iron : sulphur ratio in nitrogenase was 1:8:6 and that the iron-molybdenum cofactor was able to reconstitute nitrogenase in Azotobacter and Klebsiella but not N. crassa nit-1 nitrate reductase, while the molybdenum cofactor showed the opposite behaviour. Differences between the two cofactors have been compared by Alikulov et al. (1980).

Dubourdieu et al. (1976) studied the subject from another point of view. Using E. coli K-12 grown with ⁹⁹Mo on nitrate they found the same forms in both particulate and soluble fractions of low protein content exhibited very high radioactivity and were distinct from ⁹⁹Mo labelled proteins like FDH and nitrate reductase. They suggested that the former could represent

molybdenum storage molecules.

Empty cofactor was formed when, E. coli was grown on molybdenum free medium; this cofactor was found to dimerise N. crassa nit-1 nitrate reductase. The reconstituted enzyme had no activity unless molybdate was added to the complementation mixture (Amy & Rajagopalan, 1979). The authors pointed out the possibility of the presence of two pools of molybdenum cofactor in E. coli: one represented by a small dialysable, low molecular weight cofactor and the other by a cofactor bound to a high molecular weight species excluded from Sephadex G-25. The smaller cofactor might have been formed by dissociation during extraction. Different forms can be produced according to the purification procedures used; for example a number of forms of xanthine dehydrogenase have been copurified including a desulpho form unstable even in fresh samples and identified by its characteristic Mo V signals in EPR spectra. The so called "rapid" molybdenum centre was found in the functional enzyme, while the "slow" molybdenum centre was found in the desulpho enzyme. The structural difference between the two is that the former has a thiolate group which is replaced by an oxo-form in the "slow" molybdenum centre (Gutteridge & Bray, 1980). Both forms showed different behaviour in potentiometric titration (Porras & Palmer, 1982). Different forms of molybdenum cofactor may also arise for other reasons e.g. differences in chemical or physical treatments (see section 4.9).

4.6 Properties of molybdenum cofactor

The molybdenum cofactor from many sources is able to reconstitute nitrate reductase in N. crassa nit-1. It is unlikely that the molybdenum-containing component would occur as a large, identical molybdenum polypeptide chain in organisms ranging from microbes to mammals. Therefore the highly specific phenomenon of recognition of multimeric enzymes would suggest that the molybdenum-containing component is occurring as an identical and immutable molybdenum peptide or as a small organic molecule.

Acid treatment of molybdoenzymes is known to dissociate multimeric proteins to subunits so that the molybdenum cofactor is exposed to react with N. crassa nit-1 nitrate reductase or other demolybdoenzymes. Lee (1978), suggested that acid treated molybdenum cofactor is protein free from I¹²⁵ labelling experiments. Further confirmation came from the observation that no inactivation occurred on treatment with proteolytic enzymes (Stouthamer, 1976). Lee (1978) found that the molybdenum cofactor extracted from xanthine oxidase had a molecular weight in the range of 700-1000 daltons. Similarly N. crassa molybdenum cofactor had a molecular weight of 1000 or less and was a small dialysable molecule, highly labile to inactivation by oxygen and with an absolute requirement for molybdate to give in vitro assembly of N. crassa nit-1 nitrate reductase (Garrett & Nason, 1969; Nason et al., 1971). Active molybdenum cofactor can be preserved by keeping reduced conditions: either anaerobiosis alone or with the addition of particular reducing agents. Under such conditions Alikulov et al. (1980) showed that molybdenum cofactor can bind molybdate even after passage through a Sephadex G-75 column. Inactivation of molybdenum cofactor could be avoided partially by adding high concentrations of molybdate (Stouthamer, 1976).

In most cases both released and bound molybdenum cofactor was active at neutral pH, while alkaline or acidic conditions caused loss of activity not restored by subsequent neutralisation (Alikulov et al., 1980). On the other hand molybdenum cofactor from E. coli was relatively stable from pH 3 to pH 11 but the activity was sharply reduced below pH 3 (Amy & Rajagopalan, 1979).

The structure of molybdenum cofactors from different molybdoenzymes cannot be absolutely identical since the molybdenum centres in Mo-hydroxylases contain a cyanolysable sulphur atom as in xanthine oxidase and xanthine dehydrogenase which is shown by EXAFS and EPR studies to be due to Mo=S structure. The sulphur moiety can react with cyanide in the functional molybdenum-centre to yield thiocyanate (Barber et al., 1982), while no such sulphur atom

has been found in other molybdoenzymes like nitrate reductase.

Another common feature of molybdenum cofactor from different sources is that treatment with oxidising agents accelerates the appearance of specific fluorescence and loss of reconstitution ability, while strict anaerobic conditions prevent this (Rajagopalan *et al.*, 1982).

In addition to oxygen other agents such as arsenite and ferricyanide can inactivate the molybdenum centre of molybdoenzymes due to stabilisation of molybdenum in the Mo V state (Rajagopalan *et al.*, 1968; Rajagopalan & Handler, 1964; Coughlan *et al.*, 1969; Kessler & Rajagopalan, 1974; Barber & Siegel, 1983; George & Bray, 1983).

4.7 Molybdenum cofactor purification

The purification of molybdenum cofactor has been attempted by releasing the molybdenum cofactor moiety from molybdoenzymes after their denaturation. The latter can be achieved either by thermal denaturation (Alikulov *et al.*, 1980), acidification (Claassen *et al.*, 1982b) or by limited tryptic cleavage as used for sulphite oxidase (Johnson & Rajagopalan, 1977).

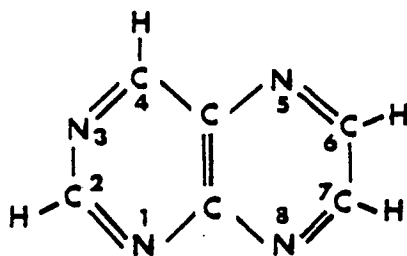
Two main approaches can be followed: the first is purification of molybdenum cofactor in an active form under anaerobic conditions in the presence of reducing agents (Lee, 1978; Alikulov *et al.*, 1980) or by using rapid purification like HPLC to overcome time-dependent inactivation (Claassen *et al.*, 1982b). The second is the isolation of molybdenum cofactor in an inactive form (Hainline, 1980).

Inactivation of molybdenum cofactor as mentioned previously leads to an increase in fluorescence with characteristics similar to that of some pteridines (Johnson *et al.*, 1980). The chemistry of pteridines is therefore discussed briefly in the following section

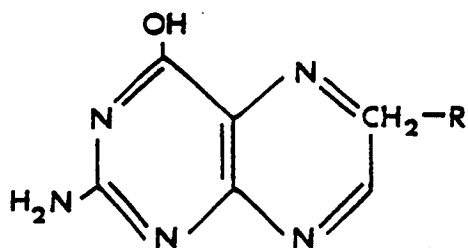
4.8 Pteridines

Pteridine is a hypothetical aromatic compound with the formula $C_6H_4N_4$ in configuration and numbering system shown in Figure 6a.

When this structure is substituted with an amino group at

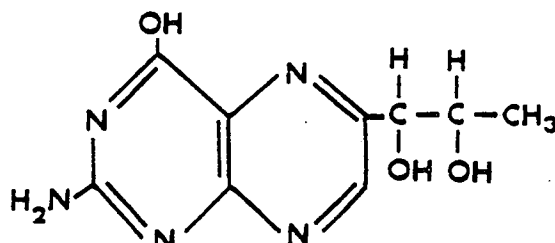


a) The structure and numbering system used for pteridines.

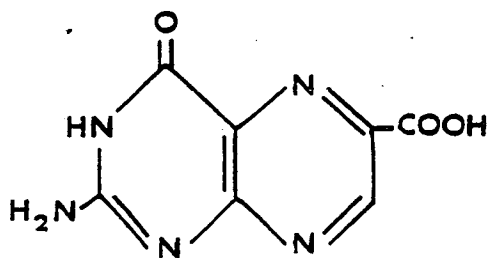


b) Conjugated pterins.

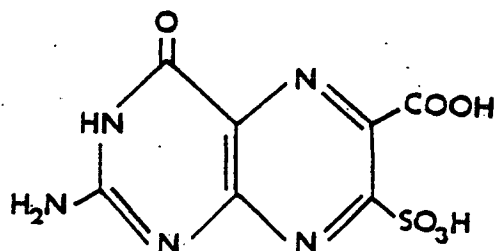
R = a *p*-aminobenzoic acid or a *p*-aminol glutamic acid residue.



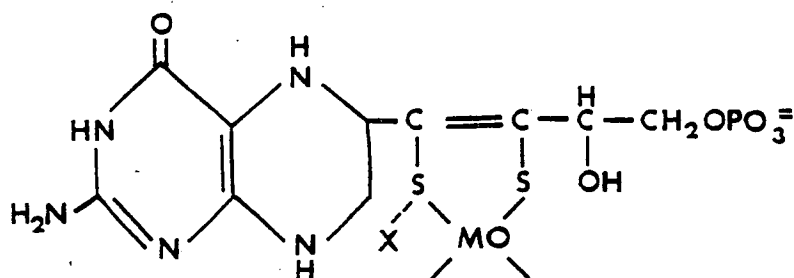
c) Biopterin.



d) Pterin-6-carboxyl.



e) 6-carboxy-7-sulphoxy pterin.



f) molybdenum cofactor
(proposed structure)

Figure 6. Pteridines and pterins

the 2-position and a hydroxy group at the 4-position, the derivatives are given the trivial name of pterins. Pterins can exist in a conjugated form as in the folic acid family or unconjugated as in bipterin (Figure 6b and c). Different pterin structures are mainly due to different substituent groups at the 6-position.

Pterins exist naturally as pigments of some organisms e.g. butterflies contain xanthopterin and leucopterin, while Drosophila melanogaster contains sepiapterin. Different forms of pterins have been identified as enzyme cofactors e.g. in methanol dehydrogenase of Methylosinus sporium and Methylosinus trichosporium (Urushibara & Forrest, 1970). The cofactor involved in the enzymatic conversion of phenylalanine to tyrosine in rat liver was identified as a dihydropterin (Kaufman, 1963; Kato & Sueoka, 1983). This natural pterin could be substituted by the artificial compound 2-amino-4-hydroxy-6-methyl pteridine only in its reduced form (Kaufman, 1959; Kaufman & Levenberg, 1959). The cofactor of phenylalanine hydroxylase of Pseudomonas species (ATCC 11299a) was identified as 2-amino-4-hydroxy-6-(L-threotrihydroxy propyl) pteridine (Guroff & Strenkoski, 1966; Guroff & Rhoads, 1969).

The synthesis of pterins has been demonstrated to begin with GTP which provides all the carbon atoms for the pterin ring (Albert, 1957; Brown, 1971). Bipterin and folic acid share the initial steps of pterin synthesis in E. coli, Lactobacillus plantarum, Pseudomonas species (ATCC 11299a) and some animals. Folic acid is synthesised in bacteria and plants and bipterin in animals; so animals need folic acid to be supplied in their diet.

4.9 Proposed molybdenum cofactor structure

The extreme instability of the molybdenum cofactor has made structural studies on this species extremely difficult. Pterins usually undergo oxidative photodecomposition particularly in dilute alkaline solution with appearance of an intense blue UV fluorescence. Denaturation of sulphite oxidase and milk xanthine

oxidase by boiling in 5% sodium dodecylsulphate resulted in gradual emergence of fluorescence, while non-molybdoenzymes failed to yield such fluorescent materials (Johnson et al., 1980).

Working on the molybdenum cofactor in an active state, Alikulov and coworkers showed that the molybdenum cofactor of milk xanthine oxidase and nitrate reductase of lupine bacteroids consisted of two parts after gel filtration. An aromatic compound associated with amino acid residues eluted after the salt fraction. Linkage was through an amino group and was disrupted on exposure to oxygen. Analysis of the peptide showed that it was composed of aspartic acid, threonine, serine, lysine and alanine in the ratio 2:1:5:3:1. The second part of the molybdenum cofactor was a protein with a molecular weight of 25,000 daltons as determined by gel filtration. This polypeptide chain was assumed to be responsible for the binding of molybdenum cofactor to the molybdoenzyme.

The production of a stable, inactive fluorescent molybdenum cofactor has been exploited by Hainline (1980) who isolated and identified the molybdenum cofactor in an inactive form rather than as an active form, on the basis that identification and structural characterisation of the inactive form would give an indication about the structure of the parent molecule. Sulphite oxidase, xanthine oxidase and Chlorella nitrate reductase were used as sources for molybdenum cofactor. Different procedures for denaturation and isolation of the molybdenum cofactor were used; including boiling with 5% sodium dodecylsulphate, acid treatment, extraction with aqueous pyridine and treatment with guanidine hydrochloride. The latter proved to be the best method. Two forms were obtained; Form A was obtained with iodine oxidation and form B with ferricyanide oxidation. Form A on oxidation with permanganate in alkaline solution yielded pterin-6-carboxyl (Figure 6d) which is similar to the oxidation product of 6-alkyl pterins (Rajagopalan et al., 1982). Chromatography of form A on a quaternary amino ethyl (QAE) ion exchange column, resulted in fractionation of form A into two species: one an acidic species was eluted with 10 mM

HCl, the other a neutral species was eluted with 10 mM acetic acid. The acidic form showed fluorescence spectra similar to biopterin except with bathochromic shifts of 10-20 nm. Both acidic and neutral forms gave single spots on cellulose TLC but with different relative mobilities that indicated a structural similarity. The acidic form was shown to contain a phosphate group since it was very sensitive to alkaline phosphatase; estimation of inorganic phosphate suggested that there was one molecule of phosphate per molecule of pterin and that these were associated with one molybdenum atom in the native sulphite oxidase. The neutral form unlike the acidic form was sensitive to periodate indicating the presence of vicinal hydroxyl group.

Form B produced on ferricyanide oxidation was found to be very sensitive to alkaline phosphatase, suggesting the presence of a monophosphate ester. Permanganate oxidation in alkaline solution gave 6-carboxy-7-sulphoxy pterin (Figure 6e) which resembled the end product of permanganate oxidation of deoxyurothione, the synthetic analogue of urothione which is a naturally occurring sulphur containing pterin.

The results have been interpreted as the cofactor possessing a sulphur bound to the pterin ring at the 7-position and perhaps elsewhere in the molecule. The side chain was tentatively identified as 1' dehydro, 3'-oxo-,4'-phospho-buten-4-ol. The presence of functional groups such as hydroxyl and phosphate on the intact native cofactor might allow stabilisation of molybdenum in the geometry needed for interaction with substrate as well as other prosthetic groups within each enzyme, and this close proximity of the reduced pterin ring might also serve to influence the electron acceptor or donor activity of the molybdenum centre.

In further studies, form B was compared with urothione and a relationship suggested between deficiency of molybdenum cofactor in patients and the absence of urothione in their urine. Hence a structure for molybdenum cofactor has been proposed as shown in Figure 6f (Johnson & Rajagopalan, 1982).

Recently, the molybdenum cofactor isolated from E. coli nitrate reductase has been shown to be similar to urothione (Ishizuka et al., 1983). A sulphur atom may be present in the iron-molybdenum cofactor of nitrogenase component I since some thiomolybdate compounds have been isolated from the nitrogenase of some clostridial species (Zumft, 1978).

5. Chlorate Resistant Mutants

The study of chlorate resistant mutants has proved to be a powerful tool for investigation of the metabolism of molybdenum in plants and microorganisms. Mutants of E. coli able to grow anaerobically in the presence of chlorate have been isolated and most lack nitrate reductase and other molybdoenzymes. These mutants designated chl or nar can be also isolated as strains unable to grow on nitrate as terminal electron acceptor (Venables & Guest, 1968; Ruiz-Herrera et al., 1969), and both methods can be used on the same mutagenised culture (Van Hartingsveldt et al., 1971; Glaser & De Moss, 1972).

Chl mutants have been extensively studied in E. coli. To date eight different classes have been isolated designated chl A to chl I. The genetic loci of some of these mutants showed no linkage, but the results obtained by Casse (1970) that the high yield (93%) of spontaneous chl mutants were linked to the gal-bio region indicated that more than one gene mapped in this region (chl A, D and E) (Bachmann & Low, 1980). Not all mutants lacking nitrate reductase are resistant to chlorate, this might indicate that chlorate resistance reflects only severe alterations in molybdoenzymes such as production of intact molybdenum cofactor and its proper binding to molybdoenzymes. This is discussed fully in relation to each class of chl mutants in the following sections.

Chl A

Chl A or nar A is a pleiotropic mutation affecting nitrate reductase, chlorate reductase, and formate hydrogenlyase activities (Venables, 1972; MacGregor & Schnaitman, 1971). These mutants produced the α and β subunits of nitrate reductase (MacGregor, 1975c) but the enzyme was poorly associated with the membrane and comprised only 0.4% of the total membrane protein compared with 3.5-4.0% for the wild type. At the same time the cytoplasm of these mutants had immature polypeptides, α' and β' which were absent in the wild type (Giordano *et al.*, 1980b). The immature α' and β' polypeptides existed in the wild type cytoplasm just before the assembly of nitrate reductase and underwent post-translational modification during the process of translocation or insertion into the membrane which included the covalent addition of fatty acids. The mature β subunit migrates normally on sodium dodecylsulphate-polyacrylamide gel compared to the faster migrating β' subunit (MacGregor & McElhaney, 1981; Chaudhry & MacGregor, 1983a). The chl A gene product has been cloned and shown to be a protein with molecular weight of 81,000 daltons (Giordano *et al.*, 1981).

The FDH produced in chl A mutants during anaerobic growth on nitrate had normal subunits but was inactive and the inactive enzyme was equally distributed between the cytoplasm and the membrane fraction as shown by rocket immunoelectrophoresis (Terrière *et al.*, 1981; Giordano *et al.*, 1982). Molybdenum cofactor production by this mutant has been studied by Amy (1981) who showed that the molybdenum cofactor of this mutant had low affinity for binding molybdenum as a high molybdate concentration was required in the reconstitution of NADPH : nitrate reductase activity of N. crassa nit-1 (30 mM molybdate compared with 10 mM for the wild type). However, the molybdenum cofactor of this mutant was still able to perform the structural function of dimerising N. crassa nit-1 nitrate reductase.

Chl B

Chl B or nar B is similar to chl A in its pleiotropy. The chl B gene product has been extensively studied by Riviere *et al.* (1975). It is a protein of molecular weight 35,000 daltons with no lipid attached and has been termed F_A . The F_A factor polymerises readily upon isolation and this leads to inactivation. Transferase is another name suggested for the chl B gene product. Absence of this factor in chl B mutants resulted in some abnormalities such as the reduction in cytochrome b content (43% of wild type content) but the α and β subunits of nitrate reductase were present in a similar ratio to that of the wild type (Giordano *et al.*, 1982). The function of this factor seems to be incorporation of molybdenum cofactor into the enzyme and probably the membrane using the molybdenum cofactor peptide as substrate (Dubourdieu *et al.*, 1976).

Immunoelectrophoretic studies showed that only a small fraction of nitrate reductase was incorporated into the membrane of chl B mutants but that the protein incorporated had the same mobility as the wild type. However, the soluble fraction contained 70% nitrate reductase protein with normal mobility but in an inactive form; the remaining 30% migrated rapidly (Giordano *et al.*, 1982). A crude extract of this mutant exhibited high activity in complementation of N. crassa nit-1 nitrate reductase, sometimes even higher than the wild type.

As a result of extensive studies of chl A and chl B mutants many authors suggest that the chl B gene product is required for membrane insertion as well as enzyme activation, while the chl A gene product is required for α' and β' subunits conversion (Giordano *et al.*, 1980b). This point has been investigated by reconstitution studies between these mutants. After reconstitution of crude extracts from chl B and chl A strains, the slow and fast migrating species still existed but all became active and the faster migrating form was found to have a reduced content of α subunit (Giordano *et al.*, 1982). Although after complementation, nitrate reductase only comprised a low percentage (0.93%) of the total particulate reconstituted

protein, the reconstituted vesicles were found to incorporate other proteins such as ATPase and cytochrome $b_{556}^{NO_3}$ and were capable of energy-linked functions as judged by quenching of acridine dye fluorescence. The orientation of the reconstituted vesicles was found to be inside-out with respect to the original cell (Giordano *et al.*, 1980b). Similarly, FDH was found to be reconstituted in an active form after mixing the cytoplasm or crude extract of the two mutants under defined conditions (Terrière *et al.*, 1981).

Reconstitution experiments, using the soluble fraction from chl A and chl B strains, have provided a good model for understanding the nitrate reductase complex system and probably other molybdo-enzyme systems but do not mimic *in vivo* assembly of this system since it has been found by MacGregor & Schnaitman (1974) that reconstitution only occurred under certain conditions: namely when the soluble lipoprotein fragments were small enough to make catalytic sites available and exposed to the molybdenum cofactor and the temperature was below the transition temperature of the membrane lipids.

Chl C

While chl A and chl B mutations have been mainly investigated by biochemical methods, chl C mutations have been investigated principally by genetic analysis. The chl C gene is very close to the trp region on the E. coli linkage map. Since chl C mutants were only affected in the formate : nitrate electron transport chain Guest (1969) inferred that chl C coded for the α subunit of nitrate reductase; this contains the nitrate reducing site (Chaudhry & MacGregor, 1983a). Mutants in this class showed varying sensitivity to chlorate perhaps indicating that nitrate reductase and chlorate reductase were not identical in structure (Glaser & De Moss, 1972). As some chl C mutants were temperature sensitive it was suggested that the chl C gene codes for at least part of the polypeptide subunits of nitrate reductase and that the locus might include other genes (De Moss, 1978). On the other hand, some chl C mutants showed fragmented α polypeptides and this has been

attributed to proteolysis (MacGregor, 1975c). In a recent report, the chl C region was considered to have an operon structure, new mutants were isolated and used to investigate nitrate reductase synthesis and regulation. It has been found that the chl C operon codes for both α and β subunits of nitrate reductase (Stewart, 1982; Stewart & MacGregor, 1982; Edwards et al., 1983).

Chl D

The characteristic feature of chl D mutants is their ability to grow anaerobically on nitrate when the medium is supplemented with high levels of molybdate (Glaser & De Moss, 1971). Therefore, the chl D gene product might be required for molybdate transport across the membrane, involved in the initial stages of molybdenum association, or be required to ensure that molybdenum is in the correct redox state for insertion into apoproteins. In this it is similar to the cnx E gene in Aspergillus nidulans (Arst et al., 1970). However, the high level of molybdate can circumvent the defect only in vivo, i.e. using intact cells with lag phase extended to 30-40 min (Speral & De Moss, 1975).

Point and deletion chl D mutants showed different phenotypes indicating that chl D mutants are of two types. However, both FDH and nitrate reductase polypeptides were found in the membrane fraction of chl D strains (Giordano et al., 1980a).

The chl D gene product is also apparently required for accumulation of inactive demolybdo nitrate reductase when cells were grown on nitrate in the presence of tungstate (Speral & De Moss, 1975). In a class of cnx mutants of Nicotiana tabacum, similar in phenotype to the chl D mutants the defect appears to reside in a lack of molybdenum as a catalytically active ligand metal for the cofactor, while the structural moiety of the molybdenum cofactor is unimpaired (Mendel et al., 1981).

Chl E

Chl E mutants have pleiotropic defects in electron transport components and cover a variety of phenotypes (Venables, 1972; MacGregor & Schnaitman, 1971). Immunological studies showed that some strains have both FDH protein and activity but lost nitrate reductase protein and activity, while others showed the opposite behaviour (Graham *et al.*, 1980). This might indicate that there is more than one gene in this locus which maps in the bio region of the E. coli genome at 18 min.

An early report suggested that the chl E gene coded for the γ subunit of the nitrate reductase complex (cytochrome $b_{556}^{NO_3^-}$) (MacGregor & Schnaitman, 1971), but later it was found that the haem-deficient mutant A1004a possessed BV^{++} linked : nitrate reductase activity (Jones & Garland, 1977) which was absent in some chl E strains.

Chl E mutants have also been isolated by transposon insertion techniques; all were chlorate resistant, failed to produce gas and appeared to be defective in TMAO reductase. They produced normal α , β , and γ subunits of nitrate reductase except that the β subunit migrated faster on sodium dodecyl sulphate-polyacrylamide gel electrophoresis due to incomplete post-translation modification. All these mutants behaved similarly to chl A in reconstitution experiments with other chl mutants and were phenotypically similar to chl A strains. The conclusion from this study was that the chl E gene product is required for insertion of molybdenum cofactor into the nitrate reductase enzyme (Stewart & MacGregor, 1982).

Chl F

Chl F mutants were first isolated by Glaser & De Moss (1972) but they are not well characterised. These mutants showed 50% of wild type activity of nitrate reductase and were unable to reduce formate. Hence it was suggested that the chl F gene codes for the FDH apoprotein. Guest (1969) suggested that at least one polypeptide of FDH is coded for by a gene in the

chl C region and that any chl C mutation exerts a polar effect on its expression and this may be the reason for reduced FDH activity.

Chl G

One mutant was originally isolated by Glaser & De Moss (1971) and had 20% of the wild type activity for overall transfer for formate to nitrate. Later it was shown that the mutant had FDH activity but reduced nitrate reductase activity although the nitrate reductase subunits were formed normally (Jenkins *et al.*, 1979).

Chl G mutants have been isolated by phage insertion and the new isolates showed full chlorate resistance, synthesised only 1.5% of wild type nitrate reductase and were defective in the expression of TMAO reductase. Addition of high molybdate (10 mM) to the growth medium enhanced the activity of several molybdoenzymes including biotin-sulphoxide reductase (Del Champillo-Campbell & Campbell, 1982). The authors suggested that chl G provides another component in addition to the chl D gene product that is required for activation of nitrate reductase (or other molybdoenzymes) at low concentration of molybdate (Stewart & MacGregor, 1982).

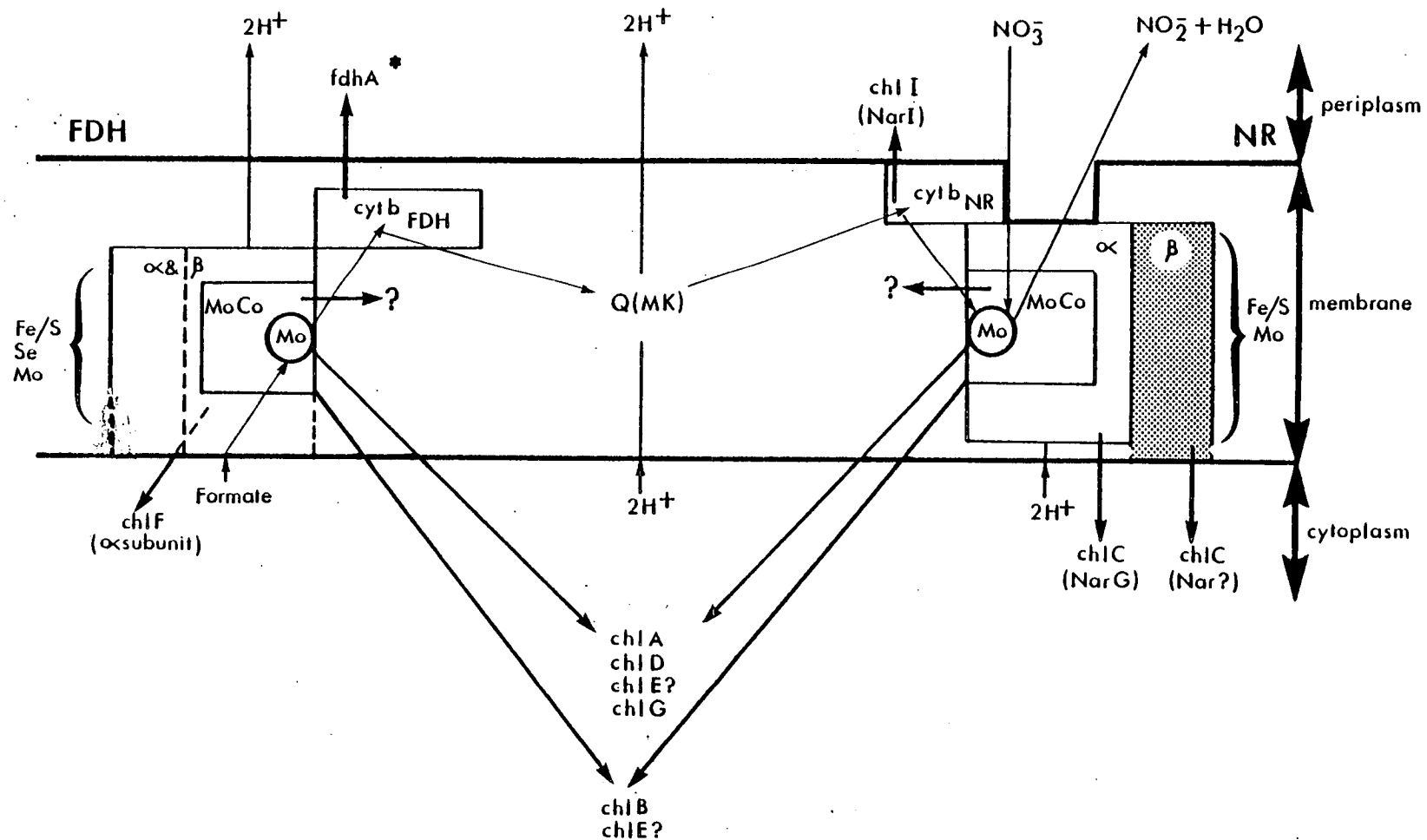
Chl I

It appears that two b-type cytochromes are induced when E. coli is grown on nitrate anaerobically as mentioned previously: one is cytochrome b_{NR} and the other cytochrome b_{FDH} (Enoch & Lester, 1974). A novel class of chlorate resistant mutants have been isolated and designated as chl I: they have reduced level (50%) of cytochrome b which is assumed to be cytochrome b_{FDH} , while cytochrome b_{NR} is absent. Despite their chlorate resistance these mutants have nitrate reductase activity and this has been attributed to the fact that a physiological donor (cytochrome b_{NR} in this case) is required to produce sufficient activity for chlorite production (Orth *et al.*, 1980). Using

Mu cts insertions, chl I was found as a part of the chl C operon; mutations in chl C showed a strong polar effect on the expression of chl I supporting the hypothesis that both genes are in the same transcriptional unit. As insertion of Mu cts in the chl I gene led to an increase in the distance between the trp locus and the chl C locus the gene order must be trp-chl I-chl C (Bonnefoy-Orth et al., 1981).

In conclusion, the pleiotropic loss of molybdoenzyme activities is not entirely due to loss or defect in the molybdenum cofactor but in other factors influencing the production of intact cofactor; accordingly the pleiotropic effects and the alterations in cytoplasmic membrane proteins or other corresponding cell fraction would be - secondary effects.

From the above discussion the following figure could be proposed (Figure 7) to illustrate the organisation of the nitrate reductase complex in the E. coli membrane and the probable gene-product relationships.



FDH, formate dehydrogenase; Fe/S, non-haem iron sulphur protein; Se, selenium; Mo, molybdenum; Cyt, cytochrome; Q, quinone; MK, menaquinone; MoCo, molybdenum cofactor; NR, nitrate reductase
 * (see Hackett & Bragg, 1983a, b)

Figure 7: Nitrate reductase complex of *E. coli* (see also Haddock & Jones, 1977; Stouthamer *et al.*, 1980)

CHAPTER 2

MATERIALS AND METHODS

1. Chemicals and Media

All chemicals were "AnalaR" and were generally obtained from Sigma London Chemical Company Ltd., (Poole) or British Drug Houses (MacFarlane Robson, Glasgow). Ready made media were obtained from Oxoid Ltd., (Basingstoke).

Hydrogen and oxygen-free nitrogen (White spot) were obtained from BOC Ltd., Glasgow.

2. Buffers

2.1 TMAO buffer

0.1M phosphate buffer (KH_2PO_4), pH 6.5 containing 0.1M NaCl and 1 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$.

2.2 MacLeod buffer (Martin & MacLeod, 1971)

10 mM Tris-HCl, pH 7.0 containing 0.3M NaCl, 50 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 10 mM KCl.

2.3 Preparation buffer

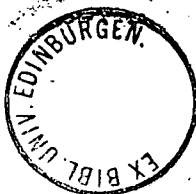
This buffer was used for preparing N. crassa crude extract, 0.1M phosphate buffer, pH 7.3 containing 1 mM Dithiothreitol (DTT); 0.5 mM EDTA; and 1 mM phenylmethanesulfonyl fluoride (PMSF); the latter was dissolved in a very small volume of absolute alcohol and added to the buffer just before use.

2.4 Breaking buffer

This buffer was used to prepare crude extract of A. putrefaciens when it was required as molybdenum cofactor source and was composed of 10 mM phosphate buffer (K_2HPO_4), pH 7.4 containing 0.5 mM EDTA.

2.5 Reaction buffer

This was used in the assay for NADPH : nitrate reductase, 0.1M phosphate buffer (K_2HPO_4), pH 7.4.



2.6 Formate dehydrogenase solubilisation buffer

20 mM Tris-HCl buffer, pH 7.3 containing 1 mM EDTA.

3. Sterilisation Methods

Growth media were autoclaved for 15 min at 103.4 KPa.

Sugar solutions were autoclaved for 15 min at 68.9 KPa.

Thermolabile compounds like vitamins were sterilised by filtration (Nuflow membrane filters, 0.22 μ m pore size, Oxoid Ltd., Basingstoke).

4. Bacterial Culture

4.1 Bacterial strains

Alteromonas spp NCMB 400, NCMB 1520, NCMB 1521, NCMB 1733, NCIB 10472 were obtained from the National Collection of Marine Bacteria (NCMB) held at the Torry Research Station, Aberdeen. E. coli, Pseudomonas aeruginosa, Bacillus subtilis were obtained within the Department of Microbiology.

4.2 Maintenance of cultures

Stock cultures of all strains were stored at 4°C on nutrient agar containing 2% NaCl slopes and subcultured every 1-2 months.

4.3 Growth media

(a) The medium described by Wood & Baird (1943) was generally used and contained (gl^{-1}): NaCl, 20; K_2HPO_4 , 1; Oxoid peptone, 5; Oxoid yeast extract, 2; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1. The medium was adjusted to pH 6.5 and autoclaved. For agar plates the medium was solidified by the addition of 15 gl^{-1} Oxoid Agar No. 3.

(b) Nutrient agar (Oxoid) was used as a solid medium after addition of 20 gl^{-1} NaCl (This medium will be referred to hereafter as NANA).

(c) Nutrient broth (Oxoid) was prepared by rehydration of Oxoid product and salt was added at 20 gl^{-1} .

(d) Maltose : TMAO medium, basically this medium prepared as in (a) but the peptone and yeast extract were reduced to 1/40, adjusted to pH 6.5 and appropriate indicator was added. Maltose, lactate and TMAO were added after sterilisation to a final concentration of 27.8 mM, 30 mM and 10 mM respectively.

4.4 Growth conditions

Cultures were incubated at their optimum temperature for growth (20°C).

Aerobic conditions were achieved by incubating the culture in a medium occupying 20-25% of the Erlenmeyer flask volume in an orbital incubator (Gallenkamp, Model INR 250-010J) at 180 rev. min⁻¹.

For microaerobic growth, the culture vessel was completely filled with the appropriate medium and incubated statically.

Anaerobic growth conditions were achieved by using anaerobic jars (Baird & Tatlock), these jars were evacuated and flushed with hydrogen in the presence of a low temperature catalyst (Oxoid BR₄₂).

For microaerobic and anaerobic growth, media were supplemented with 30 mM sodium lactate and 10 mM of the appropriate electron acceptor.

4.5 Growth measurement

(a) Turbidity

Bacterial growth was monitored as the increase in absorbance at 660 nm ($A_{660}^{1\text{cm}}$) using a single beam spectrophotometer (Shimadzu UV-120-02).

(b) Viable count

Samples (1 ml) were removed aseptically from the growing culture and serially diluted into sterile 2% NaCl. Aliquots (0.1 ml) of the dilutions were spread onto duplicate NANA plates and the plates were incubated at 20°C for 48h. Plates with 50-200 colonies were counted and the results expressed as viable cell ml⁻¹. The relationship between the log of A_{660} and viable count for aerobic culture is shown in Figure 1.

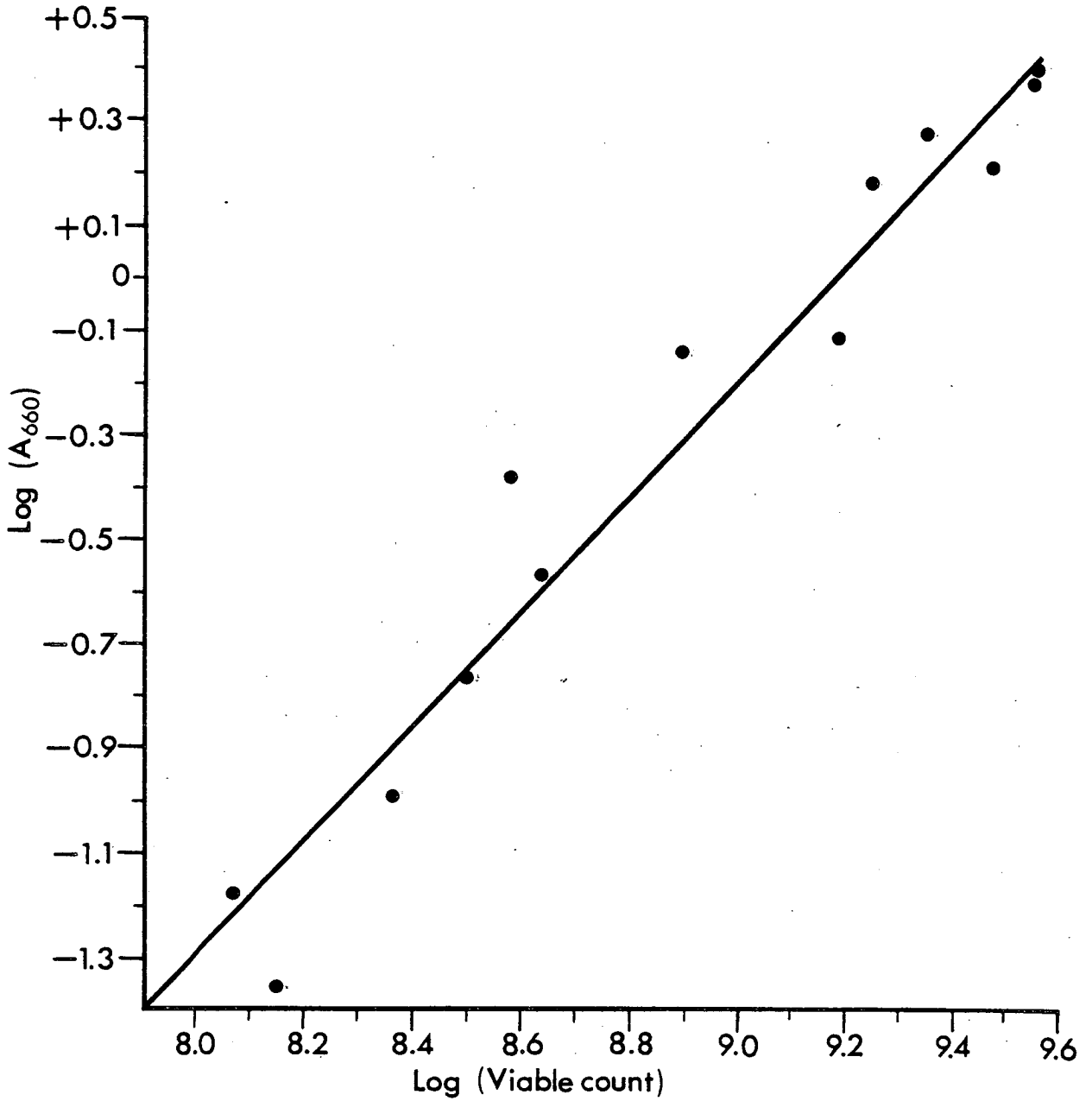


Figure 1: Relationship between the log of A_{660} and the log of viable count of A. putrefaciens for aerobic culture

5. Fungal Culture

5.1 Fungal strains

N. crassa wild type (STA4) and nit-1 mutant (34547 stock No. 54 and 32) were obtained from the Fungal Genetics Stock Centre (FGSC), Humboldt State University Foundation, Arcata, California.

5.2 Growth media

The solid medium used for subculturing and maintenance was either one quarter strength potato dextrose agar (PDA) or solidified modified Fries basal medium (Nicholas & Nason, 1954b). The latter has the following composition (gl^{-1}): sucrose, 2; NH_4Cl , 4.3; sodium tartrate, 6.2; KH_2PO_4 , 1; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5; NaCl , 0.1 and CaCl_2 , 0.1. The medium was supplemented with biotin and trace elements at the following concentrations (μgl^{-1}): biotin, 5; Na_2BO_7 , 88; $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$, 64; $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 960; ZnSO_4 , 8800; CuCl_2 , 270; and $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 72.

Sucrose was prepared as a 40% (w/v) solution, sterilised and added to the sterile basal medium. Biotin was sterilised by filtration and added. The trace elements solution was prepared as a stock. The solution of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ was prepared separately in 0.1N H_2SO_4 and sterilised by filtration. The pH of the resultant medium ranged from 5.5-6.0.

5.3 Maintenance of strains

Agar discs were cut out from hyphal growth on one quarter strength PDA plates, transferred to PDA slopes and incubated at 25°C for 5 d until full sporulation developed. The vials were filled with sterile mineral oil (or liquid paraffin) to a depth 1 cm above the top of the agar. These slopes were kept at 4°C.

5.4 Growth conditions and induction of nitrate reductase

A spore suspension or agar discs were used to inoculate the modified Fries minimal medium (1 mm agar disc used per 100 ml of medium). Cultures were incubated at 30°C for 48h aerobically with shaking at 180 rev. min^{-1} for full growth.

Induction of nitrate reductase was achieved as follows: Mycelial mats, fully grown in the above medium, were separated by vacuum filtration using Whatman No. 1 filter paper and a Buchner funnel. The mycelia were washed three times with cold distilled water and transferred to fresh Fries minimal medium in which the NH_4Cl was replaced by $6.9 \text{ g l}^{-1} \text{ NaNO}_3$. The cultures were incubated at 30°C for 3-4h with good aeration to induce nitrate reductase. The mycelia were washed thoroughly with cold distilled water, squeezed to remove excess moisture, divided into 1g portions and kept at -80°C until required.

6. Bacterial Cell Fractionation

6.1 Harvesting and preparation of cell suspensions

Bacterial cells were harvested by centrifugation at 12,000g for 15 min at 4°C (MSE 18 centrifuge). Cells were washed once by resuspension and homogenisation in TMAO buffer using a Uni-form glass homogeniser (Jencons, Leighton Buzzard, Bedfordshire). After recentrifugation the cell pellet was resuspended in TMAO buffer or other buffer as required to give an approximate final protein concentration of $5-10 \text{ mg ml}^{-1}$.

6.2 Preparation of cell free extract

Cells were harvested, washed and resuspended as above then passed through the French pressure cell at 27.6 MPa. Unbroken cells and cell debris were removed by centrifugation at 12,000g for 10 min at 4°C . The supernatant is referred to as the cell free crude extract.

6.3 Preparation of periplasm

Periplasm was prepared essentially according to Birdsell & Cota-Robles (1967) by lysozyme : EDTA treatment of cells to produce spheroplasts. The method was modified for use with A. putrefaciens. The cells were harvested, washed once with TMAO buffer and resuspended in 10 mM Tris-HCl, pH 8.0 containing 0.5M sucrose and left for 10 min at room temperature with continuous stirring. Lysozyme was added to a final concentration of $30 \text{ } \mu\text{g ml}^{-1}$.

and the suspension incubated for 10 min before dilution with an equal volume of 10 mM Tris-HCl, pH 8.0. EDTA solution was added gradually to a final concentration of 1 mM and spheroplast formation was monitored by phase contrast microscopy. Spheroplast formation (90-100%) was usually completed within 10-15 min after EDTA addition. DNAase (EC 3.1.4.5) ($10 \mu\text{g ml}^{-1}$) was added. If formation of spheroplasts was very slow the suspension was incubated for 5-10 min at 37°C . Generally $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ as a solution was added to a final concentration of 50 mM after spheroplasts formation was completed. The periplasm and spheroplast were separated by centrifugation at 35,000g for 25 min at 4°C .

6.4 Preparation of cytoplasm and membrane

Spheroplasts were prepared as in the previous section and disrupted in the French pressure cell. After removal of unbroken cells and cell debris, the crude extract was subjected to ultracentrifugation at 180,000g for 100 min at 4°C (MSE 65 ultracentrifuge). The supernatant was decanted gently and retained as the cytoplasmic fraction. The reddish-brown pellet was termed the membranes and resuspended in the TMAO buffer.

6.5 Solubilisation of formate dehydrogenase

FDH was solubilised from the membranes according to Ruiz-Herrera & Figueroa (1972) and Ruiz-Herrera *et al.* (1972). Membranes were prepared as in section 6.4 except that membranes were resuspended in FDH solubilisation buffer (section 2.6) instead of TMAO buffer so that the protein concentration was approximately 10 mg ml^{-1} . Equal volumes of membrane preparation and solubilising agent solution were mixed thoroughly and kept at 0°C for 1h, then centrifuged at 150,000g for 40 min at 4°C . The supernatant was decanted and retained as the soluble fraction. The pellet was resuspended in the same buffer to the original volume of the membrane preparation. Both fractions were assayed for FDH activity.

7. Preparation of *Neurospora crassa* Crude Extract

Fresh or frozen mycelia (1g) were ground in 3 ml of preparation buffer using a tissue grinder (Griffith's tube Corning Ltd., Stone) until a homogenous sample was obtained. The homogenate was centrifuged at 12,000g for 20 min at 4°C; the supernatant, a turbid, orange-yellow solution, was used as a crude extract of *N. crassa* nit-1. The extract was always prepared freshly.

8. Preparation of Mammalian Succinate Dehydrogenase

Crude succinate dehydrogenase was prepared from sheep heart according to Morton (1955) with slight modification.

Clean heart muscles were washed thoroughly with tap water until squeezing the muslin bag released only a light pink fluid. The muscles were suspended in 10 mM K_2HPO_4 , pH 7.2 cooled to 4°C using a ratio of 200g muscle to 300 ml buffer and the suspension was blended in a blender for 5 min. The homogenate was centrifuged at 1000g for 20 min at 4°C to remove the cell debris. The resultant red-cloudy supernatant was cooled to about 0°C; proteins from the supernatant were precipitated by gradual acidification with 1M acetic acid until the pH reached 5.7 and the precipitate was collected by centrifugation at 12,000g for 30 min at 4°C. The pellet was resuspended and homogenised in 0.1M phosphate buffer (12 ml buffer: 200g muscle) to give a crude succinate dehydrogenase preparation with a protein content of approximately 3 mg ml⁻¹. The preparation was divided into aliquots and stored at -20°C.

9. Enzyme Assays

9.1 TMAO reductase

The enzyme was assayed by the anaerobic, TMAO dependent reoxidation of reduced methyl viologen ($MV^{•+}$) at 600 nm. The reaction mixture contained $MV^{•+}$, 0.3 mM; TMAO, 1 mM; enzyme

source (10-100 μ l) and TMAO buffer in a total volume of 4 ml. The TMAO buffer containing oxidised methyl viologen (MV^{++}) sparged with oxygen-free nitrogen prior to assay was placed in a Hellma 21N stoppered cuvette together with enzyme source and a few antibumping granules. The Teflon stopper, drilled to take a 10-100 μ l microsyringe was inserted, displacing excess buffer so that all air was excluded. Small amounts (30-50 μ l) of freshly prepared 25 mM $Na_2S_2O_4$ in 10 mM NaOH were added by microsyringe sufficient to raise the A_{600} to about 1.0 after thorough mixing by inversion. The endogenous rate was recorded for 1-2 min and the reaction started by addition of TMAO. The enzyme activity was determined from the rate of decolourisation of the purple dye at 600 nm assuming an extinction coefficient of $13 M^{-1} cm^{-1}$ for MV^{++} (Thorneley, 1974).

Specific activity was expressed as nmol MV^{++} oxidised min^{-1} (mg protein) $^{-1}$.

9.2 Nitrate reductase

Nitrate reductase was assayed using the viologen dye assay as in section 9.1, except that TMAO buffer was replaced by MacLeod buffer and TMAO replaced by nitrate (1 mM).

9.3 Nitrite reductase

Nitrite reductase was assayed as for nitrate reductase except that nitrate was replaced by nitrite (1 mM).

9.4 Fumarate reductase

Fumarate reductase was assayed as for TMAO reductase except that TMAO was replaced by fumarate (1 mM).

9.5 Formate dehydrogenase : DCPIP

Formate dehydrogenase : DCPIP was assayed spectrophotometrically by the method of Ellis (1959) based on following the reduction of DCPIP at 600 nm according to the reaction



The anaerobic cuvette was filled with nitrogen saturated TMAO buffer (4 ml), enzyme source (10-100 μ l), different concentrations of PMS and mixed thoroughly before addition of DCPIP to raise the A_{600} to between 1 and 2 (0.05-0.1 mM).

The endogenous rate was recorded for 1-2 min and then the reaction was started by addition of 2 mM of sodium formate. The activity was determined from the rate of decolourisation of the blue oxidised DCPIP using the molar extinction coefficient $21 \text{ M}^{-1} \text{ cm}^{-1}$ (Lester & De Moss, 1971). Specific activity expressed as nmol DCPIP reduced $\text{min}^{-1} (\text{mg protein})^{-1}$.

Since the concentration of PMS was critical for the reaction, the optimum concentration of the PMS was determined. PMS at a concentration of 1.5-2 mM was found to be sufficient for the maximum activity of the enzyme (Figure 2).

9.6 Formate dehydrogenase : BV

The assay was modified from the method of Lester & De Moss (1971) and based on following the increase in A_{600} through BV^{++} reduction.

The assay mixture in an anaerobic cuvette contained anaerobic TMAO buffer containing BV^{++} . The BV^{++} was partially reduced by the controlled addition of freshly prepared $0.25 \text{ M Na}_2\text{S}_2\text{O}_4$ in 0.52 M NaHCO_3 until the A_{600} increased to approximately 0.01. After recording the endogenous rate, the reaction was started by addition of 15 mM sodium formate. The activity was calculated from the rate of reduction of BV^{++} at 600 nm using the molar extinction coefficient of $7.4 \text{ M}^{-1} \text{ cm}^{-1}$ and expressed as nmol BV^{++} reduced $\text{min}^{-1} (\text{mg protein})^{-1}$.

The optimum concentration of BV^{++} for this assay was determined. BV^{++} at a concentration of 1.5 mM was found to be sufficient to give the maximum activity of the enzyme (Figure 3).

9.7 NADPH : nitrate reductase (*Neurospora crassa*)

The assay was carried out as described by Nason & Evans (1953), with modification. The final reaction mixture (2 ml) was composed of the following components: 20 mM KNO_3 ; 0.1 mM FAD; 0.2 mM NADPH; 50 mM K_2HPO_4 , pH 7.2; 5 mM Na_2SO_3 . The

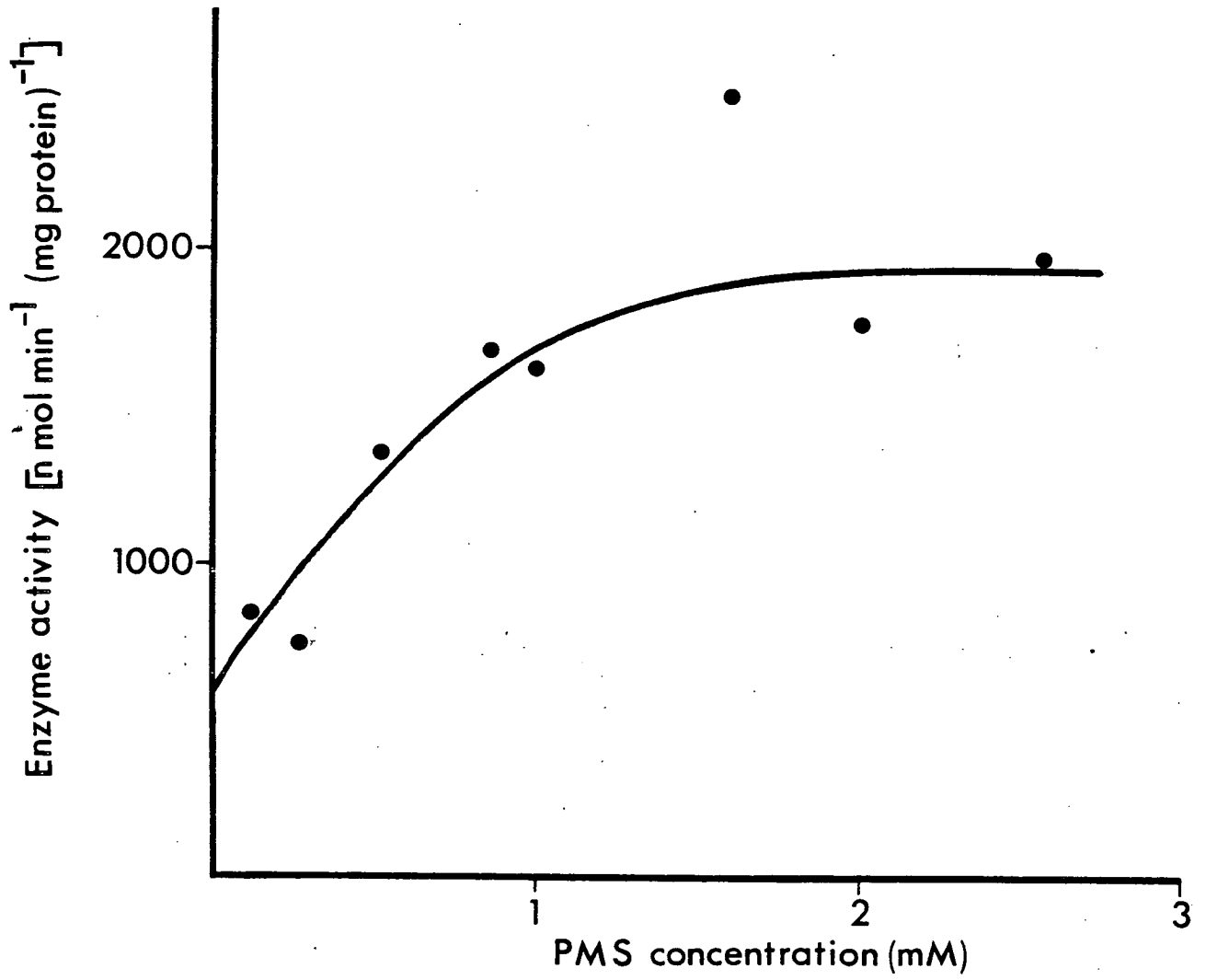


Figure 2: Optimum concentration of PMS for FDH activity

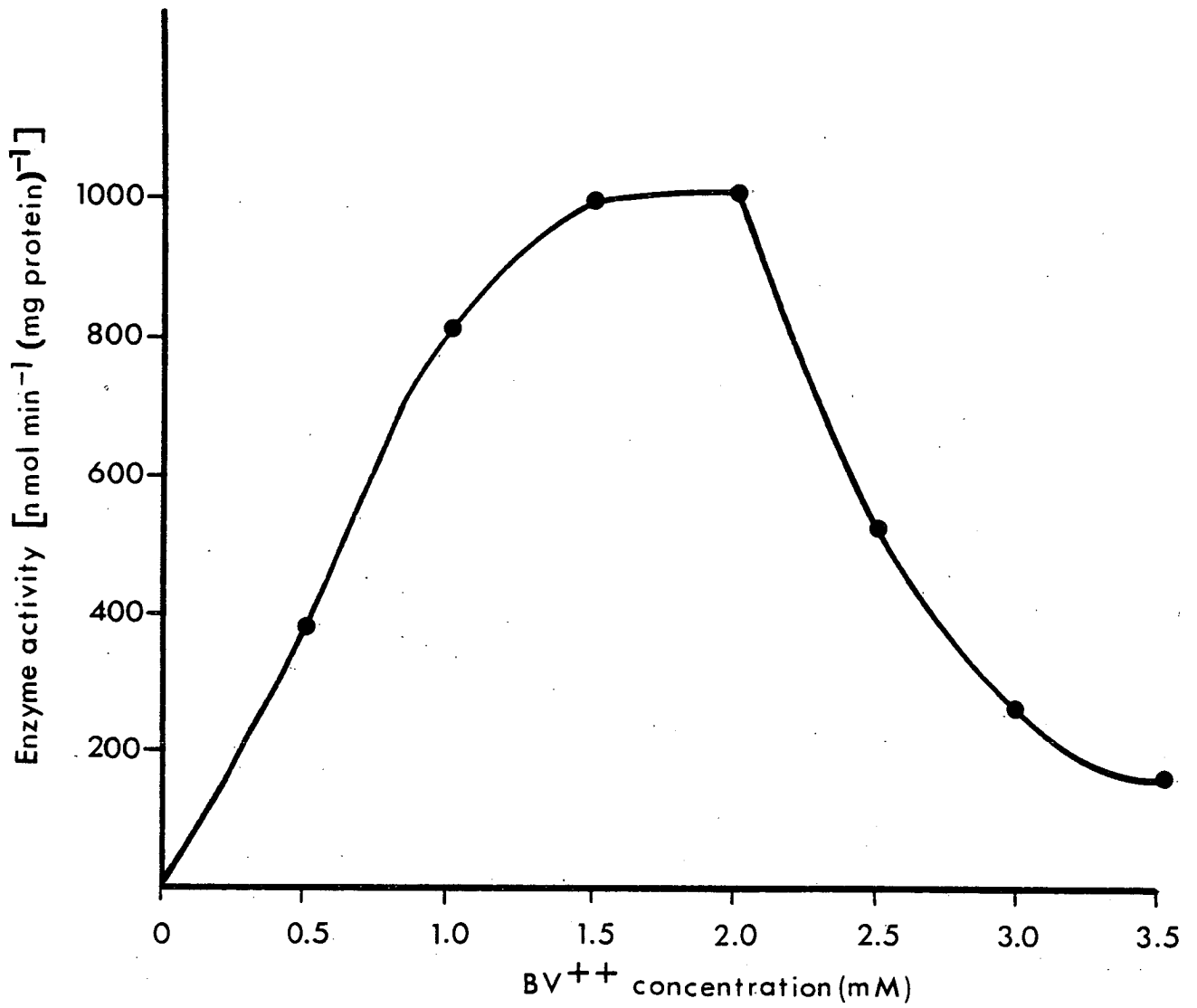


Figure 3: Effect of BV⁺⁺ concentration on FDH activity

sodium sulphite, NADPH, and FAD solutions were always prepared freshly.

Enzyme source (0.2 ml) was added to the above mixture, and the assay mixture was incubated at 25°C for 40-60 min. Then the resultant nitrite was estimated as described in section 11.2(b). The specific activity was expressed as nmol nitrite produced min^{-1} (mg protein) $^{-1}$.

9.8 Mammalian succinate dehydrogenase

The activity was determined from the time course for production of reduced iodonitrotetrazolium (INT) in the presence of excess succinate using the assay of Clark & Porteous (1964).

The solutions used were:

- (a) 0.1M sodium phosphate buffer, pH 7.6 containing 5 mM EDTA; 50 mM sucrose and 1 mM semicarbazide (Rodgers, 1961).
- (b) 0.5% (w/v) INT.
- (c) 10% (w/v) trichloroacetic acid.
- (d) Ethyl acetate (bench grade).

Aliquots (0.4 ml) containing 400 nmol neutralised sodium succinate were placed in acid washed McCartney bottles containing 1 ml solution (a); 0.4 ml solution (b) was added and the mixture incubated in a 37°C water bath for 2h. The bottles were removed and placed in an ice bath for 5 min. Crude succinate dehydrogenase preparation (0.2 ml) was added and the samples were kept for a further 15 min on ice. The bottles were transferred to a 37°C water bath. At intervals, samples were removed onto ice after stopping the reaction by addition of 2 ml reagent (c). The formazan was extracted with ethyl acetate and measured as described in section 11.3.

The results of a typical run are shown in Figure 4. A similar assay was carried out using a fixed incubation period and varying the amount of succinate dehydrogenase. From these experiments a fixed time interval and fixed volume of succinate dehydrogenase preparation were chosen for the estimation of succinate.

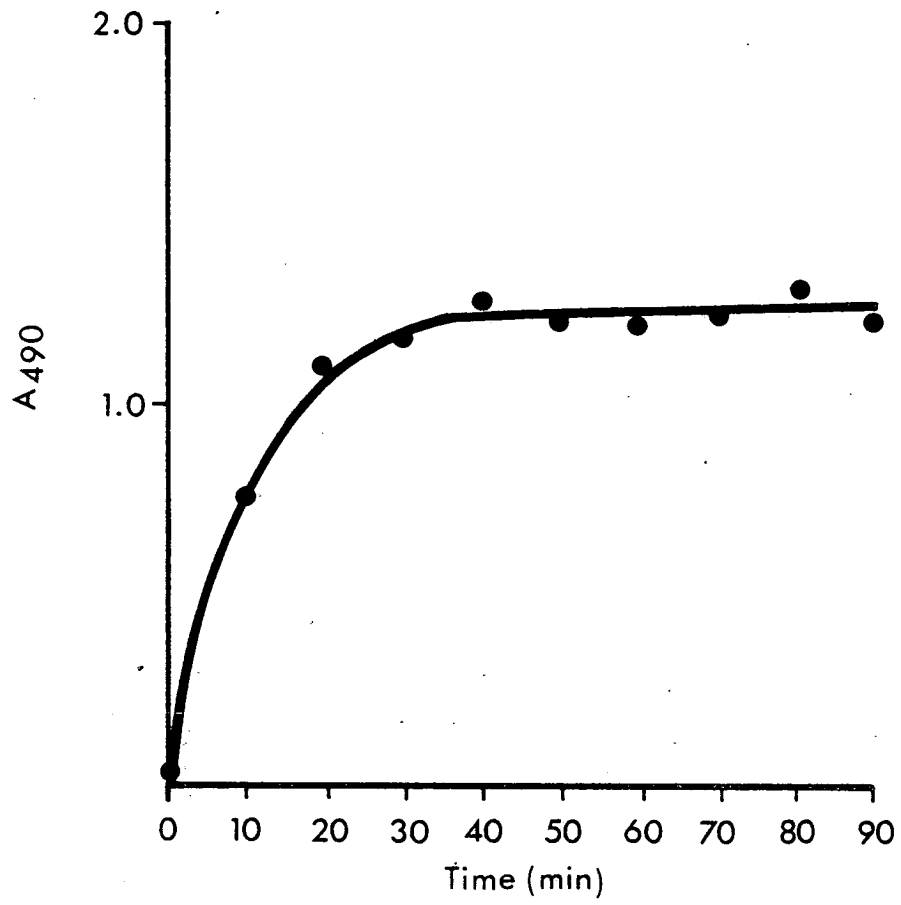


Figure 4: Assay of succinate dehydrogenase

10. Assay for Respiratory Chain Activity

10.1 Formate : TMAO reductase

The final reaction mixture volume of 50 ml was composed of TMAO buffer, pH 6.5; 10 mM sodium formate and cell suspension (2-4 mg protein). This mixture was sparged for 10 min with oxygen-free nitrogen and the reaction started by addition of 2 mM TMAO, pH 6.5. Aliquots (5 ml) were withdrawn at 1 min intervals and the reaction stopped by addition of 1 ml of 25% trichloroacetic acid. The precipitated protein was removed by centrifugation and the TMA in the supernatant was estimated. The specific activity was expressed as $\text{nmol TMA produced min}^{-1} (\text{mg protein})^{-1}$.

10.2 Formate : nitrate reductase

The assay mixture (10 ml) was composed of anaerobic TMAO buffer, pH 6.5; 10 mM sodium formate and cell suspension (2-4 mg protein). The reaction was started by addition of 10 mM KNO_3 . Samples (0.2 ml) were withdrawn at 1 min intervals for nitrite estimation. The specific activity was expressed as $\text{nmol nitrite produced min}^{-1} (\text{mg protein})^{-1}$.

10.3 Formate : fumarate reductase

Fumarate reductase with formate as donor was assayed similarly to nitrate reductase except that the reaction was started by addition of 10 mM sodium fumarate, pH 6.5, and succinate was estimated as the product.

Aliquots (1.5 ml) were removed and the reaction stopped by placing test-tubes in a boiling water bath. Precipitated proteins were removed by centrifugation and 0.4 ml samples of the supernatant were used to estimate the succinate. Specific activity was expressed as $\text{nmol succinate produced min}^{-1} (\text{mg protein})^{-1}$.

11. Chemical Estimations

11.1 Trimethylamine

TMA was determined colourimetrically using the method described by Murray & Gibson (1972a, b) except that no formaline

was used and 45% KOH used instead of the Na_2CO_3 solution. A calibration curve for TMA was determined and shown to be linear over the range 0-600 nmol TMA as shown in Figure 5.

11.2 Nitrite

Nitrite was estimated by two methods: the first used for measurement of formate : nitrate reductase activity and the second for measurement of NADPH : nitrate reductase activity.

(a) The reagents used were: 1% (w/v) sulphanilamide in 1N HCl (solution 1) and 0.02% (w/v) aqueous N-1-naphthylethylene diamine dihydrochloride (solution 2). Samples (0.2 ml) containing 0-200 nmol nitrite were mixed thoroughly with 8.8 ml solution 1. Solution 2 (1 ml) was added with rapid mixing and the pink colour was allowed to develop at ambient temperature for 20 min. The absorbance at 540 nm was measured against a distilled water blank. A standard curve was determined and the assay was shown to be linear in the range 0-200 nmol NaNO_2 as in Figure 6a (Radcliffe & Nicholas, 1968).

(b) Nitrite also was determined according to Garrett & Nason (1969) with slight modifications. Standard solution or unknown sample (1 ml) was added to 1 ml of 1% (w/v) sulphanilic acid in 20% (w/v) concentrated HCl followed by addition of 1 ml distilled water and 1 ml of 0.12% (w/v) aqueous N-(1-naphthyl) ethylene diamine dichloride. After rapid mixing, the pink colour was allowed to develop at ambient temperature for 20 min. Samples were clarified by a short centrifugation using a MSE bench centrifuge at full speed and the A_{540} measured against a blank which contained all components except the enzyme source. A nitrite standard curve was determined and the assay was shown to be linear in the range 0-200 nmol NaNO_2 (Figure 6b).

11.3 Succinate

Succinate was determined as described in section 9.8 except that the volume of succinate dehydrogenase preparation and the 37°C incubation period were fixed and the succinate concentration varied. Samples (0.4 ml) containing 0-400 nmol

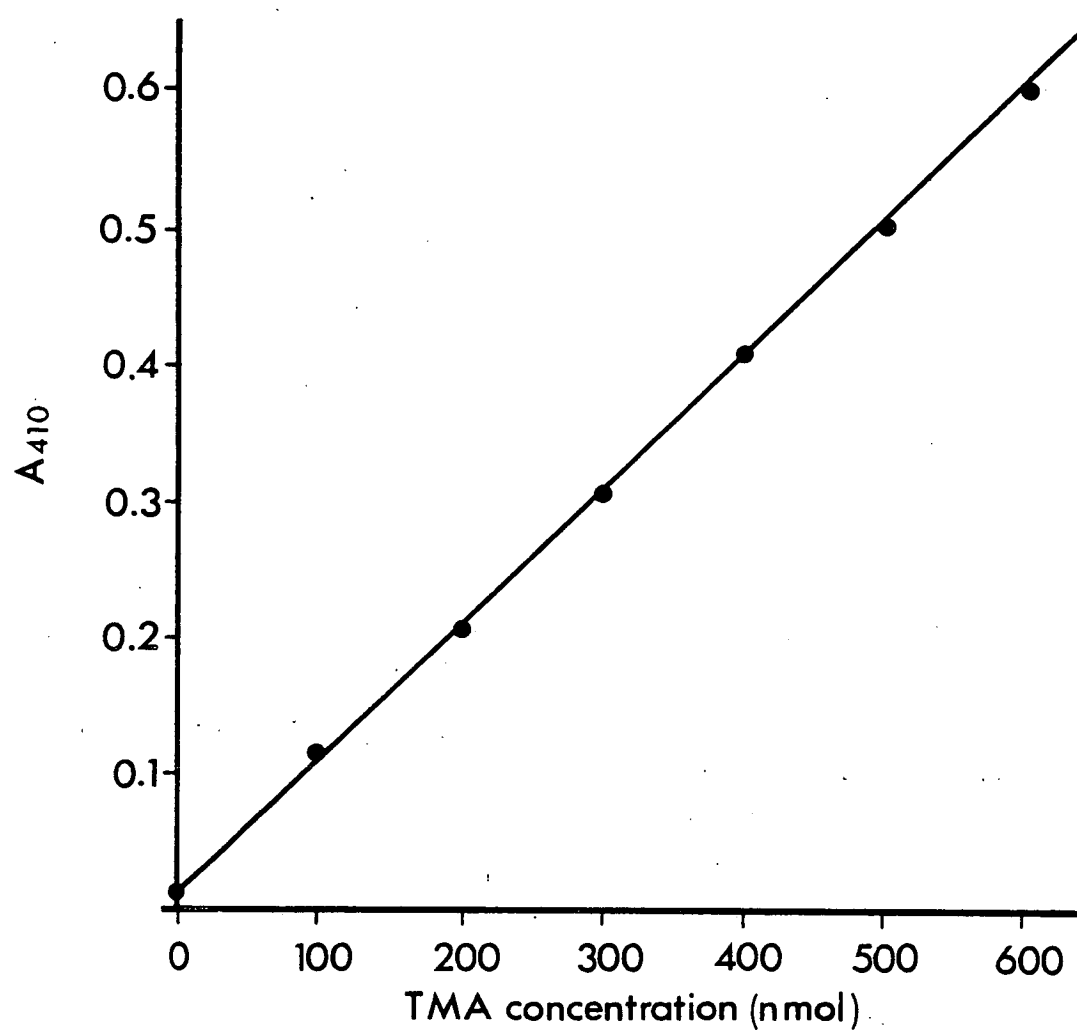


Figure 5: Calibration curve for TMA

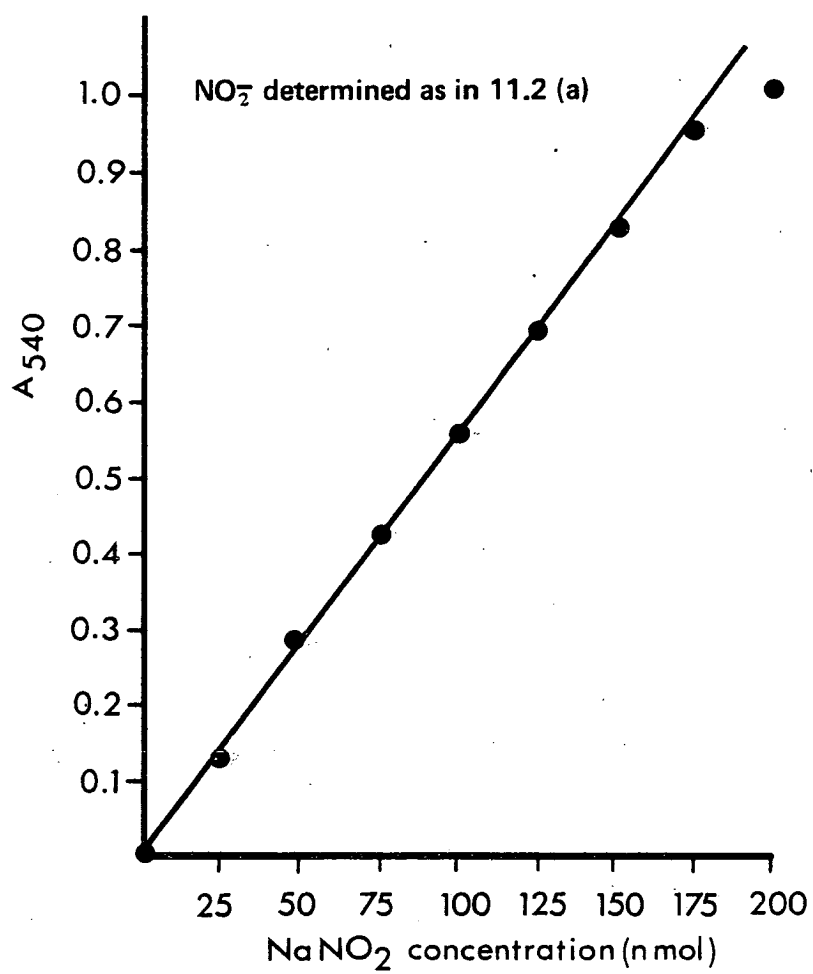


Figure 6a : Calibration curve for nitrite

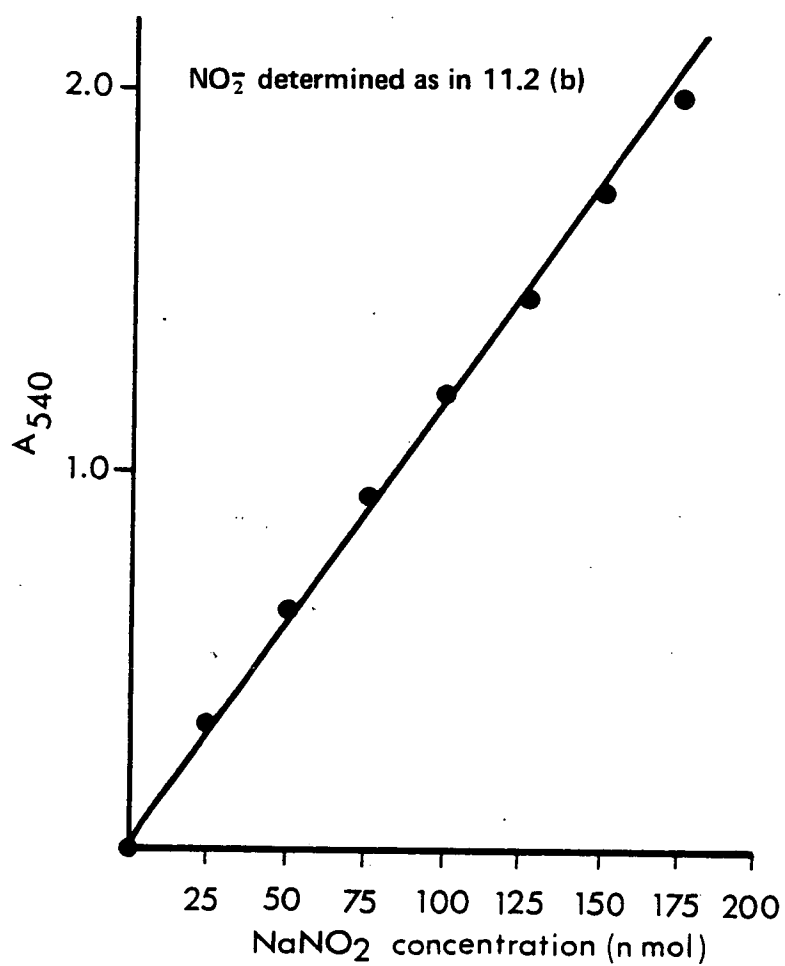


Figure 6b: Calibration curve for nitrite

succinate were assayed, typically using 0.2 ml succinate dehydrogenase preparation and an incubation period of 40 min. The formazan was extracted by vigorous shaking with ethyl acetate (4 ml), the layers separated by centrifugation for 5 min and the A_{490} of the ethylacetate measured against a reagent blank. A standard curve was determined for each experiment and the assay was shown to be linear in the range 0-600 nmol succinate (Figure 7).

11.4 Protein estimation

Protein concentration was determined with Folin-Ciocalteu phenol reagent using the method of Lowry et al. (1951). Bovin serum albumin fraction V was used as standard. In the presence of interfering substance, protein was precipitated using Peterson's (1977) modification of the method described by Lowry et al. (1951). Protein were precipitated out first using 72% (w/v) trichloroacetic acid in the presence of sodium deoxycholate, redissolved in 0.1N NaOH and determined as previously.

12. Mutagenesis Procedures

12.1 Mutagenesis by ultra violet light

An early log phase aerobic culture (10 ml) of A. putrefaciens NCMB400, grown in Wood & Baird medium was washed once in TMAO buffer and resuspended in the same buffer to the original volume. The suspension was exposed to UV light at a dose rate of $1.05 \text{ J sec}^{-1} \text{ cm}^{-1}$ (Honovia model 12 gerimicidal lamp, Honovia Lamps Ltd., Slough, Bucks). Samples (1 ml) were removed at different time intervals, diluted and plated onto NANA plates. The results are depicted in Figure 8. Resistance to rifampicin ($20 \mu\text{g ml}^{-1}$) was used as an indication of mutation frequency.

12.2 Mutagenesis with N-methyl-N'-nitro-N-nitrosoguanidine (NTG).

A log phase aerobic culture of the wild type strain was harvested, washed once in TMAO buffer and resuspended in TMAO buffer pH 5.9. NTG was added to a final concentration of $30 \mu\text{g ml}^{-1}$

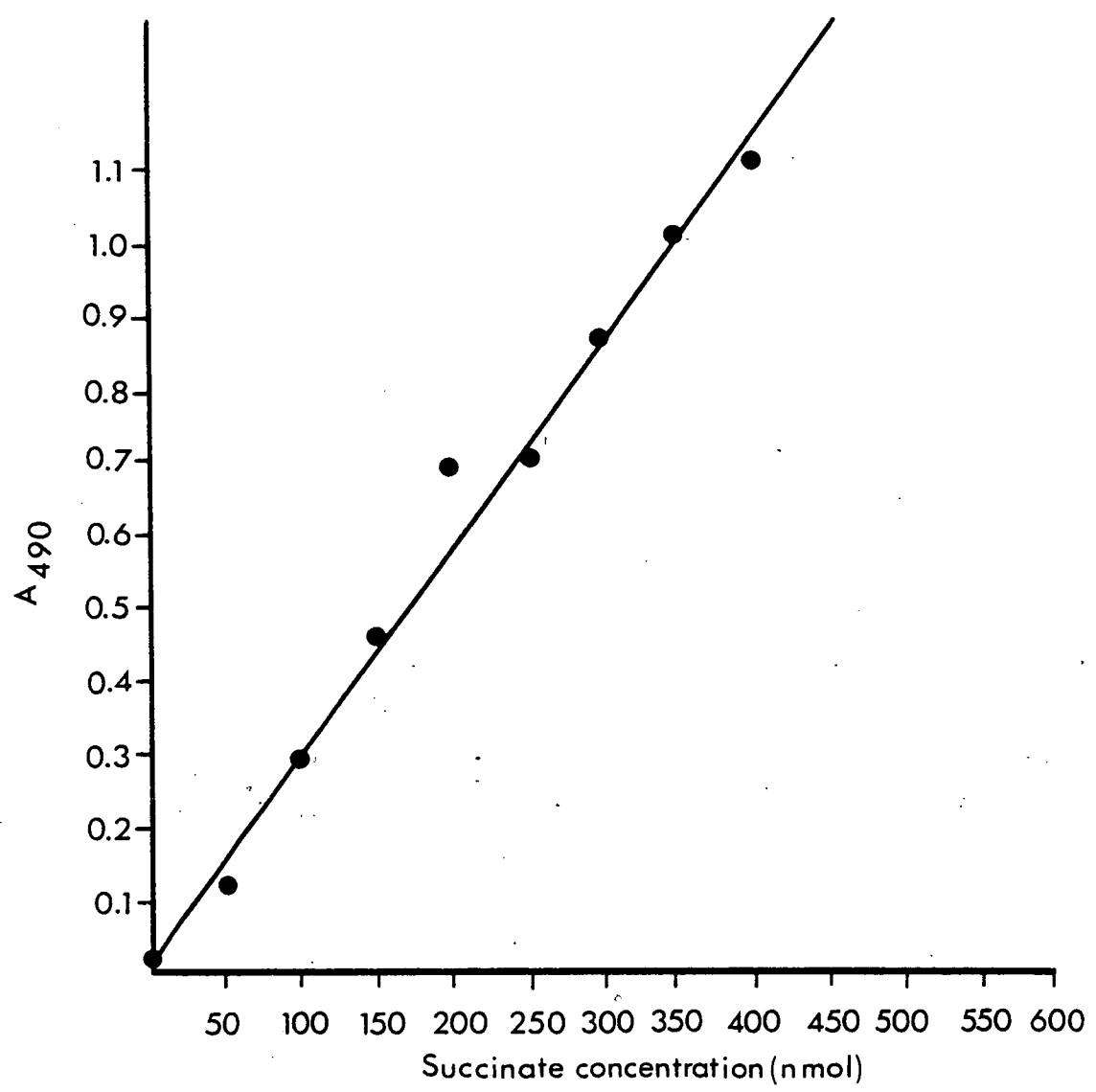


Figure 7: Standard curve for succinate

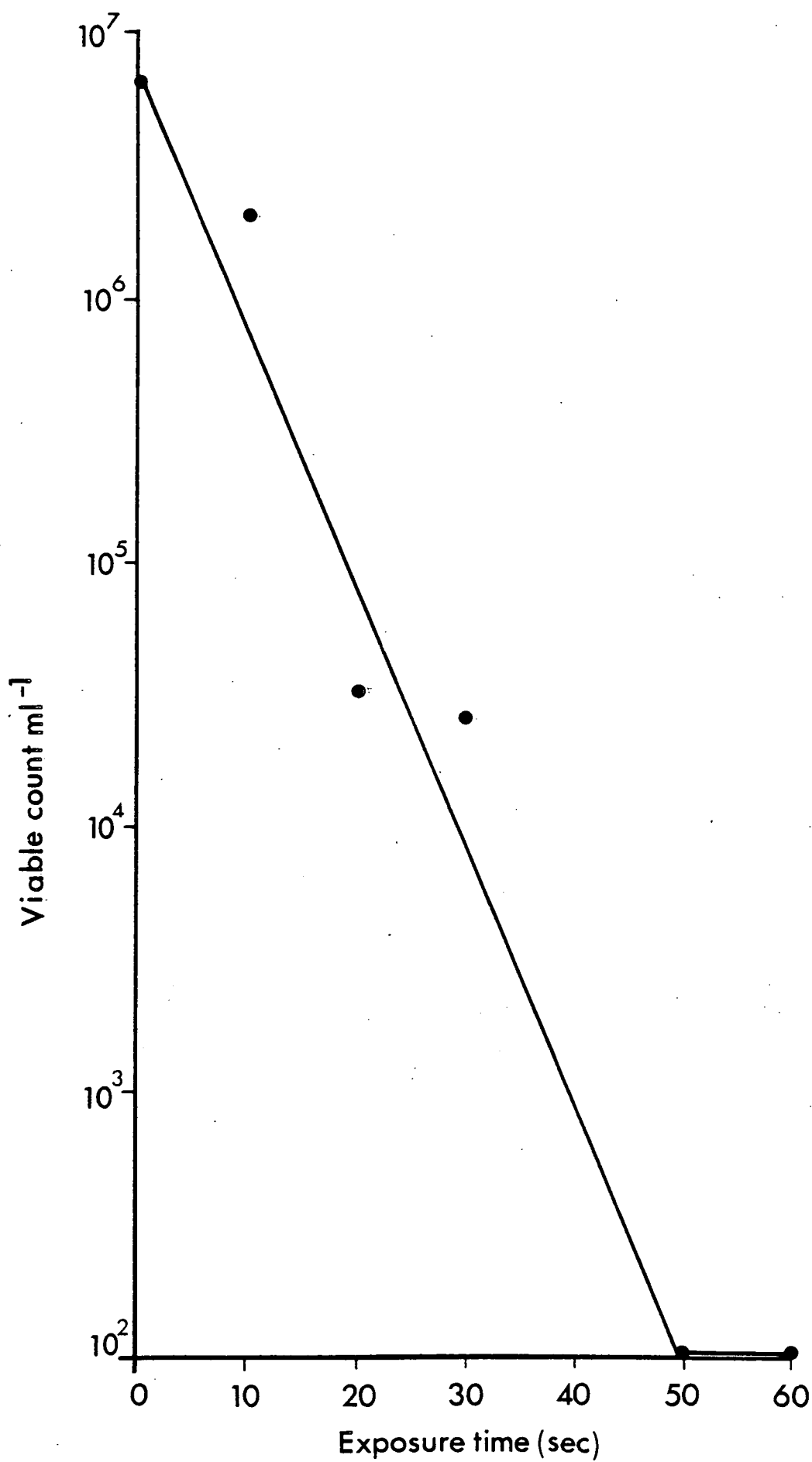


Figure 8: UV survival curve of *A. putrefaciens*

and the culture incubated aerobically at 20°C. Aliquots (1 ml) were withdrawn at 10 min intervals up to 60 min, serially diluted in 2% NaCl and plated onto NANA. Single colonies were allowed to develop at 20°C and then picked, patched onto fresh NANA plates and grown overnight at 20°C. Patches were replica plated onto NANA plates with the following supplements: (a) Lactate and TMAO, (b) Lactate and nitrate, and (c) None (control). The control plates were incubated aerobically overnight and the other plates anaerobically for 3 d at 20°C. Colonies unable to grow anaerobically on TMAO or nitrate were picked and subjected to further characterisation.

13. Penicillin Enrichment

Mutated cultures were incubated aerobically overnight for phenotypic expression and then incubated anaerobically in Wood & Baird medium containing lactate and TMAO for 1h before adding benzyl penicillin to 200 µg ml⁻¹. Cultures were left to grow in the presence of penicillin for 4h and the penicillin was removed by centrifugation and washing the cells with TMAO buffer. Figure 9 shows the effect of penicillin on the viability of cells. Figure 10 shows the isolation procedure.

14. Screening Procedures for Electron Transport Mutants

14.1 Anaerobic growth test

Putative mutants were grown on NANA plates and then replica plated onto NANA medium supplemented with the appropriate electron acceptor and incubated anaerobically for 4 d. Growth on the original and the anaerobically incubated plates were compared; mutants unable to grow anaerobically were picked.

14.2 Plate assay for benzyl viologen : TMAO reductase

This system was adopted from that originally used for isolation of FDH mutants by Mandrand-Berthelot et al. (1978).

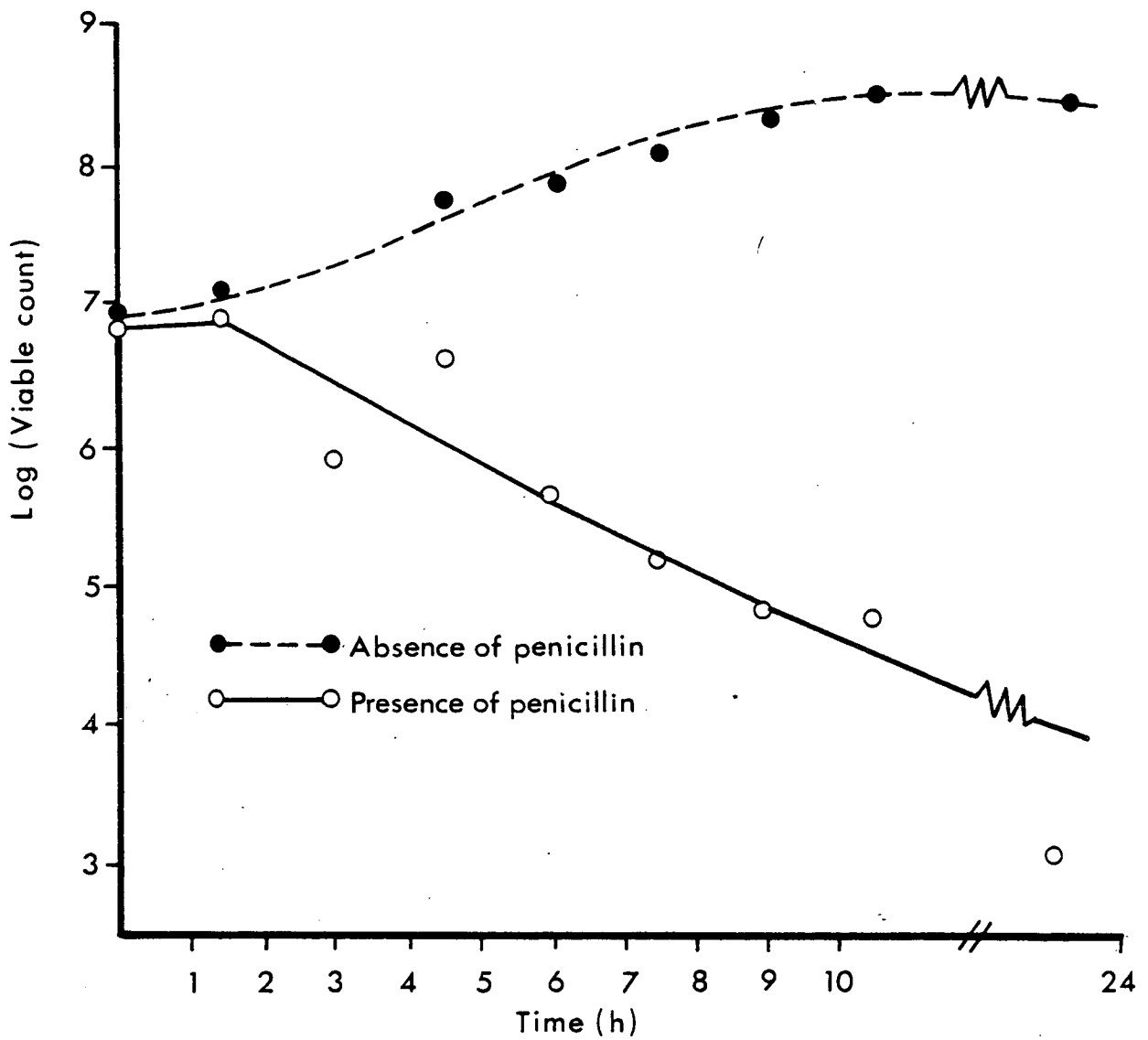


Figure 9: Effect of penicillin on cell viability during microaerobic growth of *A. putrefaciens*

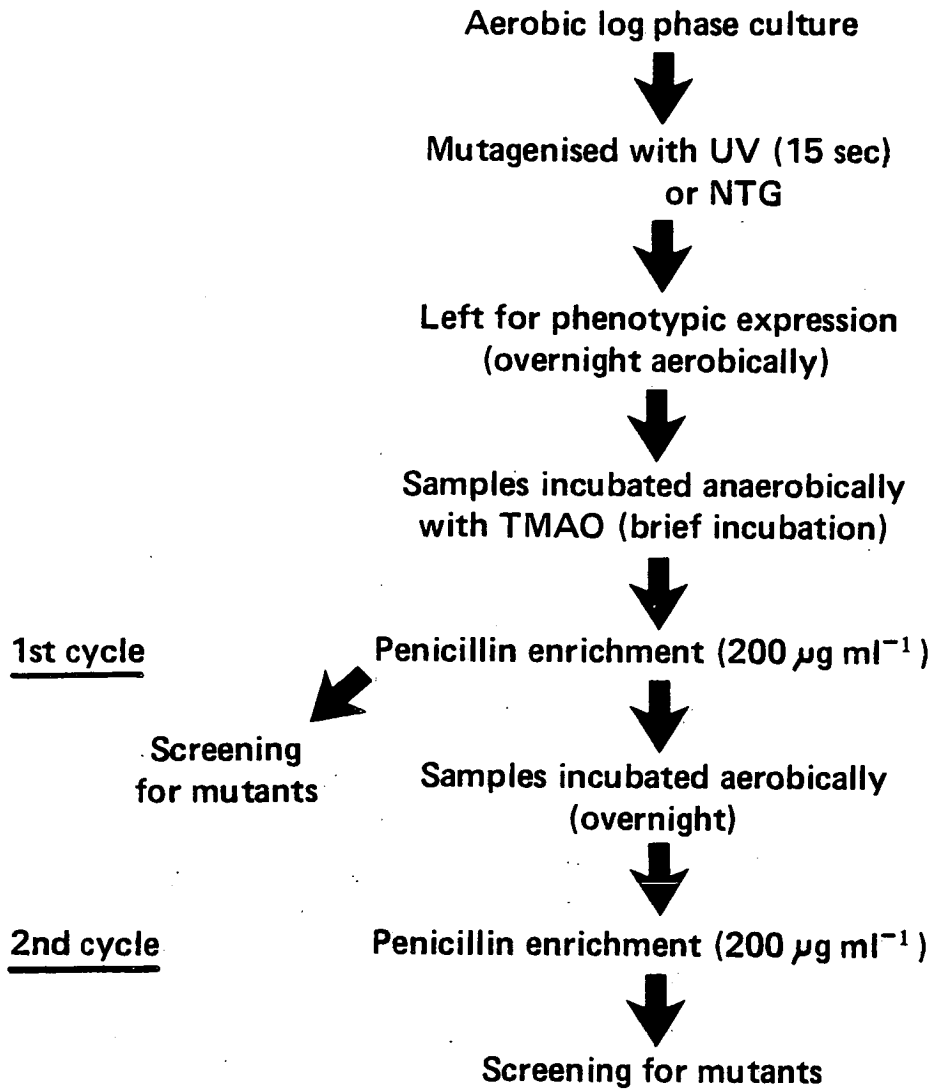


Figure 10. Mutagenesis procedure for isolation of electron transport mutants.

The overlay system used was 0.75% (w/v) Oxoid Agar No. 3; 1 mg ml⁻¹ BV; and 0.25M sodium formate in 25 mM phosphate buffer, pH 7.0. The reduction of BV was accomplished by making small addition of solid Na₂S₂O₄ just prior to use and mixing thoroughly. The overlay was kept at 45°C and 10 ml added to each plate. Colonies remaining violet after 2-3 min were noted and the corresponding colonies picked from the original plates.

15. Biochemical Tests

The following tests were performed as described by Cruickshank (1968) using appropriate organisms as control: catalase, citrate utilisation, nitrate reduction, urease, litmus milk reaction, starch hydrolysis, gelatin liquefaction, indol production, methyl red and Voges-Proskauer.

The ability of strains to grow at different temperatures was tested by incubation of cultures on NANA plates in oven incubators set at the desired temperatures.

Antibiotic sensitivity was tested using multiple antibiotic discs (Oxoid 30-1H); discs were placed aseptically at the centre of each plate and the plates were incubated at 20°C.

The oxidase test was done according to Marrs & Gest (1973). The "NADI" reagent was made by mixing equal volumes of a 1% (w/v) aqueous solution of N,N-tetramethyl-p-phenylene diamine HCl (kept in dark) and a freshly prepared solution of 1% (w/v) 1-naththol in 95% ethanol. Cultures, grown aerobically overnight on NANA plates, were taken and the plates covered with NADI reagent. The excess reagent was poured off and the plates exposed to air. Oxidase negative colonies failed to develop a blue-purple colour. E. coli and Pseudomonas aeruginosa were used as negative and positive controls respectively.

Motility was checked by stabbing semi-solidified Wood & Baird medium and the soft agar stabs were incubated for 48h at 20°C. Motile bacteria were diffused out through the medium.

The TMAO reduction test was carried out according to Laycock & Reiger (1971).

For carbohydrate utilisation test, the strains were inoculated into tubes of modified Leifson medium (1963) containing 1% (w/v) of the carbohydrate to be tested and 0.0001% (w/v) phenol red or bromocresol purple as indicator. Durham tubes were included to test gas production.

The O-F test was carried out using Leifson medium (1963) supplemented with 1% (w/v) glucose or maltose. Two tubes for each strain were used, one was incubated aerobically and the other incubated anaerobically.

16. Morphological Tests

All slides were examined at a magnification of X1000 under oil immersion using a Vickers microscope.

16.1 Gram stain

Heat-fixed smears from liquid or solid culture were stained with an aqueous solution of crystal violet for 1 min, treated with Gram's iodine for another 1 min, decolourised with alcohol for 30 sec and then counterstained with alcohol-phenolic carbol fuchsin solution for 1 min.

16.2 Flagella stain

Flagella were stained according to Cruickshank (1968) except that the smears were left to react with tannic-carbol fuchsin for 12 min.

17. Complementation Assays of *Neurospora crassa* nit-1 Nitrate Reductase

17.1 *N. crassa* wild type crude extract as molybdenum cofactor source

Crude extract of uninduced wild type *N. crassa* was complemented with nit-1 crude extract at different ratios and temperatures in the presence of 10 mM molybdate (Nason et al., 1970).

17.2 A. putrefaciens crude extract as molybdenum
cofactor source

A. putrefaciens samples for molybdenum cofactor detection was grown aerobically in Wood & Baird medium containing 2 mM molybdate, the cells were harvested and washed twice with 0.25M sucrose and the pellets either used immediately or kept at -80°C (Amy & Rajagopalan, 1979). For complementation cells were resuspended in the breaking buffer at a ratio of 1g to 10 ml buffer and broken by passing through a French pressure cell at 27.6 MPa, unbroken cells were removed by centrifugation at 12,000g for 10 min at 4°C .

The complementation mixture was composed of 0.3 ml nit-1 crude extract and 50 μl (or different amounts) of A. putrefaciens crude extract (or other samples) in the presence of 10 mM molybdate. The mixture was incubated at $20-25^{\circ}\text{C}$ for 20 min. Then 0.2 ml samples were taken for assay of NADPH : nitrate reductase activity.

Molybdenum cofactor activity was expressed as nmol nitrite produced min^{-1} (mg protein of A. putrefaciens extract) $^{-1}$ under the above conditions provided that nit-1 extract was in excess.

CHAPTER 3

RESULTS AND DISCUSSION

SECTION I

ANAEROBIC RESPIRATION OF ALTEROMONAS PUTREFACIENS

1. Anaerobic Respiration with TMAO

(a) TMAO reductase

A. putrefaciens was grown under a variety of conditions and cell suspensions were assayed for $MV^{•+}$: TMAO reductase activity. The results in Table 1 show* that aerobic culture had the lowest $MV^{•+}$: TMAO reductase activity; lowering the oxygen tension as in microaerobic condition resulted in markedly increased activity, but the greatest activity was found in cultures grown microaerobically after addition of TMAO in the presence or absence of nitrate or fumarate. However, TMAO reductase activity of cells grown with fumarate plus nitrate was lower even than that of cells grown microaerobically without any added electron acceptor.

(b) Formate : TMAO respiratory chain activity

Activity was measured in cultures grown with TMAO and also with the combinations of TMAO plus nitrate and TMAO plus fumarate (Table 2). The whole chain activity of formate : TMAO reductase was enhanced for cells grown with TMAO plus fumarate, but not for cells grown with TMAO plus nitrate.

(c) Cellular distribution of TMAO reductase

TMAO reductase activity of a culture grown with TMAO, as measured by oxidation of $MV^{•+}$, was determined in different cellular fractions, the results shown in Table 3. The highest TMAO reductase activity was recovered in the periplasmic fraction.

Table 1. $MV^{•+}$: TMAO reductase activity in cultures grown under different growth conditions

Growth condition	Electron acceptor added to the growth medium	Specific activity*
Aerobic	None	22
Microaerobic	None	847
"	TMAO	5937 —
"	Nitrate	1070
"	Fumarate	547
"	TMAO & fumarate	5076
"	TMAO & nitrate	6210
"	Fumarate & nitrate	100

* Specific activity, $\text{nmol } MV^{•+} \text{ oxidised min}^{-1} (\text{mg protein})^{-1}$

Table 2. Formate : TMAO respiratory chain activity

Electron acceptor added to the growth medium	Activity *
TMAO	380
TMAO & fumarate	574
TMAO & nitrate	329

* Activity, nmol TMA produced min^{-1} (mg protein) $^{-1}$

Table 3. The distribution of TMAO reductase in different cellular fractions

Cellular fraction	Specific activity *	Total activity *
Whole cell	4.50 —	102
Periplasm	10.47	290
Spheroplast	0.43	10
Spheroplast crude extract	0.69	7
Cytoplasm	1.58	4
Membrane	0.36	3

* Activity, $\mu\text{mol MV}^{++}$ oxidised min^{-1} (mg protein) $^{-1}$

2. Anaerobic Respiration with Nitrate

(a) Nitrate reductase

MV^{o+} : nitrate reductase activity was measured in suspensions of cells grown under different conditions. Activity was absent or below the limits of detection in aerobic cultures, but like TMAO reductase, nitrate reductase was synthesised under the other conditions tested (Table 4). However, the maximum activity was recorded when nitrate alone was included in the culture medium. Almost similar activities were detected with TMAO alone or in combination with nitrate, but these were lower than the activity for nitrate grown cells. Fumarate grown cultures showed low activity, moreover, the inclusion of fumarate in combination with nitrate or TMAO resulted in lower activity than with TMAO plus nitrate.

(b) Formate : nitrate respiratory chain activity

Activity of the chain was measured for cultures grown under different growth conditions; the results are shown in Table 5. The maximum activity was found in culture grown with nitrate plus TMAO, while inclusion of fumarate lowered the activity to 8% of the nitrate grown culture activity.

(c) Cellular distribution of nitrate reductase

MV^{o+} : nitrate reductase activity of a culture grown microaerobically with nitrate was estimated in different cellular fractions (Table 6). The highest nitrate reductase activity was recovered in the periplasm as previously found for TMAO reductase.

3. Anaerobic Respiration with Fumarate

(a) Fumarate reductase

MV^{o+} : fumarate reductase activity was determined in cultures grown under different growth conditions (Table 7). As fumarate

Table 4. $MV^{•+}$: nitrate reductase activity in cultures grown under different growth conditions

Growth condition	Electron acceptor added to the growth medium	Specific* activity
Aerobic	None	0
Microaerobic	None	26
"	TMAO	743
"	Nitrate	905 —
"	Fumarate	79
"	TMAO & fumarate	304
"	TMAO & nitrate	790
"	Fumarate & nitrate	303

* Specific activity, $\text{nmol } MV^{•+} \text{ oxidised min}^{-1} (\text{mg protein})^{-1}$

Table 5. Formate : nitrate respiratory chain activity

Electron acceptor added to the growth medium	Activity*
Nitrate	92
TMAO	44
Fumarate	7
Nitrate & TMAO	178
Fumarate & TMAO	45

* Activity, nmol nitrite produced min⁻¹ (mg protein)⁻¹

Table 6. The distribution of nitrate reductase in different cellular fractions

Cellular fraction	Specific* activity	Total* activity
Whole cell	0.52	198
Periplasm	1.39	78
Spheroplast	0	0
Spheroplast crude extract	< 0.01	2
Cytoplasm	< 0.01	1
Membrane	0.04	5

* Activity, $\mu\text{mol MV}^{•+}$ oxidised min⁻¹ (mg protein)⁻¹

Table 7. $MV^{•+}$: fumarate reductase activity in cultures grown under different growth conditions

Growth condition	Electron acceptor added to the growth medium	Specific* activity
Aerobic	None	183
Microaerobic	None	1099
"	TMAO	3716
"	Nitrate	371
"	Fumarate	5157 —
"	Fumarate & TMAO	10624
"	Fumarate & nitrate	243

* Specific activity, $\text{nmol } MV^{•+} \text{ oxidised min}^{-1} (\text{mg protein})^{-1}$

Table 8. Formate : fumarate respiratory chain activity

Electron acceptor added to the growth medium	Activity*
Fumarate	181
TMAO	75
Fumarate & TMAO	217

* Activity, $\text{nmol succinate produced min}^{-1} (\text{mg protein})^{-1}$

acted as an electron acceptor to support the anaerobic growth of A. putrefaciens this was associated with induction of high fumarate reductase with high activity. Like other anaerobic enzymes, fumarate reductase activity was enhanced by partial anaerobiosis as the activity increased to 6 times of the aerobic culture. Addition of TMAO in combination with fumarate resulted in enhancing the fumarate reductase activity more than the enhancement of TMAO reductase caused by fumarate (Table 1). The maximum activity was recorded for cultures grown with a combination of TMAO plus fumarate, while growth with fumarate plus nitrate resulted in 95% reduction in activity compared with that of fumarate grown cultures.

(b) Formate : fumarate respiratory chain activity

The whole chain activity was estimated, but as TMAO respiration was the aim in this investigation, the activities were measured only in cells grown with fumarate (control), with TMAO and with both electron acceptors added. The results are shown in Table 8. The activity of TMAO grown cells was about 42% of the control culture activity, but growth with both TMAO plus fumarate resulted in an increase in the activity to 120% of that of the control culture activity.

(c) Cellular distribution of fumarate reductase

The distribution of fumarate reductase in different fractions of cells grown microaerobically with fumarate was estimated in a similar manner to those of nitrate reductase and TMAO reductase (Table 9). Fumarate reductase also seems to be located principally in the periplasm as the bulk of the activity was recovered in this fraction.

4. Formate Dehydrogenase

FDH is the first component in many anaerobic respiratory chains. Its activity was determined by using the two electron

Table 9. The distribution of fumarate reductase in different cellular fractions

Cellular fraction	Specific* activity	Total* activity
Whole cell	3.70 —	203
Periplasm	53.75	446
Spheroplast	0.20	32
Spheroplast crude extract	0.95	57
Cytoplasm	1.03	22
Membrane	0.24	6

* Activity, $\mu\text{mol MV}^{\bullet+}$ oxidised min^{-1} $(\text{mg protein})^{-1}$

acceptor DCPIP with PMS as mediator and by using the one electron acceptor BV as described in sections 9.5 and 9.6 of Materials and Methods.

(a) FDH : DCPIP

Activity was determined in cultures grown under different growth conditions (Table 10). Partial anaerobiosis without the addition of any electron acceptor resulted in an increase in activity of 2.7 fold over the activity of aerobically grown cells. However, nitrate grown cells showed the highest activity.

(b) FDH : BV

Activity of FDH with BV as electron acceptor was also determined in different cultures (Table 11). Anaerobiosis did not enhance the FDH : BV activity; addition of different electron acceptors to the growth medium caused a slight increase in activity, except that the presence of TMAO resulted in a 2.7 fold enhancement of activity over that of cells grown microaerobically without any added electron acceptor.

(c) Cellular distribution of FDHs

The intracellular location of FDH was found to be similar under different growth conditions, therefore only the results using a TMAO grown culture are shown in Table 12. The periplasmic fraction showed the lowest activity of FDH : DCPIP and no FDH : BV activity. Samples containing membrane generally showed high activities, however, the cytoplasm contained about 20% of FDH : DCPIP activity and a higher percentage of FDH : BV activity.

(d) Solubilisation of FDH from the membrane

Different reagents were used to solubilise FDH from the membrane of cells grown microaerobically with TMAO, including the chaotropic salts KSCN and $KClO_3$; the non-ionic detergents Triton X-100, Brij 35, 36 and Tween 80; the ionic detergents

Table 10. FDH : DCPIP activity in cultures grown under different growth conditions

Growth condition	Electron acceptor added to the growth medium	Specific* activity
Aerobic	None	413
Microaerobic	None	1115
"	TMAO	3280
"	Nitrate	6022
"	Fumarate	3120

* Specific activity, nmol DCPIP reduced min^{-1} (mg protein) $^{-1}$

Table 11. FDH : BV activity in cultures grown under different growth conditions

Growth condition	Electron acceptor added to the growth medium	Specific* activity
Aerobic	None	259
Microaerobic	None	231
"	TMAO	617
"	Nitrate	329
"	Fumarate	279

* Specific activity, nmol BV^{++} reduced min^{-1} (mg protein) $^{-1}$

Table 12. The distribution of FDH : DCPIP and FDH : BV in different cellular fractions

Cellular fraction	FDH : DCPIP*		FDH : BV**	
	Specific activity	Total activity	Specific activity	Total activity
Whole cell	4.13	247.20	1.27	76.89
Crude extract	6.50	346.20	2.12	112.74
Periplasm	0.58	1.40	0.00	0.00
Spheroplast	11.00	731.50	2.27	151.50
Spheroplast crude extract	5.10	277.70	2.98	162.60
Cytoplasm	2.54	50.00	3.06	60.30
Membrane	3.43	89.00	2.18	5.70

* $\mu\text{mol DCPIP reduced min}^{-1} (\text{mg protein})^{-1}$

** $\mu\text{mol BV}^{++} \text{ reduced min}^{-1} (\text{mg protein})^{-1}$

sodium deoxycholate and sodium dodecylsulphate; and finally the cationic detergent cetyltrimethylammonium bromide (CTAB). The results are shown in Table 13. It appears that CTAB is the best agent to solubilise the FDH as most of the activity was removed to the soluble fraction (99%) with no loss of activity and the treatment was selective in that only 6% of the total protein was solubilised. Triton X-100, the next most effective agent solubilised a high amount of membrane proteins (48%). However, the solubilised activity was similar to that solubilised by chlorate and sodium dodecylsulphate. Brij 36 was the least effective agent: it solubilised 68% of membrane proteins and there was 26% reduction in activity compared to the control.

As CTAB was the best solubilising agent for FDH, further investigations were done to find out the optimum concentration for solubilisation (Figure 1). The maximum solubilisation and highest selectivity was obtained with 0.5% CTAB; lower concentrations failed to solubilise FDH, while at higher concentrations the specific activity was lower due to more proteins being solubilised.

(e) Stability of formate dehydrogenase

FDH has been often reported in other organisms as being an unstable enzyme. The stability of FDH derived from A. putrefaciens cells grown microaerobically with TMAO was investigated in three different systems (Table 14). Firstly membrane was prepared in TMAO buffer containing metal ions like Mg^{++} and a high concentration of NaCl, secondly membrane was prepared in 20 mM Tris-HCl containing 1 mM EDTA as FDH stabiliser and thirdly a portion of membrane prepared in Tris-HCl/EDTA buffer was subjected to CTAB treatment to solubilise the FDH. Samples were incubated at three different temperatures 0°C, -20°C, and -80°C. It could be concluded from Table 14 that temperature had different effects and the presence of EDTA always provided some protection. Generally under all conditions there was an initial reduction in the activity. The best storage conditions was to keep the membrane with high protein content in Tris-HCl buffer containing EDTA at low temperature.

Table 13. Solubilisation of FDH from the membrane using different agents

Agent		FDH : DCPIP* specific activity	% of CTAB treatment	Selectivity** of treatment
Membrane (control)		2427.90		
0.5%	CTAB	7089.50	100	2.92
1%	Triton X-100	2671.50	37.70	1.10
0.5 mM	KClO ₃	2656.50	37.50	1.09
1 mM	SDS	2628.20	37.10	1.08
2 mgml ⁻¹	Tween 80	2460.60	34.70	1.01
3 mM	DOC	2103.10	29.70	0.87
1%	Brij 35	2036.20	28.70	0.84
1 mM	KSCN	1808.70	25.50	0.74
1%	Brij 36	1749.80	24.70	0.72

* As in Table 10

** Selectivity =
$$\frac{\text{Specific activity of soluble fraction}}{\text{Specific activity of membrane sample (control)}}$$

SDS = sodium dodecylsulphate

DOC = sodium deoxycholate

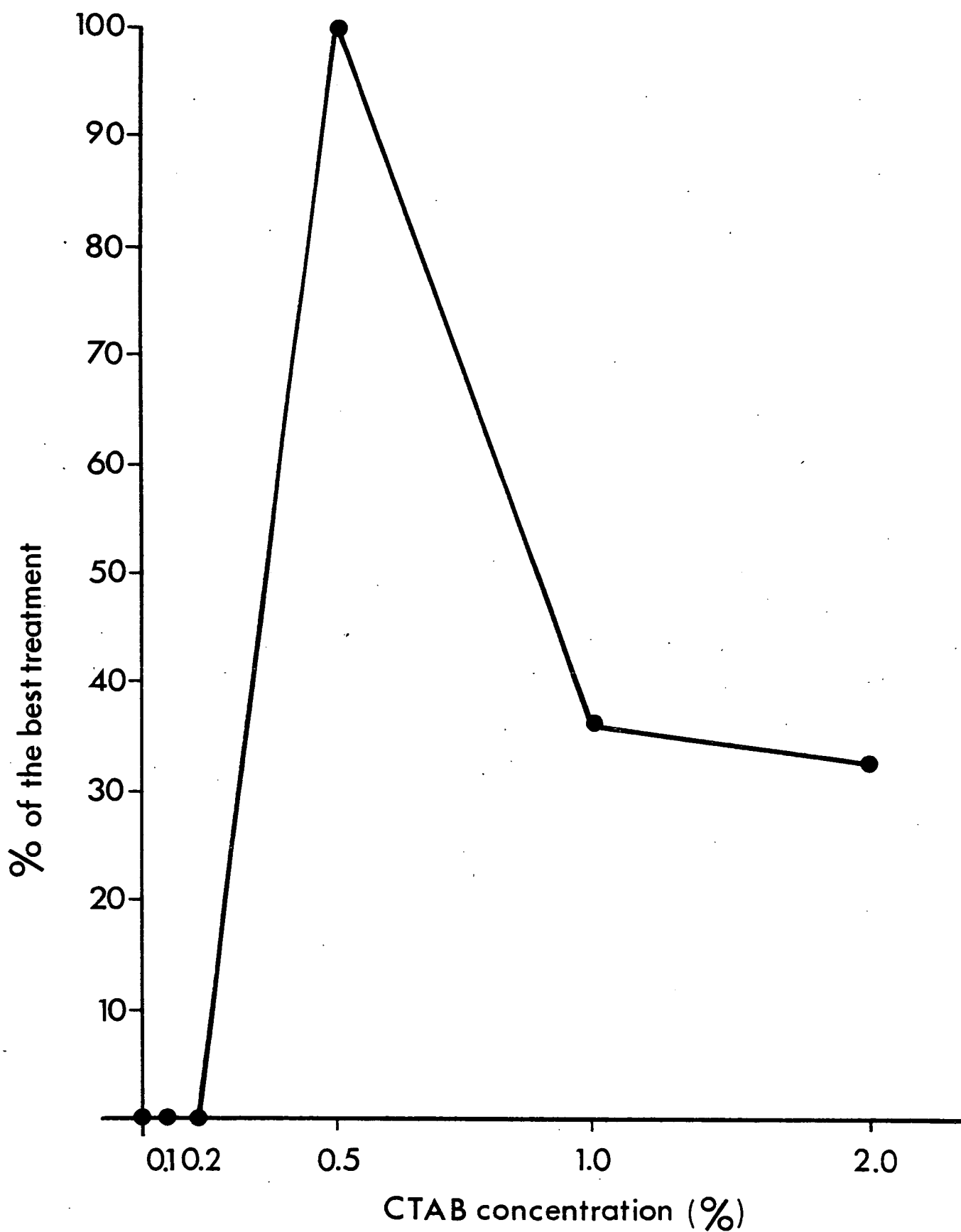


Figure 1: Optimum concentration of CTAB required to solubilise the FDH from the cytoplasmic membrane of *A. putrefaciens*

Table 14. FDH stability under different conditions

Time (d)	TMAO buffer			Tris/EDTA buffer			CTAB solubilised sample		
	0°C	-20°C	-80°C	0°C	-20°C	-80°C	0°C	-20°C	-80°C
1	100	100	100	100	100	100	100	100	100
2	72	78	38	52	61	56	47	60	90
3	73	8	N.D.	59	74	N.D.	49	52	N.D.
4	66	0	N.D.	44	70	N.D.	23	69	N.D.
5	48	0	47	36	60	71	23	50	51
6	51	0	70	31	87	74	20	66	57
7	47	0	66	36	65	87	15	42	64
8	43	0	63	35	61	84	9	65	80
9	35	0	45	28	60	77	9	56	59

N.D. = not determined

5. Discussion

(a) Regulation of anaerobic respiration

The results of Tables 1, 4 and 7 indicate that the terminal reductases involved in anaerobic respiration were absent or present at very low levels under aerobic conditions, but their activities were enhanced greatly at low oxygen tension even in the absence of any terminal electron acceptor. Addition of an electron acceptor to the growth medium resulted in a great increase in the activity of the corresponding reductase. In all three anaerobic respiratory systems investigated for A. putrefaciens, it has been found that combination of low oxygen tension with the presence of the corresponding electron acceptor resulted in the highest activity.

The effect of oxygen on the activities of terminal respiratory enzymes in anaerobic electron transport could occur at a number of levels: during transcription, translation, assembly of polypeptides, insertion into the membrane (where appropriate) and catalysis. Transcriptional control of nitrate reductase synthesis in E. coli has been studied by several authors but their conclusions differ. Stouthamer et al. (1980) suggested that induction of nitrate reductase required the presence of nitrate and absence of oxygen and this might indicate that both factors are required for repression and biosynthesis. They proposed this hypothesis upon the evidence that oxygen and possibly nitrate simultaneously affect the transcriptional and translational processes. Both nitrate and oxygen affect the synthesis of mRNA specific for nitrate reductase, but oxygen does not affect the initiation of mRNA or initiation of nitrate reductase but a step in between (see also Ruiz-Herrera & Salas-Vargas, 1976). Recent studies using chl C-lac operon fusion indicated that the principal controls of nitrate expression acts at the level of transcription (Chippaux et al., 1981; Stewart, 1982) on the other hand Showe & De Moss (1968) claimed that the synthesis of nitrate reductase is not only controlled by a repressor which is sensitive to nitrate and affects mRNA

synthesis but also by a redox sensitive repressor and that the effective intracellular redox potential would be the controlling factors. Therefore, Wimpenny & Cole (1967) suggested that the redox potential of the medium rather than the presence of a specific electron acceptor regulates the formation of the reductase. However, the intracellular redox potential is influenced by several factors such as the identity and concentration of the electron acceptor (which determines the extracellular redox potential and capability for electron flow in cells). A similar mechanism for control has been proposed by Kapralek et al. (1982).

De Groot & Stouthamer (1970a, b) assumed that nitrate reductase represses its own synthesis under all conditions in which electron flow to the redox enzyme is impossible; in such a mechanism oxidised nitrate reductase might act as a repressor for its own synthesis and this type of regulation has been called "Autogenous regulation of gene expression" (Goldberg, 1974), which is equivalent to the regulation mechanism proposed for the assimilatory nitrate reductase of Aspergillus nidulans by Cove & Pateman (1969).

The presence of the electron transport chain to oxygen might repress the formation of reductases by changing the redox state of membrane components via terminal oxidases (see Oltmann et al., 1976b; Kučera et al., 1981). The latter suggestion has been confirmed by the results of an E. coli mutant blocked in aerobic respiration (Simoni & Schalenberger, 1967); this mutant was able to produce a functional nitrate reductase when nitrate was added to the growth medium under aerobic conditions and was also chlorate sensitive.

Hackett & MacGregor (1981) showed that oxygen affected the insertion of the newly synthesised soluble nitrate reductase of E. coli into the membrane. Insertion appeared to be dependent on another protein formed under conditions which induced nitrate reductase; the identity of this protein could be the cytochrome b apoprotein (Chaudhry & MacGregor, 1983b).

The mechanisms by which oxygen affects the respiratory enzymes in A. putrefaciens are not fully understood at present but it is certain that there is more than one control mechanism. The low nitrate reductase activity under aerobic conditions could reflect repression of nitrate reductase synthesis by lack of electron transport to nitrate or through a high redox potential. On the other hand transcription did not appear to be inhibited by oxygen as the aerobic culture of A. putrefaciens had a considerable activity of anaerobic reductase when the medium contained the appropriate electron acceptor. However, the induction of nitrate reductase to a full level in A. putrefaciens required the presence of nitrate and absence of oxygen and this might indicate that both factors are required for induction and biosynthesis. Oxygen was able to inhibit the activity of TMAO reductase in A. putrefaciens, whereas no such effect was observed for E. coli. Any effect of oxygen on insertion of the enzyme into the membrane can be excluded since all the terminal reductases in A. putrefaciens were periplasmic. The effect of oxygen could be further investigated by studying the polypeptide of each reductase by using electrophoretic studies and/or immunological studies throughout the synthesis of the reductase.

The high induction of TMAO reductase at low oxygen tension and in the presence of TMAO in A. putrefaciens is in agreement with the results obtained in E. coli. Directly or indirectly TMAO appears to act as an inducer for TMAO reductase, FDH and cytochrome c552 in A. putrefaciens (this study; Easter, 1982). The mechanism of induction in A. putrefaciens is unknown but may be similar to the induction of nitrate reductase in E. coli (Stouthamer et al., 1980). Alternatively, induction may depend upon the redox potential as suggested for E. coli TMAO reductase (Sakaguchi & Kawai, 1977b). They suggested that the change in the redox potential inside and outside the cells under such conditions is necessary for commencing the enzyme synthesis which has been attributed to de novo synthesis of enzyme protein (Sakaguchi & Kawai, 1975a). The zero or low TMAO reductase activities in

cultures of A. putrefaciens grown aerobically can be explained as repression by oxygen. In E. coli both the synthesis of TMAO reductase and its activity are inhibited by oxygen and this could be a reflection of the high preference of cells for oxygen as terminal electron acceptor so that cells grown even under anaerobic conditions retained the respiratory chain linked to oxygen together with that to TMAO as terminal electron acceptor (Sakaguchi & Kawai, 1976) and this is also true for other anaerobic respiratory chain like the nitrate respiratory chain (Hackett & Bragg, 1983a, b).

In A. putrefaciens both nitrate and fumarate were able to act as terminal electron acceptors for anaerobic growth. Neither nitrate nor fumarate appeared to repress TMAO reductase synthesis and TMAO seems the preferred electron acceptor in this bacterium despite the lower redox potential of the TMAO/TMA couple (0.13V) compared with the nitrate/nitrite couple (0.42V). Both the reduction of TMAO and nitrate were estimated in cultures of A. putrefaciens grown on nitrate and TMAO, but it was not determined which product was formed first (Easter, 1982). However, in cells grown under these conditions TMAO reductase activity was not reduced at all (this study). A. putrefaciens grown on nitrate showed lower activity of fumarate reductase compared with cells grown in the absence of any terminal electron acceptor. Conversely fumarate caused a severe reduction in both MV^{++} : nitrate reductase and formate : nitrate chain activities. This could mean that in A. putrefaciens fumarate is used preferentially to nitrate probably because fumarate is more important physiologically as being produced endogenously as a metabolic intermediate and both nitrate and fumarate respiration could be regulated by the same system in a similar manner to the regulation of TMAO and nitrate respiration of E. coli. The order of preference for terminal electron acceptors in A. putrefaciens appears therefore to be oxygen > TMAO > fumarate > nitrate.

These results contrast with work on E. coli where nitrate appears to be used in preference to TMAO. Nitrate inhibited the synthesis of TMAO reductase but not its activity (Sagai & Ishimoto, 1973; Sakaguchi & Kawai, 1975c). Indeed mutants have been

isolated (Nark⁻) that are altered in the repression of TMAO reductase by nitrate. These mutants were found to have a functional nitrate reductase and at the same time to be derepressed for TMAO reductase formation in the presence of nitrate. The authors postulated that the Nark⁻ phenotype reflects the loss of a gene product(s) which responds to nitrate respiration and acts to repress the transcription of other respiratory enzymes (Stewart & MacGregor, 1982; Stewart, 1982). The repression of TMAO and fumarate reductases by oxygen and nitrate in E. coli has been interpreted as a mechanism for ensuring that the hydrogen acceptor with the highest energy yield is utilised in catabolism. In view of the results presented here for A. putrefaciens this interpretation must be modified to account for the physiological adaptation of bacterial species with time and effect of the environmental factors in their habitat. TMAO is available in the marine environment of A. putrefaciens and hence may be preferred because of its relative abundance.

In conclusion it seems that TMAO functions directly or indirectly as an inducer for TMAO reductase and other components such as cytochrome c₅₅₂ and FDH in A. putrefaciens. The second function of TMAO is to accept electrons from oxidisable substrates. This bifunctional role of TMAO in cell respiration would be reproduced in the course of marine fish spoilage: as soon as oxygen was consumed by microbial respiration the conditions for induction of TMAO reductase would occur so that Alteromonas spp could grow at the expense of strictly aerobic bacteria.

(b) Location of enzymes

The terminal component of a respiratory chain which reacts with an organic or inorganic electron acceptor is usually membrane bound. The results of Tables 3, 6 and 9 showed that all the terminal reductases of A. putrefaciens are periplasmic, but could be loosely associated with the cytoplasmic membrane. TMAO reductase and its associated cytochrome c₅₅₂ have been shown to be periplasmic in Alteromonas spp (Easter, 1982). In contrast TMAO reductase in E. coli and Vibrio parahaemolyticus is membrane

bound (see Introduction). However, a few reductases in other bacteria have been reported to be periplasmic e.g. nitrite reductase in Pseudomonas aeruginosa (Wood, 1978) and Paracoccus denitrificans (Meijer et al., 1979; Alefounder & Ferguson, 1980). Fumarate reductase is usually membrane bound with the exception of Clostridium formicoaceticum where it is periplasmic and fumarate reduction is not coupled to oxidative phosphorylation but serves merely as a hydrogen sink (Dorn et al., 1978). One advantage to a bacterium of a periplasmic location for enzymes is that the toxic end products of reduction such as nitrite and TMA would be harmful and cause damage to cell components; therefore their generation outside would minimise this effect. On the other hand such an explanation cannot hold for fumarate reductase whose reduction product is succinate. The respiratory systems of Alteromonas spp with periplasmic terminal reductases could represent another class of respiratory chain with extracytoplasmic location obviating the need for active transport.

Retention of some TMAO reductase or fumarate reductase activity by spheroplasts could be attributed to either loose association of these enzymes with the cytoplasmic membrane as for periplasmic enzymes which are not firmly bound to the structural components but can be associated with lipopolysaccharide (Costerton et al., 1974), or the reductases could be bound to other components of the respiratory chain. Alternatively, the retention of activity could be due to insufficient breakdown of the cell wall but using harsher conditions of spheroplasting caused leakage of cell components and cell lysis as judged by the high viscosity of the resultant suspension.

As shown in Table 12 FDH is essentially membrane bound; although the cytoplasm had some activity, contamination with membrane fragments could be expected as the cells were broken with the French pressure cell. This treatment has been suggested to cause aggregation of very small membrane fragments that cannot be removed by centrifugation (MacGregor, 1975a). Even using other methods like sucrose gradient differential centrifugation might not give sharp separation since the membrane bound FDH of

E. coli sedimented in a broad band on sucrose gradient indicating that derived membrane fragments were present which failed to sediment by virtue of their size or composition. These fragments could be membrane-like **lipid-protein** aggregates (Cox et al., 1981).

(c) Formate dehydrogenase

(i) FDH : DCPIP and FDH : BV

The distinction between FDH : DCPIP and FDH : BV is based partly on the electron acceptor specificity of FDH associated with the two enzymes. An attempt was made to find out the role of these enzymes in A. putrefaciens and to determine whether they were distinct enzymes or not; the results shown in Table 11 do not give any indication as FDH : BV activity was more or less similar under all growth conditions except that for TMAO grown culture which showed some enhancement of activity. It is known in other organisms that FDH : BV is depressed when cells are grown anaerobically with nitrate, but the results of Table 11 indicated that there is no depression of this enzyme in cells grown with nitrate.

E. coli metabolises formate anaerobically, firstly via the formate hydrogenlyase system, which catalyses formate oxidation to carbon dioxide and hydrogen. This is present during fermentative growth on glucose under strict anaerobic conditions and is composed of FDH, hydrogenase and unidentified electron carriers but devoid of cytochromes. The FDH involved in this system is repressed during growth in the presence of an exogenous electron acceptor and is able to use BV as an artificial electron acceptor; this enzyme is also induced by formate. Secondly formate oxidation can also be coupled to the reduction of nitrate via a formate : nitrate respiratory pathway, the FDH involved in this system is the FDH : DCPIP (Lester & De Moss, 1971; Ruiz-Herrera & Villarreal-Moguel, 1975).

Confirmation for the existence of two different FDH(s) came, firstly by using a haem-less mutant which was able to synthesis FDH : BV but not FDH : DCPIP (Gray et al., 1966), and secondly

by isolation of mutants from E. coli and Salmonella typhimurium with a functional formate hydrogenlyase system and its associated FDH : BV but lacking the FDH : DCPIP enzyme (Ruiz-Herrera & De Moss, 1969; Barrett et al., 1979). Cox et al. (1981) achieved a partial separation of the two enzyme activities and on the basis of an analysis of selenium-containing peptides, suggested that the FDH : DCPIP and FDH : BV possess different selenopeptides. Recently, it has been found that both enzymes are immunologically distinct (Giordano et al., 1983).

The cellular location of these two enzymes has not been clearly established and appears to depend on the organisms and on the conditions employed for cellular fractionation. Ruiz-Herrera et al. (1972) in E. coli reported that both FDH : BV and FDH : DCPIP are membrane bound, while Gray et al. (1966) using another E. coli strain found that FDH : BV was distributed in both the soluble and membrane fraction. In a recent study 80% of FDH : BV in E. coli was found in the soluble fraction (Giordano et al., 1983). The latter findings could be similar to the situation in A. putrefaciens (Table 12). However, in Vibrio succinogenes both FDH : BV and FDH : DCPIP are membrane bound and the workers suggested that on present evidence only one form of enzyme exists in this bacterium (Kröger et al., 1980).

It seems that the idea of two different enzymes is more likely in the fermentative organisms mentioned above in which the two enzymes showed clearly different characteristics with respect to their molecular weights, optimum temperature and optimum pH for activities (Ruiz-Herrera & Villarreal-Moguel, 1975). Moreover, both activities require molybdenum and selenium (Cox et al., 1981, see also section III), such close relation suggests that both enzymes might share the polypeptide responsible for formate oxidation and that this polypeptide might be bound into the membrane to different components each responsible for the specificity of electron acceptor. Alternatively, both enzymes may be genetically unrelated but may require a common component like the molybdenum cofactor (see section III).

(ii) FDH solubilisation

According to Maddy & Dunn (1976) membrane proteins can be categorised into three groups: (1) proteins which are loosely attached and can be washed away by physiological saline, (2) peripheral or extrinsic proteins bound to membranes under physiological conditions, but released when conditions are altered slightly e.g. washing with low ionic strength solutions, and (3) integral or intrinsic proteins, which are tightly bound. Integral proteins can be separated from membrane lipids only under conditions that disturb the hydrophobic interactions and lead to the complete disruption of membrane structure. FDH seems to belong to this third group as washing the membrane with TMAO buffer, a treatment used in preparing spheroplasts failed to release it from the membrane.

The term solubilisation can be defined operationally that certain particulate proteins can no longer be sedimented by preparative ultracentrifugation. Solubilisation can be achieved by using different treatments, for instance, heat solubilisation in diluted alkaline solution at 60°C has been used successfully in solubilisation of nitrate reductase system from E. coli membranes (MacGregor, 1975a). Under such conditions a specific membrane bound protease was activated and cleaved the hydrophobic region. The other means is to use chemical reagents and under such circumstances the loss of biological activity from the membrane can be used as an indicator of solubilisation for those proteins with overt biological activity. As chaotropic salts have a wide range of effectiveness in solubilising proteins $KClO_3$ and KSCN were used to solubilise the FDH from the membranes of A. putrefaciens. Neither reagent was particularly effective in solubilising FDH. Also KSCN caused loss of about 26% of the activity (Table 13). As the effect of these salts depends upon their ability to alter the charge distribution of membrane components, FDH in A. putrefaciens may be bound to the membrane by hydrophobic interactions.

The other group of chemicals were detergents, which usually interact with both hydrophilic and lipophilic regions of proteins in an essentially disruptive fashion. An important point in

using detergents for solubilisation is that two sets of interactions are involved in the process: the interaction of detergent molecules with membrane components and interactions between detergent molecules. The former is important at low detergent concentrations and the latter at high concentrations. The interaction of detergent molecules with a membrane component depends on both the number of binding sites and the affinities of those sites towards the detergent molecules; usually sites of high affinity are few and saturated at low detergent concentration, such that binding has a minimal effect on protein conformation. The second set of interactions depends on the critical micellar concentration (cmc) of detergent, if this cmc is sufficiently low, the concentration of free molecules will only be sufficient to saturate protein sites of high affinity for detergent with minimal effect on protein conformation, accordingly, the detergents with low cmc will tend to be less disruptive than those with a high cmc.

In solubilising the A. putrefaciens membrane proteins some ionic detergents were used. Sodium deoxycholate was found to be less efficient in solubilising the FDH as can be seen from Table 13. In E. coli both nitrate reductase and FDH were found to be cosolubilised by $1 \text{ mg (mg protein)}^{-1}$ of sodium deoxycholate (Enoch & Lester, 1974). In general a disadvantage of using such detergents is that they tend to be more denaturing than a non-ionic detergent. Another ionic detergent sodium dodecylsulphate was also used, but it was not very efficient. This is in agreement with the known characteristics of sodium dodecylsulphate which has a high cmc value, exhibits a high level of binding and causes disruption of the native conformation of proteins.

A non-ionic detergent Triton X-100 (1%) was used to solubilise A. putrefaciens FDH. It was found to solubilise 48% of the membrane protein giving a low specific activity for FDH. Higher concentrations of Triton X-100 were avoided since it is known that Triton X-100 at high concentration would be disruptive for both outer and inner membranes (Filip et al., 1973). However, Triton X-100 is widely used as its cmc is very low (0.25 mM) and it has been found that the proteins solubilised by this detergent have high molecular weights. This may indicate that the solubilised materials are present in the form of small vesicles or aggregates and can be

useful for isolating intact respiratory chain components. For example the nitrate reductase complex of E. coli was released in an intact state using Triton X-100 (MacGregor & Schnaitman, 1974). Triton X-100 was used also to solubilise fumarate reductase of Vibrio succinogenes (Kröger et al., 1980) and in E. coli has been used successfully to solubilise the fumarate reductase but as it also solubilised about 60-80% of the membrane protein (Plas et al., 1983) further studies were precluded.

The Brij series of detergents are linear primary alcohol polyoxyethylene compounds and are thought to break the hydrophobic bonds between the lipid and protein of the cell membrane. Two detergents were used to solubilise the A. putrefaciens FDH. Brij 36 solubilised 68% of the membrane protein and at the same time it caused about 26% loss in activity (Table 13). In E. coli Brij 36 was found to be the best treatment to solubilise FDH : DCPIP and FDH : BV (Ruiz-Herrera et al., 1972) and solubilised 30-40% of membrane proteins without loss in activity.

From the results presented in Table 13, it can be concluded that the best treatment for FDH solubilisation of A. putrefaciens was achieved with CTAB. Generally such cationic detergents are used as bacteriostats at low concentration and as bacteriocides at high concentrations and the cell death results from disruption of the bacterial cell membrane. Effective contact between these detergents and bacterial cells is facilitated by the negative charges normally present at the cell surface. The CTAB effect may be inhibited by the presence of cations such as Ca^{++} and Mg^{++} competing for the anionic sites of the cell surface, so care was taken to minimise the presence of such cations.

In an extensive study on the mechanism of action of CTAB Nozaki et al. (1974) suggested that there are four sites for CTAB attachment to the protein used in this study (bovine serum albumin) and they are close to glutamate or aspartate residues. It has been found that solubilisation of proteins from membranes with such cationic detergents resulted in denaturation of those proteins, but in some cases it has been reported that CTAB had no denaturing effect on the solubilised bovine rhodopsin (Heller, 1968; Hong &

Hubbell, 1972). A striking point is that CTAB has been found to inhibit the FDH : DCPIP of E. coli (Ruiz-Herrera & Villarreal-Moguel, 1975), whereas an activation of FDH activity was found in this study using A. putrefaciens.

The maximum solubilisation occurred at 0.5% concentration, higher concentrations were less effective. Nozaki et al. (1974) suggested that for CTAB at high concentrations the interaction becomes similar to the corresponding process for sodium dodecyl sulphate, but as CTAB has a longer alkyl chain, it has correspondingly greater intrinsic hydrophobicity than sodium dodecylsulphate. In this study there was no loss in FDH activity at high concentration of CTAB but as larger amounts of proteins were solubilised, the specific activity was reduced.

(iii) FDH stability

FDH from many sources is unstable and this is true for both FDH : DCPIP and FDH : BV. EDTA and DTT have been used as protective agents in addition to the use of anaerobic buffers (Ruiz-Herrera & Villarreal-Moguel, 1975; Cox et al., 1981). Many factors have been reported to cause FDH inactivation particularly, molecular oxygen as 80% of FDH : DCPIP and FDH : BV activities of E. coli were lost when bubbled with air for 1 min (Giordano et al., 1983). Other factors could account for FDH inactivation like buffer ions, pH, protein concentration, temperature and light (Leohardt & Andreessen, 1977). Metals are known to inactivate the FDH, if they are present in excess over the protecting EDTA. This was observed for A. putrefaciens FDH (Table 14). From the results of Table 14 FDH inactivation probably occurred by combination of more than one factor. For the first buffer used (TMAO buffer) the effect of temperature is very clear and a very low temperature afforded good protection; and the loss of activity at -20°C could be due to mechanical effects from short freezing-thawing cycles which can occur at this temperature especially when very small amounts of samples are used. Inclusion of EDTA gave more protection but it seems

to be more effective when used at a lower temperature in conjunction with a high protein concentration. DTT was not included in the system used in Table 14 due to chemical interference of this agent with the reaction mixture used for FDH : DCPIP assay.

FDH is known to be a very complicated enzyme and one aspect of the relationship of its structure to inactivation has focused on the sulphydryl group content. It has been demonstrated that the FDH of one Gram-negative methane utilising bacteria contains 12-SH groups; as two of them are essential and inactivated very rapidly it has been proposed that these are involved in the active site of the enzyme. Modification of two other -SH groups led to 15-20% loss in activity. In the latter modification, formation of internal disulphide bounds and conformational changes could be reversed by adding mercaptoethanol. The remaining eight -SH groups were less important and the differences in the reactivity of these groups is evidently due to their different accessibility with respect to the modifying agents and their different microsurroundings (Dikov et al., 1980). The enzyme needs to be in an active conformation for optimum activity and the conformation could be changed by freezing-thawing process which consequently affected the activity as shown in Table 14. Most likely FDH inactivation occurs at the active site where formate is oxidised. In a recent study Egorov et al. (1982) has shown that modification can occur at this site, using agents like pyridoxal that affect the formate binding site of FDH. These agents may act by modifying the lysine residue thought to be involved in the binding of formate through its carboxyl group to the amino group of lysine

SECTION II

MOLYBDENUM COFACTOR EXPERIMENTS

1. Molybdenum Cofactor in Wild type *Neurospora crassa*

As the molybdenum cofactor is a constitutive component in *N. crassa*, an uninduced culture for nitrate reductase was used to complement an extract derived from an induced culture of the *nit-1* mutant; incubations were at 4°C for 4h.

Figure 2a shows the effect of addition of exogenous molybdate (10 mM) in the complementation mixture for a ratio of *nit-1* extract : wild type extract of 1 : 1. Figure 2b shows the effect of varying the ratio of *nit-1* extract to wild type extract (i.e. molybdenum cofactor source) in the complementation mixture; the ratio of 3 : 1 (*nit-1* : wild type) gave the highest activity, and this ratio was used in subsequent experiments.

The effect of time on the complementation process is shown in Figure 2c; complementation was independent of time over the intervals used and a period of 4h incubation at 4°C was used for further work. The effect of different molybdate concentrations on the complementation process is shown in Figure 2d; there was no significant difference between different concentrations and 10 mM molybdate was chosen for further work.

2. Optimum Conditions for *Alteromonas putrefaciens* Molybdenum Cofactor Complementation

Aerobic crude extract (20 µl) of *A. putrefaciens* grown to late log phase in Wood & Baird medium containing 2 mM molybdate was mixed with 0.3 ml *nit-1* extract in the presence of 10 mM molybdate and kept for 4h at 4°C as previously described for wild type *N. crassa*, other samples were incubated at room temperature (20 - 25°C) for 10 min and 4h. The results are depicted in Figure 3. As incubation at room temperature for

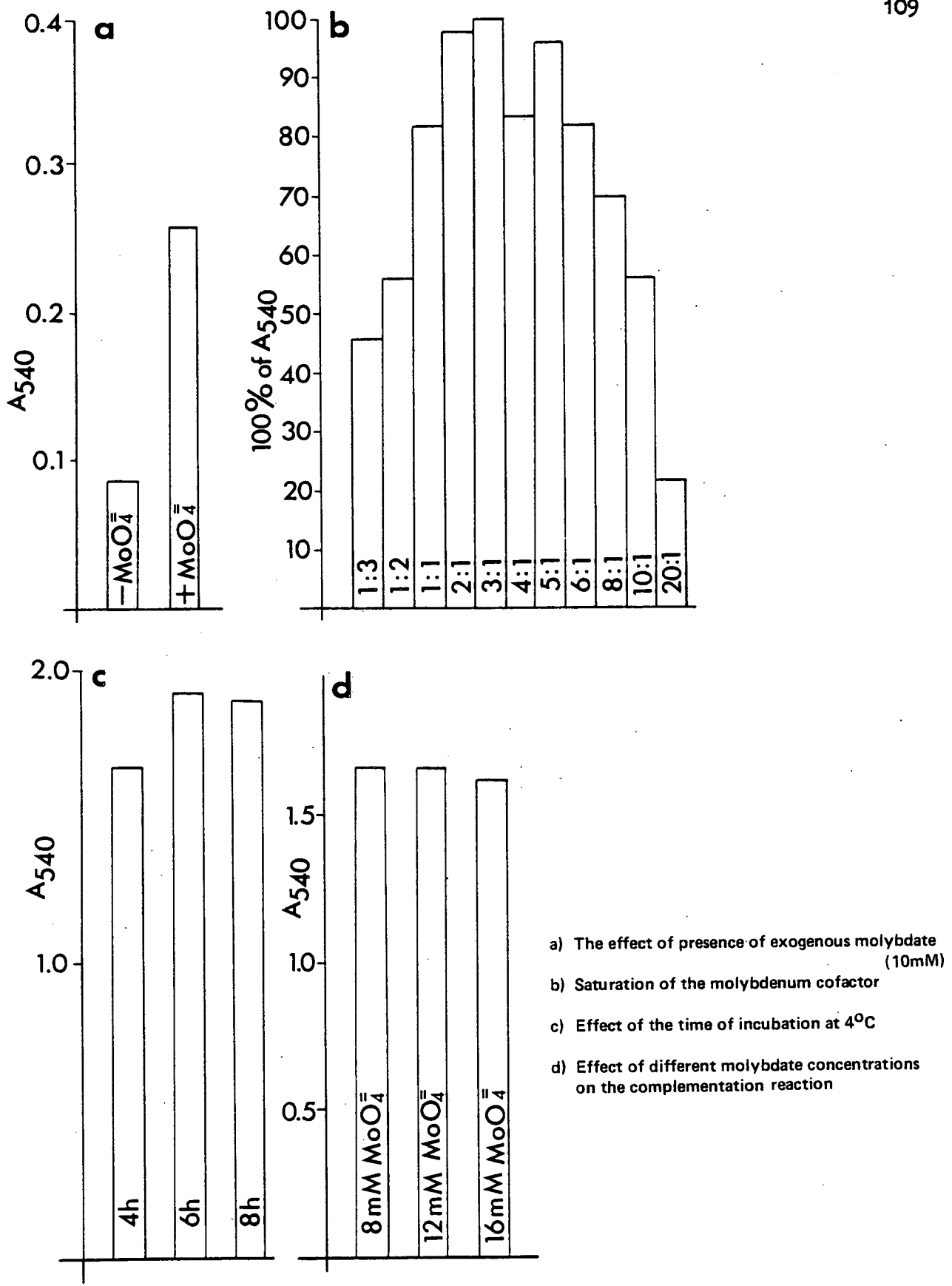


Figure 2. Complementation of nit-1 extract with extract of uninduced wild type N. crassa extract

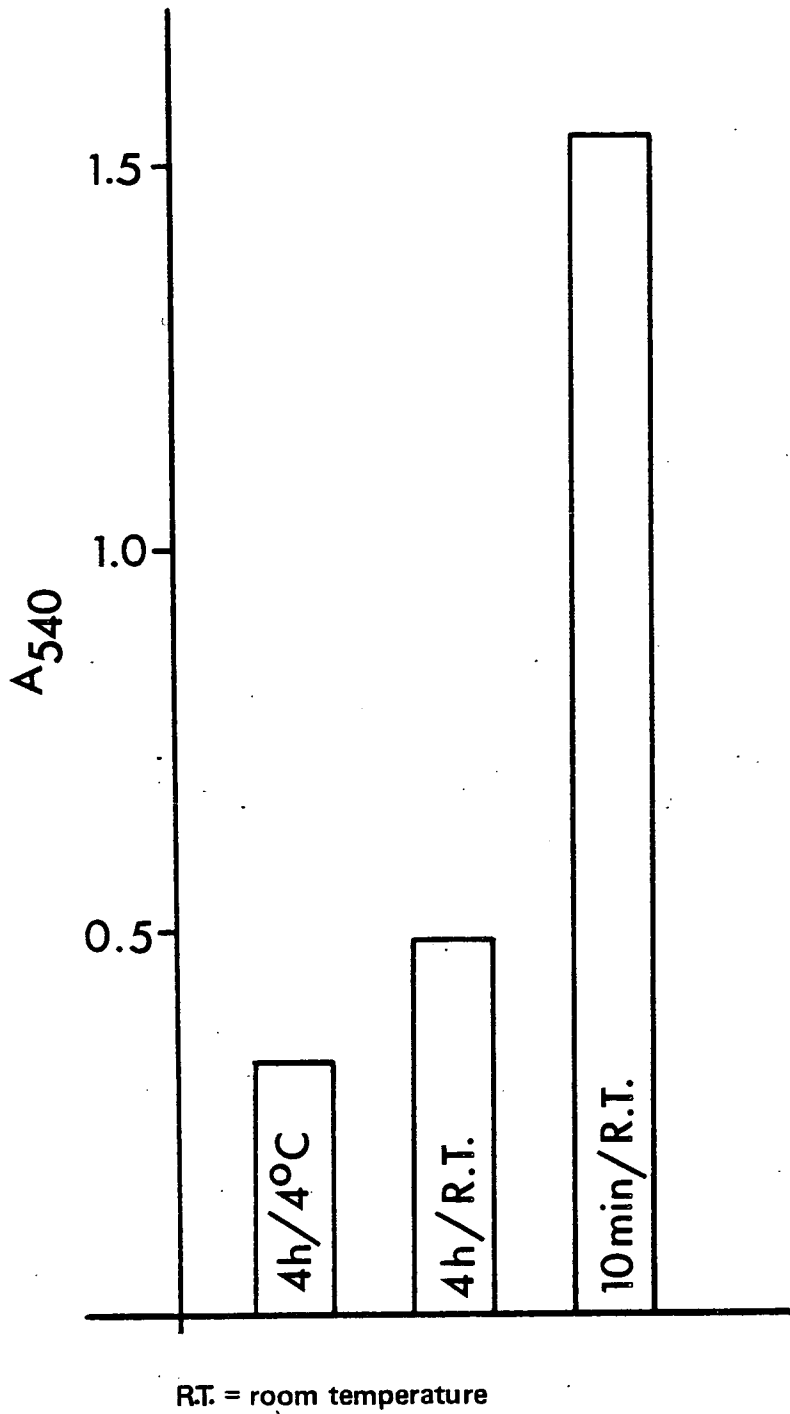


Figure 3. Effect of temperature and time on *nit-1* complementation with *A. putrefaciens* molybdenum cofactor

10 min gave the highest nitrate reductase activity, this treatment was chosen for further work.

3. Molybdenum Cofactor Saturation and Time Course of Complementation

(a) Using aerobic crude extract from A. putrefaciens for complementations, different amounts of molybdenum cofactor source (3 mg ml^{-1} protein) were mixed with 0.3 ml nit-1 crude extract in the presence of 10 mM molybdate and incubated at room temperature for 10 min. The reconstituted activity of nit-1 nitrate reductase increased linearly at the beginning with the amount of bacterial extract but tailed off with increasing amounts of extract (Figure 4), for further experiments 50 μl was used.

(b) The time course for complementation was examined using 50 μl of molybdenum cofactor source. The mixture was incubated at room temperature for different intervals and assayed for nitrate reductase activity (Figure 5). Maximum complementation activity was reached after 20 min, the activity remained at the same level for a further 20 min and then started to decrease with time. Therefore, the best complementation time was 20 min which was used subsequently.

(c) Complementation of nit-1 was carried out using the cytoplasm of cells grown microaerobically with nitrate and the previously defined conditions for complementation; the saturation of N. crassa nit-1 nitrate reductase with bacterial molybdenum cofactor was studied using different amounts of molybdenum cofactor source (1.27 mg ml^{-1} protein). The results are represented in Figure 6, and were similar to those shown in Figure 4.

4. Molybdenum Cofactor Stability

The stability of molybdenum cofactor was studied by assaying the complementation activity during storage under various conditions. Initially cells were broken in 10 mM phosphate buffer, pH 7.4 and samples were kept statically in sealed containers at 0°C . The

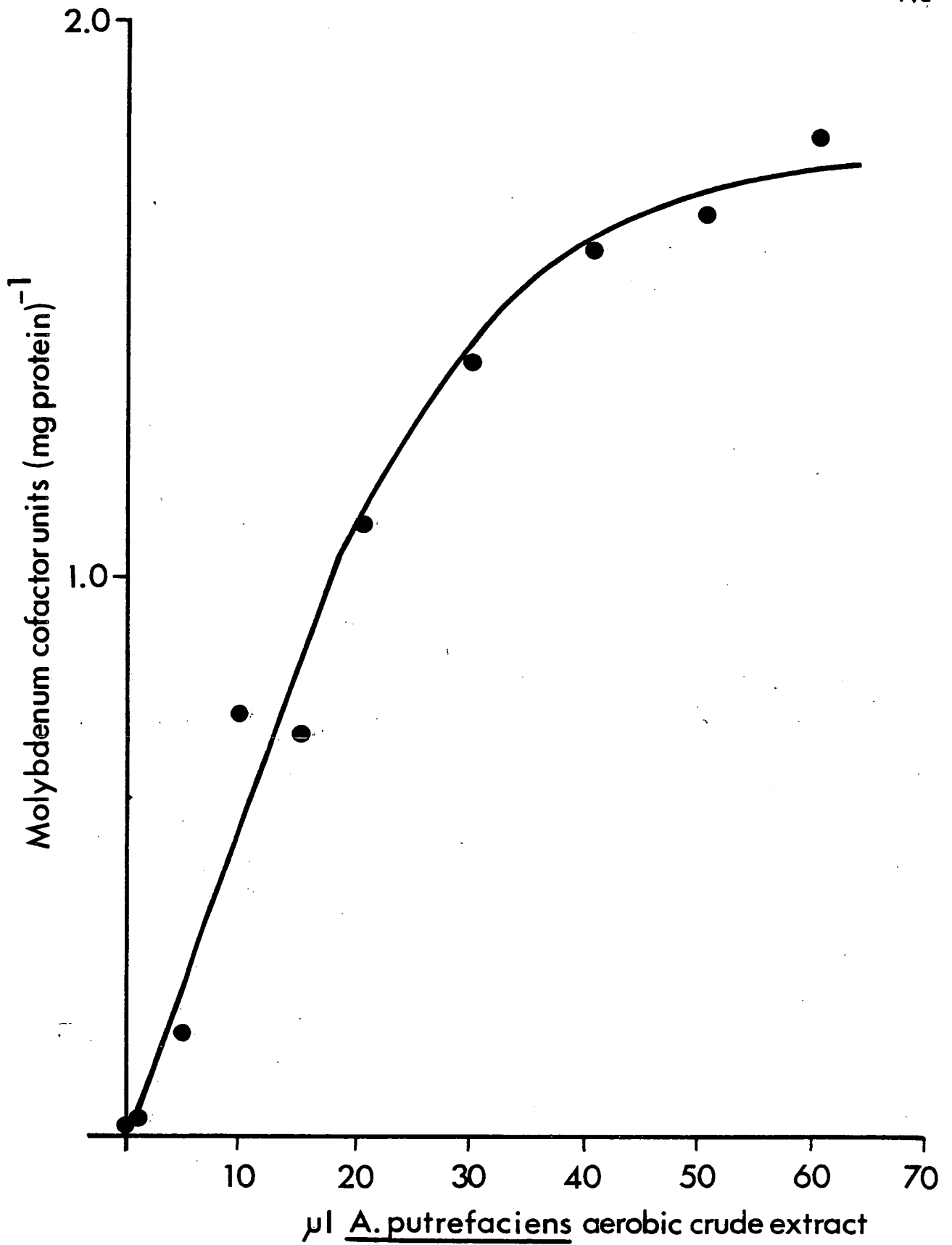


Figure 4: Saturation of the molybdenum cofactor using aerobic crude extract of *A. putrefaciens*

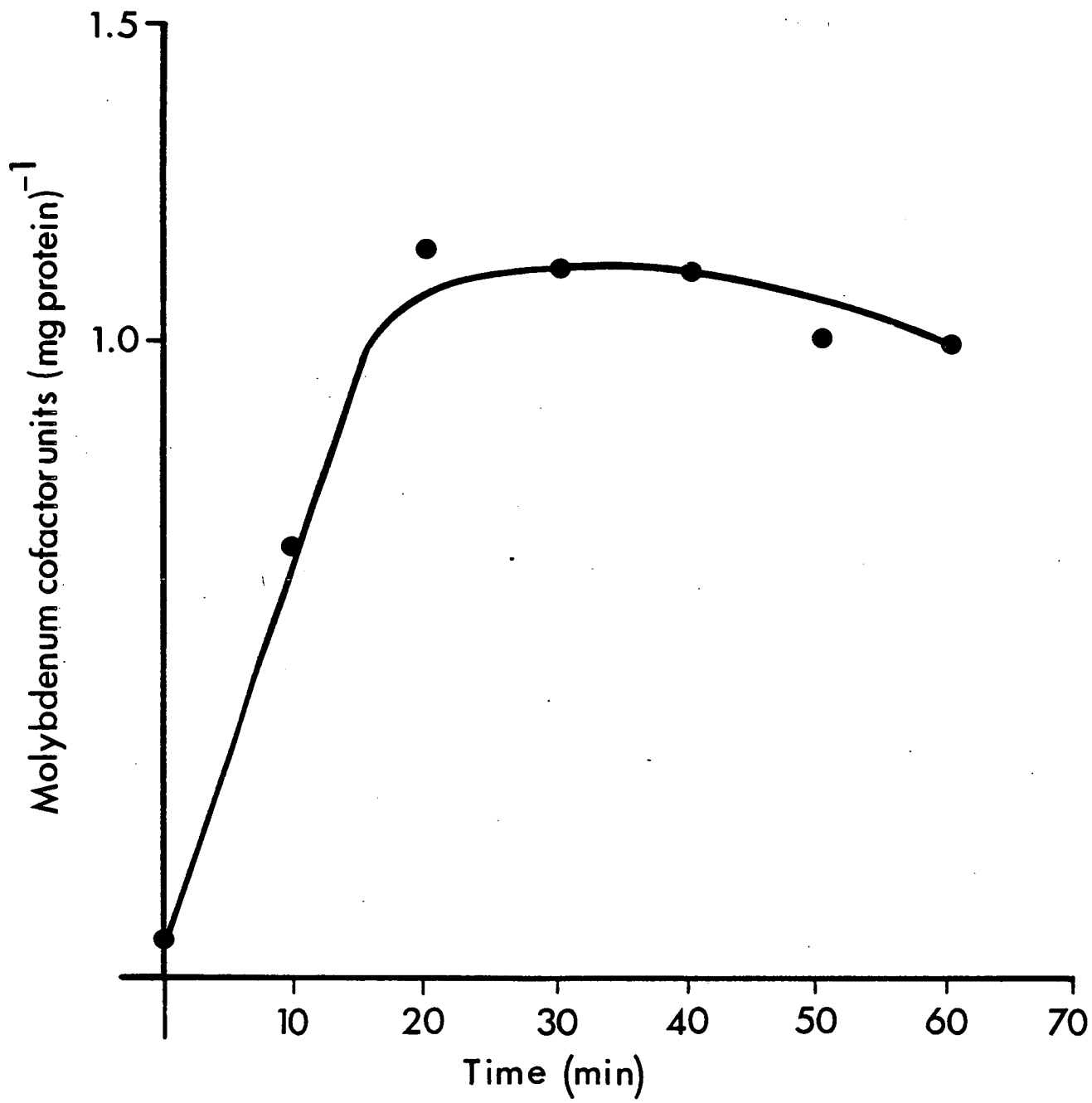


Figure 5: Time course of the complementation reaction

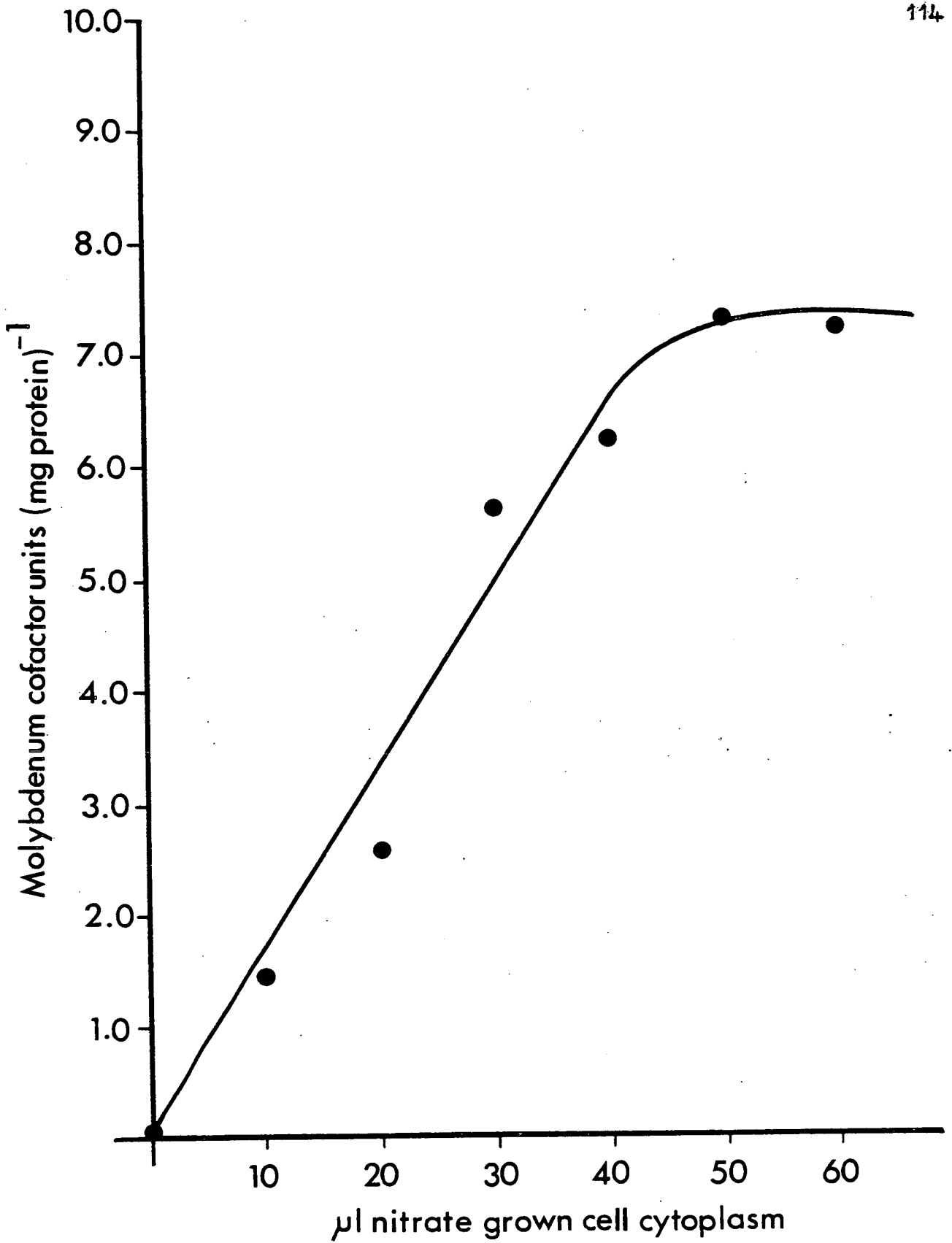


Figure 6: Saturation of the molybdenum cofactor using cytoplasm from nitrate grown cells

time dependent inactivation is shown in Figure 7; the activity was reduced to 43% after 2h and gradual inactivation was observed. The lowest activity was detected after 6h, at this time the activity was only 8% of the initial activity. Some restoration of activity was observed after 24h.

The effect of different reducing agents was also studied. The reducing agents used were DTT (1 mM); sodium ascorbate (5 mM); sodium sulphide (10 mM) and sodium dithionite (2 mM). The effects of the chelating agent EDTA (0.5 mM) and the protease inhibitor PMSF (1 mM) on molybdenum cofactor stability were also tested (Table 15), with all treatments there was a gradual loss in activity but at various degrees; the maximum activity loss occurred at 6h, thereafter some activity was restored. The best treatment was addition of DTT, as after 2h there was a loss in activity of about 38%, a maximum loss of 76% at 6h and 47% of the initial activity was detected after 24h. Therefore the effect of DTT was investigated further to find out the optimum concentration of DTT required for protection. As the peak of inactivation was at 6h, samples treated with different concentrations of DTT were assayed at 0h, 6h, and 72h (Table 16). As previously observed, activity was least after 6h; the minimal loss was recorded with 10 mM DTT. In all samples activity was regained after 72h and the final activities with concentrations of 5, 10, and 15 mM DTT were $\geq 85\%$ of the initial activity.

Addition of molybdate to the buffer in which molybdenum cofactor was released appeared to have no protective effect.

5. Distribution of Molybdenum Cofactor in Cellular Fractions under Different Growth Conditions

It was noticed that during aerobic growth no molybdenum cofactor was synthesised during the lag phase and early log phase, but that the culture produced molybdenum cofactor in the late log phase and in the stationary phase.

Cultures were grown under different conditions: aerobically to the late log phase and microaerobically without any terminal

Table 15. Molybdenum cofactor stability using different treatments

Treatment	% Molybdenum cofactor activity					
	0h	2h	4h	6h	8h	24h
Phosphate buffer (control) (10 mM)	100	43	29	8	16	36
Phosphate buffer + sodium ascorbate (5 mM)	100	49	30	11	13	25
Phosphate buffer + sodium sulphide (10 mM)	100	53	27	8	14	60
Phosphate buffer + sodium dithionite (2 mM)	100	48	27	6	10	13
Phosphate buffer + PMSF (1 mM)	100	41	14	5	6	4
Phosphate buffer + DTT (1 mM)	100	62	39	24	28	47
Phosphate buffer + EDTA (0.5 mM)	100	41	24	8	11	29

Table 16. Protection of molybdenum cofactor with different concentrations of DTT

DTT (mM)	% Molybdenum cofactor activity		
	0h	6h	72h
0.1	100	7	19
1	100	24	59
5	100	20	86
10	100	47	88
15	100	38	98

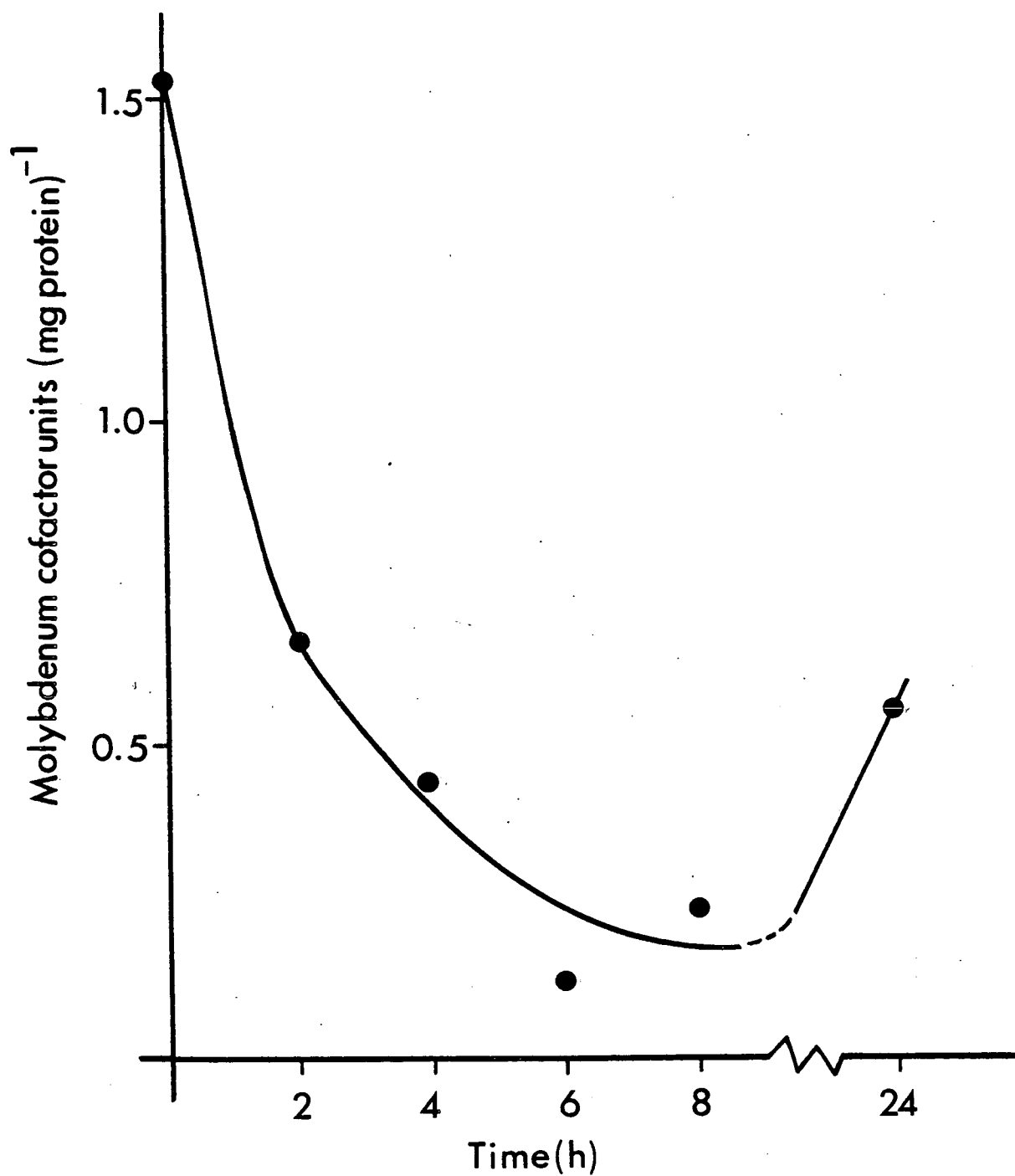


Figure 7: Effect of time on the stability of the molybdenum cofactor

electron acceptor, with TMAO, with nitrate, and with fumarate. A periplasmic fraction was prepared and the spheroplasts disrupted to give a crude extract. This was separated into cytoplasm and membrane by ultracentrifugation, the membranes were washed once before assaying for molybdenum cofactor activity. Samples were assayed for molybdenum cofactor (Table 17). Acid treatment (Lee, 1978) of periplasm failed to increase the activity more than untreated samples. Heat shock treatment of the membrane fraction (Amy, 1981) also failed to release molybdenum cofactor from the membrane. Therefore, all samples were assayed directly as for the soluble fraction.

The cellular distribution of molybdenum cofactor was dependent on the growth conditions; usually the membrane fraction contained little activity (0 - 2.5%) except for the fumarate grown culture. According to the total activity recovered in the periplasm the cultures can be arranged in order of decreasing molybdenum cofactor activity as following: microaerobic, fumarate, nitrate, TMAO, and aerobic. The aerobic periplasm contained little or undetectable molybdenum cofactor. However, for TMAO grown cultures, both the spheroplast crude extract and cytoplasm exhibited low specific activities compared with cells grown under other conditions.

6. Discussion

Molybdenum cofactor is generally assayed by its ability to complement the inactivate assimilatory nitrate reductase of N. crassa mutant nit-1 which lacks the molybdenum-containing moiety. The induced culture of this mutant represents the early portion of the electron transport sequence of the enzymatic pathway and the latter part, the molybdenum-containing moiety supplied by the molybdenum cofactor source (see Introduction Figure 5). It is known that the association of molybdenum cofactor with nitrate reductase is universal, this cofactor can be exchanged between several enzymes and thus appears not to be very specific which may mean that the molybdenum cofactors associated with various

Table 17. Molybdenum cofactor distribution in cellular fractions under different growth conditions

Culture	Periplasm		Spheroplast crude extract		Cytoplasm		Membranes	
	Specific* activity	Total* activity	Specific activity	Total activity	Specific activity	Total activity	Specific activity	Total activity
Aerobic	0	0	2.24	198.20	1.77	31.30	0	0
Microaerobic	13.62	230.20	4.90	479.50	6.36	276.80	0.014	2.81
" (TMAO)**	1.21	33.70	0.27	77.50	1.50	29.50	0.05	1.25
" (Nitrate)**	1.47	82.90	2.18	333.50	4.66	557.20	0.06	8.20
" (Fumarate)**	11.01	91.20	0.98	62.10	1.68	35.87	0.50	11.20

* Activity nmol nitrite produced min⁻¹ (mg protein of A. putrefaciens sample)⁻¹

** Electron acceptors added to microaerobic culture

enzymes in a particular organism are similar if not identical. In support of this hypothesis, the molybdenum cofactor from bacterial sources like E. coli and Proteus mirabilis has been reported to have physical properties identical to those cofactors released from mammalian molybdoenzymes like sulphite oxidase (Amy, 1980; Claassen et al., 1982a; Ishizuka et al., 1983).

Molybdenum cofactor in soluble fractions does not need to be released by use of chemical or physical treatments, instead these treatments could lead to decrease in activity (Amy & Rajagopalan, 1979). Indeed extracts from different bacteria have been used without acid treatment or other treatments (Nason et al., 1970; Ketchum & Sevilla, 1973; Ketchum & Swarin, 1973; see also Introduction Table 2), this means that the cofactor in the extracts is loosely associated with some carriers. However, bound molybdenum cofactor needs to be released for assay by the N. crassa nit-1 complementation, as in nature the molybdenum cofactor is tightly bound to the parent enzyme and requires acidification or other treatment for release. Generally the bound molybdenum cofactor can be released by acidification; another way to release the cofactor is the heat shock which has been applied especially to release the molybdenum cofactor from membrane proteins (Amy & Rajagopalan, 1979; Claassen et al., 1982a; Mendel, 1983). Acid and heat treatments were used additively to release the molybdenum cofactor in Nicotiana tabacum since the molybdenum cofactor in this organism was found to be predominantly in bound form (95%) (Mendel et al., 1982a). However, molybdenum cofactor has been released by treatment of the enzyme with sodium dodecylsulphate as for the respiratory nitrate reductase of Bacillus licheniformis and Klebsiella aerogenes; but the released cofactor was active only when mercaptoethanol was present (Van't Riet et al., 1979). The above evidence indicates that the molybdenum cofactor is not covalently bound to one of the enzyme subunits.

The complementation system used in this work was composed of N. crassa nit-1 extract (induced culture) and a microbial molybdenum

cofactor source. For nit-1 complementation with an uninduced culture of wild type N. crassa a low temperature and a long incubation time were required for optimum activity (Figure 2) in agreement with previous work by Nason & coworkers, while for complementation with A. putrefaciens molybdenum cofactor short incubation (10-20 min) was sufficient and activity decreased with prolonged incubation (4h). In E. coli the optimum temperature for complementation was 23-30°C for 10 min and molybdenum cofactor activity was greatly decreased at 0°C (Amy & Rajagopalan, 1979) which is similar to the complementation of A. putrefaciens at 4°C for 4h (Figure 3).

The buffer used to break the cells to obtain the molybdenum cofactor was 10 mM phosphate buffer as a high phosphate concentration (e.g. 0.1 M) has been repeatedly reported to be inhibitory (Lee, 1978), though phosphate buffer was more effective than other buffers like Tris-HCl (Lee et al., 1974b). Routinely the preparation buffer contained EDTA and a reducing agent DTT to prepare the N. crassa nit-1 extract (Materials and Methods section 2.3). Both EDTA and DTT have been found to be indispensable for the viability of molybdenum cofactor (Lee, 1978). The requirement for EDTA might be to remove the heavy metal ions and by so doing to protect the sulphydryl groups of the molybdenum cofactor from the harmful effects of these cations; EDTA is a well known non-specific chelating agent and the readiness of -SH groups present in most molybdoenzymes to react with heavy metal ions is well documented. Alternatively, EDTA might directly participate in molybdenum cofactor by binding to the molybdenum moiety so as to enhance the activity of the in vitro formed N. crassa nitrate reductase in a manner similar to the riboflavin-EDTA system found in wheat (Stoy, 1955).

Addition of molybdate was found to enhance the in vitro formation of NADPH : nitrate reductase as shown in Figure 2a using uninduced wild type N. crassa as molybdenum cofactor source. All workers dealing with nit-1 complementation have found maximal

activity when 8-10 mM molybdate was included in the complementation mixture. This observation strongly indicates that molybdenum can be removed without causing undue damage to the molybdenum cofactor and that it can be replenished with exogenous molybdenum by insertion. Although molybdate increased the complementation activity, its effect varied with the source of molybdenum cofactor, as can be seen in Figure 2a, that the activity increased to 3 times when molybdate was included in the complementation mixture using wild type N. crassa as molybdenum cofactor, while using A. putrefaciens extract the activity only increased 1.4 fold with molybdate (see section IV). In other cases as in using the cell free extract of Proteus mirabilis Claassen et al. (1981) found that this molybdenum cofactor source was able to reconstitute nit-1 nitrate reductase in the absence of molybdate but this activity was at least 2 times lower than in the presence of exogenous molybdate. It has been suggested that molybdate may stabilise the molybdenum cofactor and compensate for metal lost from the cofactor on ageing (Lee et al., 1974b), but this cannot be true since fresh samples were used in this work. In addition in E. coli fresh samples grown in medium with high molybdate showed stimulation of activity if molybdate was added to the complementation mixture (Amy & Rajagopalan, 1979). On the other hand the isolated molybdenum cofactor showed an absolute dependence on the presence of exogenous molybdate (10 mM) which indicated that the molybdenum had been completely removed from the cofactor during the isolation process, which was confirmed by the elution of molybdenum in the original sample as molybdate in the salt fraction (Alikulov et al., 1980). Mendel et al. (1981) and Mendel (1983) concluded that once the molybdenum cofactor was liberated from its carrier loss of molybdenum was rapid, which explains the stimulation of molybdenum cofactor activity under in vitro conditions by addition of molybdate. Alternatively a pool of molybdenum-free cofactor might exist. An interesting observation is the requirement for a high concentration of molybdate (10 mM) in the complementation mixture to replace molybdenum in molybdenum-free cofactor but in unknown mechanism.

Lee (1978) proposed that there may be an enzyme named insertase responsible for molybdenum insertion at low molybdate concentration (see Introduction, section 4.3), therefore, under in vitro conditions in the absence of this enzyme a high molybdate concentration would probably be required for insertion in a similar way to overcome the defect in chl D mutants. Higher molybdate concentration (30 mM) might be required for expression of molybdenum cofactor activity in a modified molybdenum cofactor of a chl A mutant of E. coli which showed lower affinity for molybdate than cofactor in wild type (Amy, 1980, 1981).

NADPH which was present in the complementation system was checked and found to be unable to act as an electron donor for the dissimilatory nitrate reductase of A. putrefaciens. Na_2SO_3 was included in the complementation mixture throughout the experiments with A. putrefaciens as nitrite reductase inhibitor to avoid any variation since results with Proteus mirabilis showed a slight decrease in the complementation activity when Na_2SO_3 was omitted (Claassen et al., 1981).

Molybdenum and nitrate are specific and essential for induction and synthesis of nitrate reductase in plants (Takayshi & Nason, 1957; Notton & Hewitt, 1971), but in N. crassa, nitrate alone was sufficient to induce nitrate reductase but the enzyme was devoid of activity in the absence of molybdenum. Thus in vivo the enzyme may be made prior to metal ion insertion (Lee, 1978). In bacterial cells it has been reported that molybdenum cofactor can be synthesised aerobically under conditions in which no molybdoenzymes are induced and is then mainly found in the cytoplasm (Amy & Rajagopalan, 1979; Amy, 1981; Claassen et al., 1981), under these conditions some membrane bound molybdenum cofactor was detected in the absence of nitrate reductase and FDH and the authors suggested that this could be components of other as yet unidentified molybdoenzymes (Amy & Rajagopalan, 1979). In A. putrefaciens it is not known whether the heat shock treatment was unable to release the cofactor and a different treatment was required or whether the membrane has really very little molybdenum cofactor present (Table 17) because most of the molybdoenzymes

with exception of FDH are periplasmic (see section I). This observation is in agreement with the general findings that bacterial membranes are a poor source of molybdenum cofactor compared with purified molybdoenzymes (Claassen, et al., 1982a).

Treatment of periplasm with acid resulted in about 23.4% reduction in activity compared to untreated samples, in agreement with the results reported for treating the soluble fraction of E. coli (Amy & Rajagopalan, 1979). However in general acid treatment has been shown to be a powerful method for release of molybdenum cofactor from purified molybdoenzymes as mentioned previously.

As TMAO reductase and nitrate reductase are periplasmic molybdoenzymes (section I and III), the situation in A. putrefaciens is quite different from that in other organisms such as E. coli and Proteus mirabilis. During aerobic growth of A. putrefaciens molybdenum cofactor activity was undetectable until culture had entered the late log phase of growth when molybdenum cofactor was found in the cytoplasm, thus the synthesis of molybdenum cofactor might be induced by anaerobiosis since when the number of cells exceeds 10^8 cell ml⁻¹ the oxygen supply becomes insufficient and there is a demand for another energy source. This is in contrast to the situation for Proteus mirabilis in which it was possible to detect molybdenum cofactor activity in aerobic culture at A₆₆₀ 0.25, though higher activities were observed during the early log phase of anaerobic growth (Claassen et al., 1981).

The conclusion drawn from Table 17 is that in late log phase aerobic culture the molybdenum cofactor was concentrated in the cytoplasm and no activity was detected in the periplasm or membrane, whereas under microaerobic conditions the molybdenum cofactor was distributed intracellularly to match the molybdoenzymes requirement. In microaerobic and fumarate grown cultures the highest specific activity was recorded in the periplasm compared with lower specific activities recorded for TMAO and nitrate grown cultures when some of the molybdenum cofactor would be incorporated into molybdoenzymes and unavailable for complementation, this indicates indirectly that all the molybdenum cofactor comes from the pool

not bound to the molybdoenzymes.

In most cases, the crude extract of spheroplast and the cytoplasmic fraction of A. putrefaciens grown with TMAO exhibited lower activities than nitrate grown. Dissimilar results have been recorded for Proteus mirabilis where cells grown with nitrate exhibited higher nitrate reductase activity but less molybdenum cofactor was found in the cytoplasm (Claassen et al., 1981) and for E. coli where there was no molybdenum cofactor activity in the cytoplasm of cells grown with nitrate (MacGregor & Schnaitman, 1972). The explanation in both these cases was that the molybdenum cofactor was incorporated into the molybdoenzymes. This could be true for culture of A. putrefaciens grown with TMAO when TMAO reductase is synthesised to high level, but the high activity found in the nitrate grown cell cytoplasm could be explained as either the nitrate reductase synthesis in A. putrefaciens being very low (section I) so that not much molybdenum cofactor needed to be incorporated comparing with TMAO reductase or as nitrate stimulating the synthesis of molybdenum cofactor in a similar pattern to that observed in Nicotiana tabacum, which has a constitutive level of nitrate reductase and molybdenum cofactor during log phase growth, both of them being stimulated by addition of nitrate (Mendel et al., 1982b). The former suggestion is preferable as A. putrefaciens and Nicotiana tabacum differ in that the latter depends on the assimilation of nitrate in its growth, accordingly low molybdenum cofactor activity was detected when cells were grown on amino acid medium.

Molybdenum cofactor of A. putrefaciens is also similar to other bacterial molybdenum cofactors in being unstable to exposure to air as shown in Figure 7. It has been reported that xanthine oxidase molybdenum cofactor could not survive for longer than 30 min at 0°C in the absence of EDTA and reducing agent like mercaptoethanol but in their presence the loss in activity was 44.6% after 2h (Lee, 1978). Table 15 shows that A. putrefaciens molybdenum cofactor behaved similarly but DTT provided more protection and in all cases the loss of activity was time dependent.

Ascorbate had no protective effect when compared with the control sample, although it has been used successfully to protect the molybdenum cofactor as it is known to be a powerful scavenger of radical and singlet oxygen (Bodannes & Chan, 1979). At 1-4 mM ascorbate protected specifically the Nicotiana tabacum molybdenum cofactor and enabled the molybdenum cofactor to withstand heating at 80°C for 8 min (Alikulov et al., 1980; Mendel et al., 1982a); however, during this treatment molybdenum cofactor lost its molybdenum but not the ability to co-ordinate molybdenum. In E. coli carrier bound molybdenum cofactor was stable aerobically and anaerobically at 0°C for 2h but at 37°C anaerobiosis offered some protection; in parallel the protein free molybdenum cofactor lost 42% of activity at 0°C after 2h under both aerobic and anaerobic conditions (Amy & Rajagopalan, 1979). Van't Riet et al. (1979) showed that molybdenum is dissociated from the cofactor upon sodium dodecylsulphate treatment in the absence but not in the presence of β -mercaptoethanol suggesting that thiol group or reducing conditions are involved in binding the molybdenum to the molybdenum cofactor.

In summary, the protection of molybdenum cofactor should be directed towards its isolation from oxygen. In most treatments described in Table 15 there was some activity retained upon prolonged storage and this could be due to consumption of oxygen by extract under static conditions and at low temperature and this means that the inactivation of molybdenum cofactor is reversible. However, endeavours to avoid the initial drop in activity by combination of different treatments were unsuccessful. An interesting point is the observation that aerobically inactivated molybdenum cofactor could perform the structural function of dimerising the nit-1 nitrate reductase (Alikulov et al., 1980), this might mean that the changes or oxidation caused by air affect the site at which the molybdenum binds to the molybdenum cofactor and perform electron carrier function.

In conclusion molybdenum cofactor synthesis in A. putrefaciens is initiated by partial anaerobiosis but at present it is not known whether the molybdenum cofactor is produced as a metal-free cofactor in minimal medium lacking molybdenum.

SECTION III

ISOLATION AND CHARACTERISATION OF ANAEROBIC

ELECTRON TRANSPORT MUTANTS

1. Isolation of Mutants

Mutants of A. putrefaciens NCMB400 were isolated in this work either by using UV or NTG mutagenesis, and were selected for their inability to grow anaerobically with TMAO as terminal electron acceptor or for their inability to oxidise BV^{*+} in the BV : TMAO overlay system. The mutants were defective in their ability to use TMAO for anaerobic respiration and some of them were found to be affected in nitrate respiration as well. Table 18 lists the mutants isolated with the two mutagens and their phenotypes. In addition to the mutants described in Table 18, further mutants with either pleiotropic defects in anaerobic respiration or defective in aerobic respiration were isolated, these were not subjected for further investigation after their isolation.

2. Biochemical and Morphological Characterisation

The biochemical characteristics of A. putrefaciens NCMB400 wild type are shown in Table 19. Due to the significance of sugar utilisation in alteromonads, this property was investigated in strain NCMB400 wild type and other alteromonads (Table 20).

All the mutants isolated with UV irradiation were checked for the biochemical tests mentioned in Table 19 and found to be similar to wild type except that they were negative for the nitrate and TMAO reduction tests. Mutants 281 to 362 showed less resistance to Streptomycin, and variable results in the oxidase test were noticed for all mutants isolated with UV irradiation. Mutants isolated with NTG treatment were characterised only for the most important characteristics, such as Gram stain, growth at different temperatures, and oxidase

Table 18. Mutants isolated and used in this study

No. of mutant	Mutagen	Phenotype
281	UV	Pleiotropic
282	"	"
283	"	"
291	"	"
292	"	"
301	"	"
302	"	"
321	"	"
322	"	"
331	"	"
332	"	"
341	"	"
342	"	"
343	"	"
361	"	"
362	"	"
37	"	"
401	"	"
402	"	"
403	"	"
404	"	"
405	"	"
I4	NTG	Defective in TMAO respiration
I11	"	Pleiotropic
I31	"	Defective in TMAO respiration
I33	"	"
I58	"	"
I61	"	"
I66	"	"
I79	"	"

Table 18. (contd.)

No. of mutant	Mutagen	Phenotype
I82	NTG	Defective in nitrate respiration
I106	"	" " TMAO "
I114	"	" " nitrate "
I116	"	Pleiotropic
I119	"	Defective in TMAO respiration
I123	"	"

Table 19. Biochemical tests of A. putrefaciens NCMB400 wild type

Biochemical test	Result
Gram stain and shape	Gram-negative rods
Flagella stain	Polar flagellate
Growth at different temperatures	
20°C	Good growth
30°C	Moderate growth
37°C	No growth
Antibiotic sensitivity	
10 µg Chloramphenicol	S
10 µg Erythromycin	S
100 µg Sulphafurazole	R
1.5 U Penicillin	R
10 µg Streptomycin	R
10 µg Tetracycline	S
Presence of catalase	+
Oxidase reaction	+
Reduction of nitrate to nitrite	+
Reduction of TMAO to TMA	+
Citrate utilisation	-
Presence of urease	-
Litmus milk reaction	R → P
Starch hydrolysis	+
Gelatin liquefaction	+
Indole production	-
Methyl red reaction	-
Voges-Proskauer reaction	-
Motility	+

S = sensitive

R = resistant

+ = positive reaction

- = negative reaction

R → P = reduction followed by peptonisation

Table 20. Sugar utilisation of A. putrefaciens NCMB400
wild type and other alteromonads

Sugar	NCMB400	NCMB1520	NCMB1521	NCMB1733	NCIB10472
Sucrose	A	A	A	-	A
Maltose	A	A	A	-	A
Mannitol	-	-	-	-	-
Lactose	-	-	-	-	-
Glucose	-	-	-	-	-
O-F test	-*	N.D.	N.D.	N.D.	N.D.

A = acid production

- = no acid produced

*- = acid produced only in opened tubes when maltose used

N.D. = not determined

reaction. They were similar to the wild type strain, except that in the oxidase test some mutants were absolutely negative when compared with a typical oxidase positive control like Pseudomonas aeruginosa and using E. coli as oxidase negative control. Only mutant I106 is included in Table 18.

3. Molybdenum Cofactor Activity in Some Mutants

Selected mutants were subjected to molybdenum cofactor activity estimation. Strains were grown aerobically to the late log phase in Wood & Baird medium containing 2 mM molybdate, a crude extract was prepared and used as molybdenum cofactor source. Wild type was used as control and due to variation with different batches the activity is expressed as % of wild type activity (Table 21).

Mutants with high molybdoenzyme activities were not subjected to estimation of their molybdenum cofactor activity, these were I4, I31, I33, I61, I66, I79, I82, I106, I114, I119 and I123.

4. Analysis of Respiratory Enzymes of the Isolated Mutants

(a) TMAO reductase

As this enzyme was central to this study all the mutants were assayed for MV^{++} : TMAO reductase activity and the composite results are shown in Table 22; due to variation between different batches the activity is expressed as % of the wild type activity. Generally mutants isolated with UV irradiation had low MV^{++} : TMAO reductase activity together with inability to grow with TMAO anaerobically, while those isolated with NTG (I mutants), except mutants I82 and I114, were unable to grow with TMAO anaerobically, but retained a variable level of MV^{++} : TMAO reductase activity.

(b) Nitrate reductase

Due to some similarity between the nitrate and TMAO reduction pathways, some mutants were selected and assayed for MV^{++} : nitrate reductase activity (Table 23). Most of the UV induced mutants showed reduced nitrate reductase activity; mutants I82 and I114

Table 21. Molybdenum cofactor activity in some mutants

Mutants	% of specific activity
Wild type	100
281	4
282	47
283	55
291	70
292	89
301	80
302	91
321	0
322	0
331	0
341	<1
342	7
343	7
361	30
362	41
401	91
403	87
405	92
I11	82
I58	47
I116	103

Table 22. $MV^{•+}$: TMAO reductase activity of mutants used in this study

Mutants	% of specific activity
Wild type	100
281	2
282	< 1
283	< 1
291	0
292	0
301	0
302	0
321	0
322	0
331	0
332	< 1
341	< 1
342	< 1
343	< 1
361	< 1
362	< 1
37	2
401	3
402	0
403	0
404	< 1
405	0
I4	62
I11	109
I31	119
I33	152
I58	0
I61	86

Table 22. (contd.)

Mutants	% of specific activity
I66	64
I79	192
I82	129
I106	40
I114	150
I116	137
I119	110
I123	88

Table 23. MV^{++} : nitrate reductase activity in some selected mutants

Mutants	% of specific activity
Wild type	100
283	4
291	0
301	0
321	0
322	0
331	0
361	19
37	6
401	0
404	3
I11	6
I58	71
I82	173
I114	26
I116	41

were unable to grow with nitrate, but still had a considerable level of nitrate reductase activity.

(c) Formate dehydrogenase

As this enzyme is an important respiratory chain component (see section I), its activity was estimated with DCPIP as electron acceptor in most of the mutants grown microaerobically with TMAO (Table 24), the UV induced mutants had little or no FDH activity. NTG induced mutants I11 and I116 showed very reduced or no activity, the rest had activities ranging from 19-308% of wild type activity.

(d) Fumarate reductase

Fumarate reductase is a non-molybdoenzyme in most bacteria. Its activity was measured in some selected mutants of A. putrefaciens grown microaerobically with fumarate (Table 25). Most of the mutants had considerable fumarate reductase activity, but this was always lower than the wild type activity especially those induced with UV irradiation. The NTG induced mutants, I11 and I116 showed reduced activity. Mutants I82 and I114, defective in nitrate respiration, had very high fumarate reductase activity.

Based on the results shown in Tables 21-24, the mutants can be categorised into several groups. Group 1, includes mutants with low molybdenum cofactor activity and reduced TMAO activity: 281, 341, 342, and 343; Group 2, mutants have high molybdenum cofactor activity and reduced TMAO reductase activity: 282, 283, 361, and 401; Group 3, mutants have high molybdenum cofactor activity and undetectable TMAO reductase activity: 291, 292, 301, 302, 403, and 405; Group 4, mutants are completely defective in molybdenum cofactor activity and this is reflected on the activities of all their molybdoenzymes: 321, 322, 331; Group 5, mutants were totally unable to grow anaerobically with TMAO as judged by plate growth and by decrease in the A_{660} of their liquid culture, but exhibited considerable MV^{+} : TMAO reductase activity: I4, I31, I33, I66, I79, I106, I119, and I123;

Table 24. FDH : DCPIP activity in some mutants

Mutants	% of specific activity
Wild type	100
283	2
291	0
301	0
321	0
322	0
331	0
341	14
361	15
37	11
401	0
404	6
I4	109
I11	0
I31	174
I33	55
I58	40
I61	19
I66	308
I79	100
I82	145
I106	62
I114	242
I116	1
I119	122
I123	142

Table 25. MV^{++} : fumarate reductase activity of some
selected mutants

Mutants	% of specific activity
Wild type	100
281	38
291	36
301	24
321	61
322	75
331	67
341	41
361	45
401	57
I11	1
I58	41
I82	143
I114	163
I116	9

Group 6, represents mutants only defective in TMAO reductase, but unfortunately, it is represented by only one mutant I58; Group 7, mutants are affected in nitrate respiration only: I82 and I114; Group 8, mutants are affected mainly in their FDH activity I11, I61, and I116. The rest of the mutants listed in Tables 18 and 22 had reverted before their analysis was completed (332, 362, 37, 402, and 404).

(e) Molybdenum cofactor distribution in some mutants

As Group 3 mutants showed high molybdenum cofactor activity but no molybdoenzyme activities they could be affected in their ability to transfer the molybdenum cofactor from the cytoplasm to the periplasm, where the respective apoproteins are located. Mutants 401 from Group 2 and 291, 301, and 302 from Group 3, were grown microaerobically with TMAO, the cells fractionated and the cellular fractions assayed for molybdenum cofactor activity (Table 26). The specific molybdenum cofactor activity of periplasm is shown in Figure 8. The results indicated that the molybdenum cofactor was distributed in these mutants similarly to the wild type strain, and generally the cytoplasm exhibited low activity when cells were grown with TMAO microaerobically (see section II).

(f) Reduction sites for fumarate and TMAO with MV^{++}

Both fumarate reductase and TMAO reductase were assayed with MV^{++} as electron donor; TMAO reductase activity was absent in the molybdenum cofactor mutants (Group 4), yet fumarate reductase activity was retained. This might mean that TMAO and fumarate reduction by MV^{++} takes place at different sites. Mutants were grown microaerobically with TMAO plus fumarate, and assayed in cell suspensions for the corresponding reductase activities. On addition of TMAO to the assay mixture, no MV^{++} oxidation was detected; when fumarate was subsequently added to the same cuvette, rates of MV^{++} oxidation similar to those detailed in Table 25 were recorded (Table 27).

Table 26. Molybdenum cofactor distribution in some pleiotropic mutants

Cellular Fraction	Wild type		291		301		302		401	
	Specific* activity	Total* activity	Specific activity	Total activity	Specific activity	Total activity	Specific activity	Total activity	Specific activity	Total activity
Crude extract	0.57	41	0.51	65	0.54	39	0.75	50	N.D.	N.D.
Periplasm	1.20	34	3.60	118	3.90	76	3.69	30	6.35	118
Spheroplast crude extract	0.27	78	0.63	212	0.75	374	2.45	714	0.16	17
Cytoplasm	0.04	1.54	0.26	13	0.23	14	0.57	25	0.03	2
Membrane	0.05	0.70	0.03	0.50	0.06	0.33	0.04	0.82	0.09	1

* Unit molybdenum cofactor (mg protein⁻¹ of A. putrefaciens samples)

N.D. = not determined

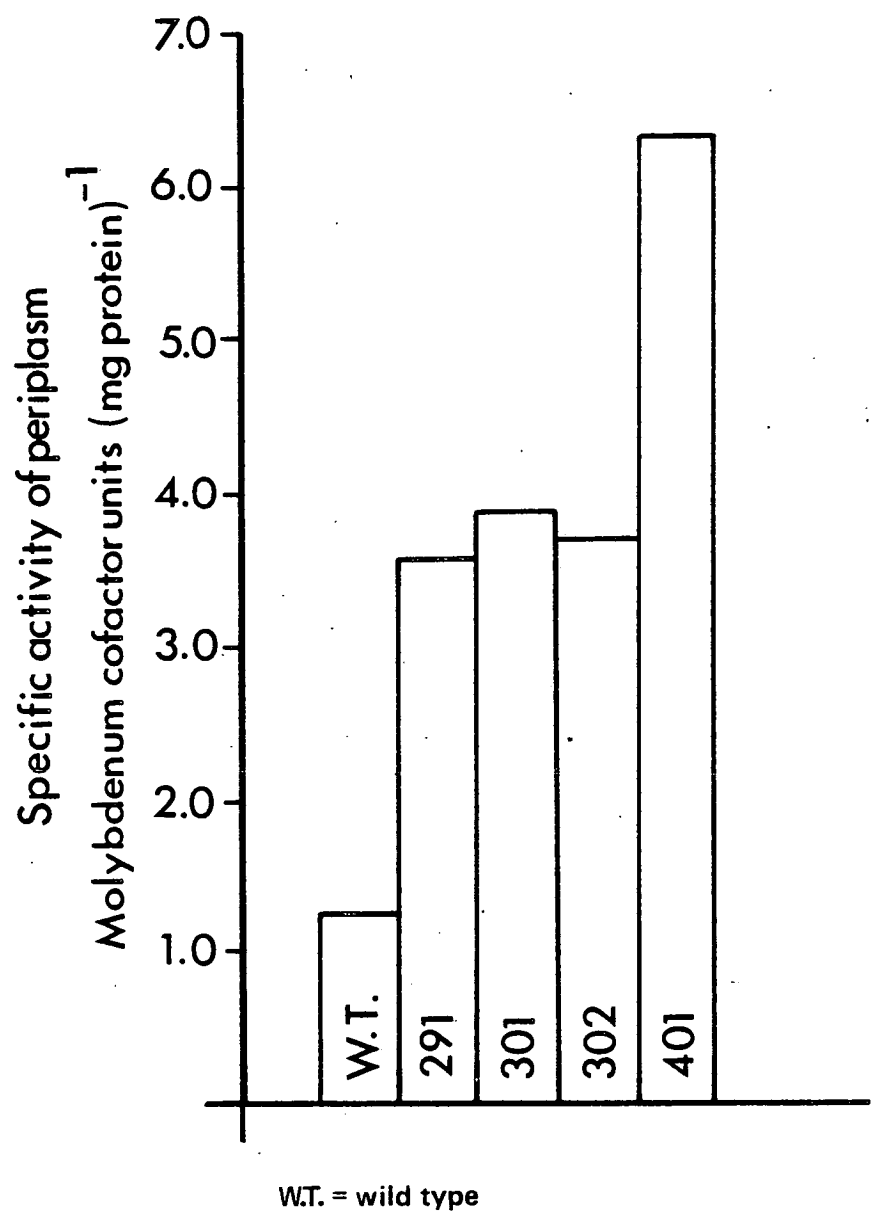


Figure 8: Specific activity of the molybdenum cofactor in periplasmic fraction of some pleiotropic mutants

Table 27. $MV^{•+}$: TMAO reductase and $MV^{•+}$: fumarate reductase activities in molybdenum cofactor mutants

Mutants	TMAO reductase*	Fumarate reductase*
Wild type	4334	1542
321	0	784
322	0	1153
331	0	1038

* Specific activity, nmol $MV^{•+}$ oxidised min^{-1} (mg protein) $^{-1}$

Table 28. $MV^{•+}$: nitrate reductase and $MV^{•+}$: nitrite reductase activities in molybdenum cofactor mutants

Mutants	Nitrate* reductase	Nitrite* reductase	Fold increase
Wild type	342	553	
321	0	44133	80
322	0	7857	14
331	0	9401	17

* Specific activity, nmol $MV^{•+}$ oxidised min^{-1} (mg protein) $^{-1}$

(g) Reduction sites for nitrate and nitrite with $MV^{•+}$

In a similar manner to that mentioned in section (f) nitrite reductase activity was estimated in molybdenum cofactor mutants. Strains were grown microaerobically with 5 mM nitrate and assayed for nitrate reductase and nitrite reductase (Table 28). In these mutants when there was no functional nitrate reductase, nitrite reductase activity was increased markedly over the wild type level. In order to investigate the regulation of nitrite reductase synthesis in A. putrefaciens, nitrite reductase was determined in mutants I82 and I114 which were unable to grow on nitrate but retained the nitrate reductase activity (Table 29). It seems that the presence of functional nitrate reductase had a control role on the nitrite reductase: with a highly functional nitrate reductase present nitrite reductase activity was less than the wild type activity (mutant I82) and vice versa for mutant I114.

(h) FDH : DCPIP and FDH : BV differentiation

As shown in Table 24, Group 8 mutants (I11 and I116) had very reduced FDH : DCPIP activity, and were chosen for the investigations described in this section. They were grown microaerobically with different electron acceptors and assayed for FDH : DCPIP activity only (Table 30). Mutant I11 showed very low FDH activity under all conditions tested. However, as this mutant also showed reduced activity for nitrate reductase (Table 23) and fumarate reductase (Table 25), it was not investigated further. As FDH : DCPIP and FDH : BV activities in many bacteria appear to depend upon the growth conditions used, mutant I116 was grown microaerobically with different electron acceptors and assayed for both FDH : DCPIP and FDH : BV activities (Table 31). The results do not distinguish clearly between the possibilities of one or two FDH enzymes in A. putrefaciens.

Table 29. MV^{*+} : nitrate reductase and MV^{*+} : nitrite reductase activities in mutants defective in nitrate respiration

Mutants	nitrate reductase*	nitrite reductase*
Wild type	461	709
I82	800	667
I114	118	2679

* As in Table 28

Table 30. FDH : DCPIP activity in Group 8 mutants grown under different growth conditions

Mutants	Growth conditions				
	Aerobic	Microaerobic			
		- TEA	+ TMAO	+ Nitrate	+ Fumarate
Wild type	100*	100	100	100	100
I11	3	5	2	0	0
I116	37	0	1	40	8

* % of wild type specific activity

TEA = terminal electron acceptor

Table 31. Activities of FDH : DCPIP and FDH : BV in mutant I116 under different growth conditions

TEA added to the growth medium	FDH : DCPIP (% of wild type activity)	FDH : BV (% of wild type activity)
TMAO	0	0
Nitrate	25	0
Fumarate	16	6

TEA = terminal electron acceptor

5. Effect of Molybdate

The results in Table 32 shows the effect of added molybdate on formate : nitrate reductase activity in wild type and mutants. The strains had been grown microaerobically in Wood & Baird medium supplemented with nitrate and molybdate (1 mM). Molybdate appeared to have no effect on the activity of the wild type or most of the mutants. For mutant 283 activity was slightly stimulated by the presence of molybdate in the growth medium but the wild type activity was not restored.

The effect of adding molybdate to the growth medium on the activities of the terminal reductases, nitrate reductase and TMAO reductase, was investigated for some of the mutants (Tables 33 and 34). Addition of extra molybdate led to enhancement of the $MV^{•+}$: nitrate reductase activity of the wild type only (Table 33). The effect of molybdate on $MV^{•+}$: TMAO reductase activity was variable (Table 34); the wild type strain showed enhancement, while for the TMAO reductase mutant (I58) addition of molybdate did not repair the defect in this mutant, for the mutants I11 and I116 affected in their regulation system the extra molybdate led to decrease in the TMAO reductase activity.

6. Chlorate Resistance

Mutants were checked for chlorate resistance by streaking an overnight culture onto NANA plates containing 30 mM lactate, 10 mM fumarate as an alternative electron acceptor and either 5 mM or 20 mM chlorate. The plates were incubated anaerobically for 4-7d and the results scored (Table 35). After a further incubation period of 2d under aerobic conditions, the results were scored again. Generally, the pleiotropic mutants of Groups 1-4 induced with UV irradiation showed high resistance to chlorate. For other groups there were different degrees of resistance within the group.

Table 32. Effect of molybdate on the activity of formate :
nitrate chain activity

Mutants	% of wild type specific activity	
	- Molybdate	+ Molybdate
Wild type	100 (227)*	100 (226)*
283	4	18
37	6	6
404	4	9

* Figures in parenthesis represent the actual activity of wild type

Table 33. Effect of molybdate on the activity of MV⁺⁺ :
nitrate reductase

Mutants	% of wild type specific activity	
	- Molybdate	+ Molybdate
Wild type	100 (297)*	100 (327)*
291	0	0
301	0	0
321	0	0
331	0	0
361	19	19
401	0	4

* Figures in parenthesis represent the actual activity of wild type

Table 34. Effect of molybdate on the activity of MV⁺⁺ :
TMAO reductase

Mutants	% of wild type specific activity	
	- Molybdate	+ Molybdate
Wild type	100 (2121)*	100 (4191)*
I11	109	60
I58	0	0
I116	147	50

* Figures in parenthesis represent the actual activity of wild type

Table 35. Chlorate resistance of mutants under anaerobic growth conditions

Mutants	Growth of mutants	
	5 mM chlorate	20 mM chlorate
Wild type	-	-
Group 1		
281	+++	+++
341	+++	+++
342	+++	+++
343	+++	+++
Group 2		
282	+++	+++
283	+++	+++
361	+++	+++
401	+++	+++
Group 3		
291	+++	+++
292	+++	+++
301	+++	+++
302	+++	+++
Group 4		
321	+++	+++
322	+++	+++
331	+++	+++
Group 5		
I4	+	-
I31	-	-
I33	+	(-)
I66	+	+
I79	+	+
I106	+	(-)
I123	++	+

Table 35 (contd.)

Mutants	Growth of mutants	
	5 mM chlorate	20 mM chlorate
Group 6		
I58	+	+
Group 7		
I82	+++	+++
I114	-	-
Group 8		
I11	-	-
I61	+	+
I116	+	+

- = no growth

+ = very small colonies

+++ = large colonies

(+) = mixture of large and small colonies

7. Reaction of Mutants on Maltose : TMAO Medium

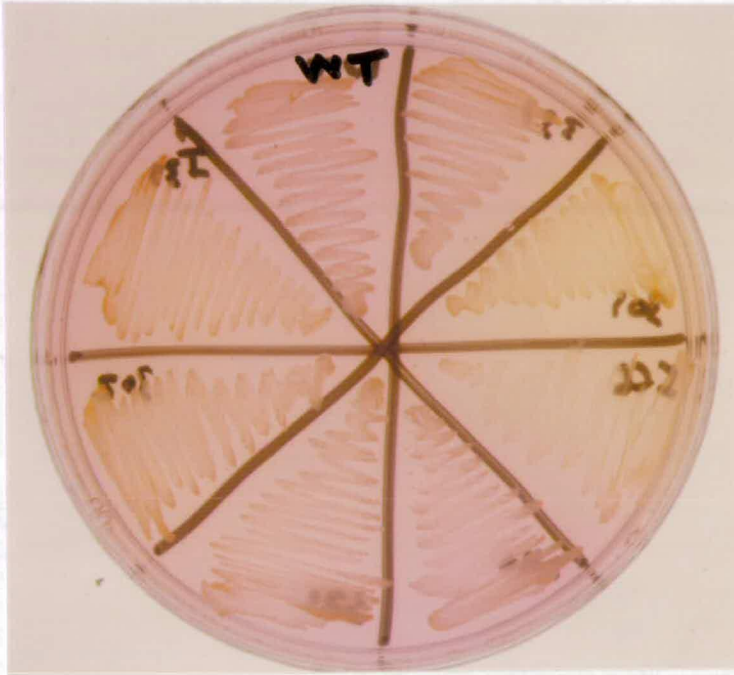
The wild type strain of A. putrefaciens utilises maltose oxidatively under aerobic conditions and produces acid (Table 20). On the other hand TMA the end product of TMAO reduction is highly basic. These two characters were exploited to develop the maltose : TMAO medium and used to test the mutants defective in TMAO respiration. Mutants unable to produce TMA from TMAO reduction anaerobically still retained the acidic colour of indicator produced under initial aerobic incubation while the wild type retained the basic colour after anaerobic incubation. The reaction of wild type and some mutants on maltose : TMAO medium is shown in Figure 9. Although the colours are not clearly differentiated in the photograph, differentiation on the actual plates was easy and the results from different cultures were reproducible.

8. Discussion

Selection of mutants based on their inability to grow anaerobically on TMAO as terminal electron acceptor yielded a complex spectrum of mutants and it proved difficult to assign specific lesions to most of the mutants partly because of their pleiotropic phenotypes.

Most of the isolated mutants were similar to the wild type strain in their biochemical tests except for nitrate and TMAO reduction tests which formed the basis of selection. The differences noticed in the antibiotic sensitivity tests could be a secondary effect of the mutation(s) produced in those strains or due to other mutations produced in another region of the genome.

All the mutants induced by UV irradiation and isolated as TMAO respiratory mutants were pleiotropically defective in TMAO and nitrate reduction. Their inability to grow on TMAO anaerobically was coupled with low $MV^{•+}$: TMAO reductase activity (0-2%), in



W.T. = wild type

Figure 9: Reaction of wild type and electron transport mutants on maltose : TMAO medium

contrast to the higher activities in the NTG induced mutants.

In terms of molybdenum cofactor activity (Table 21), three levels of activities were observed: no molybdenum cofactor activity as in Group 4, <10% of wild type activity as in Group 1, and a third level ranging from 40-100% of wild type activity as in the rest of the groups. The variation of molybdenum cofactor activity and the effect of mutations involving enzyme-cofactor association is reflected in some mutants giving rise to distinct phenotypes.

Fumarate reductase, as a non-molybdoenzyme, was not severely reduced in most mutants except those affected in the regulation of anaerobic electron transport like I11 and I116. The commonly observed reduced fumarate reductase activity is probably an indirect effect due to the incomplete induction of other anaerobic respiratory chain components like FDH, a molybdoenzyme which was reduced or absent in mutants affected in their molybdenum metabolism. The behaviour of each group is discussed separately below:

Group 1

The mutants had low molybdenum cofactor activity combined with low MV^{+} : TMAO reductase activity; at the same time they were unable to grow with nitrate anaerobically. These mutants seem to be affected in the synthesis of molybdenum cofactor rather than the molybdoenzyme apoproteins. These mutants could be leaky for molybdenum cofactor synthesis unlike Group 4. In other organisms such as Aspergillus nidulans, it was suggested that the cnx J gene product has a positive-acting regulatory role whose product is necessary for a high, but not a moderate or low, level of cofactor synthesis (Arst et al., 1982). Looking at the results of this group, the presence of such a regulatory gene in A. putrefaciens could be suggested. In E. coli, the only chl mutants class that showed reduced molybdenum cofactor activity was chl G (Amy, 1981). Those mutants were defective in the expression of TMAO reductase (Stewart & MacGregor, 1982), therefore, Group 1 mutant could resemble to chl G

mutants in this aspect. On the other hand, chl G mutants retained the molybdoenzyme activities when grown with high concentration of molybdate (Stewart & MacGregor, 1982; Del Campillo-Campbell & Campbell, 1982), whereas all mutants studied in this project showed no stimulation of molybdoenzyme activity when grown in the presence of high molybdate concentration. Moreover, in the estimation of their molybdenum cofactor, cells were grown in medium containing 2 mM molybdate and the complementation was carried out in the presence of 10 mM molybdate.

Group 2

The reduced TMAO reductase activity of mutants in this group is not a reflection of reduced molybdenum cofactor activity as in Group 1 (see Tables 21 and 22). Generally, these mutants had reduced activity of other molybdoenzymes, so the defect is presumably the lack of proper binding of molybdenum cofactor to the molybdoenzymes. In E. coli, it has been reported that the component responsible for such function is the F_A , the chl B gene product (Riviere et al., 1975); if the situation in A. putrefaciens is similar to E. coli in this respect, then Group 2 mutants could be leaky for this gene product as some activities were detectable.

Group 3

The mutants in this group are more likely to be analogous to the chl B mutants of E. coli (Riviere et al., 1975), since the transfer and distribution of the molybdenum cofactor in these mutants were not affected (Table 26 and Figure 8). The high specific molybdenum cofactor activity in their periplasm compared with wild type confirms the conclusions drawn in section II, that the molybdenum cofactor detected in the periplasm comes from molybdenum cofactor which is not bound to the enzymes, and that the lower specific activity in the wild type periplasm is due to incorporation of molybdenum cofactor into molybdoenzymes.

Group 4

In the molybdenum cofactor mutants: 321, 322, and 331, the absence of molybdenum cofactor was combined with absence of nitrate reductase, TMAO reductase, and FDH activities which provides confirmation that these enzymes require the molybdenum cofactor for activity. The lesion of these mutants was not repaired by high molybdate concentrations in the growth medium, other anaerobic non-molybdoenzymes like fumarate reductase were retained (Table 25). These mutants appear similar to the nit-1 mutant of N. crassa and to a mutant of Proteus mirabilis strain s556 (Claassen et al., 1981).

Group 5

Mutants in this group form another class in which the defect in TMAO respiration is not due to abnormal molybdenum metabolism since they retain the MV^{+} : TMAO reductase and FDH : DCPIP activities. Although these mutants have the common feature which is the inability to grow anaerobically with TMAO, they have different levels of MV^{+} : TMAO reductase and FDH : DCPIP activities indicating that they are dissimilar and that the TMAO reduction system contains components other than FDH and TMAO reductase. The biochemical lesion in Group 5 is not necessarily the loss of the terminal reductase. Some mutants in E. coli have been isolated, unable to respire nitrate, but with a functional nitrate reductase, further investigations revealed that they lacked the cytochrome ^{NR} b₅₅₆, similarly mutants in Salmonella typhimurium were unable to respire TMAO but retained the MV^{+} : TMAO reductase activity (Bonney-Orth et al., 1981; Davidson et al., 1979). The missing component(s) in Group 5 mutants led to stopping the electron flow to TMAO which was reflected as the inability to grow on TMAO. However, the induction and synthesis of both TMAO reductase and FDH in these mutants and Group 7 mutants (see below) was normal. These results contradict the suggestion of De Groot & Stouthamer (1970a, b) discussed in section I, and unless these mutants

are affected in the regulation of anaerobic electron transport, an unlikely possibility in view of the above results, then the conclusion of De Groot & Stouthamer appears invalid.

Group 6

The only mutant in this group was the TMAO reductase mutant I58 despite strenuous efforts to isolate more such mutants by selection for the inability of strains to grow with TMAO anaerobically. However, as explained earlier a high percentage of mutants were pleiotropic or affected in a component other than the TMAO reductase enzyme itself.

The evidence that I58 is affected in the TMAO reductase enzyme only is that it is still able to induce the synthesis of other enzymes such as nitrate reductase, FDH, and fumarate reductase and has a considerable level of molybdenum cofactor activity (Table 21). The reduced molybdenum cofactor ($< 50\%$) could be due to coregulation of molybdenum cofactor and the TMAO reductase synthesis, as the latter is the most important molybdoenzyme for this organism in its habitat. Such coregulation between the molybdenum cofactor and an important molybdoenzyme has been observed in other organisms like Nicotiana tabacum. Derepression of nitrate reductase in Nicotiana tabacum was accompanied by a derepression of molybdenum cofactor synthesis (Mendel et al., 1982b).

One point to be considered about mutant I58 is that, this mutant grew aerobically at a rate much lower than that of the wild type strain; this might mean that it bears a double mutation which is expected for NTG mutagenesis or the loss of TMAO reductase might influence the aerobic respiratory system as a secondary effect on other components of the chain, presumably at a regulatory level. As TMAO respiration of A. putrefaciens in its natural environment is the essential alternative system to aerobic respiration, then the two systems may be linked and coregulated genetically. This suggestion remains a challenge for the future, when mutants defective in aerobic respiration could be studied for their ability to undergo TMAO respiration. Furthermore the fact that this mutant can grow very well with

nitrate and fumarate anaerobically supports the argument that TMAO and nitrate reduction are linked only at the level of molybdenum metabolism. The FDH activity of I58 presented in Table 24 was 40% for growth with TMAO, while this activity when grown with nitrate and fumarate was 230-250% of wild type activity.

Reported isolations of TMAO reductase mutants in the literature are rare; probably because the anaerobic electron transport chain to TMAO has many components. One example of a TMAO reductase mutant is the strain S78-30 derived from E. coli K-10 isolated by Shimokawa (cited in Takagi et al., 1981). This mutant was isolated from culture grown in nutrient broth containing 0.1% KNO_3 and it has no TMAO reductase activity and also showed no proton translocation associated to TMAO reduction using intact cells. Recently other mutants of E. coli were isolated after NTG induction, they mapped at different loci associated with nitrate reductase (Takagi & Ishimoto, 1983).

Group 7

I82 and I114 represent the members of this group; they were unable to grow with nitrate anaerobically as judged by lysis of cells and decrease in A_{600} under such conditions. I82 retained high MV^{++} : nitrate reductase activity (173% of wild type), while I114 retained only 26%, both of them retained high FDH activity (Table 24). They appear similar only in their overall lack of nitrate respiration but not at a more detailed level. The information available about these mutants at present only permits the suggestion that they are missing an unidentified component(s) specific for nitrate respiration other than the dehydrogenase and reductase and that this component is not involved in TMAO or fumarate reduction since both mutants were able to grow anaerobically with TMAO and fumarate and showed normal activities for the corresponding reductases. The missing component could be a certain type of cytochrome like cytochrome b_1 in E. coli which is associated with nitrate

reductase near the electron transfer site (MacGregor, 1975b).

Group 8

The main characteristic of the mutants in this group (I11, I61, and I116) is their reduced FDH activity. Their molybdenum cofactor is not affected (Table 21), the reduced FDH activity in these mutants cannot be due to an effect in the FDH enzyme itself, but it is more likely in the regulation system since they showed reduced activity for other enzymes.

Mutant I116 showed varying behaviour depending on the growth conditions for the FDH : DCPIP activity (Table 30). However, using this mutant FDH : DCPIP and FDH : BV activities were not differentiated under several different growth conditions. In E. coli it is obvious that there are two types of FDHs (see discussion - section I); on the other hand the loss of FDH : DCPIP but not FDH : BV activity in some mutants of Salmonella typhimurium has been interpreted as PMS (DCPIP) receiving electrons from the FDH : DCPIP protein only when the enzyme is properly bound to the cytochrome (Barrett et al., 1979; Barrett & Riggs, 1982a). This might indicate that there is only one FDH enzyme in Salmonella typhimurium and that the different behaviour towards electron acceptors depends on other components associated with the FDH protein. Therefore, mutant I116 could be mutated in a regulatory gene affecting the level of FDH and associated components under different growth conditions.

Mutant I11 as described above was affected in all anaerobic respiration. Although, it exhibited higher TMAO reductase activity than the wild type and had normal molybdenum cofactor activity, its' zero FDH activity appeared to affect its growth with TMAO. It showed severely reduced nitrate reductase and fumarate reductase activity and was unable to grow on nitrate and fumarate anaerobically. A striking observation was that this mutant was very sensitive to chlorate anaerobically despite the lack of growth with nitrate (see Table 35); therefore, this mutant could be considered to resemble the fnr mutants

isolated in E. coli. This class of mutants was isolated by Lambden & Guest (1976a, b) and formerly called frd B. Mutation in this locus led to loss of expression of several anaerobic enzymes including nitrate, nitrite, and fumarate reductases (Chippaux et al., 1981; Newman & Cole, 1978). Recently, further fnr mutants were isolated in E. coli by phage Mu insertion (Stewart & MacGregor, 1982; Stewart, 1982); these mutants were found to be repressed for TMAO reductase activity which indicates that the TMAO reduction system is under the same control as those for nitrate and fumarate reduction. It was proposed that the fnr locus codes for a positive regulatory protein which inactivates the transcription of anaerobic respiratory enzymes structural genes. Most of the fnr mutants were found to be cryptic by chlorate sensitivity. The fnr mutants described by Lambden & Guest were chlorate sensitive, in addition to the severe reduction in their nitrate reductase and fumarate reductase activities, they have reduced FDH : BV activity which was combined with a reduction in formate hydrogenlyase complex activity. The defects in mutant I11 have some similarities to those found for fnr mutants of E. coli but differ in the expression of TMAO and FDH : DCPIP. The following Table shows the characteristic features of these mutants.

Table 36. Phenotypes of fnr mutants of E. coli and I11 mutant of A. putrefaciens

Character	<u>fnr</u> (<u>E. coli</u>)	I11 (<u>A. putrefaciens</u>)
Chlorate sensitivity	S	S
FDH : DCPIP	+	-
FDH : BV	-	-
Nitrate reductase	-	-
Fumarate reductase	-	-
TMAO reductase	-	+

S = sensitive

However, the suggestion that I11 is similar to fnr mutants of E. coli should be stated with caution; since the TMAO and nitrate regulation systems in E. coli and A. putrefaciens are quite different, as nitrate does not repress TMAO reductase in the latter organism, while in the former it does (see discussion of section I).

From the above discussion it can be concluded that the nitrate and fumarate reduction systems in A. putrefaciens are under the same control. The TMAO reduction system is not under this control, but it is influenced indirectly by regulation of FDH, which was reduced or absent in I11 when grown with TMAO.

The remaining mutants 332, 362, 37, 402, and 404 reverted prior to complete analysis. According to the primary characterisation these mutants were pleiotropic (Table 18). However, their reversion indicates they were affected in some common function of molybdoenzymes, which resulted in such pleiotropy or may be affected in their regulation.

As TMAO reductase was assayed with $MV^{•+}$ and this activity was absent in the molybdenum cofactor mutants, it might indicate that the molybdenum center is the site for $MV^{•+}$ oxidation by TMAO similarly to that proposed for $MV^{•+}$ oxidation of nitrate reduction to nitrite in the nitrate reductase of higher plants (Hewitt & Notton, 1980). However, these mutants showed fumarate reductase activity when assayed with $MV^{•+}$ which means that the fumarate reduction by $MV^{•+}$ takes place at another site than a molybdenum center (Table 27). It is known in Vibrio succinogenes that fumarate reductase binds FAD covalently as a prosthetic group; this prosthetic group catalyses the reduction of fumarate by $BV^{•+}$ (Kröger & Innerhofer, 1976a). These results might indicate that fumarate reductase in A. putrefaciens contains FAD.

The effects of molybdenum cofactor and nitrate reductase on nitrite reductase was investigated using molybdenum cofactor mutants and Group 7 mutants. The findings that nitrite reductase is present in molybdenum cofactor mutants (Tables 28 and 29) argues that nitrite reductase is a non-molybdoenzyme and that the lack

of molybdenum cofactor or nitrate reductase seem to derepress nitrite reductase synthesis. These results indicate that nitrite reductase can be assayed with $MV^{•+}$ acting at a non-molybdenum center. $MV^{•+}$ can be used as donor for nitrite reductase in A. putrefaciens and other organisms like Chlamydomonas reinhardtii (Barea & Cardenas, 1975).

It has been reported that some mutants defective in molybdenum processing like chl A exhibited high nitrite reductase activity when grown on nitrate (Venables et al., 1968; O'Hara et al., 1967) and this was explained as nitrite reductase induction by nitrate rather than by nitrite. However, although nitrate induced nitrite reductase only when added at low concentrations (<10 mM), at high concentrations (100 mM) no nitrite reductase was observed (Cole & Wimpenny, 1968). Moreover, nitrate was found to be able to induce nitrate reductase and nitrite reductase as well in Nicotiana tabacum without any formation of nitrite. The induced nitrate reductase activity was higher than the nitrite reductase activity (Mendel & Müller, 1979), which is in contrast to the results reported here for A. putrefaciens. When A. putrefaciens was grown on TMAO and nitrate both TMA and nitrite were found in the culture medium (Easter, 1982) and the latter was not further reduced perhaps because both nitrite and nitrate compete for the electrons supplied by formate as reported for E. coli (Abuo-Joadue et al., 1979a, b). However, the high nitrite reductase activity in molybdenum cofactor mutants may be due to the absence of molybdenum cofactor introducing conformational shifts of the reductase molecule rendering this molecule unfit for its repressor-function (Stouthamer et al., 1980).

The effects of molybdate in the growth medium on the activities of molybdoenzymes were complex and did not always result in enhancement of activity. The inclusion of molybdate in the growth medium enhanced the activities of some known molybdoenzymes like nitrate reductase and TMAO reductase but not FDH (see section IV), nor the overall activity of the anaerobic transport chain (Table 32).

The latter might be due to a balancing of individual activities within the chain, as the nitrate reductase was enhanced by molybdate whereas the FDH activity was reduced by molybdate. The reduction in the TMAO reductase activity of I11 and I116 mutants caused by addition of molybdate confirms that those mutants are affected in the regulation system but not at the same locus as they showed some dissimilarities.

The use of chlorate as a selective agent for the isolation of both dissimilatory and assimilatory nitrate reductase mutants is based on the toxicity due to nitrate reductase dependent conversion of chlorate to chlorite. Purified TMAO reductase and nitrate reductase have a significant chlorate reductase activity (Shimokawa & Ishimoto, 1979; Forget, 1974), accordingly the defect in these enzymes (especially in molybdenum processing) could lead to inability to reduce chlorate. Most bacterial chlorate resistant mutants have been found to be defective in molybdenum processing (see introduction, section 5), but the iron-molybdenum cofactor of nitrogenase was unaffected in some chl mutants (Kennedy & Postgate, 1977). However, this does not exclude the possibility that both nitrate reductase and nitrogenase share some steps in molybdenum processing.

Generally, the chlorate resistant mutants have a pleiotropic feature and this resistance is a constant feature for chl A, B, D, E, and G mutants but it is not an invariable property of mutation in the chl C region as in E. coli and Salmonella typhimurium (Chippaux et al., 1972). Thus selection of mutants by chlorate resistance although effective is not straightforward. Oltmann et al. (1976b) found that some chl mutants of Proteus mirabilis had high FDH activity in disagreement with the general properties of other chl mutants; however, a similar situation was found with mutants I79, I82, and I66. Moreover, mutant I82 in which high nitrate reductase activity was detected was unable to reduce chlorate; this could indicate that not only functional nitrate reductase is required for conversion of chlorate to chlorite but also other electron carrier(s) missing in this mutant.

Mutant I114 also in Group 7 had reduced nitrate reductase but was very sensitive to chlorate suggesting that the defect in this mutant affected only the nitrate reduction system but not the chlorate reduction system. This is supported from other studies on nitrate reductase mutants of Nicotiana tabacum, different degrees of chlorate resistance were observed and this led to the suggestion that chlorate inhibits the wild type strain not only via its nitrate reductase catalysed conversion to chlorite but also by a nitrate reductase independent mechanism (Müller & Grafe, 1978; Buchanan & Wary, 1982), a suggestion proposed earlier for E. coli (Ruiz-Herrera et al., 1969) from study of unlinked groups of mutants.

Aerobic incubation of A. putrefaciens wild type and mutants after anaerobic incubation in the presence of chlorate led to normal growth which means that chlorate is bacteriostatic anaerobically but not bactericidal as for other organisms. However, a new class of mutants Nar L⁻ was isolated in E. coli designated chlⁱ (chlorate-inhibited) (Stewart & MacGregor, 1982), these mutants were resistant to 5 mM chlorate but not 10 mM and subsequent aerobic incubation allowed full growth of these strains. In contrast to the typical chl^r strains which lost most of the molybdoenzyme activities, the strains in this class had the same activity as the wild type. In this respect E. coli and A. putrefaciens are different since the former is killed by chlorate anaerobically. Moreover, E. coli mutants that have lost ubiquinone (ubi⁻) as evidenced by accumulation of 2-octaprenylphenol the precursor of ubiquinone 8, are sensitive to chlorate when grown aerobically (Azoulay et al., 1978; Terrière et al., 1983).

The basic nature of TMA and the ability of A. putrefaciens to use maltose and produce acid were the basis of developing the maltose : TMAO medium for detection of TMAO reduction. Wood & Baird medium was used as this is not very well buffered and small changes in $[H^+]$ could be easily detected. However, this medium contains a high level of peptone and yeast extract which led to

peptonization reactions and strongly shifted the reaction towards the alkaline side. This problem was overcome by reducing these ingredients. The acidic reaction on aerobic incubation resulted from maltose utilisation, while subsequent anaerobic incubation caused alkalinisation if the cells were able to reduce TMAO to TMA, while the mutants remained in an acidic environment. Indicators like phenol red and bromocresol purple were valid for this purpose, but methyl violet which gave a very clear distinct colour in acidic and basic media unfortunately could not be used since it seemed to have some toxic effect on A. putrefaciens when applied at the required concentration.

This medium would be expected to yield TMAO reductase mutants and mutants pleiotropically defective in components of anaerobic respiration, but could also yield strains affected in maltose utilisation.

SECTION IV

THE EFFECT OF MOLYBDATE AND TUNGSTATE ON SYNTHESIS AND ACTIVITY OF RESPIRATORY ENZYMES

1. The In vivo Effect of Molybdate and Tungstate

(a) The effect of tungstate on TMAO reductase, nitrate reductase, and fumarate reductase activities

The in vivo effect of tungstate on the above enzymes was investigated by growing A. putrefaciens under different conditions in the presence or absence of tungstate. The activities of the enzymes was estimated in whole cell suspensions (Table 37). Nitrate reductase was extremely sensitive to the presence of tungstate in the growth medium, the activities of both the constitutive and nitrate induced enzyme were reduced very severely at 2 mM tungstate. The activity of TMAO reductase was not markedly affected even at high concentration of tungstate. Under aerobic and microaerobic growth conditions fumarate reductase activity decreased with increasing concentrations of tungstate, but was still detectable at 15 mM tungstate. By contrast fumarate reductase activity in fumarate grown cultures was hardly affected at all by the presence of tungstate in the growth medium.

(b) The effect of tungstate and molybdate on FDH activity

A. putrefaciens was grown under the same conditions described in the preceding section and FDH : DCPIP activity was estimated (Table 38). The activity from cultures grown with nitrate was reduced by the presence of 1 mM tungstate in the growth medium. This was not observed under any other growth conditions. Indeed the activities from aerobic and microaerobic grown cultures were enhanced by the presence of tungstate in the growth medium and only decreased at concentrations ≥ 10 mM. The FDH : DCPIP activity of culture grown with fumarate was not affected at any

Table 37. The in vivo effect of tungstate on TMAO reductase, nitrate reductase, and fumarate reductase

Culture	% Specific activity																	
	TMAO reductase						Nitrate reductase						Fumarate reductase					
	Tungstate in growth medium (mM)						Tungstate in growth medium (mM)						Tungstate in growth medium (mM)					
	0	1	2	5	10	15	0	1	2	5	10	15	0	1	2	5	10	15
Aerobic	100	66	54	90	86	57	100	29	0	0	0	0	100	75	31	46	29	9
Micro-aerobic	100	32	35	53	25	33	100	28	29	52	31	20	100	118	59	46	24	17
" + TMAO	100	50	37	32	33	31	-	-	-	-	-	-	-	-	-	-	-	-
" + Nitrate	-	-	-	-	-	-	100	10	7	0	0	0	-	-	-	-	-	-
" + Fumarate	-	-	-	-	-	-	-	-	-	-	-	-	100	85	97	101	116	97

- = not determined

Table 38. The in vivo effect of tungstate on FDH : DCPIP activity

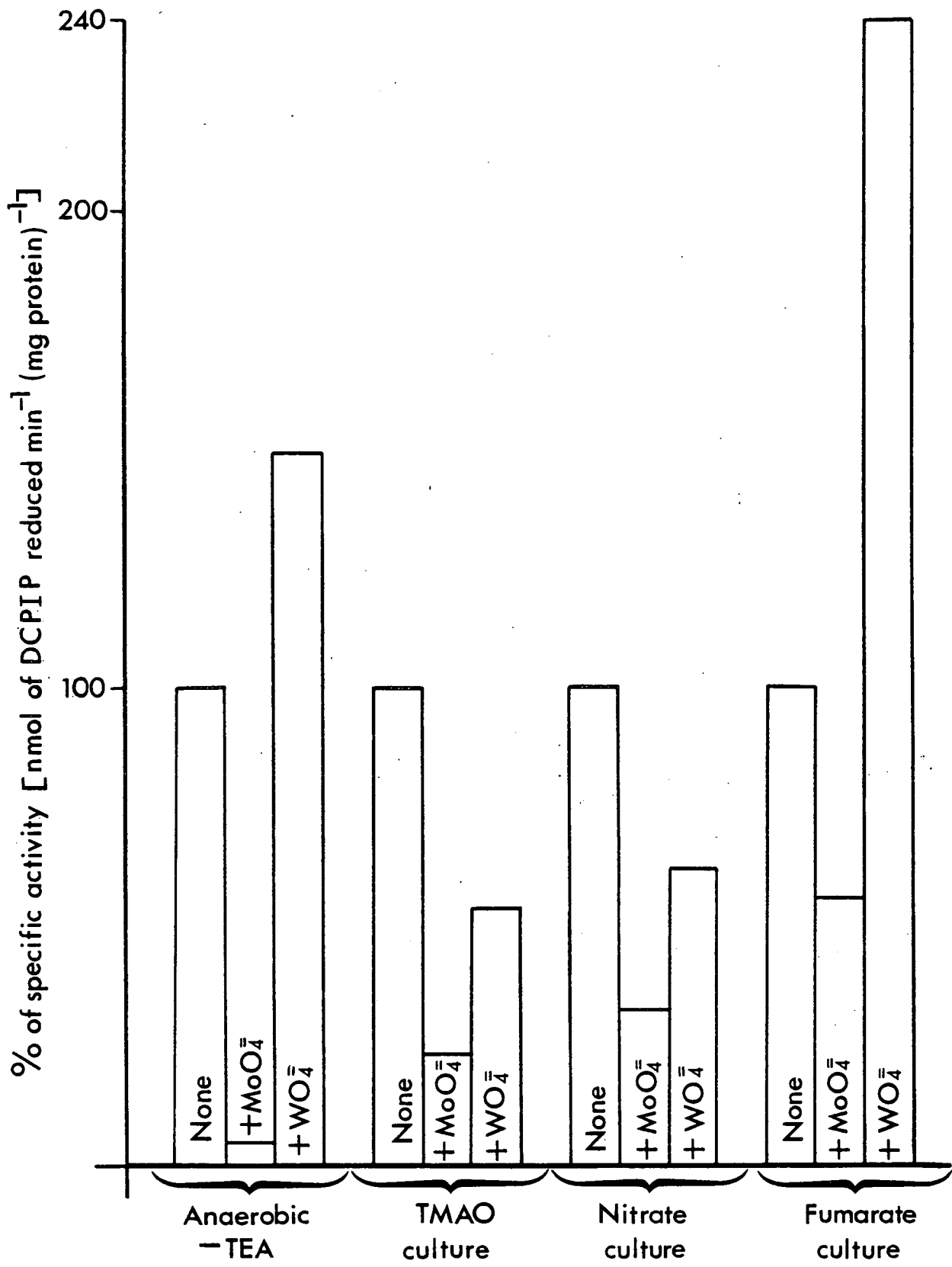
Culture	% Specific activity					
	Tungstate in growth medium (mM)					
	0	1	2	5	10	15
Aerobic	100	452	384	417	203	12
Microaerobic	100	215	109	158	28	12
" + TMAO	100	96	47	25	18	18
" + Nitrate	100	21	14	13	11	11
" + Fumarate	100	164	128	182	132	106

of the concentrations used.

The effects of molybdate on FDH activity were investigated. FDH activity was reduced under all growth conditions by the presence of molybdate in the growth medium, while it was observed repeatedly that the nitrate reductase and TMAO reductase activities were enhanced by addition of molybdate (Tables 33 and 34, section III). These effects were further investigated by growing A. putrefaciens in Wood & Baird medium and Wood & Baird medium supplemented either with 2 mM molybdate or tungstate under different growth conditions and assaying for FDH using both DCPIP and BV. The results are shown in Figure 10 (FDH : DCPIP) and Figure 11 (FDH : BV). Under all growth conditions the presence of molybdate reduced the activity of FDH compared with controls. This effect was most marked with microaerobic cultures grown in the absence of an exogenous electron acceptor. The effect of tungstate on FDH activity was qualitatively the same as in Table 38.

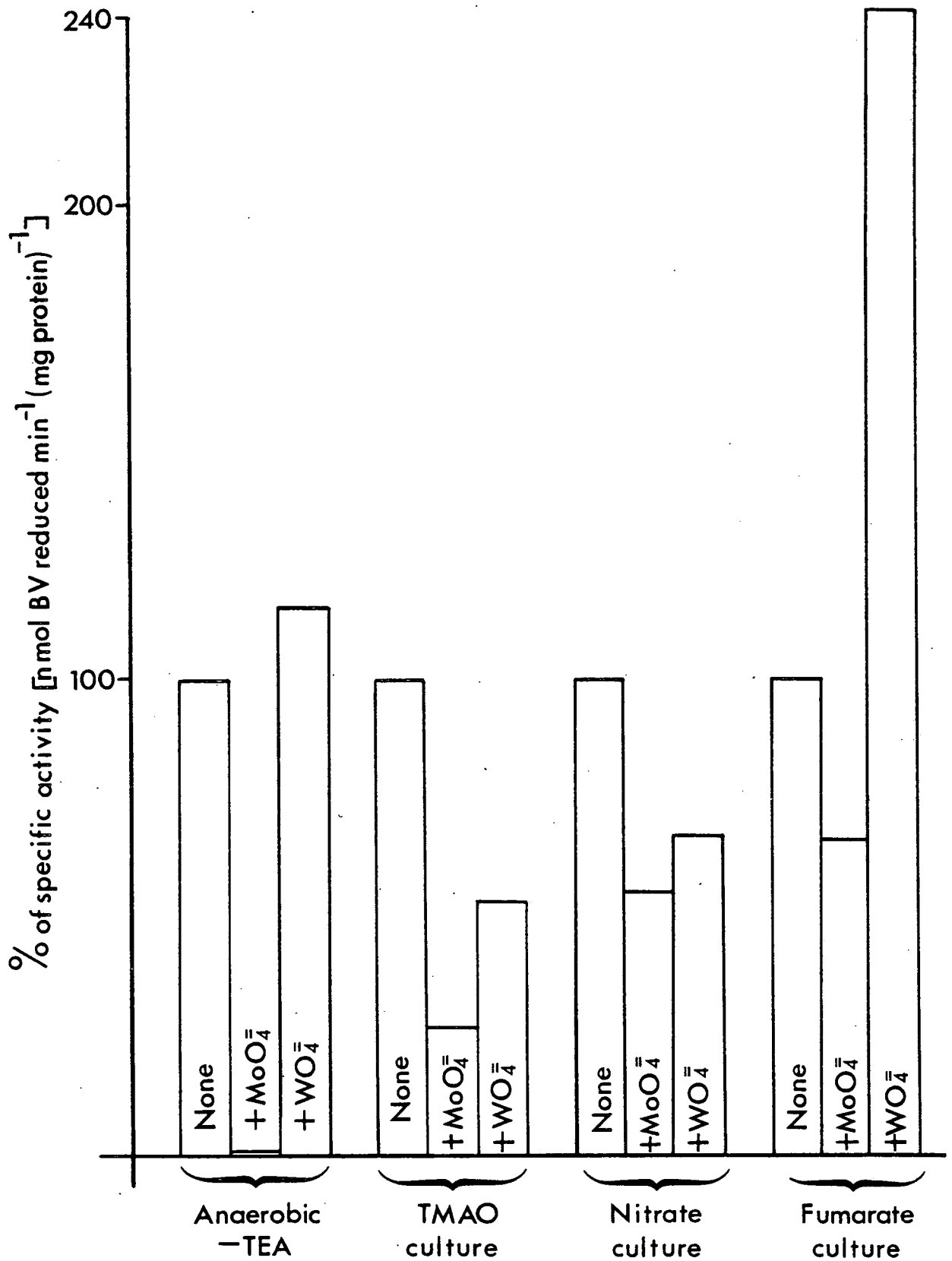
(c) The effect of molybdate and tungstate on the synthesis of the molybdenum cofactor

Cultures of A. putrefaciens wild type were grown aerobically in the presence of 2 mM molybdate or tungstate and tested for the presence of molybdenum cofactor. A control culture was grown without either molybdate or tungstate. The experiment was repeated with two mutants 301 and 302, which cannot bind the molybdenum cofactor to the molybdoenzymes (section III). Cells were grown aerobically to the late log phase and the molybdenum cofactor activity in the crude extract was measured by complementation with N. crassa nit-1 nitrate reductase in the presence of 10 mM molybdate as described in section II. The results are depicted in Figure 12. The presence or absence of molybdate in the growth medium had little effect on molybdenum cofactor synthesis in the wild type, and at the same time tungstate had no repressive effect. The same was observed for the mutant cultures.



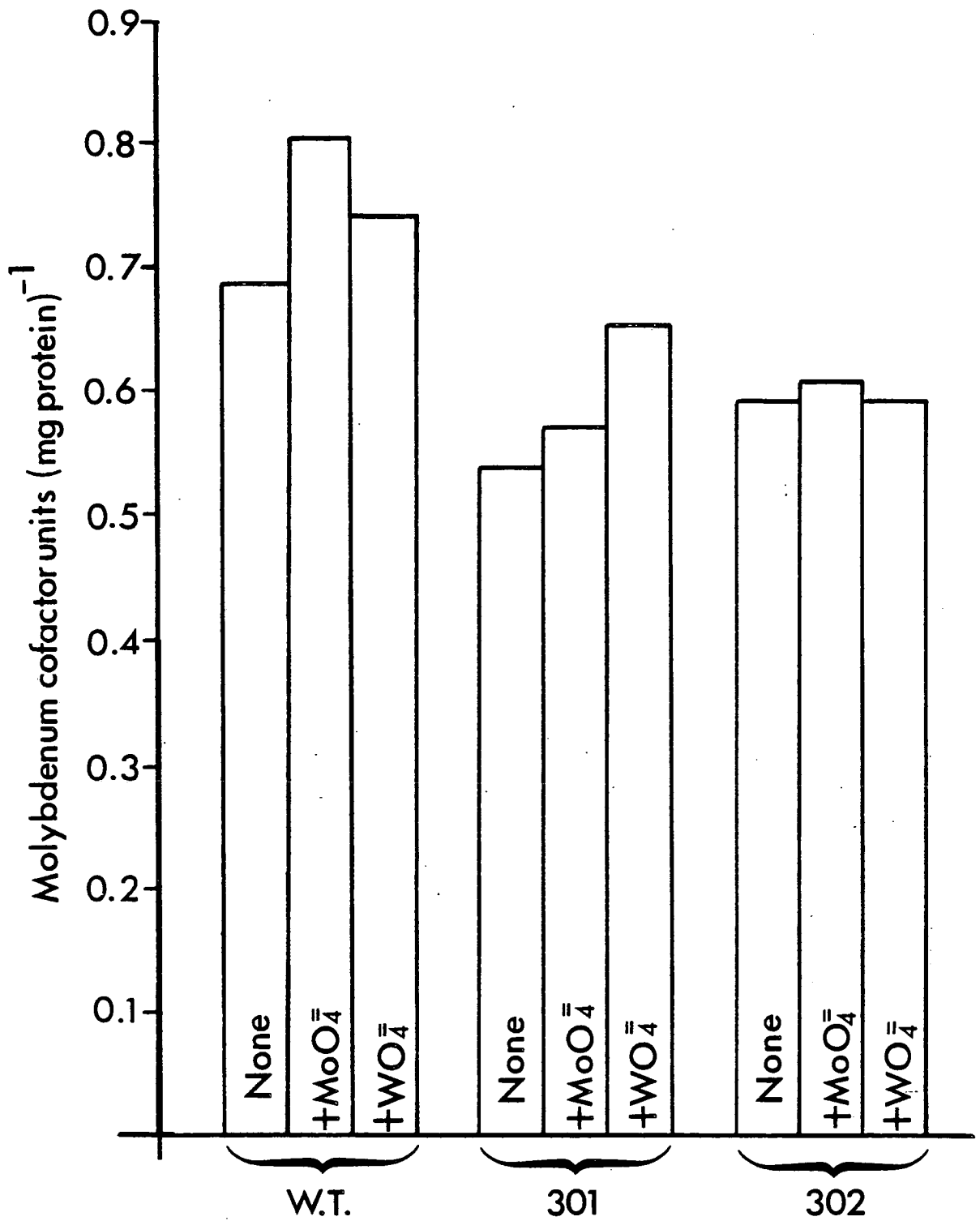
TEA = terminal electron acceptor

Figure 10: The in vivo effect of molybdate and tungstate on the FDH:DCPIP activity



TEA = terminal electron acceptor

Figure 11: The in vivo effect of molybdate and tungstate on the FDH:BV activity



W.T. = wild type

Figure 12: The in vivo effect of molybdate and tungstate on synthesis of the molybdenum cofactor

2. The In vitro Effect of Tungstate

In these experiments the wild type strain was grown under different growth conditions in the absence of tungstate, and the whole cell suspensions were prepared. These suspensions were incubated with different concentrations of tungstate for 10 min at room temperature and then assayed for the corresponding enzymes. The results for TMAO reductase, nitrate reductase, and fumarate reductase are shown in Table 39 and for FDH in Table 40. Constitutive TMAO reductase activity was not affected by tungstate, while the inducible TMAO reductase showed slight inhibition when incubated with tungstate prior to assay. Constitutive and inducible nitrate reductase were both affected at 1 mM tungstate and the activity was about 50% of the control sample. Fumarate reductase of both microaerobic and fumarate culture was unaffected. Using the periplasm as source of TMAO reductase, nitrate reductase, and fumarate reductase, the results were more or less similar to that of using whole cell samples.

Overall FDH from cells grown under a variety of conditions was not strongly inhibited by tungstate. Tungstate had no effect on FDH from nitrate grown cells and only reduced FDH activity from fumarate grown cells to about 70% of the control value.

3. The Effect of Molybdate and Tungstate on Complementation of *Alteromonas putrefaciens* Molybdenum Cofactor with *Neurospora crassa nit-1* Nitrate Reductase

A. putrefaciens was grown aerobically in Wood & Baird medium containing 2 mM molybdate to the late log phase and the derived crude extract used as a molybdenum cofactor source to complement *N. crassa nit-1* nitrate reductase. Complementation was carried out with different combinations of molybdate and tungstate : with molybdate (as control treatment); without molybdate; with tungstate; 1 : 1 ratio of molybdate : tungstate; 10 : 1 molybdate :

Table 39. The in vitro effect of tungstate on TMAO reductase, nitrate reductase, and fumarate reductase

Culture	% Specific activity																							
	TMAO reductase								Nitrate reductase								Fumarate reductase							
	Tungstate in assay mixture (mM)								Tungstate in assay mixture (mM)								Tungstate in assay mixture (mM)							
	0	0.1	0.2	0.3	0.4	0.6	0.8	1.0	0	0.1	0.2	0.3	0.4	0.6	0.8	1.0	0	0.1	0.2	0.3	0.4	0.6	0.8	1.0
Micro-aerobic	100	110	206	147	142	203	129	213	100	60	72	78	85	50	63	62	100	116	206	142	90	77	164	194
" + TMAO	100	102	86	93	75	53	75	85	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
" + Nitrate	-	-	-	-	-	-	-	-	100	53	78	56	39	66	73	48	-	-	-	-	-	-	-	-
" + Fumarate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	100	155	246	246	222	76	111	83

- = not determined

Table 40. The in vitro effect of tungstate on FDH activity

Culture	% Specific activity							
	Tungstate in assay mixture (mM)							
	0	0.1	0.2	0.3	0.4	0.6	0.8	1.0
Microaerobic	100	112	96	84	112	96	94	102
" + TMAO	100	63	116	127	106	59	81	151
" + Nitrate	100	118	105	179	289	137	223	126
" + Fumarate	100	67	89	77	68	74	75	72

tungstate; 10 : 0.1 molybdate : tungstate. The results are shown in Figure 13. The results indicated that complementation by A. putrefaciens molybdenum cofactor can be carried out without adding molybdate to the complementation mixture, but that addition enhanced nitrate reductase activity by about 30-40%. Tungstate showed antagonistic effects towards molybdate in complementation: little or no molybdenum cofactor activity was observed in the presence of tungstate and this was not relieved by addition of an equal concentration of molybdate.

The effect of molybdate and tungstate was also studied after completion of the complementation reaction. Complementation was carried out in the presence of molybdate, tungstate, and without addition of anions. Then molybdenum cofactor was assayed. Molybdenum cofactor activity was also assayed after incubation with molybdate or tungstate for 20 min at 20-25°C post-complementation (Table 41). Inclusion of tungstate in the complementation mixture caused severe reduction in the activity of the complemented enzyme. Both molybdate and tungstate had no effect after the completion of complementation process.

4. Discussion

In as much as tungsten is in the same chemical group in the periodic table as molybdenum, one might expect tungsten to serve as a partial substitute for molybdenum or as a competitive inhibitor of molybdenum function in molybdoenzymes (see introduction, section 4). Generally tungsten has been considered biologically inactive, while molybdenum is biochemically important and is a constituent of several enzymes (see introduction Table 1). The competitive inhibition in vivo of molybdenum function by tungstate is well documented (Hewitt & Notton, 1980).

The effect of tungstate on A. putrefaciens enzymes depended upon the nature of the molybdoenzyme. The activities of constitutive and inducible forms of TMAO reductase were not abolished

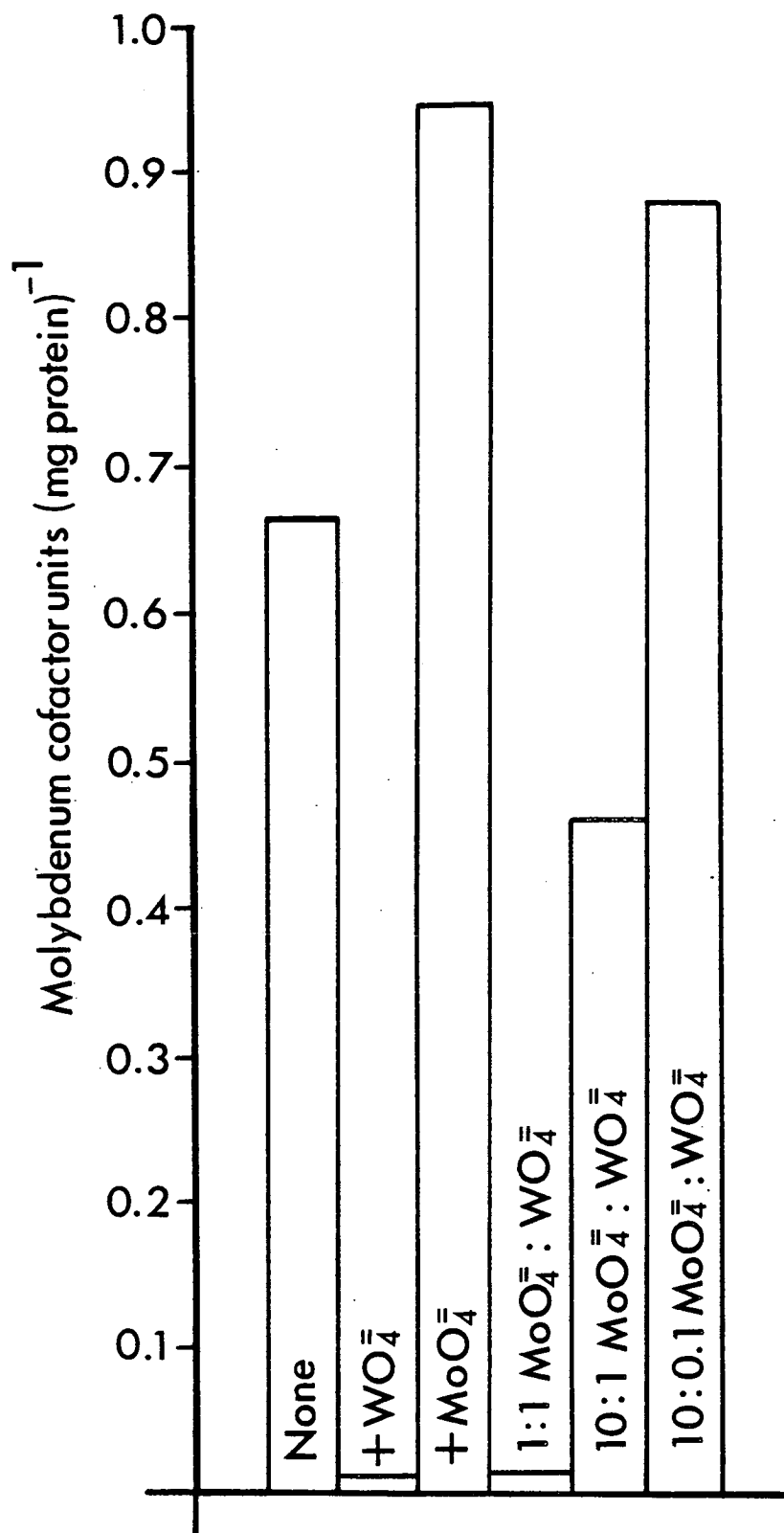


Figure 13: The effect of molybdate and tungstate on the nit-1 complementation reaction

Table 41. The effect of molybdate and tungstate on the nit-1 complementation reaction

Type of complementation	% Activity*
(1) Complementation without molybdate	100
(2) Complementation with molybdate	142
(3) Complementation with tungstate	2
(4) Complementation without molybdate, then 10 mM molybdate was added	73
(5) Complementation without molybdate, then 10 mM tungstate was added	62
(6) Complementation with molybdate, then 10 mM tungstate was added	99
(7) Complementation with tungstate, then 10 mM molybdate was added	3

* Activity units molybdenum cofactor (mg protein)⁻¹

even in the presence of high concentration of tungstate in the growth medium. This might be because TMAO reductase has a greater affinity to incorporate the molybdate present in the growth medium, or it is more specific in its recognition of molybdate. Both constitutive and inducible forms of TMAO reductase of E. coli grown in complex medium showed about 90% inhibition by 0.1 mM tungstate (Shimokawa & Ishimoto, 1979). In A. putrefaciens the enzyme most susceptible to tungstate inhibition was nitrate reductase. These results are in agreement with other organisms: when nitrate reduction or nitrogen fixation are involved, tungstate has an overt antagonism. However, the constitutive form seemed to be more resistant to 1 mM tungstate (Table 37). In soyabean (Glycine max) the constitutive form of nitrate reductase showed more resistance to tungstate than the inducible form (Aslam, 1982). The explanation was that the formation of the constitutive nitrate reductase preferentially used the molybdenum present in the seed and therefore, its formation was not affected by tungstate, or that the constitutive nitrate reductase did not require molybdenum for activity. The first explanation seems more likely and could account for the results found in this study for A. putrefaciens, since the molybdenum cofactor is required for the constitutive nitrate reductase as evidenced by the absence of this form or its activity in the molybdenum cofactor mutants. It has been suggested that tungstate inhibition or the inability of the tungsten analogue to be enzymatically active was not due to altered spatial relationship within the protein but from other factors probably redox potential as tungsten differs significantly from molybdenum in several redox systems (Hewitt & Notton, 1980). However, presently it is not possible to relate data from other organisms to those obtained from A. putrefaciens since more steps are required to find out whether tungsten affects only the activity or the synthesis of enzyme protein.

The effect of tungstate on A. putrefaciens fumarate reductase was not as severe as for TMAO reductase and nitrate reductase.

At 1 mM tungstate the constitutive form was enhanced by 18%, while at the same concentration this form showed 14% inhibition in Proteus mirabilis, but at 0.1 mM tungstate the enzyme also showed activation by 8% (Oltmann et al., 1979). The constitutive fumarate reductase of A. putrefaciens at high tungstate concentrations was not abolished but showed a gradual decrease with increasing concentrations, probably as an indirect effect of tungstate toxicity. The inducible form of fumarate reductase was unaffected by higher tungstate concentrations.

Surprisingly, the FDH activity of aerobic culture was higher in the presence of tungstate than in its absence. At 5 and 10 mM tungstate the aerobic growth was slightly reduced compared with the control culture, but as shown in Table 38 FDH activity was not affected at these concentrations. Thus tungstate probably affects other metabolic processes. The only conditions under which tungstate reduced the FDH activity was when cells were grown with nitrate or TMAO. Again this was probably an indirect effect of the reduction in nitrate reductase and TMAO reductase activities by tungstate. Enoch & Lester (1972) found in E. coli that aerobic FDH was inhibited by 94% when tungstate was added to the growth medium at 10^5 molar excess over molybdate. For a nitrate culture with selenium supplied at 10^{-6} M and molybdate at 10^{-7} M, FDH activity was $2610 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$; addition of 1 mM tungstate to this culture resulted in 96% reduction of the activity, but when the molybdate concentration was increased and the ratio of tungstate : molybdate was only 10, the reduction in activity was only 12%. From these results it is obvious that the tungsten inhibition could be reversed by increasing the molybdate concentration. In the Coli-aerogenes group, Pinsent (1954) showed that only with the nitrate grown culture was FDH affected by tungstate, which is in agreement with the results of Table 38.

On the other hand tungsten has been found to be biologically active in the FDH from obligate anaerobes such as clostridial spp (Andreesen, 1980) and in Methanococcus vannielii (Jones & Stadtman, 1977). In FDH from Clostridium formicoaceticum, tungstate was

found to be incorporated preferentially to molybdate (Ljungdahl & Andreessen, 1976). Recently, this enzyme has been purified and shown to have 2 tungsten per mol of enzyme (Yamamoto *et al.*, 1983) and it has been found that there is a releasing tungsten moiety of molecular weight 5000 daltons which could be equivalent to the molybdenum cofactor. A similar phenomenon has been observed in other *Clostridial* spp and all the supplied tungstate was found to be taken up by cells and incorporated into a soluble protein (Elliot & Morteson, 1975); this protein molecule could represent a storage form similar to the molybdenum storage protein found in *E. coli* (Dubourdieu *et al.*, 1976).

It is known that the reduction of CO_2 to formate is thermodynamically very unfavourable and perhaps tungsten is more efficient in catalysing this reaction compared with molybdenum, possibly by forming a tungsten- CO_2 complex at the initial step of CO_2 reduction to formate (Ljungdahl, 1976; Chisholm & Extine, 1974). On the other hand those FDHs containing tungsten such as in *Clostridium formicoaceticum* and *Clostridium thermoaceticum* and the molybdo form FDH of *Clostridium pasteurinum* were all found to catalyse both the oxidation of formate to CO_2 and the reduction of CO_2 to formate (Leonhardt & Andreessen, 1977). Yet, within the genus *Clostridium* tungstate and molybdate are used differentially: tungstate had an antagonistic effect on the FDH from *Clostridium cylindrosporum* (Wagner & Andreessen, 1977), but not on the molybdo form of FDH in *Clostridium pasteurinum*, while molybdate had no effect on the tungsto form of the FDHs as in *Clostridium formicoaceticum* (Leonhardt & Andreessen, 1977). In the latter organism the authors concluded from molybdate and tungstate incorporation studies that FDH is a native tungsto-protein.

In *Methanococcus vannielii*, FDH catalyses the fermentation of formate to CO_2 and methane. Molybdenum had no effect on the growth rate, while tungsten increased the growth rate markedly (Jones & Stadtman, 1977).

In most *Clostridial* spp and *Methanococcus vannielii*, the

addition of molybdate either had no effect or increased the incorporation of tungsten into FDH. This could mean that either the level of tungsten increased by addition of molybdate because analytical molybdate contains tungsten as a contaminant or that the molybdate may function as co-inducer for the production of FDH (Yamamoto et al., 1983). These results contrast with those obtained for A. putrefaciens FDH, in which the addition of molybdate always resulted in reduction of FDH activity (Figure 10). In fact A. putrefaciens was grown in complex medium (Wood & Baird medium) which might contain low concentrations of tungsten essential for FDH if it is assumed to be a tungsto-enzyme; the reduction of activity by molybdate might be evidence that FDH in A. putrefaciens is a tungstoenzyme rather than molybdoenzyme. Molybdate added to the growth medium would increase the ratio of molybdate : tungstate several fold, and this increased the activity of other molybdoenzymes, while it reduced the FDH activity. The effect of molybdate and tungstate on FDH : DCPIP and FDH : BV was similar and the absence of both activities in molybdenum cofactor mutants, might suggest that there is only one form of FDH in A. putrefaciens.

The results from section III showed that molybdenum cofactor mutants and other mutants affected in molybdenum processing lacked FDH activity. Thus if FDH were a tungstoenzyme then tungsten would probably need to coordinate the molybdenum cofactor molecule. Accordingly this molecule or ring can coordinate both molybdenum and tungsten and perform enzymatic activities depending on the reaction and the microsurrounding environment. In such a situation, both a tungstoenzyme and a molybdoenzyme would exist in the same electron transport chain, and as both elements act antagonistically towards each other, regulation of their metabolism would have to be strictly controlled. This hypothesis could explain the observation that when molybdate was added to the growth medium the overall activity of formate : nitrate respiratory chain was not enhanced (Table 32 section III), while at the enzymic level nitrate reductase activity was

enhanced (Table 33) and FDH activity depressed (Figures 10, 11). The presence of a molybdoenzyme and a tungstoenzyme in the same chain has been observed in some Clostridial spp particularly Clostridium acidurici. During growth on uric acid, FDH together with xanthine dehydrogenase mediate the electron transport from formate to uric acid : xanthine dehydrogenase is a molybdoenzyme in this organism and FDH is a tungstoenzyme (Wagner & Andreesen, 1977). In Clostridium barkeri Imhoff & Andreesen (1979) reported that the nicotinic acid hydroxylase can be activated by tungsten; recently the fluorescence spectral studies on this enzyme after the aerobic denaturation indicated the presence of fluorescent component with properties similar to those reported for the "pterin-like" cofactor from xanthine oxidase and several other molybdo proteins (Dilworth, 1983). This might give an indication that molybdenum and tungsten can coordinate the same cofactor under certain circumstances.

The participation of selenium in A. putrefaciens FDH was not investigated during this work since no minimal medium had been developed, but its involvement cannot be ruled out, since the Wood & Baird medium would probably contain selenium. The investigation of selenium in FDH could lead to a clearer picture about this enzyme, since that it has been found as an integral part in the FDHs from different organism and particularly in the form of selenocystein in those FDHs that incorporate tungsten rather than molybdenum as in Methanococcus vannielii and Clostridium thermoaceticum (Jones, 1978; Jones & Stadtman, 1981; Yamamoto et al., 1983).

The effect of tungstate on the complementation process of nit-1 nitrate reductase indicated that only the free molybdenum cofactor is able to exchange its molybdenum as reported by other authors (Lee et al., 1974a, b; Alikulov et al., 1980; Mendel et al., 1981; Mendel, 1983). The results of Table 4.1 indicated that A. putrefaciens molybdenum cofactor can complement nit-1 nitrate reductase without addition of extra molybdate to the

complementation mixture, this means that a high percentage of the molybdenum cofactor has already incorporated the molybdenum since the cells were grown in a medium containing high molybdate. The abolition of activity by inclusion of tungstate in the complementation mixture was due to the replacement of molybdenum by tungsten which indicates that the free molybdenum cofactor only binds molybdenum loosely. However when the cofactor is locked in the enzyme then such replacement comes impossible and this explains the results that there was no effect for either molybdate or tungstate after the completion of complementation. This conclusion is supported by the work done by Lee (1978) who showed that molybdenum can be replaced in the exposed molybdenum cofactors but not the tightly locked cofactor of the induced nitrate reductase of N. crassa wild type, and the results of Alikulov et al. (1980) using another source of molybdenum cofactor. These results are in disagreement with the results obtained by Amy & Rajagopalan (1979) using E. coli extract as molybdenum cofactor source. They found that the empty cofactor, or cofactor incorporating the tungsten were able to perform the structural function of molybdenum cofactor and the resulted 7.9S species of complemented nit-1 nitrate reductase retained the activity upon incubation with 1 mM molybdate. However, the reduction noticed when tungstate was added after complementation (Table 4: No. 5 and 6) is clearly due to prolonged incubation as optimum molybdenum cofactor activity was obtained at 20 min (Figure 5, section II) and thereafter decreased with time. This was confirmed by the fact that a similar addition of molybdate resulted in a similar decrease in molybdenum cofactor activity. The results shown in Figure 13 indicate that tungsten competes strongly with molybdenum for the active site of the exposed molybdenum cofactor. At the same concentrations, tungsten competes the molybdenum and the presence of molybdate at a very high ratios of molybdate : tungstate was required for molybdenum cofactor activity. This is in contrast to findings in E. coli, where no inhibition of activity was detected when 5 mM tungstate was added in the presence of 1 mM molybdate in

the complementation mixture (Amy & Rajagopalan, 1979).

Neither molybdate or tungstate appeared to have any effect on the synthesis of molybdenum cofactor. Proteus mirabilis when grown in the presence of tungstate showed 2-3 times higher molybdenum cofactor activity than when grown in the absence of tungstate. This high activity was combined with an increased amount of demolybdo nitrate reductase. The latter could be filled by molybdate during reactivation (Claassen et al., 1981). A similar effect of tungstate on the formation of demolybdoenzymes has been observed in E. coli wild type and chl D mutant. These observations led to the suggestion that tungstate might enhance the expression of the nitrate reductase structural genes or it could stabilise the demolybdo form of the enzyme, since in all cases higher levels of demolybdo-inactive nitrate reductase was produced (Giordano et al., 1980a). This may indicate that tungsten as an analogue of molybdenum can act as a co-inducer. At the level of molybdenum cofactor synthesis, A. putrefaciens showed no such behaviour, which could mean that A. putrefaciens is better adapted to tungsten available as tungstate in the marine environment at $0.1 \mu\text{g l}^{-1}$ (MacKenzie, 1969; Brewer, 1975).

GENERAL DISCUSSION

AND

CONCLUSIONS

The results obtained from this study indicate that many factors are involved in the induction and synthesis of terminal reductases and the components of an anaerobic respiratory chain. Among these factors is the presence of the appropriate electron acceptor in the growth medium which contributes to a large extent to the induction of its respiratory chain components. The natural habitat and environmental adaptation are other factors as has been observed with other organisms from different sources (Jones *et al.*, 1975).

Anaerobic nitrate respiration has received much attention in *E. coli*, in this organism the nitrate respiration causes cessation of TMAO respiration, which is in contrast to the results obtained for *A. putrefaciens* probably due to the differences in regulation of anaerobic electron transport in the two organisms. However, absence of molybdenum cofactor in both organisms resulted in the loss of nitrate reductase and TMAO reductase as well as FDH. It is known that the nitrate reduction system is very complicated and at present its regulation is not fully understood. It seems that the nitrate reduction system is the essential one for *E. coli* and the systems for fumarate or TMAO reduction are regulated accordingly as evidenced by results using *fnr* and *NarX* mutants (Stewart, 1982; Stewart & MacGregor, 1982). Such a situation does not exist in *A. putrefaciens*, and it is possible that TMAO respiration is the favoured anaerobic electron transport system in this bacterium. Although, more studies are required on the TMAO reduction system in *A. putrefaciens*, the results obtained here and the wide spectrum of mutants isolated indicate that this system is very complex.

Most of the genetic studies on molybdoenzymes like nitrate reductase were done using chlorate resistant mutants which have a pleiotropic phenotype. This selection probe was avoided in this study since the aim was the study of TMAO reductase and related components. However, although isolation of mutants was based on their inability to grow with TMAO anaerobically

a very high percentage of strains isolated were found to have pleiotropic defects. This pleiotropy is essentially due to defects in some steps of molybdenum metabolism resulting in a lack of the active molybdenum cofactor required for molybdoenzymes involved in different systems.

It is thought that nitrate reductase and TMAO reductase require molybdenum cofactor in E. coli (Takagi & Ishimoto, 1983), but it seems that this requirement is not quite the same. Takagi et al. (1981) found that chl G and chl E mutants have TMAO reductase activity. Chl E was suggested to have a role in the association of molybdenum cofactor to nitrate reductase (see introduction, Figure 7), while the chl G :: Mu cts and chl E :: Mu cts mutants were found to be defective in expression of TMAO reductase (Stewart & MacGregor, 1982). This might indicate that chl G and chl E have more than one gene and code for products specific for nitrate reductase and TMAO reductase. These results emphasise differences in molybdenum processing between the two enzymes in E. coli, although the same cofactor appears to be used. As most of the mutants in Group 1, 2, and 3 of A. putrefaciens (section III) have lost both nitrate reductase and TMAO reductase, it is more likely that the lesions are general for all molybdoenzymes rather than specific to only one of the two enzymes.

Lack of a genetic map and gene transfer systems for A. putrefaciens have precluded investigation of several points. Consequently use of mutagenic agents like NTG led to uncertain conclusions as in the case of I58 which is affected in TMAO reductase and has a second defect in its' inability for full aerobic growth; so it is uncertain whether the absence of TMAO reductase has a regulatory role for aerobic growth or whether this mutant bears another mutation. Lack of a minimal medium for this bacterium has hampered investigations on the involvement of tungsten and molybdenum in the enzymes studied and which metal is incorporated preferentially.

Molybdoenzymes from different sources contain a common structure, a molybdenum cofactor which binds the metal in a

catalytically active configuration. This structure is thought to consist of a pterin ring with a side chain which might provide sites for interaction with molybdenum as well as the strong non covalent interaction with various molybdoenzyme apoproteins. The participation of pterin in oxidation-reduction reactions raises the possibility that the pterin ring of the cofactor might have a role in the modulation of the oxidation-reduction state of the molybdenum during catalysis. As a phosphate group was estimated in the side chain of the molybdenum cofactor (Hailine, 1980; Rajagopalan et al., 1982; Johnson & Rajagopalan, 1982), the charges of the cofactor contributed by the phosphate molecule may be involved in binding the cofactor to the enzyme. Alternatively the phosphate may be directly involved in forming oxo-ligands to the molybdenum.

The synthesis of intact molybdenum cofactor involves more steps than the synthesis of the pterin ring as evidenced by the finding that the chl mutant of Proteus mirabilis (s556) showed no molybdenum cofactor activity to complement nit-1 nitrate reductase even with addition of high concentration of molybdate to the complementation mixture, yet extracts of this mutant showed the same behaviour as wild type extracts in yielding fluorescent material upon inactivation (oxidation of pterin ring) (Claassen et al., 1982a). As molybdenum cofactor and folic acid are structurally similar (Hailine, 1980), molybdenum cofactor mutants of Group 4 were grown in medium supplemented with folic acid with the idea of supplying the aromatic nucleus of pteridine. There was no increase in TMAO reductase activity. This could mean: (i) that the cells might be unable to take up the folic acid from the growth medium or (ii) that the bacterium is unable to split out the side chain (para-amino benzoic acid) of the folic acid and free the aromatic ring for cofactor synthesis or (iii) that the cells were able to produce the aromatic ring of the molybdenum cofactor but lacked other steps in producing the molybdenum cofactor. The latter possibility could be investigated by auxotrophic studies; requirement for folic

acid would mean that the aromatic ring was not synthesised.

As the substrate for molybdoenzymes have chemically stable bonds the activation energy for reaction is high; molybdoenzymes might have evolved to provide a low energy electron flow between the substrate and acceptor (Ljundahl, 1976). The proposed model for mechanism action of molybdenum in molybdoenzymes suggested by Stiefel (1973) is based on a mechanism of coupled proton and electron transfer. He suggested that molybdenum is the best element to fit this purpose, since the metal complex has a redox potential in the appropriate range and there is a tendency for deprotonation of molybdenum ligands as the oxidation state changes from IV to VI. A tungsten complex would display the correct acid-base behaviour but would be too stable in the higher (VI) oxidation state.

The results obtained from section III showed that active FDH required a molybdenum cofactor. The results of section IV indicated that FDH activity was enhanced by tungstate but not by molybdate. The conclusion from these results is that molybdenum cofactor could coordinate tungsten in a similar way to nicotinic acid hydroxylase (Imhoff & Andreesen, 1979; Dilworth, 1983). It seems that every bacterium has developed its particular FDH, thus a variety of FDH enzymes exist. More investigations are required to clarify the nature of the catalytic centre of FDH in A. putrefaciens. Purification of this enzyme would enable determination of whether this enzyme contained molybdenum or tungsten. Membrane bound FDHs differ in their protein-lipid interactions which is reflected by different degrees of solubility with a particular detergent and also with different detergents. CTAB seems to be very selective in solubilising A. putrefaciens FDH, while the same detergent both inactivates the E. coli FDH and fails to release it from the membrane (Ruiz-Herrera et al., 1972). Differentiation of FDH : DCPIP and FDH : BV was not clear throughout this study, but isolation of mutants affected solely in FDH could be helpful. However, the available evidence indicates that there is only one form of FDH in A. putrefaciens as indicated by results of Group 8 mutants and

also the results of section I. In A. putrefaciens formate seems to be the principal physiological donor during anaerobic growth with TMAO, nitrate, and fumarate. Unfortunately no FDH mutant was isolated and all FDH defective mutants were either also lacking activities of other molybdoenzymes or showed reduced FDH activity due to defects in regulatory genes affecting other anaerobic enzymes as shown by the results with mutants I11 and I116. However, the high level of induction of FDH by partial anaerobiosis and anaerobic growth with different electron acceptors (section I) confirms the role of formate, although further investigation is required. This includes isolation of FDH mutants and other mutants affected in the control of electron transport system(s), study of metabolic pathways and the assay of the phosphoroclastic reaction during anaerobic growth.

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