THE PURIFICATION AND PROPERTIES OF TRIMETHYLAMINE N-OXIDE REDUCTASE FROM *ALTEROMONAS* SP.NCMB 400

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To my parents

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The Purification and Properties of Trimethylamine N-Oxide Reductase from *Alteromonas* sp. NCMB 400

The enzymes of respiration-linked trimethylamine N-oxide reduction were studied in the marine bacterium *Alteromonas* sp. NCMB 400. Two trimethylamine N-oxide reductases were identified by gel electrophoresis and zymogram staining with molecular weights of 90 000 and 47 000 daltons. The high molecular weight enzyme was maximally induced under microaerobic conditions in the presence of either trimethylamine N-oxide or dimethylsulphoxide but not with nitrate or fumarate. The low molecular weight enzyme was synthesized constitutively under microaerobic conditions. The two enzymes were released into the periplasmic cell fraction upon spheroplasting, suggestive of a loose association with the outer surface of the cytoplasmic membrane. Enzyme activity was stable for periods in excess of two weeks at 4°C and six months at -18°C and -80°C, with no evidence of protease activity or proteolytic damage to the enzyme.

The high molecular weight trimethylamine N-oxide reductase was purified to homogeneity by ammonium sulphate precipitation, DEAE Sepharose CL 6B, hydroxyapatite and Sephacryl S-300 chromatography. Specific activity was increased about 60 fold and recovery was approximately 7%. Gel filtration and polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate indicated a single polypeptide that was not dissociated into the second low-molecular weight trimethylamine N-oxide reductase. The purified enzyme reduced a restricted range of N-oxide analogues including the N-oxides of trimethylamine, N,N-dimethyldodecylamine, pyridine and picoline with K_m values of 0.02, 2.41, 6.83 and 6.95mM respectively. Adenosine N-oxide was also a substrate. Methyl viologen and to a lesser extent benzyl viologen were the only effective electron donors to the purified enzyme from a range of physiological and non-physiological compounds tested. Activity was inhibited by thiol modifying agents suggesting the presence of a sulphydryl group(s) in the enzyme and by high concentrations of molybdate and tungstate but not by metal chelating

compounds or by known respiratory inhibitors.

The absorption spectrum of the purified enzyme indicated the possible presence of flavin, but activity was not stimulated by the presence of added flavin. No haem or non-haem iron was detected in the enzyme but molybdenum was present at 1.3g atoms mol^{-1}. This is in support of a previous genetic analysis of trimethylamine N-oxide reduction implicating the functioning of a molybdenum cofactor in catalysis (Nasser, 1983).

An immunological comparison of various trimethylamine N-oxide reductases was made using polyclonal antibodies raised against the purified enzyme from *Alteromonas* sp. NCMB 400. This enzyme was unrelated immunologically to the second trimethylamine N-oxide reductase in *Alteromonas* sp. NCMB 400, or to the enzymes present in *Escherichia coli* and *Salmonella typhimurium*.

The results are discussed in relation to other bacterial enzymes capable of trimethylamine N-oxide reduction and show some essential structural and functional differences.

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Chapter 1

Introduction

1.1 Bacterial Energy Generation

A feature common to the diverse mechanisms by which biologically useful energy is generated is their ability to conserve energy in the form of adenosine 5'-triphosphate (ATP). Analysis of the thermodynamics involved has shown that between -41.8 and -50.2 kJ are required for the synthesis of one mole of ATP from adenosine 5'-diphosphate (ADP) and inorganic phosphate (Pi) (Thauer *et al.*, 1977). The standard free energy ($\Delta G^{\circ\prime}$) of ATP hydrolysis is estimated at -31.8 kJ mol^{-1}, the energy being used to drive a variety of energy dependent cellular processes such as metabolite or ion transport, biosyntheses and active movement. In heterotrophic bacteria, energy is generated by the coupling of the biological oxidation of organic molecules to the phosphorylation of ADP. This conservation of energy is brought about by two distinct mechanisms; substrate level phosphorylation and oxidative phosphorylation (Haddock and Jones, 1977).

Substrate level phosphorylation is a relatively simple process involving oxidation or reduction reactions with large and negative free energy changes that can be coupled to the phosphorylation of ADP. Despite the wide range of organic substrates available to heterotrophic bacteria only a few reactions are capable of conserving energy by substrate level phosphorylation. In fermentative metabolism, the final acceptor of the reducing equivalents is an organic molecule generated in the terminal steps of the fermentative pathway (Cole, 1981). Fermentation leads to the accumulation of partially oxidized/reduced substrates making it an energetically inefficient process (Morris, 1975). Further energy may be obtained by a more complete oxidation of the carbon substrate in cells capable of "electron acceptor limited growth", in which electrons released from the oxidation are transferred to an exogenous inorganic or organic electron acceptor (Cole, 1981). An increase in the molar growth yield is often observed in the presence of a suitable electron acceptor (Stouthamer, 1969) due to an increase in ATP production from the fall in free energy as electrons are transferred to acceptors of higher

mid-point redox potential (E_o'). The terminal electron acceptor is seen as serving as a high redox potential "electron sink" for the reducing equivalents, functioning to drive substrate level phosphorylation that would not occur in its absence (Kröger, 1977). In *Streptococcus faecalis*, fumarate can accept the reducing equivalents of glucose in place of pyruvate and is associated with an additional site for substrate level phosphorylation (Deibel and Kvetkas, 1964).

The second method of ATP synthesis in heterotrophic bacteria is oxidative phosphorylation. The synthesis of ATP is coupled to electron transport reactions driven by the oxidation of organic compounds of negative redox potential (Haddock and Jones, 1977). This overall redox process is subdivided into a series of smaller redox steps forming a series of coupled reactions constituting a respiratory chain. A range of redox couples are presented in Table 1.1. Assuming that two electrons are transferred between each redox couple, a potential difference of approximately 0.25V is required for the synthesis of one molecule of ATP from ADP and P_i, corresponding to a free energy change of -48 kJ mol^{-1}.

The exact mechanism of oxidative phosphorylation is still under debate but the most widely accepted theory is the chemiosmotic hypothesis originated by Mitchell (1961). Haddock and Jones (1977) have described it in simple terms:

> "the chemiosmotic hypothesis requires that a proton translocating electron transport chain and a proton translocating ATPase coexist in a membrane that is essentially impermeable to most ions, including both OH^- and H^+ ions. The end result of either electron transport or ATP hydrolysis is the generation across the membrane of gradients of both pH (ΔpH) and electrical potential ($\Delta\psi$), with the soluble phase on one side of the membrane alkaline and electrically negative relative to the other. The sum of these two components in electrical units (usually millivolts) is known as the protonmotive force (ΔP) and, although these components are not identical, they are all related and interconvertable as described by the expression:

Table 1.1 Redox Potential of some Electron Donors and Acceptors in Oxidative Phosphorylation

	E'_0 (V)
CO_2/formate	-0.432
H^+/H_2	-0.414
$S_2O_3^{2-}/HS^- + HSO_3^-$	-0.402
NAD/NADH	-0.320
FAD/FADH	-0.220
$Pyruvate^-/lactate^-$	-0.190
Fumarate/succinate	+0.033
TMAO/TMA	+0.130
NO_2^-/NO	+0.350
NO_3^-/NO_2^-	+0.433
O_2/H_2O	+0.818
NO/N_2O	+1.175
N_2O/N_2	+1.355

(Castell, 1950; Thauer *et al.*, 1977)

$$\Delta P = \Delta\psi - Z\Delta pH$$

where $Z = 2.3RT/F$

1.2 Bacterial Respiratory Chains

An essential feature of the chemiosmotic hypothesis is the presence of an intact membrane and a topographical arrangement of components within the membrane (Thauer *et al.*, 1977). In contrast to the soluble enzymes catalysing fermentation reactions, the enzymes responsible for oxidative phosphorylation are generally arranged asymmetrically in the plasma membrane of the cell so as to catalyse vectorial chemical reactions, and in order of increasing redox potential to allow the transfer of electrons from donor to acceptor (Haddock and Jones, 1977).

Bacterial respiratory chains bear some resemblance to the respiratory chains of higher organisms in that they contain the same type of components as in the mitochondria. These components include iron-sulphur flavoproteins as dehydrogenases, quinones, cytochromes and terminal oxidases. Indeed, some bacteria such as *Paracoccus denitrificans* and *Alcaligenes* spp. possess components so like those of mitochondria that an evolutionary link between the two has been proposed (John and Whatley, 1975; Jones, 1977). This apparent unity of composition is however largely superficial, with the majority of heterotrophic bacteria possessing respiratory chains very different in composition, both between species, and between bacteria and the mitochondrion. Perhaps the major characteristic of bacterial respiratory systems as a whole is their immense variety of redox component combinations (Stouthamer, 1978). At least nine different cytochromes have been identified in *Escherichia coli*, though not all are involved in oxidative phosphorylation (Haddock and Jones, 1977). The ability of a single species to synthesize a variety of components allows some degree of adaptability to changing environmental conditions. A particular example of variation in redox carrier patterns is seen with a change in the availability of oxygen.

1.3 Aerobic Respiration

When oxygen is used as a terminal electron acceptor electron transport to oxygen can be coupled to oxidative phosphorylation in the process termed aerobic respiration. Changes in the availability of oxygen often induce different terminal oxidases, these being accommodated in the organism as branches of the respiratory chain (Figure 1.1). This is exemplified by *Azotobacter vinelandii*, an aerobic nitrogen-fixing bacterium which possesses two major branches of the respiratory chain to two different terminal oxidases, functioning under different relative concentrations of oxygen. Under high concentrations of oxygen, the oxygen labile nitrogenase is protected from damage by the induction of cytochrome *d* as terminal oxidase, resulting in the absence of a third site of energy conservation. The loss of a phosphorylation site, termed "energy slip" (Haddock and Jones, 1977), allows oxygen to be scavenged from the nitrogen-fixing site through a process of "respiratory protection" (Stouthamer, 1977). The second major branch of the respiratory chain is to a cytochrome *a,o* oxidase, present under low oxygen tension conditions (Figure 1.1). This branch resembles the electron transport chain of *P.denitrificans* and is associated with a third energy conservation site (Robson and Postgate, 1980).

1.4 Anaerobic Respiration

In the absence of oxygen, many facultatively and obligately anaerobic bacteria can utilize a wide variety of components both inorganic and organic as terminal electron accepters including nitrate, nitrite, sulphate, sulphite, fumarate (Konings and Michels, 1980) and trimethylamine N-oxide (TMAO) (Yamamoto and Ishimoto, 1977). This process is termed anaerobic respiration and is accompanied by changes in the respiratory chain, particularly with respect to the appropriate terminal reductases. These features are discussed in more detail in the subsequent sections.

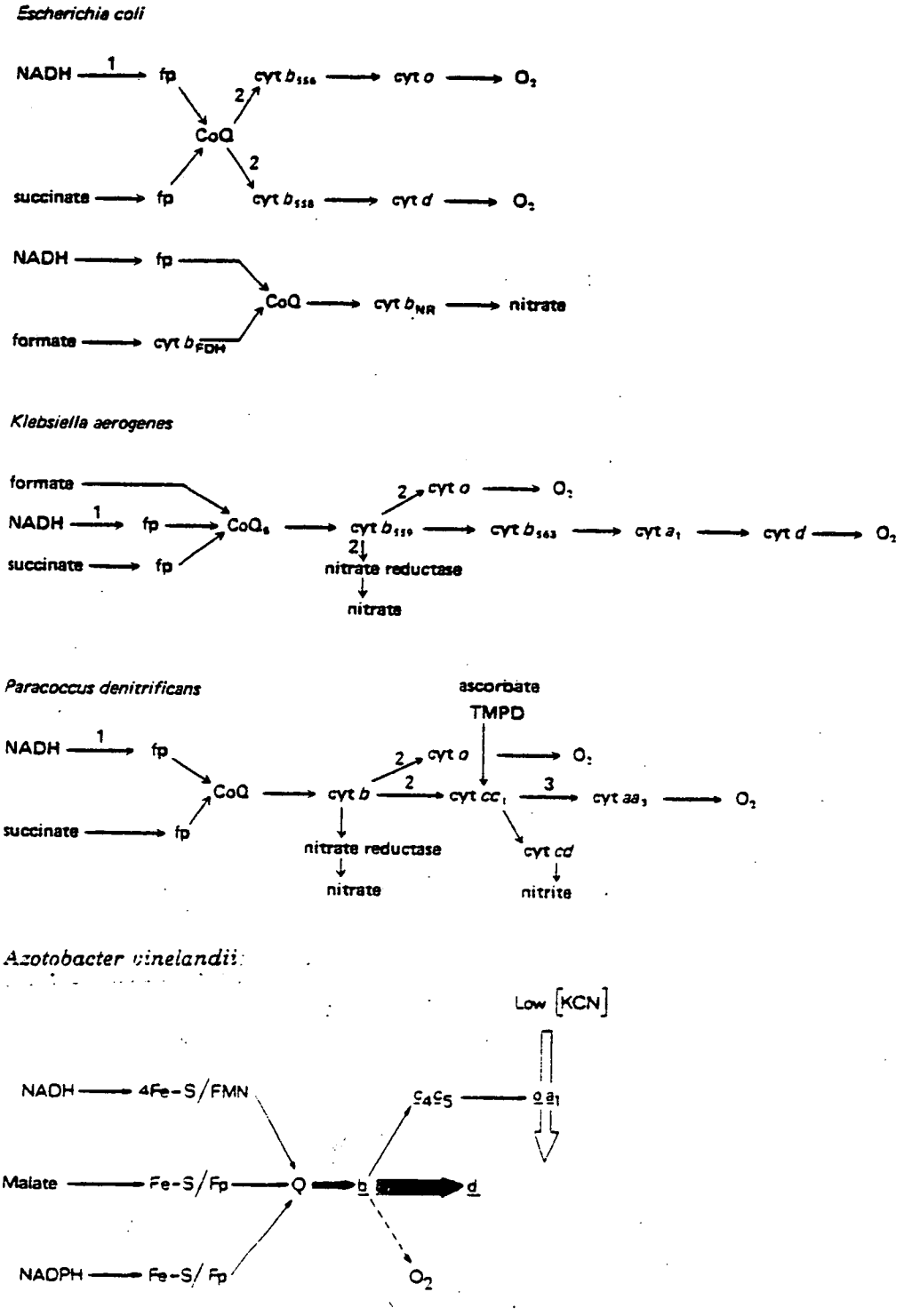


Figure 1.1 The Respiratory Chain of a Number of Selected Bacteria to Oxygen, Nitrate and Nitrite

FP, fp, flavoprotein; CoQ, Q, coenzyme Q (ubiquinone); FMN, flavin mononucleotide; Fe-S, iron-sulphur protein; b, c_4c_5, d, oa_1, respective cytochromes.
Phosphorylation sites in the respiratory chains of *Escherichia coli*, *Klebsiella aerogenes* and *Paracoccus denitrificans* are indicated by 1,2 and 3 respectively.
(from Haddock and Jones, 1977; Stouthamer *et al.*, 1980)

1.5 Nitrate Respiration

The metabolism of nitrate by microorganisms has been divided into assimilatory or dissimilatory processes (Stouthamer, 1976). The former results in the net uptake of nitrogen into cellular material, the latter, also known as nitrate respiration takes place in the absence of oxygen with nitrate acting as terminal electron acceptor. A third mode of nitrate utilization has been proposed as a self defence mechanism in *Rhodopseudomonas sphaeroides*, nitrate being reduced to nitrite which is toxic to other microorganisms in a competitive environment (Kerber and Cardenas, 1982).

The nitrate respiratory system represents the most studied and best characterized bacterial electron transport chain (Haddock and Jones, 1977). Respiratory nitrate reductase (EC 1.7.99.4) is generally associated with the cytoplasmic membrane of bacteria (Stouthamer *et al.*, 1980), an exception being *Alteromonas* sp.NCMB 400 in which it is periplasmic (Nasser, 1983). As a consequence of its membrane location, purification of nitrate reductase usually involves membrane solubilization as the primary step. Taniguchi and Itagaki (1960) released nitrate reductase from the membrane of *E.coli* by heat treatment followed by alkaline extraction and subsequent purification to yield an enzyme of 1 000 000 daltons molecular weight. Alternative purification protocols for the nitrate reductase of *E.coli* have given rise to different physical properties. For example, acetone extraction followed by deoxycholate treatment (Forget, 1974) gave a molecular weight of 320 000 daltons. This variation in molecular weight has led to the suggestion that nitrate reductase may exist in different forms depending upon the method of solubilization (Lund and De Moss, 1976). The enzyme has been purified from a number of bacteria including; *P.denitrificans* (Lam and Nicholas, 1969), *Pseudomonas aeruginosa* (Fewson and Nicholas, 1961) and *Klebsiella aerogenes* (van't Riet and Planta, 1975), and is composed of two subunits A and B. However, depending upon the isolation procedure, the enzyme may contain an additional subunit. In *K.aerogenes* there is a subunit of 52 000 daltons

(van't Riet and Planta, 1975) and when solubilized with Triton X 100, the nitrate reductase from *E.coli* contains a third subunit C (Enoch and Lester, 1974; MacGregor, 1975b). The *E.coli* nitrate reductase is therefore composed of three subunits, having a stoichiometry of 2A:2B:4C, and respective molecular weights of 142 000, 60 000 and 19 500 daltons, giving a molecular weight for the enzyme complex of approximately 500 000 daltons (Enoch and Lester, 1975; MacGregor and Christopher, 1978). Antibodies raised against one subunit do not cross react with the other subunits, while there is some cross reactivity between the same subunit in different genera, for example *E.coli* and *K.aerogenes (Enterobacteriaceae)* but not between *E.coli* and *Bacillus licheniformis* (van't Riet *et al.*, 1979).

Tentative functions have been assigned to the subunits of the nitrate reductase complex. Subunit A contains the active site of the enzyme (MacGregor, 1975a; De Moss, 1977; Graham and Boxer, 1980; Chaudhry and MacGregor, 1983a), subunit B has been implicated in binding of the enzyme complex to the membrane (De Moss, 1977), and subunit C is a b-type cytochrome (Cytochrome b^{NR}_{556}) (Enoch and Lester, 1974; MacGregor, 1975b) of two distinguishable kinetic types (Hackett and Bragg, 1982). The biosynthesis of the subunits and their assembly into the membrane has recently received attention. Cytoplasmically synthesized subunits are only inserted into the membrane in the presence of cytochrome *b* (MacGregor, 1976). On insertion of all three subunits to form a complete enzyme complex, subunits A and B are post-translationally modified to proteins of higher molecular weight (MacGregor and McElhaney, 1981; Chaudhry and MacGregor, 1983a,b) by covalent addition of fatty acyl residues (unpublished data, Chaudhry and MacGregor, 1983a).

The chemical labelling studies of Boxer and Clegg (1975) using ^{125}I/lactoperoxidase and MacGregor and Christopher (1978) using transglutaminase have shown labelling of subunit A and that it is exposed to the cytoplasm. This is supported by the immunofluorescence data of Graham and Boxer (1978). Boxer and Clegg (1975) have also shown the labelling of subunit C from the periplasmic aspect of the membrane, suggesting a

transmembraneous orientation for the enzyme complex. Subunit B was not labelled in any of these studies with *E.coli* nitrate reductase, suggesting a location within the membrane or masking of this subunit by subunit A (Stouthamer *et al.*, 1980). The nitrate reductase from *K.aerogenes* is also transmembraneous, but it is uncertain which of the subunits A and B span the membrane (Wientjes *et al.*, 1979; Stouthamer *et al.*, 1980).

A common feature of nitrate reductase from many bacteria is the presence of iron, acid-labile sulphide and molybdenum (Stouthamer, *et al.*, 1980). *E.coli* cytochrome-less nitrate reductase contains 12 Fe/S groups (Forget, 1974; Chaudhry and MacGregor, 1983b), whereas the enzyme from *B.licheniformis* (van's Riet *et al.*, 1979) and *K.aerogenes* (van't Riet and Planta, 1975) contain 8 Fe/S groups per molecule. Molybdenum is present at one atom per molecule in each case (Forget, 1974; Bosma *et al.*, 1978) as a molybdenum cofactor (Chaudhry and MacGregor, 1983b). In *E.coli*, the non-haem iron, acid-labile sulphide and molybdenum cofactor are associated with subunit A only (Chaudhry and MacGregor, 1983b). The evidence for participation of these metals in nitrate reductase catalysis has come from low temperature EPR studies (Forget and Der Vartanian, 1972; Bosma *et al.*, 1978; Vincent and Bray, 1978). These indicate the presence of two or three 4Fe-4S iron-sulphur clusters and a redox couple for the molybdenum of oxidation state IV/VI for a two electron reduction of nitrate to nitrite (Stiefel, 1973) or alternatively two sequential one electron transfers to nitrate involving Mo IV and Mo V (Vincent and Bray, 1978). Further studies are required to clarify the interaction between the iron-sulphur clusters and the molybdenum, and the *in vivo* mechanism of catalysis.

The respiratory chain to nitrate reductase in *E.coli* contains as the primary electron donor formate dehydrogenase, formate being the preferential donor for nitrate reduction (Ruiz-Herrera and De Moss, 1969). The formate dehydrogenase associated with nitrate respiration has been purified and is composed of three subunits, α, β and γ in molar ratio 1:1.2:0.55 and respective molecular weights 110 000, 32 000 and 20 000 daltons, associating into a tetramer of approximately 600 000 daltons (Enoch and Lester, 1975). The enzyme was shown to

contain (in molar amounts) 1.0 haem, 0.95 molybdenum, 0.96 selenium, 14 non-haem iron and 13 acid-labile sulphide (Enoch and Lester, 1974 and 1975). Subunit γ is a b-type cytochrome, genetically and kinetically distinct from that present in nitrate reductase (Haddock and Jones, 1977). In addition to formate, NADH (Ishimoto and Yamamoto, 1977), L,α-glycerophosphate (Haddock and Jones, 1977), D-lactate (Giordano *et al.*, 1980) and L-lactate (Nishimura *et al.*, 1983) can serve as hydrogen donors to nitrate via their respective dehydrogenases. A quinone component is also present in the nitrate respiratory chain; both menaquinone and ubiquinone are synthesized during anaerobic growth on nitrate, with ubiquinone being more efficient in electron transport to the cytochrome b_{556}^{NR} (Enoch and Lester, 1974).

Oxidative phosphorylation has been shown to be coupled to nitrate reduction in *E.coli* (Ota *et al.*, 1964) and *Pseudomonas denitrificans* (Ohnishi, 1963) with evidence for the formation of one mole ATP coupled to electron transfer from formate to nitrate in *E.coli* (Yamamoto and Ishimoto, 1977). Two types of respiratory nitrate reduction can be distinguished (van't Riet *et al.*, 1979). That present in *E.coli* and *K.aerogenes* is coupled to oxidative phosphorylation, a phosphorylation site being present between cytochrome b_{556}^{NR} and nitrate (Stouthamer, 1976), with nitrate reduced to nitrite which accumulates. In the second type, present in *P.dentrificans* and *B.licheniformis* (van't Riet *et al.*, 1979), there is no phosphorylation site between cytochrome b_{556}^{NR} and nitrate; instead the reduction products of nitrate respiration, nitrite and nitric oxide, are coupled to oxidative phosphorylation at a site present between cytochrome *b* and these acceptors.

Nitrate dependent proton translocation associated with the oxidation of various substrates has been demonstrated in *E.coli* resulting in $\rightarrow H^+/NO_3^-$ ratios of 4 for the oxidation of L-malate and 2 for succinate, D-lactate and glycerol, formate giving a ratio of greater than 2 (Garland *et al.*, 1975). A proposed functional organization of components in nitrate respiration is given in Figure 1.2.

The studies of Hackett and MacGregor (1981) have revealed some of the features of synthesis, insertion and degradation of

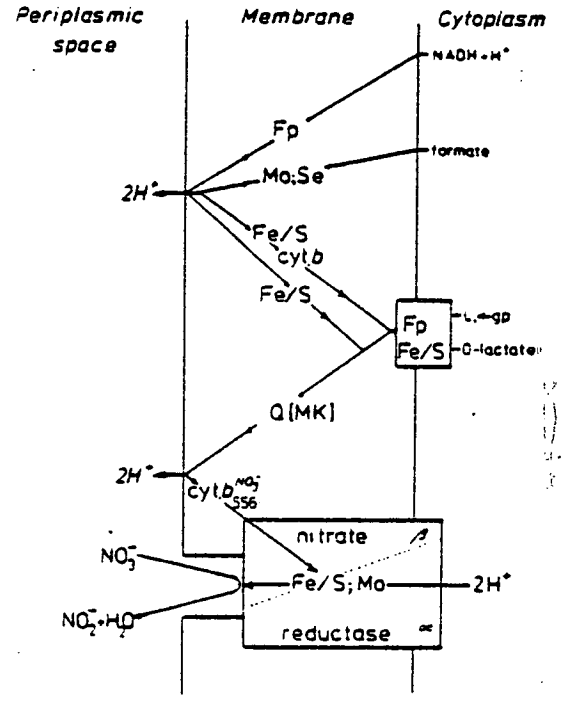


Figure 1.2 Proposed Functional Organization of the *Escherichia coli* Nitrate Respiratory Chain

Fp. flavoprotein; Mo, molybdenum containing polypeptide; Se, selenium containing polypeptide; α,β, subunits of nitrate reductase; Fe/S, iron-sulphur protein; cyt b, cytochrome *b*; L α-gp, Lα-glycerophosphate; Q[MK], menaquinone

(from Haddock and Jones, 1977)

nitrate reductase in *E.coli*. Insertion of subunits A and B into the membrane is dependent upon the induction of subunit C and in the absence of inducing conditions no further insertion is observed. Once inserted into the membrane, nitrate reductase is apparently stable, even under non-inducing conditions when activity is rapidly lost; regulation of nitrate reductase is not achieved by degradation.

It is known that in facultative anaerobic bacteria the synthesis of enzymes of anaerobic transport is repressed by aerobiosis in a phenomenon termed the "oxygen effect" (Haddock and Jones, 1977). Kaprálek *et al.* (1982) concluded that oxygen inhibits the synthesis of nitrate reductase at the level of transcription with some evidence for translational or later control of enzyme formation. However, regulation of nitrate respiration may be more complicated than induction under anaerobic conditions in the presence of nitrate. Nitrate reductase and the components of nitrate respiration can be synthesized under aerobic conditions as shown by mutants in the aerobic respiratory chain of *E.coli* (Giordano *et al.*, 1977; Azoulay *et al.*, 1978) indicating that oxygen itself does not repress nitrate reductase. Similarly, nitrate reductase can be induced in the absence of nitrate under anaerobic conditions in the presence of nitrite or azide (De Groot and Stouthamer, 1970a; Stouthamer *et al.*, 1980). From such evidence, a regulatory model incorporating electron flow through the nitrate respiratory chain as the regulatory factor in the formation of nitrate reductase has been proposed (De Groot and Stouthamer, 1970a,b; Oltmann *et al.*, 1976). In this model, nitrate reductase and cytochrome *b* or their precursors function as repressors of their own biosynthesis. Interaction with nitrite or azide (substrates) may induce conformational changes in the enzyme preventing repressor function. It is suggested that the effect of oxygen may be to repress the formation of other components of the respiratory chain. Although this mechanism provides a working hypothesis for regulation, there are some observations that remain unexplained and some of the detailed molecular aspects are difficult to envisage.

1.6 Fumarate Respiration

The reduction of fumarate to succinate in catabolic redox processes is a feature common to many bacteria (Kröger, 1977). In addition, this reduction serves an anabolic function in strict anaerobes for the provision of succinate, an important metabolic intermediate (Thauer *et al.*, 1977). The relatively high standard redox potential of the fumarate/succinate couple (E'_o = +33mV) in comparison to other redox couples of metabolism allows the oxidation by fumarate of various metabolic electron donors. The $\Delta G'_o$ released by these reactions, for example, formate to fumarate ($\Delta G'_o$ = -86 kJ mol^{-1}) is sufficient for the phosphorylation of ADP to ATP, requiring -42 to -50 kJ mol^{-1} (Kröger, 1977; Thauer *et al.*, 1977; Jones, 1976; Kröger and Winkler, 1981).

The respiratory chain to fumarate is considered one of the simplest electron transport chains and has therefore been the subject of much study as a model for oxidative phosphorylation (Lemire *et al.*, 1983; Simpkin and Ingledew, 1984). The terminal enzyme of the respiratory chain to fumarate in *E.coli*, fumarate reductase (EC 1.3.99.1) consists of four subunits of 69 000, 27 000, 15 000 and 13 000 molecular weight (Lemire *et al.*, 1982) and has been purified as a catalytically active dimer of the two large subunits (Dickie and Weiner, 1979) or as the tetrameric holoenzyme (Lemire *et al.*, 1982). The genes encoding the subunits (*frdA*, *frdB*, *frdC*, *frdD*) form an operon and are transcribed and translated coordinately (Lohmeier *et al.*, 1981; Cole and Guest, 1979a).

Analysis has shown the FrdA subunit to be a flavoprotein with the flavin adenine dinucleotide (FAD) carried by an 8 α [N-3] histidyl covalent linkage (Weiner and Dickie, 1979) and to contain a single sulphydryl group essential for activity (Robinson and Weiner, 1982). The FrdB subunit contains the 4-5 mol/mol enzyme non-haem iron and acid-labile sulphide present in the enzyme (Robinson and Weiner, 1982) with amino acid sequence data suggesting the presence of two [2Fe-2S] ferredoxin-type centres (Cole *et al.*, 1982). EPR evidence has confirmed the identity of two ferredoxin-type iron-sulphur centres, with fixed orientation on the cytoplasmic face of the plasma

membrane (Simpkin and Ingledew, 1984). The same study also revealed a HiPIP-type iron-sulphur centre within the enzyme. It was concluded that the measured iron-sulphur content of previous studies may have been underestimates due to the lability of HiPIP-type centres when isolated from membranes and that the FrdA subunit may also contain an iron-sulphur centre.

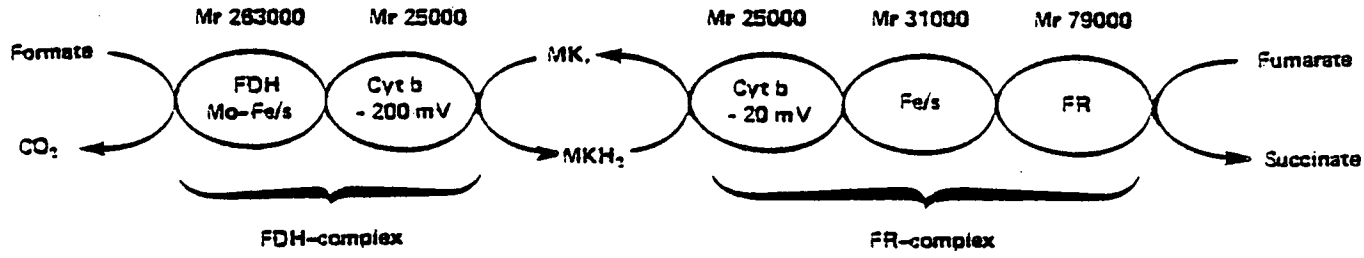
In cells of *E.coli* carrying a multicopy recombinant plasmid encoding the fumarate reductase operon, fumarate reductase has been visualized on the cytoplasmic membrane by electron microscopy. The enzyme was shown to contain a membrane extrinsic domain composed of the catalytic FrdA and FrdB subunits and a proposed membrane intrinsic portion composed of the FrdC and FrdD proteins functioning to anchor the "catalytic head" to the membrane (Lemire *et al.*, 1983). Sequence analysis of subunits FrdC and FrdD has identified regions of very hydrophobic aminoacyl residues that are sufficient to span the membrane (Gundstrom and Jaurin, 1982; Lemire *et al.*, 1983). These two subunits also serve to maintain the structure of the two large subunits (Lemire *et al.*, 1983), the tetrameric enzyme being anion independent and alkaline stable in comparison to the more labile two-subunit enzyme form (Robinson and Weiner, 1981). It has been proposed that by analogy with the membrane intrinsic component of H^+ATPase, F_o, subunits FrdC and FrdD may function in proton translocation or in other aspects of electron transport (Cole, 1984) but as yet no evidence for this idea has been presented. Levels of fumarate reductase in these cells may exceed 30 fold that present in wild-type cells, with more than 50% of total membrane protein accounted for by the enzyme. The levels of other membrane bound proteins remain constant however and the increase in fumarate reductase is accommodated by an increase in membrane lipid content. Production of excess fumarate reductase results in the enzyme being released into the cytoplasm of the cell as a lipid-enzyme aggregate visible by electron microscopy as tubules branching from the inner membrane (Weiner *et al.*, 1984).

In contrast to *E.coli* fumarate reductase, the enzyme complex of *V.succinogenes* is composed of three subunits of

79 000, 31 000 and 25 000 molecular weight in respective molar ratio 1:1:2 (Unden *et al.*, 1980; Unden and Kröger, 1980). The 76 000 dalton protein contains one molecule of FAD bound by an 8 α [N-3] histidyl covalent linkage (Kenney and Kröger, 1977), has a [4F-4S] iron-sulphur centre, and an essential sulphydryl group (Unden and Kröger, 1980). This subunit contains the catalytic site for fumarate reduction as shown with artificial but not *in vivo* electron donors, suggesting the requirement of other components for *in vivo* catalysis (Unden and Kröger, 1981; Albracht *et al.*, 1981). The 25 000 dalton subunit is a *b* type cytochrome with a mid-point potential of -15mV (Unden and Kröger, 1981) functioning to oxidize the quinone component of the electron transport chain (Kröger and Innerhofer, 1976; Kröger *et al.*, 1980). The 31 000 dalton subunit contains [2Fe-2S] iron-sulphur centre and functions as a redox mediator between the b-type cytochrome and the flavoprotein (Unden and Kröger, 1981; Albracht *et al.*, 1981).

Studies on the relatedness of bacterial fumarate reductases have been carried out using DNA/DNA hybridization and immunological cross-reactivity techniques (Unden and Cole, 1983). Members of the *Enterobacteriaceae* possessing fumarate reductase had a high degree of *E.coli* fumarate reductase DNA hybridization and immunological cross-reactivity, whereas that between *E.coli* and the distantly related *V.succinogenes* and *Pseudomonas aeruginosa* was absent. It is suggested that this is evidence for a strong selective pressure to conserve the *frd* genes within a family with possible convergent evolution between families (Unden and Cole, 1983).

Depending upon the organism and growth condition, non-fermentative electron donors to fumarate reductase include formate, NADH, lactate, glycerol and malate (Miki and Lin, 1973, 1975; Lambden and Guest, 1976; Kröger, 1977; Thauer *et al.*, 1977; Nishimura *et al.*, 1983). Associated with each donor is a specific membrane bound dehydrogenase. The best characterized electron transport chain to fumarate is with formate as hydrogen donor in *V.succinogenes* (Figure 1.3). The first component of this respiratory chain is a formate dehydrogenase, isolated as a dimer of identical subunits of 110 000 molecular weight, each containing one atom of molybdenum and about 20 iron-sulphur groups (Kröger *et al.*, 1979;



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

Figure 1-3 Electron Transport System for Fumarate.
(See Kröger et al., 1979; Unden & Kröger, 1982)

Kröger, 1980). In contrast to the formate dehydrogenase of *E.coli* neither flavin or selenium are detectable in the enzyme of *V.succinogenes*. The molybdenum of this enzyme is probably the primary acceptor of reducing equivalents from formate with the iron-sulphur groups serving as mediators between the molybdenum and the low potential cytochrome *b* (-200mV) associated with the enzyme (Kröger and Innerhofer, 1976; Kröger, 1980). Experiments with artificial non-permeant electron donors to formate dehydrogenase using membrane particles in both the right-side-out and inside-out orientation have located the substrate site for the enzyme on the outer aspect of the cytoplasmic membrane and on the opposite side to fumarate reductase (Kröger *et al.*, 1980; Kröger, 1980) (Figure 1.4a). In contrast to *V.succinogenes*, similar experiments revealed the substrate site of *E.coli* formate dehydrogenase to be on the cytoplasmic face of the membrane and on the same side as fumarate reductase (Jones and Garland, 1977; Haddock and Jones, 1977) (Figure 1.4b). However, a second formate dehydrogenase can be induced under certain conditions. This enzyme, termed a formate hydrogen lyase, present on the outer aspect of the membrane may function in a similar manner to the formate dehydrogenase of *V.succinogenes* (Jones and Garland, 1977; Bernard and Gottschalk, 1978; Kröger, 1980). The requirement of quinone for functional fumarate respiration has been revealed by extraction procedures leading to a loss of fumarate reduction, the activity being restored upon re-insertion of quinone. The nature of this quinone was shown to be a naphthoquinone, typically menaquinone, having a redox potential low enough to couple the oxidation of formate dehydrogenase associated cytochrome *b* (-200mV) with the reduction of the fumarate reductase associated cytochrome *b* (-15mV) (Kröger and Innerhofer, 1976; Kröger, 1978; Kröger, 1980).

Electron transport to fumarate has been shown to be coupled to proton translocation (Kröger, 1977; 1978; Miki and Wilson, 1978) and ATP synthesis (Macey *et al.*, 1976), with stoichiometries for the $H^+/2e^-$ ratio of 2 (Miki and Wilson, 1978) and for the P/e^- or P/fumarate ratios of 1 (Kröger, 1978; 1980; Kröger and Winkler, 1981). An alternative reduction

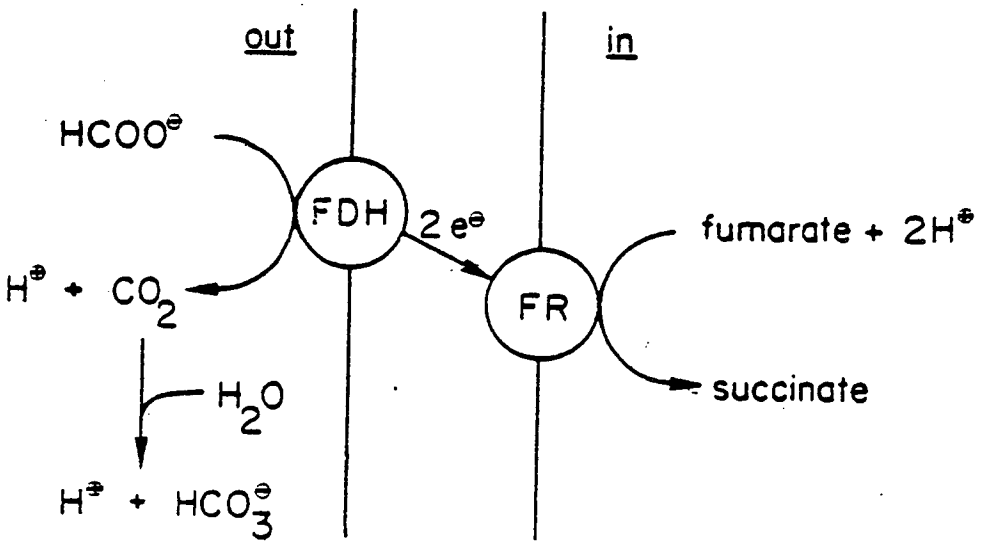


Figure 1.4a Proposed Mechanism of Generating an Electrochemical Proton Gradient in *Vibrio succinogenes*

FDH, formate dehydrogenase; FR, fumarate reductase (from Kröger, 1980)

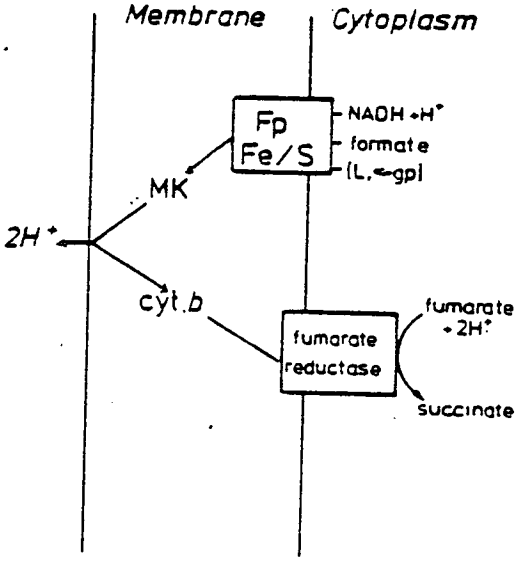


Figure 1.4b Proposed Functional Organization of the Components in Fumarate Respiration of *Escherichia coli*

FP, flavoprotein; Fe/S, iron-sulphur centre; [Lα-gp], Lα-glycerophosphate; MK, menoquinone; cyt b, cytochrome *b*

(from Haddock and Jones, 1977)

of fumarate involving electron transport not coupled to proton translocation or ATP synthesis has been demonstrated in haem deficient mutants of *E.coli* (Singh and Bragg, 1976). In this organism, ATP is generated by substrate level phosphorylation with fumarate acting as an electron sink for the reoxidation of NADH (Haddock and Jones, 1977) (Figure 1.5).

As is typical of many terminal enzymes of anaerobic electron transport, fumarate reduction is repressed by oxygen and other alternative electron acceptors and derepressed under anaerobic conditions (Spencer and Guest, 1973). In strains of *E.coli* carrying a λ*frdA* transducing phage capable of producing amplified levels of fumarate reductase anaerobically, the enzyme was found to be synthesized aerobically in levels greater than that of the wild-type anaerobically. From this it has been suggested that the repression of fumarate reductase under aerobic conditions may be overcome by amplified levels of FrdA (Cole and Guest, 1978) possibly diluting out a specific repressor protein (Cole and Guest, 1979b; 1980).

The isolation of *E.coli* mutants at the *fnr* locus, phenotypically defective in nitrate and fumarate respiration (Lambden and Guest, 1976) has led to the suggestion that this locus may have a regulatory function in fumarate reduction. This has been extended by the finding that the *fnr* gene product is typical of many DNA binding proteins and that it may regulate fumarate reductase at the level of transcription (Shaw and Guest, 1982a,b; Cole, 1984).

1.7 Trimethylamine N-Oxide Respiration

Physiological reduction of TMAO to trimethylamine (TMA) by various hydrogen donors (X) occurs according to the general equation:

$$(CH_3)_3NO + XH_2 \rightarrow (CH_3)_3N + X + H_2O$$

and by analysis of the appropriate E_o' values, the coupling of this reduction to oxidative phosphorylation appears to be thermodynamically feasible. The E_o' for the TMAO/TMA couple

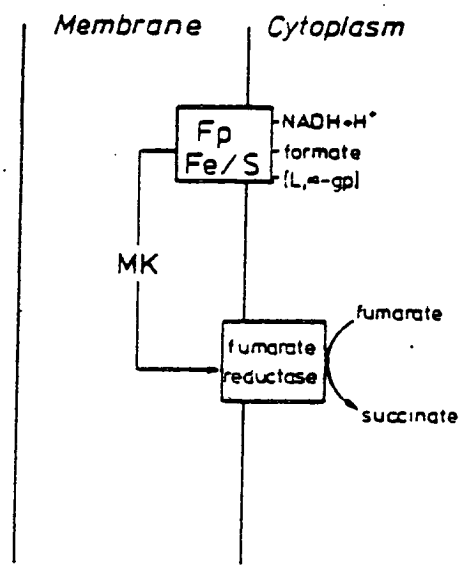


Figure 1.5 Proposed Functional Organization of Components in Fumarate Respiration of Haem Deficient Mutants of *Escherichia coli*

Fp, flavoprotein; Lα-gp, Lα-glycerophosphate; Fe/S, iron-sulphur centre; MK, menaquinone

(from Haddock and Jones, 1977)

is approximately +0.13V (Castell, 1950) whereas that for CO_2/formate is -0.43V (Thauer *et al.*, 1977), NAD/NADH is -0.32V and for pyruvate/lactate is -0.19V (Lehninger, 1970). The $\Delta G^{o\prime}$ available from the reduction of TMAO by formate, NADH and lactate is therefore approximately -108, -87 and -62kJ mol^{-1} respectively, sufficient for oxidative phosphorylation (section 1.1).

Studies on the mechanism of TMAO reduction began with Neilands (1945) and the predicted existence of a redox carrier or series of carriers between oxidizable substrates and TMAO in bacterial cells isolated from fish muscle. Anaerobic respiration with TMAO as terminal electron acceptor has been demonstrated in a number of bacteria including members of the *Enterobacteriaceae* (Yamamoto and Ishimoto, 1977; Strøm *et al.*, 1979; Kim and Chang, 1974) and *Alteromonas* spp. (Easter *et al.*, 1983; Ringø *et al.*, 1984). *Rhodopseudomonas capsulata* and *R.rubrum* have been shown to possess an anaerobic respiratory system for TMAO, with growth on non-fermentable carbon sources in the presence of TMAO (or dimethylsulphoxide, DMSO) (Shultz and Weaver, 1982) and the generation of a membrane potential coupled to electron transport (McEwan *et al.*, 1983). Reduction of TMAO in the *Rhodospirillaceae* has also been attributed to a soluble NADH-dependent system (Cox *et al.*, 1980) with TMAO functioning as an electron sink for the removal of reducing equivalents generated by fermentation, rather than as a terminal electron acceptor for electron transport driven phosphorylation. It is in the genera of the *Enterobacteriacea* however that TMAO respiration has been most studied and best characterized.

1.7.1 TMAO Respiration in the *Enterobacteriaceae*

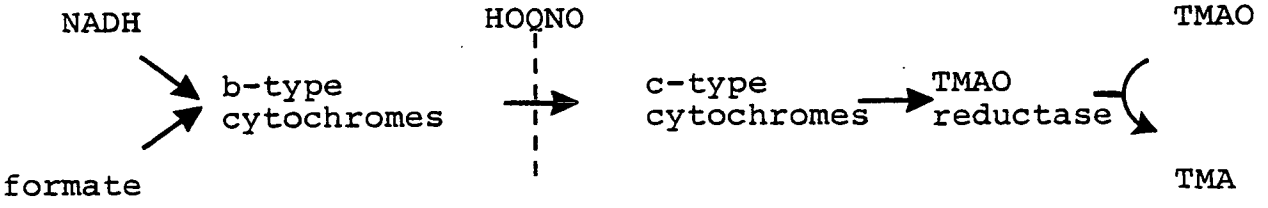
An increase in the anaerobic growth of cultures of *E.coli* has been observed when TMAO was included in the medium. It is thought that TMAO functions as part of an electron transport chain coupled to oxidative phosphorylation rather than as a metabolite for cell carbon or nitrogen (Sakaguchi and Kawai, 1975a). In *S.typhimurium*, TMAO has been shown to enhance the growth and yield of cells under anaerobic conditions and electron transport to TMAO can be coupled to oxidative

phosphorylation rather than as a metabolite for cell carbon or nitrogen (Sakaguchi and Kawai, 1975a),

resulting in increased levels of ATP production (Kim and Chang, 1974). In cells (Takagi *et al.*, 1981) and membrane vesicles (Cox *et al.*, 1980) of *E.coli*, the electron flow from formate or NADH to TMAO has been shown to produce a membrane potential as measured by the quenching of fluorescence of indicator dyes and sensitivity to the respiratory chain inhibitor 2-heptyl-4-hydroxyquinoline N-oxide (HOQNO). A transmembrane proton gradient has been demonstrated in *Proteus* sp. NTHC 153 as shown by the accumulation of serine against a concentration gradient, the accumulation being sensitive to agents that collapse the membrane potential (Stenberg *et al.*, 1982).

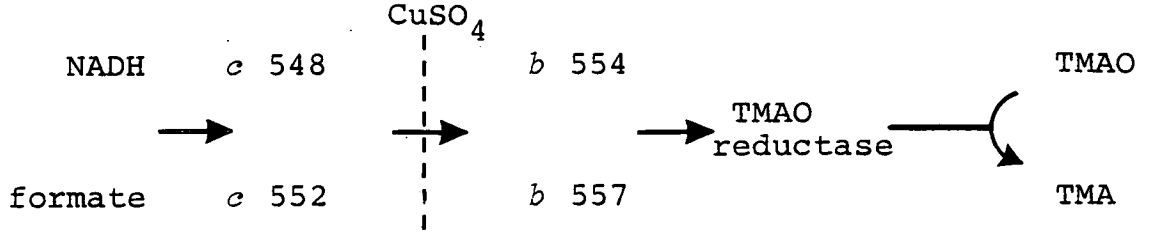
The components involved in the electron transport from a substrate to TMAO reductase have been partially characterized in *E.coli* and bear some similarity to the nitrate and fumarate reductase systems. Formate, NADH, NADPH (Sakaguchi and Kawai, 1977a; Ishimoto and Shimokawa, 1978) and L-lactate (Nishimura *et al.*, 1983), together with their respective dehydrogenases have been shown to be capable of electron donation to the TMAO reductase respiratory chain, NADH being the most effective donor (Sakaguchi and Kawai, 1977a). The existence of cytochromes as intermediary carriers linked to TMAO reduction by a TMAO reductase has also been demonstrated in *E.coli* (Sakaguchi and Kawai, 1978a,b; Sakaguchi *et al.*, 1979) and mutants defective in cytochrome biosynthesis are incapable of respiration with TMAO (Cox and Knight, 1981). Low temperature absorption spectrophotometry of respiratory components has identified a b-type cytochrome, distinct from the cytochrome *b*, functioning in the nitrate reductase system of *E.coli* (Sakaguchi and Kawai, 1978a). Further evidence has been presented by Sakaguchi and Kawai (1978b) showing HOQNO inhibition of electron transport from NADH to TMAO with accumulation of cytochrome in the reduced form and pyridine haemochromogen analysis of membranes identifying protohaem (cytochrome *b*) and haem *c*. Multiple cytochrome components were characterized with

α band absorption peaks at 549 and 551 nm (c-types) and 557.5 and 558 nm (b-types). A scheme for the sequence of components was proposed on the basis of the above evidence (Sakaguchi and Kawai, 1978b).



The c-type cytochromes and the TMAO reductase were induced by the presence of TMAO under anaerobic conditions, whereas the b-type components were synthesized constitutively (Sakaguchi *et al.*, 1979).

Recent work by Bragg and Hackett (1983) has extended the information known about the components of the respiratory chain in *E.coli* by quantifying the numbers of cytochromes present and re-assessing their sequence. By restricting the analysis to those cytochromes that were rapidly reoxidized by TMAO, two c-type cytochromes and two b-type cytochromes with α band absorption maxima at 548, 552, 554 and possibly 557 nm respectively were identified. Using $CuSO_4$ as an inhibitor of electron flow to TMAO, it was observed that the two c-type cytochromes remained in a reduced condition whereas the two b-type cytochromes were oxidized. This suggested an alternative scheme for the respiratory chain to TMAO, as follows:



This differs from that presented by Sakaguchi and Kawai (1978b) as the c-type cytochromes are placed earlier in the sequence than the b-types. Determination of the E_o' of these components to corroborate the proposed sequence was attempted but proved inconclusive. Oxidation of the cytochromes was achieved with

the membrane impermeant oxidant ammonium persulphate suggesting a location for the cytochromes at the periplasmic surface of the membrane (Bragg and Hackett, 1983).

Mutants of *E.coli* deficient in quinone biosynthesis were unable to grow at the normal levels associated with the wild-type under conditions suitable for the induction of the TMAO reductase system (Cox and Knight, 1981), suggesting the functioning of quinones in the respiratory chain. A possible location for quinone as the immediate donor for the c-type cytochromes has been proposed (Bragg and Hackett, 1983). Similar studies with mutants of *S.typhimurium* lacking a functional TMAO respiratory chain, identified a deficiency in menaquinone biosynthesis as the phenotype and a role for menaquinone in TMAO reduction (Kwan and Barrett, 1983a).

A characteristic of bacterial TMAO reduction appears to be the possession within the cell of multiple terminal enzymes capable of the reduction. Four enzymes have been identified in *E.coli* (Shimokawa and Ishimoto, 1979) and three in *S.typhimurium* (Kwan and Barrett, 1983a,b). In common with their function in anaerobic respiration, these enzymes are in some way associated with the plasma membrane, either as integral membrane proteins such as in *S.typhimurium* (Kwan and Barrett, 1983b), the enteric bacterium *V.parahaemolyticus* (Unemoto *et al.*, 1965) and the 200 000 and 70 000 dalton enzymes of *E.coli*, or loosely bound and easily released into the periplasmic cell fraction as with the 100 000 and second 70 000 dalton enzymes of *E.coli* (Shimokawa and Ishimoto, 1979). Of the four enzymes in *E.coli*, the 100 000 dalton enzyme is synthesized constitutively whereas the remaining enzymes are inducible. The 200 000 dalton enzyme has been purified by conventional techniques including: membrane solubilization, protein precipitation and gel filtration, ion exchange and hydroxyapatite chromatography, and is composed of two 80 000 and 60 000 dalton subunits. Of the three enzymes in *S.typhimurium*, two are inducible and one constitutive (Kwan and Barrett, 1983a). The inducible enzyme having the major activity associated with it has been purified by a similar protocol to that for *E.coli*, also employing chromatofocusing and has a native molecular weight of 332 000 daltons, being a tetramer of an 84 000

dalton subunit.

The physical and biological properties of the partially or wholly purified enzymes show a fairly wide range of features. The purified enzyme from *E.coli* has a broad range of substrate specificity and has been described as a tertiary amine N-oxide (acceptor) oxido-reductase (EC 1.7.99) or amine N-oxide reductase (Sagai and Ishimoto, 1973). Substrates utilized in addition to TMAO include the N-oxides of nicotinic acid, nicotinamide, α and γ picoline, together with hydroxylamine, chlorate, bromate and with the constitutive enzyme, nitrate (Sagai and Ishimoto, 1973; Shimokawa and Ishimoto, 1979). The partially purified enzyme from *V.parahaemolyticus* has only been studied in terms of its use of TMAO as substrate, but that from *S.typhimurium* has an activity with chlorate and slight activity with nitrate but not with nitrite, thiosulphate or thiosulphite. The possession of alternative N-oxide substrate use was not investigated (Kwan and Barrett, 1983b). The affinities of the enzymes for TMAO as measured by their half maximal substrate concentration ($K_{m\ TMAO}$) values vary in *E.coli* from 0.2-1.5mM (Sagai and Ishimoto, 1973; Shimokawa and Ishimoto, 1979) with 0.9mM for *S.typhimurium* (Kwan and Barrett, 1983b) and 1.7mM for *V.parahaemolyticus* (Unemoto *et al.*, 1965).

Compounds capable of acting as immediate electron donors to the purified TMAO reductase in *E.coli* include: FAD, flavin mononucleotide (FMN) (Shimokawa and Ishimoto, 1979) and with the constitutive enzyme, cytochrome *c* (Sagai and Ishimoto, 1973). In *S.typhimurium* only FMN at high concentration was an effective donor (Kwan and Barrett, 1983b). Methyl and benzyl viologen were effective artificial donors to each of the isolated enzymes.

The purified inducible enzyme from *E.coli* was shown to contain iron at 1.8 mol/mol and activity was stimulated by added Fe^{3+}. Surprisingly the enzyme was not inhibited by iron chelators such as α,α'-dipyridine (Shimokawa and Ishimoto, 1979). The enzyme from *V.parahaemolyticus* was sensitive to the agent p-chloromercuribenzoate (PCMB) suggesting the presence of an essential thiol group within the enzyme. The TMAO reductase of *E.coli* was shown to be sensitive to inhibition by added tungstate, the inhibition being relieved by addition of

molybdate and suggesting that the enzyme is a molybdoprotein. A certain class of mutants resistant to chlorate have been isolated from *E.coli* and *S.typhimurium* that are pleiotropic in their lack of both TMAO and nitrate reductases (Takagi *et al.*, 1981; Davidson *et al.*, 1979). The mutants have been characterized as being defective in molybdenum cofactor, a component of the enzyme essential for catalysis. Although both TMAO and nitrate reductases are affected by the loss of molybdenum, the two enzymes in *E.coli* have been shown to possess no common polypeptides (Takagi *et al.*, 1981; Cox and Knight, 1981).

Cultures of *E.coli* grown under aerobic conditions possess negligible TMAO reductase activity, but with the commencement of anaerobiosis, enzyme induction occurs, the level of the enzyme in the cell being further enhanced by the presence of TMAO (Sakaguchi and Kawai, 1975a). With highly grown cultures of *E.coli* in the absence of TMAO, addition of the substrate causes an almost immediate synthesis of the enzyme with the product of the reduction TMA, appearing after approximately 20 min (Sakaguchi and Kawai, 1977b). The increase in the level of TMAO reductase has been attributed to induction as opposed to post-translational modification, as evidenced by the absence of activity in cells prevented from *de novo* protein synthesis by the presence of the inhibitors actinomycin D or chloramphenicol (Sakaguchi and Kawai, 1975a). Aeration of cultures of *E.coli* already induced for TMAO reductase, inhibited the further formation and activity of the enzyme without any effect on the stability (Sakaguchi and Kawai, 1976). Addition of nitrate to a culture induced for TMAO reductase caused inhibition of the formation of the enzyme in a non-competative manner, without inhibiting the activity (Sakaguchi and Kawai, 1975c). These results have been explained as a preferential use of nitrate in anaerobic respiration over TMAO (Sagai and Ishimoto, 1973; Sakaguchi and Kawai, 1975c).

1.7.2 The Ecological Significance of TMAO reduction

The apparent preferential use of nitrate and the broad specificity of the enzymes catalysing the reduction of TMAO in the *Enterobacteriaceae* suggests a minor role for TMAO

respiration under anaerobic conditions. This is supported by the relatively rare occurrence of TMAO in the natural habitat of enterobacteria. Although fairly widely distributed in nature (Sigurdson and Wood, 1942) little information is available on the distribution of TMAO within the digestive tract. The presence of TMA monooxygenases in mammalian liver samples (Baker *et al.*, 1963) has implicated TMAO as a detoxification product of TMA produced from choline by the bacterial flora of the gut. TMAO has been detected in the urine of rats fed on TMA, following absorption of TMA and its conversion to TMAO by a monooxygenase (Benoit and Norris, 1945).

It is the fairly wide distribution of TMAO in aquatic ecosystems that has been the subject of most attention. It is a characteristic component of many marine organisms, particularly elasmobranch species (cartilagenous fish) and comprises 1-7% of the dry weight of marine fish (Groninger, 1959; Strøm, 1979). The only known mechanism for the formation of TMAO in the natural environment is by oxidation of TMA by monooxygenase enzymes (Baker and Chaykin, 1962; Zeigler and Mitchell, 1972). Although some fish species have been shown to possess TMA monooxygenase activity, the major source of TMAO within fish is believed to be dietary, particularly from zooplankton (Benoit and Norris, 1945; Baker *et al.*, 1963).

Little is known about the physiological function of TMAO within marine organisms (Strøm, 1979). An osmoregulatory role particularly within elasmobranches has been suggested, as plasma levels of the compound are subject to a fine degree of control (Shewan, 1951; Groninger, 1959). The presence of TMAO within fish tissue and its ability to act as a terminal electron acceptor in bacterial respiration has provoked most interest, particularly in its association with fish spoilage bacteria. One of the characteristic odours associated with fish spoilage is that of TMA which is normally produced in increasing amounts during putrefaction and TMA measurement has been used as a means of following microbial deterioration of iced marine fish (Shewan, 1962).

The spoilage flora of cold water marine fish is composed of Gram negative bacteria (Tarr, 1954; Shewan, 1962) including *Pseudomonas* spp., *Moraxella* spp. and in particular *Alteromonas*

spp. (Gillespie, 1983). The genus *Alteromonas* comprises heterotrophic non-fermentative bacteria, once classified as pseudomonads (Baumann *et al.*, 1972; Lee *et al.*, 1977) and is subdivided into three phenons C, D and E (Lee *et al.*, 1977). Due to the involvement of both *Alteromonas* sp. and TMAO in fish spoilage and the likely association between the two under anaerobic conditions, TMAO respiration in *Alteromonas* sp. has received attention.

1.7.3 TMAO Respiration in *Alteromonas* spp.

Alteromonas sp. NCMB 400 (National Collection of Marine Bacteria) and *Alteromonas* sp. NCMB 1735 have been examined for their ability to carry out anaerobic respiration with TMAO. An increase in the growth yield of cells incubated anaerobically has been demonstrated on the addition of TMAO. The substrate was shown to be acting as a terminal electron acceptor rather than as a cell metabolite with no change in the sum of TMAO and TMA in the medium (Easter, 1982; Stenberg *et al.*, 1984; Ringø *et al.*, 1984). As *Alteromonas* sp. NCMB 400 was able to grow with formate as an electron donor, a compound that cannot give rise to ATP synthesis by any known substrate level phosphorylation mechanism it indicated that the reduction of TMAO is coupled with energy conservation by a respiratory mechanism (Ringø *et al.*, 1984). Formate and TMAO-dependent anaerobic uptake of serine has been shown to be sensitive to ionophores that collapse the membrane potential and to be inhibited by the electron transport inhibitor HOQNO, indicative of anaerobic respiration (Stenberg *et al.*, 1984).

Substrates capable of electron donation to the respiratory chain of *Alteromonas* sp. NCMB 400 include formate, pyruvate, lactate (Easter, *et al.*, 1982) and with *Alteromonas* sp. NCMB 1735, amino acids such as serine and cysteine that are easily converted to pyruvate, or glutamate and aspartate that can form tricarboxylic acid cycle intermediates (Ringø *et al.*, 1984). The respiratory chain to TMAO in *Alteromonas* spp. is probably composed of a formate dehydrogenase, a cytochrome c_{552} and TMAO reductase, as these components are simultaneously induced by the presence of TMAO under anaerobic conditions (Easter,

1982; Nasser, 1983). Two c_{552} cytochromes have been identified in *Alteromonas* sp. NCMB 400, having a periplasmic and membrane bound location respectively, with the membrane bound component being the inducible cytochrome and functioning in TMAO reduction (Easter, 1982). Using anaerobic difference spectrophotometry and pyridine haemochromogen analyses, both b- and c-type cytochromes have been observed in membrane vesicles that are oxidizable by TMAO, having α-band absorption maxima at 554 and 552 nm respectively (Stenberg *et al.*, 1984). A membrane bound formate dehydrogenase has been solubilized by detergent treatment, but a full characterization of the enzyme has been prevented by problems associated with the lability of the protein (Nasser, 1983). Studies on the presence of other cytochromes and quinones in the respiratory chain are at present being undertaken (Morris, unpublished data).

The activity of TMAO reductase in *Alteromonas* sp. NCMB 400 under aerobic conditions is negligible but when transferred to anaerobic conditions enzyme activity becomes apparent and is enhanced by the addition of increasing amounts of TMAO to the growth medium (Nasser, 1983). With the restoration of aerobic conditions to such cultures, further formation and activity of the enzyme is inhibited (Nasser, 1983). In similar experiments, no repression of TMAO reductase formation in cells grown in the presence of TMAO by either nitrate or fumarate was evident (Nasser, 1983). Nitrate was shown to repress fumarate reductase formation and vice-versa, suggesting a different method of control between TMAO and either nitrate or fumarate than in *E.coli* (Nasser, 1983). The lack of repression of TMAO reductase formation by either nitrate or fumarate suggests that TMAO is the preferred electron acceptor in *Alteromonas* sp. NCMB 400 despite the lower redox potential of the TMAO/TMA couple (+0.13V) compared with the nitrate/nitrite couple (+0.42V) (Nasser, 1983).

Two TMAO reductase activities have been identified in *Alteromonas* spp. having a loosely membrane bound or periplasmic location (Easter *et al.*, 1983; Stenberg *et al.*, 1984). One of the enzymes has been partially purified and has an estimated native molecular weight of 400 000-450 000 daltons composed of

250 000-300 000 dalton subunits (Easter, 1982). The enzyme from *Alteromonas* sp. NCMB 400 has been shown to utilize adenosine N-oxide in addition to TMAO but was inhibited by the N-oxide of pyridine and nicotinamide. The K_{mTMAO} was estimated at 90-150μM, a K_m much lower than that observed for the enzymes from enteric bacteria. The partially pure preparation of enzyme was slightly inhibited by PCMB and by KCN (Easter, 1982).

Mutants defective in nitrate and TMAO reductases have been isolated from *Alteromonas* sp. NCMB 400 and lack functional molybdenum cofactor (Nasser, 1983). This cofactor appears to be typical of other cofactors isolated from respiratory systems in that it can complement the *nit*1 mutant of *Neurospora crassa* to produce a functional *N.crassa* nitrate reductase (Nasser, 1983).

The evidence so far presented for TMAO reduction in *Alteromonas* spp. suggests some similarity with the respiratory systems of other bacteria. These cells possess more than one enzyme that can reduce TMAO, allowing electron transport to the substrate to be coupled with oxidative phosphorylation. However the ecological link between *Alteromonas* spp. and TMAO, together with the observed increased specificity and higher affinity for the substrate, imply a greater metabolic significance for TMAO reduction in *Alteromonas* spp. than in the *Enterobacteriaceae* The aim of this study is to further elucidate the structural and functional properties of TMAO reductase in *Alteromonas* sp. NCMB 400 such that the process of TMAO respiration may be better understood.

Chapter 2

Materials and Methods

2.1 Chemicals and Materials

Unless otherwise specified, chemicals were obtained from either British Drug Houses Ltd., or Sigma Chemical Company Ltd. and were of reagent grade or better. Media were obtained from Oxoid Ltd. Gel media for liquid chromatography were from Pharmacia Fine Chemicals Ltd. except Hydroxyapatite HA which was obtained from BioRad Laboratories Ltd. Glass chromatography columns were from either Pharmacia or LKB Instruments Ltd.

2.2 Bacterial Culture

2.2.1 Bacterial Strains

Alteromonas sp. NCMB 400 was obtained from the National Collection of Marine Bacteria (NCMB) held at the Torry Research Station, Aberdeen. *E.coli* K12 and *S.typhimurium* LT2 were obtained from within the Department of Microbiology, Edinburgh.

2.2.2 Growth Media

Alteromonas sp. NCMB 400 was maintained on nutrient agar containing 2% NaCl at 4°C and subcultured every 2 months. Growth in liquid culture was carried out using the medium of Wood and Baird (1943) containing (gl^{-1}): NaCl, 20; K_2HPO_4, 1; $MgSO_4.7H_2O$, 1; peptone, 5; yeast extract, 2; pH7.2. For microaerobic growth, this medium was supplemented with 30mM sodium lactate and 10mM of the appropriate electron acceptor.

E.coli K12 and *S.typhimurium* LT2 were maintained on nutrient agar at 4°C and cultured on nutrient broth supplemented as for *Alteromonas* sp. NCMB 400 for microaerobic growth.

2.2.3 Growth Conditions

Cultures of *Alteromonas* sp NCMB 400 were incubated at their optimum growth temperature of 20°C. *E.coli* K12 and *S.typhimurium* LT2 were incubated at 37°C. Aerobic conditions for liquid

cultures were obtained in 250 ml or 2 l Erhlenmeyer flasks containing 20-25% medium in a Gallenkamp Orbital Cooled Shaker (INR 250-010J) at 180 rev.min^{-1}.

Microaerobic growth conditions were obtained by static incubation of a culture vessel completely filled with medium.

2.2.4 Examination of Cultures

Culture examination was carried out using a Vickers Instruments microscope at a magnification of x1000.

Culture purity was determined by Gram staining and plating out of culture samples. Gram staining was according to Nasser (1983) as follows: (a) crystal violet, 0.5% (w/v) aqueous solution, 1 min; (b) Grams iodine, 0.3% (w/v) iodine and 0.7% (w/v) KI aqueous solution, 1 min; (c) alcohol, 70% aqueous solution, 30 sec; (d) basic fuschin, 0.1% (w/v); phenol, 0.5% (w/v); 10% alcohol in aqueous solution, 1 min. Culture samples were plated onto solid media and incubated at 20°C and 37°C for 2 d. Growth at 37°C indicated contamination as *Alteromonas* sp. NCMB 400 cannot grow at this temperature.

2.2.5 Measurement of Bacterial Growth

Growth of the bacterial cultures was monitored by the increase in absorbance at 660nm (A_{660}) in a 1 cm path length cuvette using a single beam spectrophotometer (Shimadzu UV 120.02).

2.3 Bacterial Cell Fractionation

2.3.1 Harvesting and Preparation of Cell Suspensions

Cells were harvested from the culture medium by centrifugation at 12 000 g for 15 min at 4°C (MSE 18 centrifuge). The cell pellet was resuspended in TMAO buffer (100mM K_2HPO_4 pH 7.2 containing 100mM NaCl and 1mM $MgSO_4.7H_2O$) using a uni-form glass/teflon homogeniser (Jencons). After re-centrifugation, the cell pellet was ready for periplasm preparation.

2.3.2 Preparation of Periplasm

The method of Birdsell and Cota Robles (1967) was used as modified by Easter (1982). Washed cells were resuspended in 10mM Tris-HCl. pH 8.0 containing 500mM sucrose and incubated statically at room temperature for 10 min. Lysozyme (30 µg ml^{-1} final concentration) was added and incubation continued for a further 10 min before addition of an equal volume of 10mM Tris-HCl, pH8.0 with continuous stirring. After 10 min incubation 100mM sodium EDTA, pH8.0 was added to the suspension to a final concentration of 1mM. Spheroplast formation, monitored by phase contrast microscopy of wet mount suspensions, was evident after approximately 15 min and usually complete after 30 min incubation. Addition of $MgSO_4.7H_2O$ to a final concentration of 5mM was omitted to maintain EDTA inhibition of divalent cation requiring proteases. The periplasm was separated from the spheroplast suspension by centrifugation at 35 000 g for 30 min at 4°C.

2.4 TMAO Reductase Assay

The assay for TMAO reductase was based on the method of Jones and Garland (1977), as used by Easter (1982) and Nasser (1983). Enzyme activity was determined from the TMAO dependent re-oxidation of the reduced bipyridylium compound methyl viologen ($MV^{\cdot +}$). The reaction was carried out under anaerobic conditions and monitored spectrophotometrically at 600nm following the oxidation of $MV^{\cdot +}$ (purple) to MV^{++} (colourless).

A Pye Unicam SP6-400 UV spectrophotometer (Philips) was connected to a Servoscribe 1S RE541 chart recorder with 10mV input and full scale deflection from A_{600}^{1cm} 0.0 to 1.0. The assay was carried out in glass cuvettes fitted with teflon stoppers drilled to take microsyringes of capacity between 25 and 100 µl.

Into a cuvette was placed an aliquot of enzyme extract (5-100 µl) and some antibumping granules to aid mixing. The cuvette was filled (4.5 ml) with TMAO buffer pH6.5 containing

MV^{++} (0.3mM) sparged with oxygen free nitrogen (BOC) to remove oxygen. The stopper was carefully fitted to the cuvette so that no air remained within the cuvette and sufficient freshly prepared sodium dithionite (25mM in 10mM NaOH) was added to raise the A_{660} to approximately 0.75 (20-50 µl). The cuvette was placed in the spectrophotometer and the endogenous enzyme rate recorded. The reaction was started by addition of TMAO (1M) to a final concentration of 6.7mM and the rate of oxidation recorded.

The specific activity was expressed as nmol.$MV^{\cdot +}$ oxidized min^{-1} (mg protein)$^{-1}$ equivalent to one unit of activity (U) assuming an extinction coefficient of 13 $M^{-1}cm^{-1}$ for MV^{+} (Thorneley, 1974).

Where alternative electron donors or electron acceptors were being tested, the appropriate compound replaced either MV^{++} or TMAO respectively.

2.5 Protein Estimation

Protein concentrations were determined using the method of Peterson (1977) based on the Folin-Ciocalteu reagent used by Lowry *et al.* (1951). This method avoids errors in protein determinations due to interfering substances present in the sample solution, by prior precipitation of protein followed by estimation with the Folin-Ciocalteu reagent.

The solutions used were: copper-tartrate-carbonate (0.1% (w/v) $CuSO_4.5H_2O$, 0.2% (w/v) sodium potassium tartrate in 10% (w/v) Na_2CO_3), 10% sodium dodecyl sulphate (SDS), 0.8N NaOH and distilled water. These were mixed in equal parts to prepare fresh reagent A. Reagent B was composed of 16.7% aqueous solution Folin-Ciocalteu reagent.

(a) Precipitation step. To a sample containing 10-100 µg protein in a volume of 1 ml was added 0.1 ml 0.15% (w/v) sodium deoxycholate; the solutions were mixed and incubated at room temperature for 10 min. Then 0.1 ml 72% (w/v) trichloroacetic acid was added and mixed. The precipitated protein was pelleted by centrifugation at 8 800 g at room temperature for 15 min

using an Eppendorf 5413 centrifuge. The supernatant was discarded leaving the precipitated protein for assay.
(b) Spectrophotometric step. To the sample of precipitated protein was added 1 ml distilled water followed by 1 ml reagent A. On mixing, the pellet was solubilized and allowed to stand at room temperature for 10 min. Reagent B (0.5 ml) was added and after 30 min the A_{750}^{1cm} was read against a reagent blank. Protein concentrations in unknown samples were determined by reference to a standard curve produced for each assay using bovine serum albumin (BSA) fraction V.

2.6 Liquid Chromatography

All procedures were carried out at 4°C.

2.6.1 Gel Filtration

Two gel types were used as gel filtration media: Sepharose 4B and Sephacryl S-300 superfine in columns of bed dimensions 2.6x47 cm and 1.6x100 cm respectively. The columns were equilibrated in gel filtration buffer (10mM Tris-HCl, pH7.2 containing 200mM NaCl), the flow being provided by a peristaltic pump (Pharmacia PI).

Samples for gel filtration were loaded in volumes not exceeding the recommended limit of 5% column volume and proteins were eluted isocratically with gel filtration buffer. The eluate was collected in acid-washed test tubes in an Ultrorac Fraction Collector (LKB) and assayed for TMAO reductase activity and protein at A_{280}.

The molecular weight calibration curve for proteins eluted from the gel filtration columns was prepared using a series of molecular weight standard proteins: thyroglobulin (669 k daltons), ferritin (440 k daltons), catalase (232 k daltons), aldolase (158 k daltons), bovine serum albumin (BSA, 67 k daltons) and chymotrypsinogen A (25 k daltons). All standards were prepared as 5 mg ml^{-1} and loaded in 1 ml aliquots. Phage P22 was used to determine the void volume (Vo) of the Sepharose 4B column and Blue Dextran ($M_r > 2x10^6$ daltons) for

the Sephacryl S-300 column. The calibration curve was plotted as Kav (partition coefficient) against $\log_{10} M_r$, where

$$Kav = \frac{V_e - V_o}{V_t - V_o}$$

V_e = elution volume of protein and V_t = total column volume

2.6.2 Ion Exchange Chromatography

The cation exchanger DEAE Sepharose CL 6B was used in a column of bed dimensions 2.6x10 cm. The gel was equilibrated with ion exchange buffer (50mM Tris-HCl, pH7.0) and following the loading of samples dialysed against this buffer (1000 volumes), the column was washed with two column volumes to remove unbound protein. Bound protein was eluted with an increasing linear gradient of NaCl (300 ml) present in the ion exchange buffer. Fractions were collected and assayed as for gel filtration.

2.6.3 Hydroxyapatite Chromatography

Hydroxyapatite HA was prepared and equilibrated in hydroxyapatite buffer (10mM K_2HPO_4, pH7.2) to produce a column of bed dimensions 2.6x30 cm. Samples were loaded onto the column and unbound material washed off with buffer in the same way as for ion exchange chromatography. Bound material was eluted using an increasing linear gradient of K_2HPO_4 concentration (10mM-500mM, 300 ml) and fractions collected and assayed as for gel filtration.

2.6.4 Affinity Chromatography

Affinity chromatography was performed according to Easter (1982). The chosen support was AH-Sepharose 4B to which was attached betaine-HCl (Aldrich) such that the trimethylammonium group was the effective ligand. The method for coupling the ligand was that suggested by Pharmacia (Affinity Chromatography, Guide and Applications).

AH-Sepharose 4B (5 g) was washed in 500mM NaCl (1 l.) and distilled water (1 l.) before being resuspended in 300mM Betaine-HCl, pH4.5. The coupling agent 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide (EDC) was added as an aqueous solution dropwise, with gentle stirring, to give a final concentration of 100mM. The suspension was left at room temperature for 24 h with the pH maintained between 4.5 and 6.0 for the first 2 h. The gel was washed with alternating 50 ml aliquots of 100mM $NaHCO_3$ buffer, pH8.3 containing 500mM NaCl (500 ml) and 100mM sodium acetate buffer, pH4.5 containing 500mM NaCl (500 ml), followed by washing with distilled water (500 ml). The affinity gel was resuspended in the equilibration buffer (10mM Tris-HCl, pH7.0) and packed to form a column of bed dimensions 1.0x20 cm.

After dialysis, protein samples were loaded onto the gel against equilibration buffer and unbound material washed out as for ion exchange chromatography. Bound material was eluted with the equilibration buffer containing an increasing linear gradient of NaCl (0.0-1.0M NaCl, 300 ml). Samples were collected and assayed as for gel filtration.

2.7 Polyacrylamide Gel Electrophoresis

The method of polyacrylamide gel electrophoresis (PAGE) and in the presence of SDS (SDS-PAGE) was that of Laemmli (1970). Stock acrylamide solution was prepared from acrylamide 29.2% (w/v). NN'-Methylenebisacrylamide, 0.8% (w/v) (Electran grade) in aqueous solution and stored at 4°C in the dark. In general, linear gradient resolving gels were prepared (20.5x14.0x0.1 cm) using the solutions given in Table 2.1. The two solutions were placed in separate chambers of a gradient mixer and the mixture poured into the gel cassette such that the gradient ran from high % acrylamide at the base, to low % acrylamide at the top. This was overlayed with water saturated isobutanol to a depth of 1 cm to ensure a level top to the gel during

polymerization. Total pouring time was approximately 10 min with gel setting taking 30-40 min. The stacking gel (2.5x14.0x0.1 cm), containing 4.5% (w/v) acrylamide, was prepared as given in Table 2.1. The solution was added onto the polymerized resolving gel, after removal of the isobutanol layer. Samples for electrophoresis were prepared in sample buffer (10mM Tris-HCl, pH6.8 containing: glycerol, 10% (v/v); ±SDS, 1% (w/v); ± dithiothreitol, 5% (w/v) and bromophenol blue, 0.005% (w/v) as tracking dye). Electrode buffer contained: 25mM Tris, pH8.3; 192mM glycine; ±SDS, 0.1% (w/v). Gels were pre-electrophoresed at 100V constant voltage (LKB model 2197 power supply) for 1 h to remove ammonium persulphate. Samples were loaded in 5-100µl aliquots and the gel run at 10mA constant current for 1 h followed by 20mA constant current until the tracking dye had migrated to the base of the gel (ca.4 h). All operations were at room temperature and gels were stained for protein (2.12.1 or 2.12.2) or enzyme activity (2.12.3).

2.8 Isoelectric Focusing

Isoelectric focusing was achieved using the Ultrophor flat-bed isoelectric focusing apparatus (LKB), the power supply used for PAGE and either commercial isoelectric focusing gels (LKB PAG-Plate, pH3.5-9.5) or laboratory prepared gels (10 ml stock acrylamide (2.7), 7 ml glycerol, 3 ml Ampholine pH3.5-9.5 (LKB), 40 ml distilled water and 0.15 ml 10% (w/v) ammonium persulphate). Cooling of the flat-bed apparatus to 5°C was provided by a refrigeration unit (Tecam 1000 heat exchanger) and a water circulator (Tecam C400).

Contact between electrodes and gel was made with wicks prepared from four sheets Whatman 3MM filter paper cut to appropriate size. The anode and cathode electrolyte solutions were 1M H_3PO_4, and 1M NaOH respectively. Samples were applied to the gel on 3x5 mm pieces of Whatman 3MM filter paper (with loads up to 50 µl) placed in various

Table 2.1 Solution Volumes (ml) for Gradient PAGE and SDS-PAGE

Solution	Gel Concentration 20%	15%	10%	7.5%	5%	4.5% (Stacking gel)
1.5M Tris, pH8.8±0.4% SDS	3.75	3.75	3.75	3.75	3.75	-
0.5M Tris, pH6.8±0.4% SDS	-	-	-	-	-	2.5
Stock acrylamide	10	7.5	5	3.75	2.5	1.5
Distilled water	-	2.1	6.25	7.5	8.6	6.0
Glycerol	1.25	1.5	-	-	-	-
TEMED[1] (µl)	20	20	20	20	20	20
			DEGAS			
Ammonium persulphate (10%, µl)	20	20	20	20	20	20

[1] N,N,N',N'-Tetramethylethylenediamine

positions across the gel. The gel was run at 20W constant power for 0.5 h after which the sample application pieces were removed and electrophoresis continued for a further 1.5 h. On completion of electrofocusing the gel was sectioned for protein staining (2.12.1 and 2.12.2)., zymogram staining (2.12.3) and pH gradient determination.The pH measurement was made by sectioning the gel across its width in 0.5 cm increments, each piece being placed into a separate small test tube containing 1 ml degassed 0.5% NaCl. The tubes were stoppered, mixed and left overnight at room temperature. Measurement of the pH in each tube was carried out using a Probion 215 combination pH electrode and an Orion Research 701A Digital Ionalyser pH meter.

2.9 Agarose Gel Electrophoresis and Immunoelectrophoresis

Agarose electrophoresis was carried out using the Holm-Nielsen (Mercia Brocades) electrophoresis apparatus with power supply and cooling equipment as for isoelectric focusing. Gels for electrophoresis were prepared from 3 ml 1% (w/v) melted agarose (Mercia Brocades) on 5x5 cm glass plates. Agarose was dissolved in Tris-barbiturate buffer. The stock buffer comprised (g l^{-1}) diethylbarbituric acid, 22.4; Tris, 44.3; calcium lactate, 0.533; sodium azide, 0.65; and was diluted 1:5 in distilled water before use (final pH 8.6). Electrical contact between buffer and gel was provided by four pieces of Whatman 3MM filter paper cut to size.

Samples for agarose electrophoresis (2-10 µl) were loaded into wells cut to one edge of the gel (cathodic end) and electrophoresis carried out at 7 V cm^{-1} at 10°C until the tracking dye of bromphenol blue included in the sample (0.005% (w/v), final concentration) had migrated to the anodic edge of the gel. Gels were dried by the method described in section 2.12.

2.9.1 Rocket Immunoelectrophoresis (R.I.E.)

Agarose gels for R.I.E. were prepared as above (2.9)

with the inclusion of appropriate amounts of antiserum (2.11) sufficient to produce precipitin arcs. Samples were electrophoresed at 3 V cm^{-1} for 24 h. The washing, drying and staining procedures are described in section 2.12.

2.9.2 Crossed Immunoelectrophoresis (C.I.E.)

Samples for C.I.E. were initially subjected to agarose gel electrophoresis (2.9). Gels were sectioned and a strip (1x5 cm) containing a separated sample was transferred to the cathodic end of a second glass plate (5x5 cm). An agarose gel (2.3 ml) containing antiserum was cast onto the remainder of the second plate and electrophoresed under the same conditions as for R.I.E. (2.9.1).

2.10 Electroblotting-Immunodetection/Western Blotting

Western blotting (Burnette, 1980) was performed after the method of Towbin *et al.* (1979). Samples resolved by SDS-PAGE were transferred to nitrocellulose sheets (Schleicher and Schuell) using either the BioRad Transblot system or LKB Transphor electroblotting apparatus and the recommended protocols for each. Staining of the blotted nitrocellulose sheets was carried out by following the method given in Table 2.2.

2.11 Preparation of Antiserum against TMAO Reductase

Antibodies against TMAO reductase were raised in seven mice, two on one occasion, five on a second. Before immunization, a sample of blood was taken from each animal (0.2 ml) as a pre-immune serum control. Mice were immunized with a primary dose of purified TMAO reductase (50 µg each animal) in equal parts complete Freunds adjuvant and 2% (v/v) Tween 80 (total volume 400 µl) administered in the abdomen. On week 9 after the primary dose, animals were given a booster dose of antigen (80 µg of purified TMAO

Table 2.2 Detection of Antigen bound to Nitrocellulose after Western Blotting

(a) Place nitrocellulose in wash buffer (10mM Tris, pH7.4 containing 0.15M NaCl) for 5 min.

(b) Block nitrocellulose with wash buffer containing 5% ovalbumin for 1 h.

(c) Wash nitrocellulose with wash buffer, five times over 30 min.

(d) Add first probe; mouse anti TMAO reductase antiserum (100 µl) in wash buffer (30 ml) containing 5% ovalbumin, and incubate overnight.

(e) Wash nitrocellulose as for (c).

(f) Add second probe; rabbit anti mouse 1 g G (100 µl) in wash buffer containing 5% ovalbumin (30 ml) for 4 h.

(g) Wash nitrocellulose as for (c).

(h) Add third probe; goat anti rabbit peroxidase conjugated (Sigma) 40 µl in 30 ml wash buffer containing 5% ovalbumin for 1 h.

(i) Wash nitrocellulose as for (c).

(j) Develop in 30 ml freshly prepared; 0.01M imidazole pH7.4 containing, O-dianisidine dihydrochloride (250 $\mu g\ ml^{-1}$) and hydrogen peroxide (0.3%, v/v).

(k) Stain appears after approximately 5 min and is stored after washing in distilled water and blotting dry in the dark.

All procedures were carried out at room temperature with constant shaking.

reductase to each animal) administered as previously but with incomplete Freunds adjuvant. On weeks 10 and 12, samples of blood (0.25 ml from each animal) were taken and the serum separated from cellular material by centrifugation (5000 g, 10 min, MSE Centaur bench centrifuge). The anti-serum obtained was not purified further and was stored in the presence of 0.1% sodium azide either in aliquots of 10 µl at -20°C or in bulk at 4°C.

2.12 Staining of Material

Polyacrylamide gels were stained directly using the appropriate methods given below. Agarose gels, following electrophoresis, were washed (R.I.E. and C.I.E. only) and dried prior to staining. Gels were first pressed by wetting with distilled water and covering with one wet and five dry filter papers. A glass plate and a weight (1 kg) were placed on top of the filter papers and the pressing repeated twice at 3 min intervals. The gel was dried to a fine film on the glass plate using hot air from a hair dryer. Washing of C.I.E. and R.I.E. plates was achieved by soaking in 0.1M NaCl (2x15 min) and the pressing repeated once or twice more.

2.12.1 Silver Stain for Protein

Ultrasensitive staining for proteins on polyacrylamide gels was carried out using the BioRad silver stain method as detailed in Table 2.3.

2.12.2 Coomassie Blue Stain for Proteins

Coomassie Brilliant Blue R (0.1%) in water:methanol: glacial acetic acid (5:5:2 by volume) was filtered through Whatman No.1 filter paper to remove any insoluble material before use. Polyacrylamide gels were placed in the staining solution and incubated with shaking for 2 h or statically overnight. Dried agarose gels were incubated for 5 min in the stain solution. Excess stain was removed and the gels

Table 2.3 Method for Bio-Rad Silver Staining of Protein

A. Solutions. The following solutions were prepared freshly for each gel using "Analar" quality chemicals.

(a) Fixative A: 40% methanol, 10% acetic acid (v/v) in distilled water.

(b) Fixative B: 10% ethanol, 5% acetic acid (v/v) in distilled water.

(c) Oxidizer: Potassium dichromate, 0.2 g in 200 ml distilled water containing 0.04 ml concentrated nitric acid.

(d) Silver reagent: Silver nitrate, 0.4 g in 200 ml distilled water.

(e) Developer: Sodium carbonate, 18 g in 600 ml distilled water containing 0.30 ml formaldehyde.

(f) Stop: 0.5% acetic acid (v/v) in distilled water.

B. Protocol. Reagents were added in the order given below.

	Reagent	Volume (ml)	Incubation time
(1)	Fixative A	400	30 min/overnight
(2)	Fixative B	400	15 min
(3)	"	"	"
(4)	Oxidizer	200	5 min
(5)	Distilled water	400	5 min
(6)	" "	"	"
(7)	Silver reagent	200	20 min
(8)	Distilled water	400	1 min
(9)	Developer	200	30 s
(10)	"	"	"
(11)	"	"	until developed
(12)	Stop	400	for storage

were destained in frequent changes of destain solution (30% methanol (v/v) and 10% glacial acetic acid (v/v) in aqueous solution) and stored in 0.5% (v/v) glacial acetic acid (aqueous solution).

2.12.3 Zymogram Stain for TMAO Reductase Activity

Detection of TMAO reductase activity after PAGE, SDS-PAGE and agarose gel electrophoresis was carried out using the method of Shimokawa and Ishimoto (1977). The gel to be stained was immediately immersed in a solution containing: 50mM Tris, pH7.4; 40mM TMAO; 7.5mM $KHCO_3$; 2mM methyl viologen and 5mM sodium dithionite (added just prior to use). The gel was shaken gently in the solution until the whole gel was blue-purple (approximately 5 min). The stain was drained off and the gel allowed to stand at room temperature for 5-10 min until zones of TMAO reductase activity were visible, as clear areas against the purple background. The gel was either photographed, or fixed using 2,3,5-triphenyl-tetrazolium chloride (2.5% (w/v) aqueous solution) for 1-2 min.

2.12.4 Cytochrome Detection

Cytochrome detection was achieved by using the peroxidase activity stain of Thomas *et al.* (1976). A 6.3mM 3,3', 5,5'-tetramethylbenzidine (TMBZ) solution was freshly prepared in methanol. Immediately before use, 3 parts of the TMBZ solution were mixed with 7 parts of 0.25M sodium acetate pH5.0 and the gel immersed in this mixture at room temperature, in the dark, for 15 min. To the solution was added H_2O_2 to a final concentration of 2% with staining becoming visible within 3 min and increasing up to 30 min. The stain solution was discarded and replaced by 3 parts isopropanol, 7 parts 0.25M sodium acetate pH5.0 to store the gel. Cytochrome bands were visible as blue areas on a clear background.

2.13 Ammonium Sulphate Precipitation

Ammonium sulphate fractionation of periplasm was achieved using a saturated solution of ammonium sulphate prepared by heating 1 l. distilled water containing 800 g ammonium sulphate (specially low in heavy metals for enzyme work). The resultant solution was stored at 4°C and the pH adjusted to 7.2 by addition of dilute ammonium hydroxide. This constituted a 100% saturated solution (Jakoby, 1971). Fractionation of periplasm in terms of % saturation ammonium sulphate was achieved by use of the formula:

$$Y = \frac{S_2 - S_1}{1 - S_1}$$

where Y equals the volume (ml) of saturated ammonium sulphate to be added to 1 ml of initial saturation S_1 to give a final saturation S_2. Samples for ammonium sulphate precipitation were incubated with appropriate amounts of the saturated solution at 4°C for 2 h. Precipitated protein was removed by centrifugation at 30 000 g for 30 min at 4°C (Sorval RC5B).

2.14 Cytochrome Spectra Analysis

Reduced minus oxidized difference spectra of periplasmic fractions were determined using a Pye Unicam SP 1800 recording spectrophotometer (Philips) with 1 cm path length quartz cuvettes at room temperature. Samples were oxidized with potassium ferricyanide and reduced with sodium dithionite. Cytochrome *c* was quantified using the extinction coefficient of $24.1 mM^{-1} cm^{-1}$ (Fugita, 1966).

2.15 Assay for Neutral Protease

The assay of Levy *et al.* (1976) was used to determine neutral protease activity in periplasm. The reaction mixture contained 4 mg azoalbumin in 0.65 ml buffer (100mM Tris-HCl,

pH8.0; 1.33mM 2-mercaptoethanol; 1.33mM EDTA; and 0.08% (v/v) Triton x 100). The assay was initiated by addition of 0.1 ml sample for analysis and incubation was carried out at 20°C for 60 min. The reaction was terminated by addition of 0.1 ml ice-cold TCA (55% (w/v)) and the precipitated protein removed by centrifugation (Eppendorf 5414 bench centrifuge, 8 800 g, 15 min). The supernatant was carefully removed and adjusted to pH9.0 by the addition of 150 µl 2M NaOH. The absorbance of the solution was determined spectrophotometrically at 520 nm.

2.16 Ultrafiltration

Concentrations of samples by ultrafiltration was carried out using an Amicon ultrafiltration stirred cell (Model 8010, 10 ml capacity), and 30 000 daltons cut-off membrane filters. The cell was pressurized with nitrogen at 172.4 kPa.

2.17 Non-haem Iron Determination

The method of Doeg and Ziegler (1962) as adapted by Brumby and Massey (1967) was used for the determination of non-haem iron. The method was further modified by replacement of bathophenanthroline in 95% alcohol with water soluble bathophenanthroline sulphonate. All samples were prepared in distilled and deionized water or dialysed against the same, with concentrated nitric acid cleaned glassware used throughout. A series of standard solutions of iron between $1x10^{-6}$ and $1x10^{-4}$M were prepared from electrolytic iron in dilute nitric acid. To 0.1 ml aliquots of sample or standard containing 2-30 µmol of non-haem iron was added 0.1 ml 0.2% (w/v) degassed dithionite solution, prepared freshly and 0.7 ml water. After mixing, 0.05 ml 0.2% (w/v) bathophenanthroline sulphonate was added together with 0.05 ml 1M sodium acetate, pH4.6. Samples were mixed and incubated at 37°C for 5 min before recording the absorbance of the solution at 535 nm against a reagent blank.

2.18 Molybdenum Determination

The molybdenum content of TMAO reductase was estimated by furnace atomic absorption spectroscopy using a Varian AA 1475 model with GTA 95 graphite tube atomizer. Samples (20 µl) were placed in the furnace, dried, ashed at 950°C and atomized up to 2 850°C. Absorption due to molybdenum was monitored at 313.3 nm. Samples were prepared in 10mM Tris buffer pH10.0 to allow full release of the metal for atomization. The sensitivity of the equipment allowed detection of 8×10^{-12}g molybdenum. The content of the metal in TMAO reductase was estimated in comparison with standard solutions (0-60 ng Mo. ml^{-1}) prepared in the same manner.

Chapter 3

Results

3.1 Characterization of Periplasmic TMAO Reductase

3.1.1 Preparation of Periplasm

The isolation of the periplasmic fraction from a culture of *Alteromonas* sp. NCMB 400 was carried out after microaerobic growth at 20°C (2.2.3). Typically, the absorbance of a culture increased from A_{660} = 0.1 to 0.25-0.30 overnight and from a 7.5 l. culture of *Alteromonas* sp. NCMB 400, 150-180 ml of periplasm at approximately 1.5 mg protein ml^{-1} was obtained.

3.1.2 Stability of TMAO Reductase

Several physical and chemical methods of maintaining enzyme activity were investigated as a means of preserving the long term stability of TMAO reductase. As purification procedures often result in samples of reduced protein concentration, leading to an increase in water activity and possible protein denaturation, samples of periplasm were diluted ten-fold in TMAO reductase buffer, pH6.5 prior to the stabilization experiments. Several potential stabilizing compounds were added to separate aliquots of diluted periplasm which were then stored at 4°C, -18°C and -80°C for periods up to 6 months, followed by measurement of TMAO reductase activity. Glycerol (20% v/v, final concentration), was added to the diluted periplasm to reduce the water activity so that the effect of dilution could be assessed. The reducing agents dithiothreitol (10mM) and glutathione (10mM) were included as protective agents against sulphydryl-group oxidation and the substrate for the enzyme, TMAO (10mM) was added as a possible means of affording protection of the active site. Replicates of each treatment were set up such that each sample was frozen and thawed only once. The results obtained from these experiments are presented in Figures 3.1, 3.2 and 3.3.

For each addition and each storage temperature there was an initial decrease in TMAO reductase specific activity of approximately 50%. This occurred during the first 2 d of incubation at 4°C and one week when stored at -18°C or

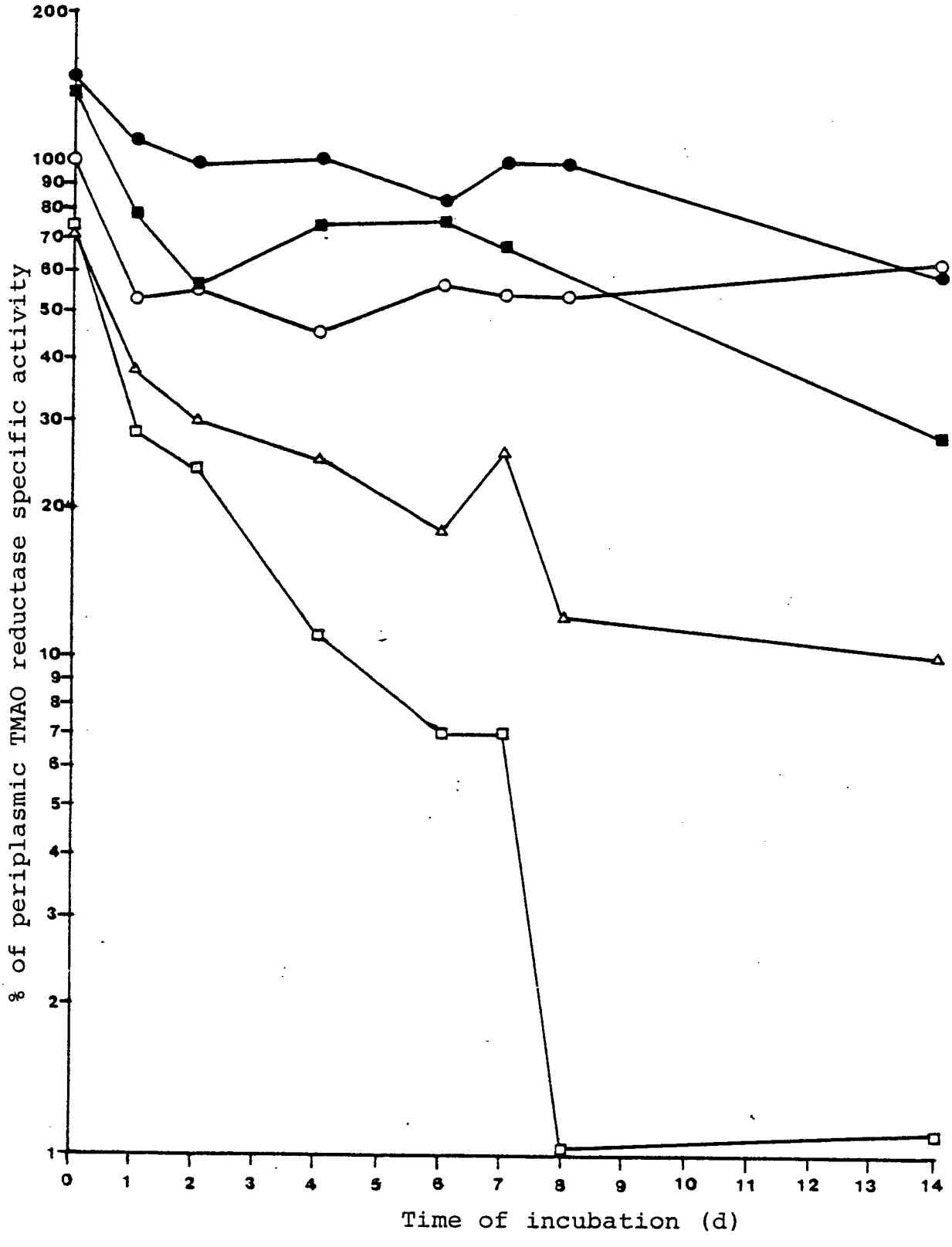


Figure 3.1 Stability of Periplasmic TMAO Reductase with Additives at 4°C.

Periplasm diluted 1:10 (P) with the following additives:

- ○ P + none
- ● P + 20% (v/v) glycerol
- □ P + 10mM glutathione
- ■ P + 10mM TMAO

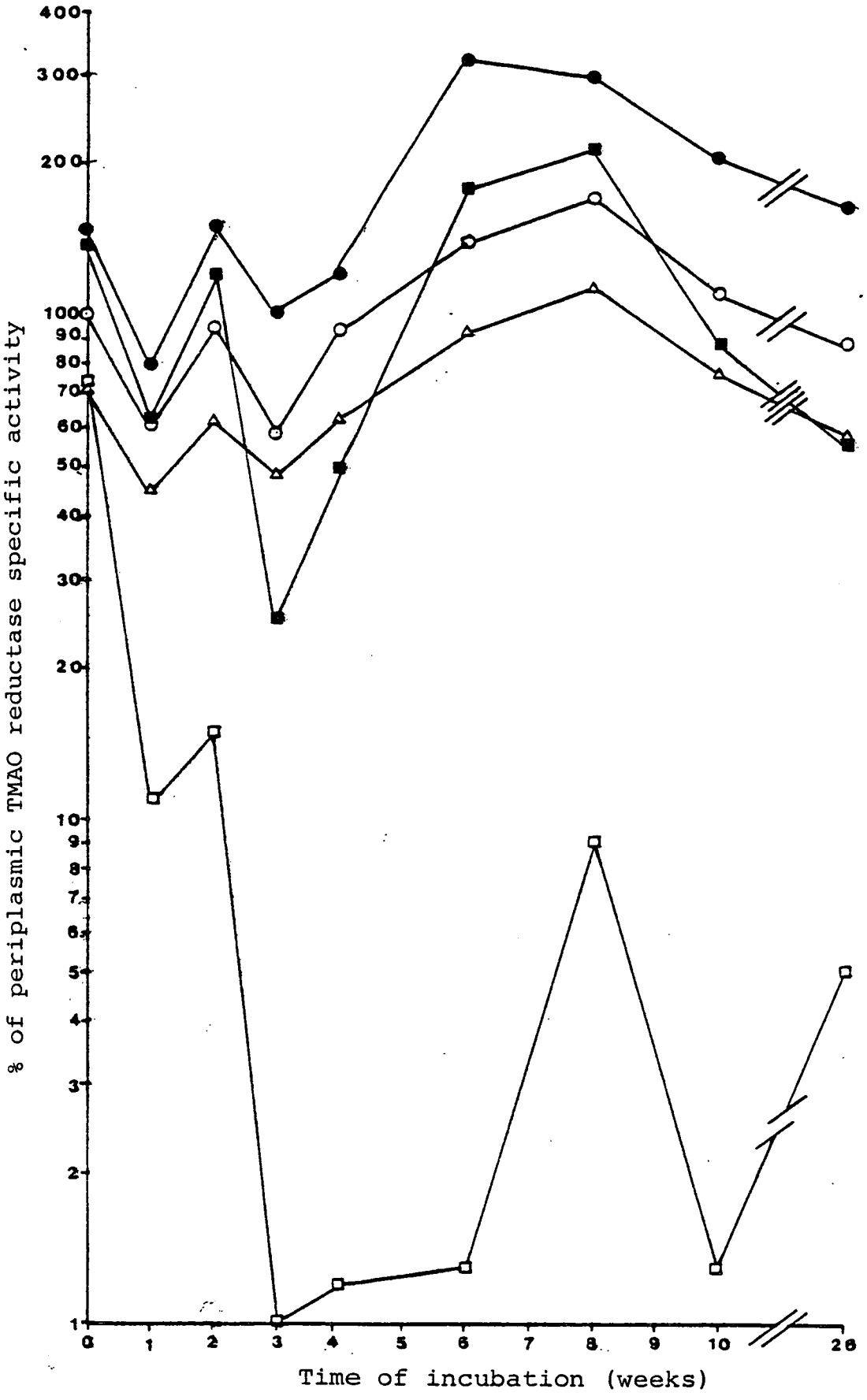


Figure 3.2 Stability of Periplasmic TMAO Reductase with Additives at -18°C.

Legend as for Figure 3.1

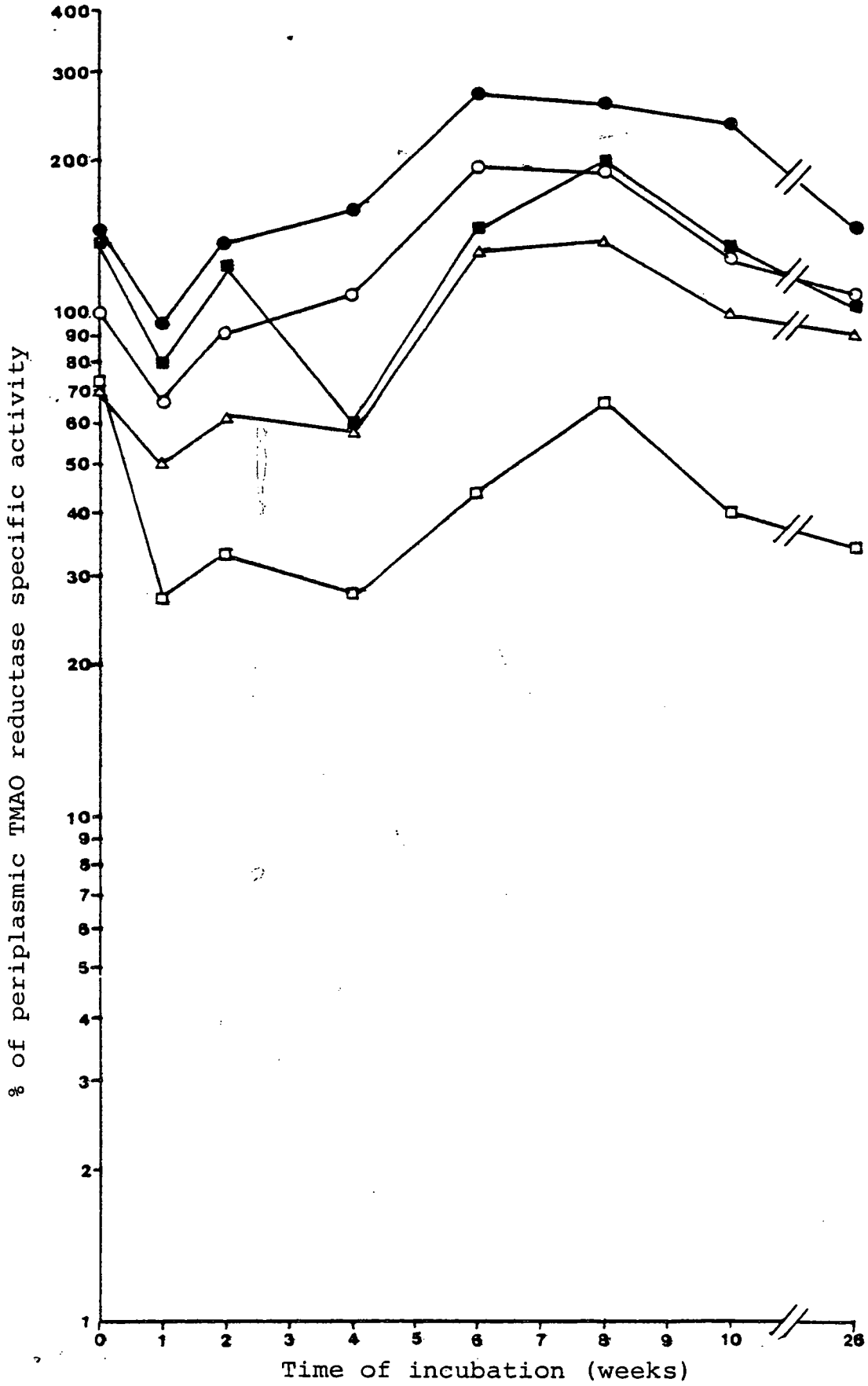


Figure 3.3 Stability of Periplasmic TMAO Reductase with Additives at -80°C

Legend as for Figure 3.1

-80°C. With increased time of incubation the specific activity remained approximately constant over two weeks at 4°C with an increase occurring between weeks 3 and 10 in samples stored at -18°C and -80°C. Incubation at 4°C was not continued after two weeks due to microbial contamination.

Glycerol was the only compound added to periplasm which conferred any advantage over untreated periplasm. The effect of glycerol was to increase the specific activity of TMAO reductase by 20-50% throughout the time course of the experiments and with each temperature of incubation. Glycerol did not provide any added stability for TMAO reductase, the specific activity curves obtained at each temperature paralleling those of periplasm alone. Addition of TMAO to the periplasm gave no increase in either specific activity or stability at each temperature. The assay of periplasmic TMAO reductase in the presence of previously added TMAO was complicated by the high endogenous activity giving rise to greater variation in measured activity than with the other additives. Dithiothreitol and in particular glutathione were detrimental to TMAO reductase, causing a decrease in specific activity between 10% and 60% of that present initially and providing no stabilization of the enzyme. It was observed that glutathione at 10mM concentration caused occasional precipitation of protein in the periplasm. No major differences in specific activity or stability of TMAO reductase were observed after prolonged storage at either -18°C or -80°C; the latter giving a smaller reduction in activity after 6 months than at -18°C. Storage at 4°C gave reasonable stability of TMAO reductase after the initial drop in specific activity during the first 2 d; activity falling by 0-20% over two weeks.

In a separate series of experiments, the effect of freezing and thawing of samples stored at -18°C and -80°C was investigated. Samples of periplasm with the same additions as made in the long term stability experiments were repeatedly frozen and thawed followed by measurement of specific activity, over a 5 d period (1 freeze-thaw cycle d^{-1}). The results are shown in Figures 3.4 and 3.5. As with the experiments on TMAO reductase stability, glycerol

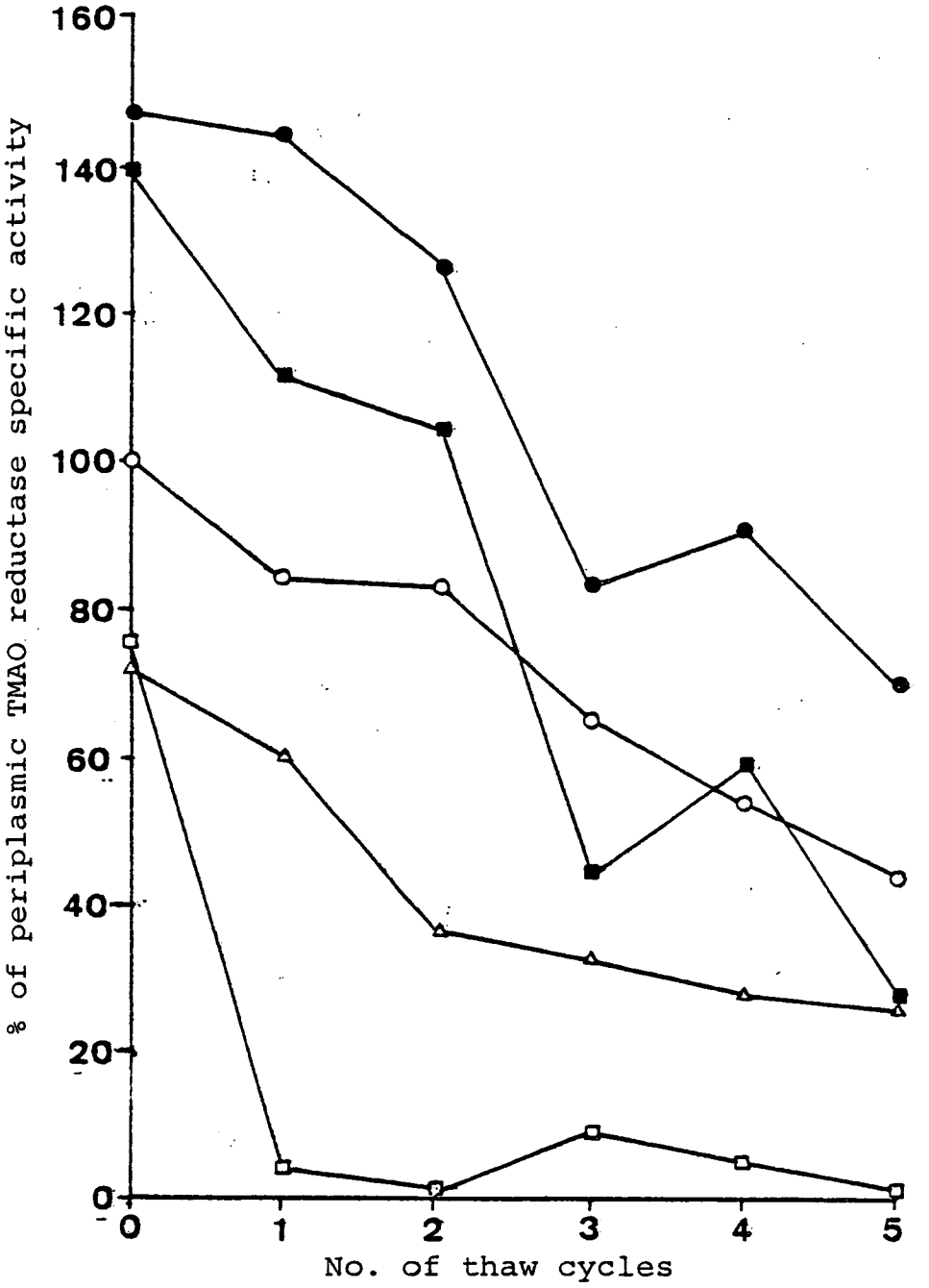


Figure 3.4 The Effect of Repeated Freezing (-18°C) and Thawing of Periplasm in the Presence of Various Additives

Legend as for Figure 3.1

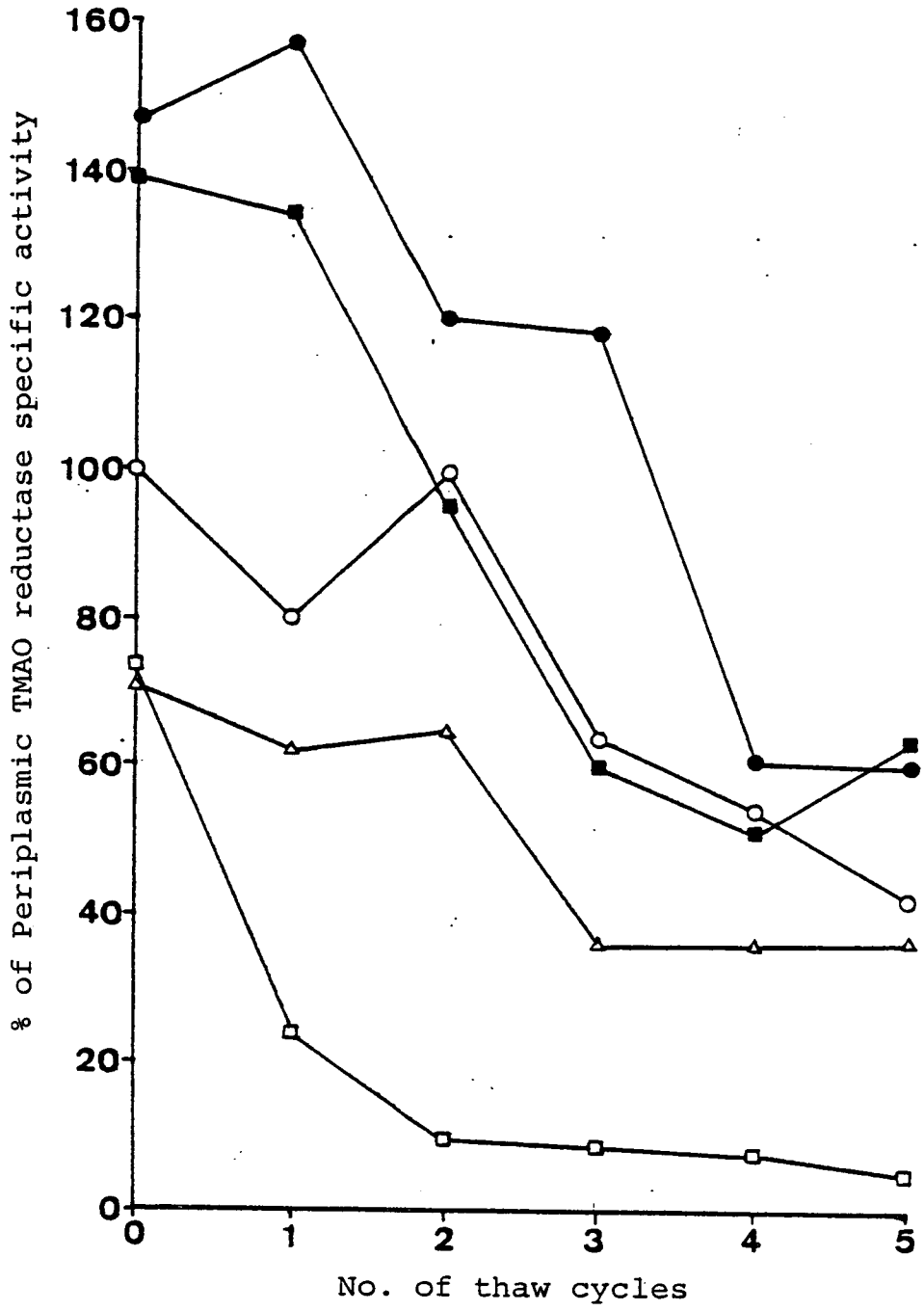


Figure 3.5 The Effect of Repeated Freezing (-80°C) and Thawing of Periplasm in the Presence of Various Additives

Legend as for Figure 3.1

maintained an increase in specific activity of 30-50% over that present in normal periplasm. Periplasm containing TMAO gave values of TMAO reductase specific activity similar to those obtained with normal periplasm after each freeze-thaw cycle. Added dithiothreitol and glutathione were again detrimental to specific activity, dithiothreitol reducing activity by approximately 10% after each freeze-thaw cycle and glutathione reducing activity to almost zero after the third cycle. No protection against freeze-thaw damage of TMAO reductase was offered by any of the additions made to the periplasm.

3.1.3 Proteolytic Activity in Periplasm

Evidence of proteolysis in periplasm was investigated by incubation of samples in wells cut into 1% casein-1% agar petri dish plates, followed by visible examination for clearing of opacity and by staining for protein (2.12.2). Tris-sucrose spheroplasting buffer and commercial protease (Sigma) were used as negative and positive controls respectively and all samples were incubated at 20°C for 7 d. The results are presented in Table 3.1. No clearing of the opaque agar was visible around the wells containing periplasm or buffer and the stain for protein with these samples was positive up to the well margin. The wells containing protease gave a prominant zone of clearing around the sample and the lack of protein staining was indicative of proteolysis.

The neutral protease assay of Levy *et al.* (1976, 2.15) in which protease activity releases an albumin linked azo dye into free solution was used to determine the presence or absence of neutral proteases, part of the metalloproteinase sub-class EC 3.4.24. The same controls were used as for the casein agar assay. No absorbance at A_{520} due to azo dye in the protein free supernatant was recorded for the periplasm and negative control samples, indicating no neutral protease activity. The positive control sample gave a detectable absorbance indicative of proteolysis.

To determine phenylmethylsulphonyl fluoride (PMSF)

Table 3.1 Evidence of Proteolytic Activity in Periplasm Using the Casein Agar Detection Method

Sample	Detection Method*	
	Coomassie blue protein stain	Visible clearing
Periplasm	+++	-
Tris-sucrose spheroplasting buffer	+++	-
Protease	-	+++

* +++ presence around well

\- absence around well

effected inhibition of proteolysis, giving evidence of serine type protease activity (EC 3.4.21), periplasm was treated with and without PMSF and the sample subjected to SDS-PAGE. PMSF (100mM) was prepared in ethanol immediately before use and added to periplasm at a final concentration of 1mM. Ethanol was added to samples of periplasm to the same concentration as used in the PMSF treated sample as control. Proteins resolved by SDS-PAGE were stained for protein and TMAO reductase activity. On examination of the stained gels, no variation in band position or intensity was evident between samples with and without PMSF or ethanol, for either the protein or zymogram stains. These experiments were repeated using samples of periplasm containing the reducing agent 2-mercaptoethanol in the presence of PMSF. It has been reported that protease-polypeptide inhibitor complexes are denatured under reducing conditions releasing active protease (Pringle, 1975). No change in band intensity or position was recorded for protein and zymogram stained samples after SDS-PAGE.

3.1.4 Gel Electrophoresis of Periplasm

SDS-PAGE was carried out on samples of periplasm incubated with reducing agent (dithiothreitol) at 100°C for 5 min. Included in adjacent wells of the gel were molecular weight standard proteins to provide a calibration of the gel. Following staining of the gel for protein, the periplasm was analysed for polypeptides present in the sample. Plate 3.1 shows the results obtained for the stained gel and Figure 3.6 the molecular weight calibration curve as derived from the mobility of the standard proteins. The results indicate a large number of polypeptides (>50) present in the sample, with an approximate molecular weight range of 10 000-200 000 daltons. When these experiments were repeated using non-denaturing PAGE in the absence of SDS, fewer bands of protein were visualized (not shown) indicating an oligomeric nature of some of the proteins present in periplasm. Using samples of periplasm prepared without dithiothreitol and boiling,

Plate 3.1 SDS-PAGE of Periplasm and Molecular Weight Standard Proteins (Protein Stain)

a Periplasm
b Cytochrome c
c Ovalbumin
d Bovine serum albumin
e Thyroglobulin

(7.5-15% acrylamide)

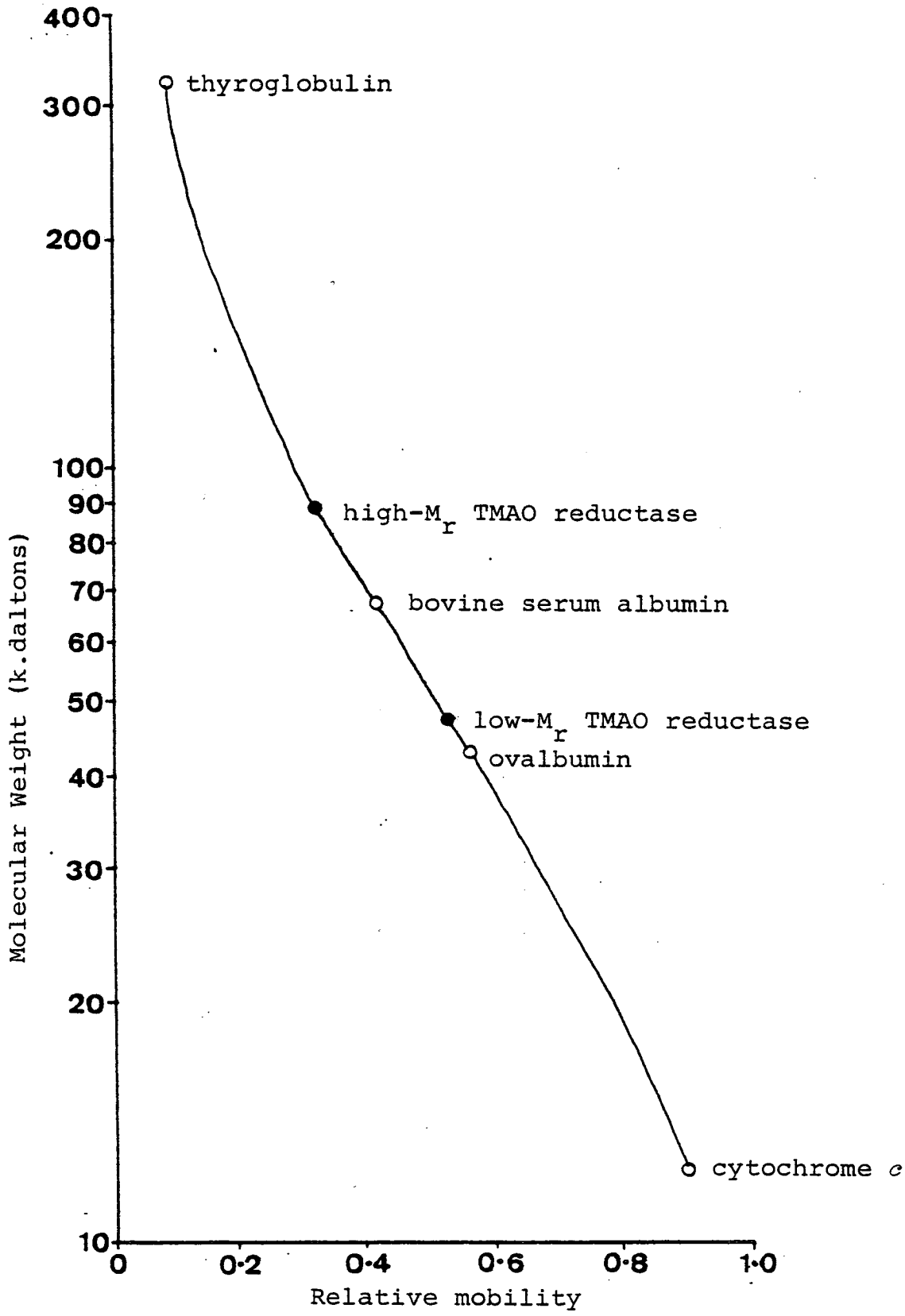


Figure 3.6 Molecular Weight Calibration Curve for SDS-PAGE

o Molecular weight standard proteins

● TMAO reductase activities

Polyacrylamide gradient 7.5-15%

similarly prepared PAGE and SDS-PAGE gels were stained for TMAO reductase activity (Plate 3.2) with bands present in both gels, even in the presence of the denaturing detergent SDS. Two zones of decolourization were identified in each gel and were designated high- and low-molecular weight (M_r) TMAO reductase. The intensity of the activity differed between the two bands on the same gel and between the two gel types. Following PAGE, the high-M_r TMAO reductase had a greater stain intensity than the low-M_r enzyme whereas the converse was true following SDS-PAGE.

Experiments were devised to relate the two activities present after PAGE with the two activities present after SDS-PAGE. This was done on a semi-preparative scale using polyacrylamide gels of double thickness (2mm) to obtain a greater loading of periplasm. Following PAGE, the gel was stained for TMAO reductase activity and the two active bands were excised with minimum contamination of non-enzymic material. The two separate pieces of gel containing TMAO reductase activity were emulsified by passage through 5 ml disposable syringes (without needles) into tubes containing 2 ml TMAO reductase buffer pH6.5. The tubes were incubated overnight at 4°C and the supernatant obtained after centrifugation (5 000 g) concentrated by ultrafiltration. The two activities from PAGE were applied to separate wells of an SDS containing polyacrylamide gel and following electrophoresis the gel was stained for TMAO reductase activity (Figure 3.7). The bands of activity after PAGE migrated to the same relative position after SDS-PAGE; the high-M_r activity remaining as the high-M_r band and the low-M_r activity remaining as the low-M_r band. The inclusion of SDS in the sample appeared to have been more detrimental to the activity of the high M_r enzyme than the low M_r enzyme, the intensity of the stain being reduced after SDS-PAGE.

An estimate of the M_r of the two TMAO reductase activities revealed by SDS-PAGE was made by a comparison of the Rf values for each activity with the molecular weight calibration curve (Figure 3.6). Values of 90 000 and 47 000 daltons were obtained for the high- and low-M_r activities respectively.

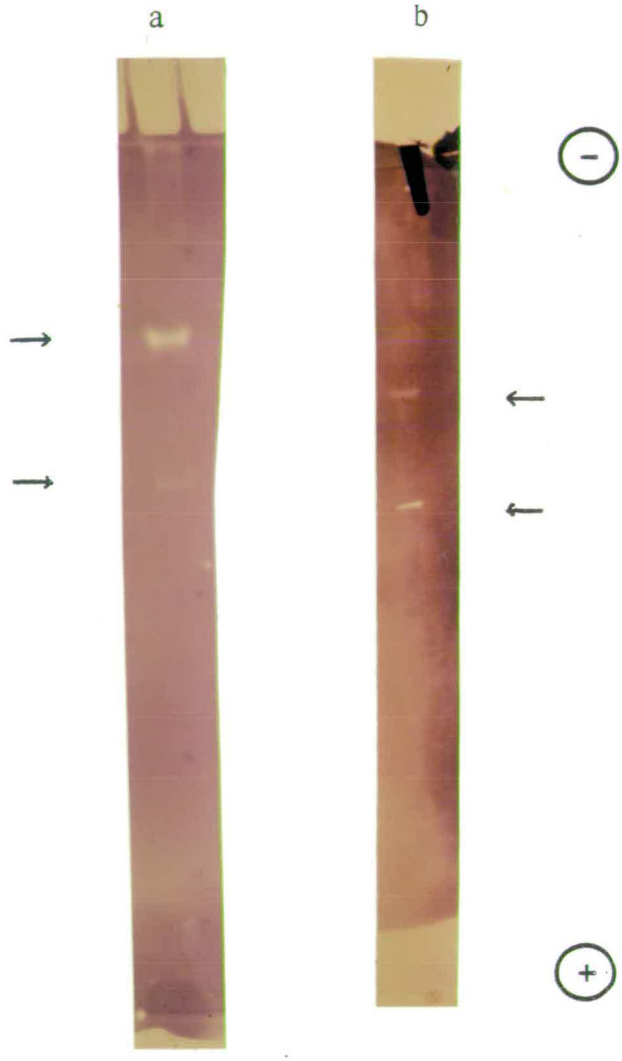


Plate 3.2 PAGE and SDS-PAGE of Periplasm (Zymogram Stain)

a PAGE

b SDS-PAGE

→ enzyme activity

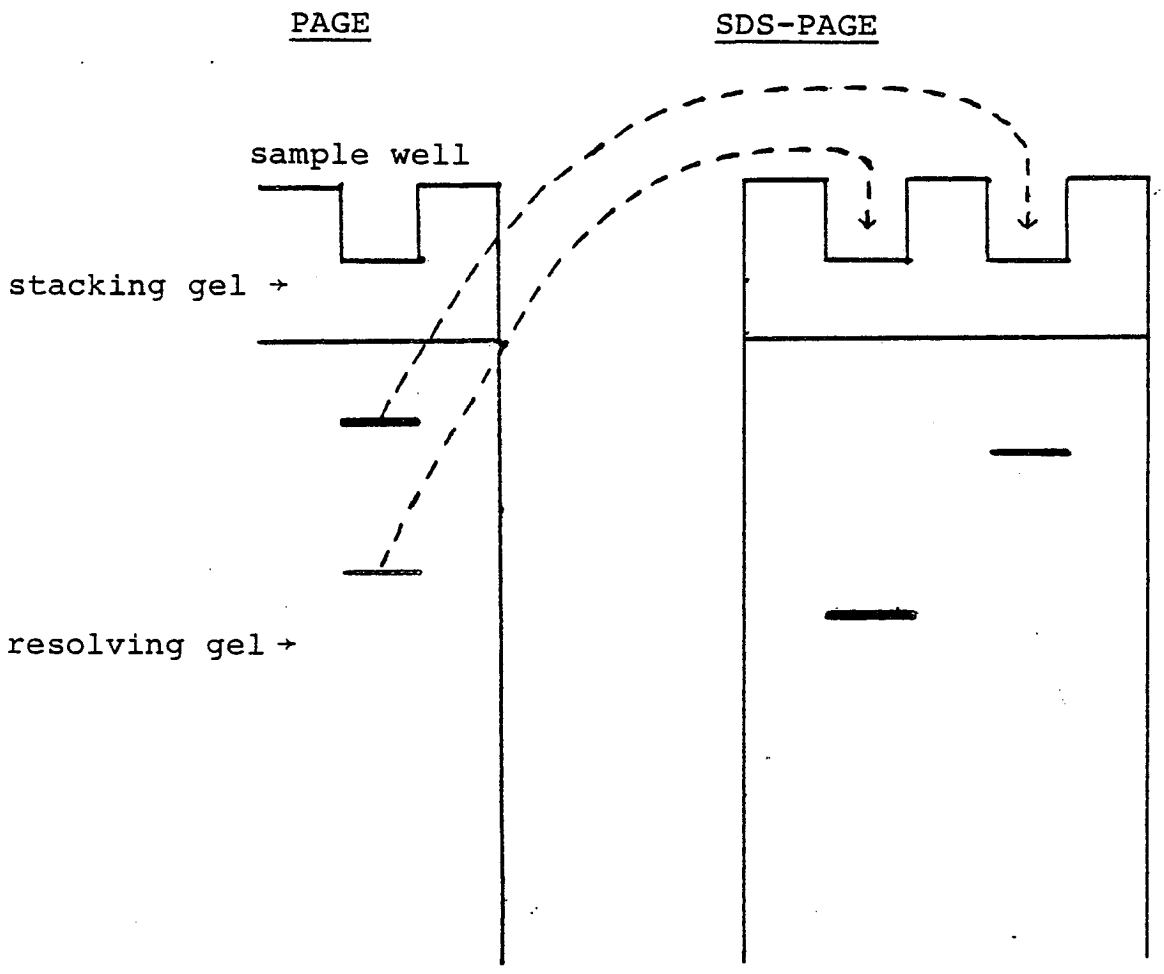


Figure 3.7 SDS-PAGE of TMAO Reductases Isolated after PAGE

Zymogram stains of 7.5-15% polyacrylamide and SDS-polyacrylamide gels. TMAO reductase activities after PAGE were eluted from the gel and loaded separately for SDS-PAGE.

3.1.5 Induction of TMAO Reductase

The ability of various analogues of TMAO to induce TMAO reductase was assessed in order to provide information on the mechanism and control of TMAO reduction. The analogues studied are listed in Table 3.2 and are characterized by the possession of either multiple alkyl groups or an N-oxide moiety. The analogues were prepared in aqueous solution, the pH adjusted to 7.2 and the solutions sterilized by membrane filtration. Inducers were added to Wood and Baird growth medium at 10mM concentration instead of TMAO. The analogue N,N-dimethyldodecylamine N-oxide (LDAO, Prochinor Ceca S.A., France) was added to 1mM final concentration as higher concentrations were found to cause cell lysis. The periplasm from each culture was isolated and assayed for TMAO reductase activity. The specific activities obtained for each analogue and their values expressed as a percentage of that obtained with TMAO as inducer, are given in Table 3.2.

The levels of TMAO reductase activity in the presence of the various potential inducers were in general lower than that obtained with TMAO as an inducer and were similar to the levels present in an uninduced control. Pyridine N-oxide was the only analogue to give a high level of induced TMAO reductase activity (85% of that with TMAO).

In a similar series of experiments, various known alternative terminal electron acceptors in anaerobic respiration were studied for their ability to induce TMAO reductase activity. The compounds used (Table 3.3) were sterilized and added to the growth medium at a final concentration of 10mM in place of TMAO. Periplasm was prepared from each culture after microaerobic growth and assayed for enzyme activity (Table 3.3). Growth in the presence of DMSO induced TMAO reductase activity to the same level as with TMAO. Nitrate and fumarate as terminal electron acceptors gave levels of enzyme activity equal to or below that obtained in the control lacking inducer.

Gel electrophoresis was used to visualize the effect of cell growth in the presence of the alternative electron

Table 3.2 Induction of TMAO Reductase Activity by Analogues of TMAO

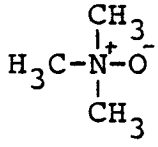
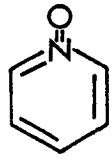
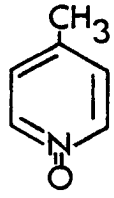
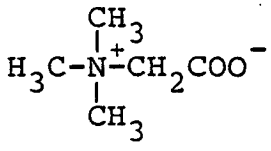
Inducer (10mM)	Structure	TMAO Reductase Specific Activity (U)	% of TMAO Induced Specific Activity
TMAO		2273	100
Pyridine N-oxide		1952	85
Picoline N-oxide		353	15
Betaine		107	5
Tetramethylammonium chloride	$(CH_3)_4NCl$	105	5
Tetraethylammonium chloride	$(C_2H_5)_4NCl$	72	3
Carnitine	$(CH_3)_3N^+CH_2CH(OH)CH_2COO^-$	192	8
LDAO (1mM)	$CH_3(CH_2)_{11}N(O)(CH_3)_2$	72	3
None		72	3

Table 3.3 Induction of TMAO Reductase Activity by Alternative Electron Acceptors

Electron Acceptor	TMAO Reductase Specific Activity (U)	% of TMAO Induced Specific Activity
TMAO	30604	100
$NaNO_3$	4850	16
Sodium fumarate	607	2
* DMSO	30740	100
None	4020	14

* DMSO = Dimethylsulphoxide

acceptors on the two enzyme activities in periplasm. Approximate equal loadings of total activity from the samples of periplasm prepared in the previous experiments were applied to an SDS containing polyacrylamide gel and after electrophoresis the gel was stained for TMAO reductase activity (Plate 3.3). Nitrate induced periplasm contained only the low-M_r activity with no evidence of the high-M_r enzyme. In contrast to this, the samples of periplasm from cells grown in the presence of DMSO or without added electron acceptor gave two bands of enzyme activity typical of TMAO induced periplasm. Fumarate induced periplasm contained a series of activities (approximately 4) none of which corresponded in their Rf value with the two normal TMAO reductase activities.

To analyse the effect of oxygen on the induction of TMAO reductase activity, cells were grown under aerobic and microaerobic conditions in the presence and absence of TMAO. The specific activities in the periplasmic fractions derived from these cells are given in Table 3.4. Growth in the absence of TMAO under aerobic and microaerobic, or with TMAO aerobically gave very low levels of TMAO reductase specific activity (0.2%, 1% and 11% of the microaerobic + TMAO value respectively). After sample concentration, equal amounts of TMAO reductase activity were electrophoresed on an SDS-polyacrylamide gel and stained for enzyme activity. Periplasm from cells grown microaerobically in the absence of TMAO had predominant staining at the low-M_r activity with reduced activity at the high-M_r band, whereas the aerobically grown cells in the presence of TMAO induced the high-M_r TMAO reductase activity to a greater extent than the low-M_r activity. Two activities typical of the TMAO + microaerobically induced enzymes were present in periplasm isolated from cells grown aerobically in the absence of TMAO.

The inhibitor of terminal oxidases, sodium azide, when added to cell cultures has been reported to increase the induction of terminal enzymes during anaerobic growth (De Groot and Stouthamer, 1970a; see introduction 1.2). Sodium azide was added to 1mM concentration during microaerobic

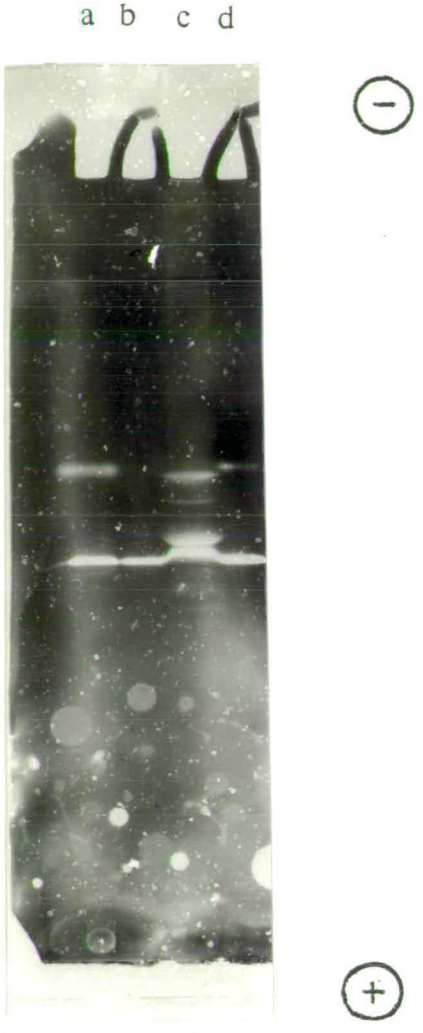


Plate 3.3 SDS-PAGE of Alternative Electron Acceptor Induced Periplasm (Zymogram Stain)

		Protein load (µg)	TMAO reductase activity load (U)
a	DMSO induced	9	285
b	Nitrate induced	164	228
c	Fumarate induced	202	247
d	Without electron acceptor	165	130

(7.5-15% acrylamide)

Table 3.4 Induction of TMAO Reductase Activity in the Presence of TMAO and Oxygen

Growth condition	TMAO Reductase Specific Activity (U)	% of TMAO + Microaerobic Induced Specific Activity
Microaerobic + TMAO	12 461	100
Microaerobic - TMAO	141	1
Aerobic + TMAO	1 380	11
Aerobic - TMAO	19	0

rowth of a culture of *Alteromonas* sp. NCMB 400 with the im of increasing the induction of TMAO reductase. Growth f the culture as measured by A_{660} was inhibited by azide t this concentration with values of A_{660} decreasing during vernight incubation (Table 3.5). The isolated periplasm hen assayed for TMAO reductase activity gave values of pproximately 28% that obtained from normally induced peri- lasm.

Table 3.5 Effect of Sodium Azide on the Growth of *Alteromonas* sp. NCMB 400 and the Induction of TMAO Reductase

Growth Condition	Initial A_{660}	Final A_{660}	TMAO Reductase Specific Activity (U)	% of TMAO Reductase Specific Activity
TMAO	0.090	0.217	15 679	100
TMAO + NaN_3	0.090	0.062	4 467	28

3.2 Purification of TMAO Reductase

3.2.1 Ultrafiltration and Ammonium Sulphate Precipitation of Periplasm

Due to the large volume of periplasm obtained from a 7.5 l. culture of microaerobically grown *Alteromonas* sp. NCMB 400 (150-180 ml), a method was sought to reduce the amount to a level that could be more easily processed. Ultrafiltration of periplasm was investigated as a procedure suitable for both sample concentration and enzyme purification. Using an Amicon Stirred Cell (model 202, 200 ml capacity) and 10 000 dalton cut-off filter a 50% reduction in periplasm volume was achieved after >4 h filtration at 4°C. This was considered unsatisfactory due to the slow processing time and the known loss in activity during a 2 d incubation at 4°C (3.1.2). In addition, little purification of TMAO reductase was obtained due to the retention of most of the protein in the sample, few proteins being less than 10 000 daltons in size. Filters with 30 000 daltons cut-off were not used due to losses of up to 25% in proteins of this size or greater. For these reasons, ultrafiltration was not used at this stage of purification.

Selective $(NH_4)_2SO_4$ precipitation of protein was studied as a method of periplasm concentration and a first step in TMAO reductase purification. Precipitation was carried out according to section 2.13 by taking separate aliquots of periplasm to different degrees of saturation in 10% increments. The protein concentration together with the specific and total activities of TMAO reductase were measured for the precipitate and supernatant obtained at each saturation (Figures 3.8, 3.9 and 3.10). The periplasmic TMAO reductase activity was precipitated from 50-60% up to 70-80% saturation. SDS-PAGE was used to analyse the fate of each of the two TMAO reductase enzymes in the precipitate and supernatant fractions at 60% saturation. The zymogram stain (Plate 3.4) identified the high-M_r enzyme as remaining in the supernatant whereas the low-M_r enzyme was precipitated. At 80% saturation, the high-M_r TMAO reductase was precipitated giving a reasonable yield of enzyme. The protein, activity and yield

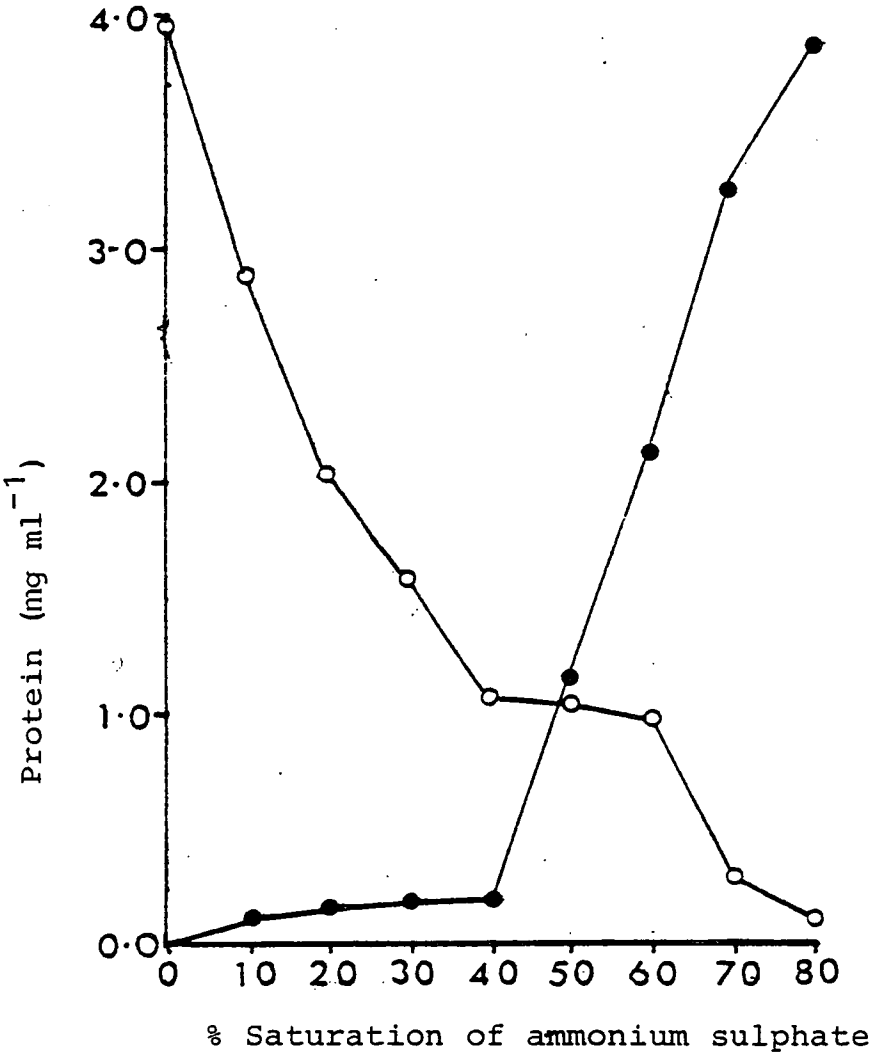


Figure 3.8 Precipitation of Periplasmic Protein by Ammonium Sulphate

o Supernatant

● Precipitate

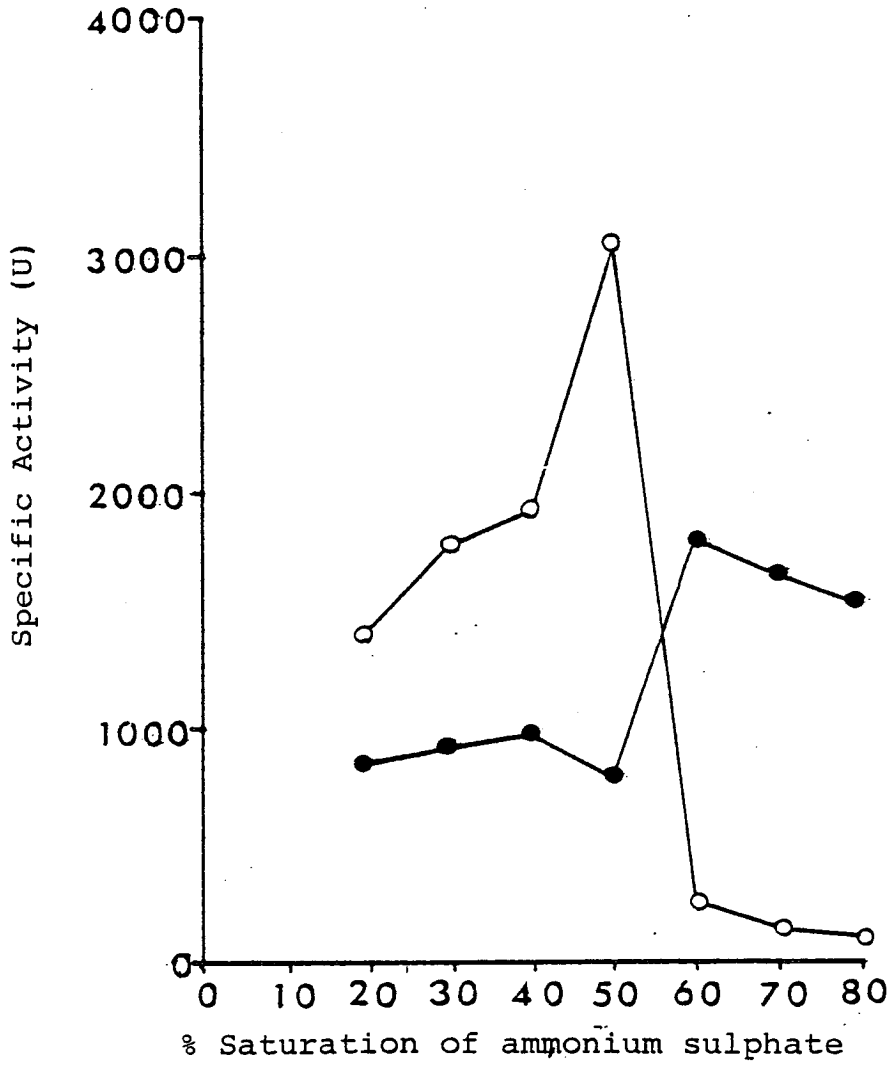


Figure 3.9 Specific Activity of TMAO Reductase in Fractions after Ammonium Sulphate Precipitation

○ Supernatant

● Precipitate

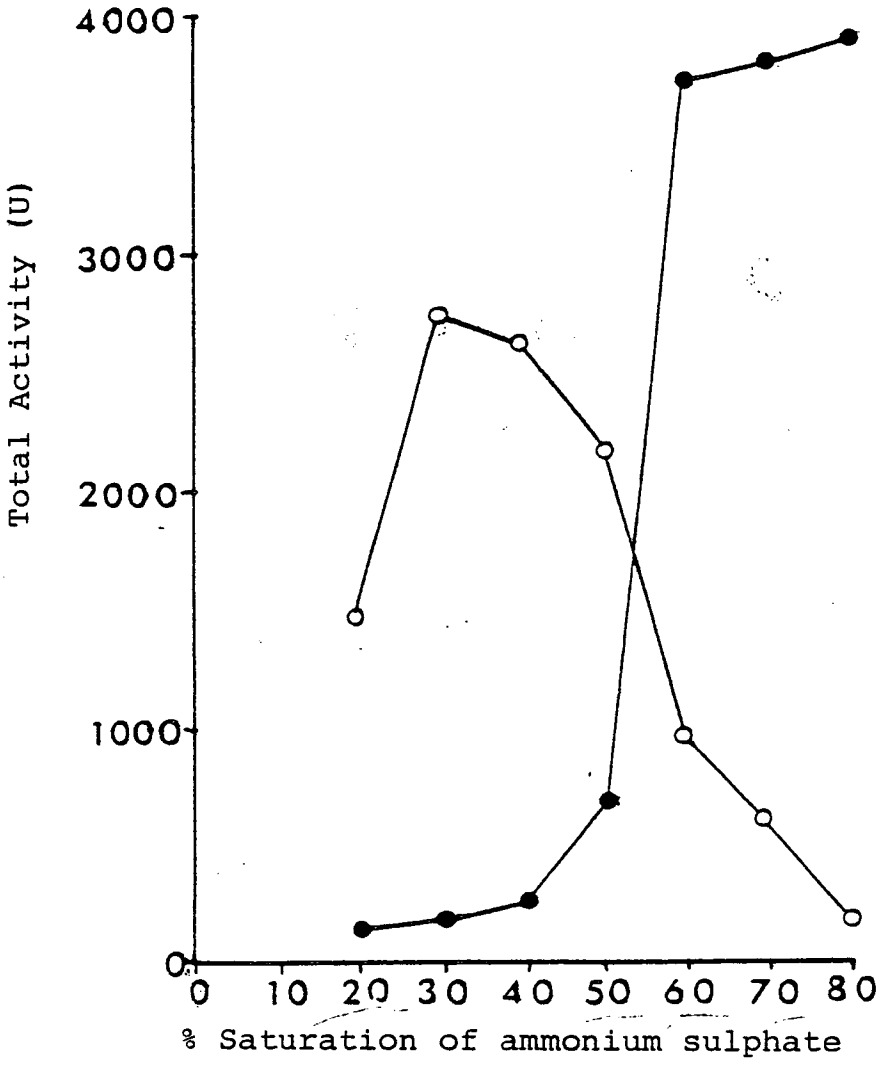
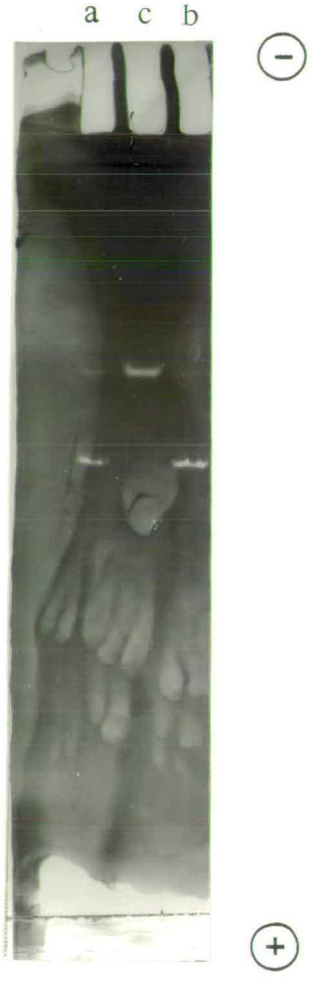


Figure 3.10 Effect of Ammonium Sulphate Fractionation on Total TMAO Reductase Activity in Periplasm

○ Supernatant

● Precipitate



ate 3.4 SDS-PAGE of $(NH_4)_2SO_4$ Fractionated Periplasm (Zymogram Stain)

		TMAO reductase activity load (U)
a	Periplasm	229
b	0-60% precipitate	286
c	60-80% precipitate	271

(7.5-15% acrylamide)

balances are given in Table 3.6. An almost 4 fold purification of high-M_r TMAO reductase was obtained by 60-80% fractionation of periplasm yielding approximately 30% of the total activity. The total protein recovered from the fractionation approached the 100% level as did the recovered activity (84%). Thus losses in protein and particularly activity were minimal after $(NH_4)_2SO_4$ precipitation. To analyse the effect of fractionation on individual proteins, samples from the 60% and 60-80% precipitates were subjected to SDS-PAGE followed by staining for protein (Plate 3.5). In comparison with an unfractionated periplasm sample, fewer bands were visualized in each of the two precipitates. A different protein profile was seen in the 60-80% precipitate compared with that in the 60% precipitate with some bands, including a protein at the same Rf as the high-M_r TMAO reductase activity, being more prominant than in either the 60% precipitate or periplasm. Precipitation at 60-80% saturation allowed the proteins to be resuspended in a reduced volume compared to that of the original periplasm sample.

3.2.2 Ion Exchange Chromatography

Previous work (Easter, 1982) estimated the pI of TMAO reductase in periplasm at approximately pH5.0. At pH values >5.0, TMAO reductase would therefore possess a net negative charge and would be bound to the immobilized positive charges on an anion exchange resin. DEAE Sepharose CL 6B was chosen as a suitable resin, having the capacity for relatively high flow rates and good resolution, giving speed in purification. As the ease of elution of bound protein is aided by the use of a solvent at a pH approaching the pI of the protein of interest, 50mM Tris-HCl, pH7.0 was chosen as the ion exchange buffer. This provided reasonable buffering capacity, at a pH near to the estimated optimum for enzyme activity (6.5-6.9, Easter, 1982).

Separate samples of dialysed periplasm were loaded onto

Table 3.6 Effect of Ammonium Sulphate Precipitation on TMAO Reductase

$(NH_4)_2SO_4$ (%)	Purification[1] (fold)	Yield[2] (%)
Precipitate (0-60)	0.6±0.1 (0.2)	46.0±5.6 (11.1)
Precipitate (60-80)	3.7±0.9 (1.8)	30.5±4.8 (9.3)
Supernatant (80)	2.3±0.9 (1.8)	8.0±3.7 (7.2)

Value ± standard error (95% confidence limits)

Number of replicates (n) = 5

1 Increase in TMAO reductase specific activity

2 Total recovered activity

Plate 3.5 SDS-PAGE of $(NH_4)_2SO_4$ Fractionated Periplasm (Protein Stain)

		Protein Load (µg)
a	0-60% precipitate	57
b	60-80% precipitate	24

(7.5-15% acrylamide, silver protein stain)

the ion exchange column, washed and eluted with a linear gradient of 0.0-1.0M NaCl within the buffer. The elution profile of protein and TMAO reductase activity is presented in Figure 3.11. Two peaks of TMAO reductase activity were eluted from the column at approximately 0.3M and 0.5M NaCl. When fractions from each peak were analysed by SDS-PAGE and zymogram staining both contained activity from the two TMAO reductase enzymes. Similarly prepared gels stained for protein showed a reduction in the number of protein bands present in each of the active peaks in comparison with periplasm. Due to the power of the resolution obtained by ion exchange chromatography of periplasm, the technique was used to resolve TMAO reductase from the 60-80% $(NH_4)_2SO_4$ precipitate of periplasm. Separate dialysed samples were loaded and eluted from the ion exchange column and the fractions monitored for protein, TMAO reductase activity and haem as the absorbance at 410 nm (Figure 3.12). TMAO reductase activity eluted as a major peak at approximately 0.25M NaCl, with a minor peak of activity at approximately 0.45M NaCl. The major peak co-eluting with two peaks of protein and a peak of haem. A large peak of non-enzymic protein was eluted after the TMAO reductase peaks providing evidence for the resolution of the enzymes from other proteins. To further improve the resolution during the early phase of elution, a shallower gradient of 0.0-0.3M NaCl was used to elute similar samples of $(NH_4)_2SO_4$ precipitate (Figure 3.13). The TMAO reductase activity eluted as a peak at approximately 0.1M NaCl with a second peak, of lower activity at 0.25M NaCl occasionally present. Eluting between the two peaks of enzyme activity were fractions containing the haem component observed in the previous experiments. This haem was partially characterized by reduced minus oxidized spectrophotometry (2.14) as a c-type cytochrome with an α-band maximum at 552 nm (Figure 3.14).

SDS-PAGE and zymogram staining of samples from the two peaks of TMAO reductase identified the activities as the same high-M_r enzyme. The major peak of activity corresponded

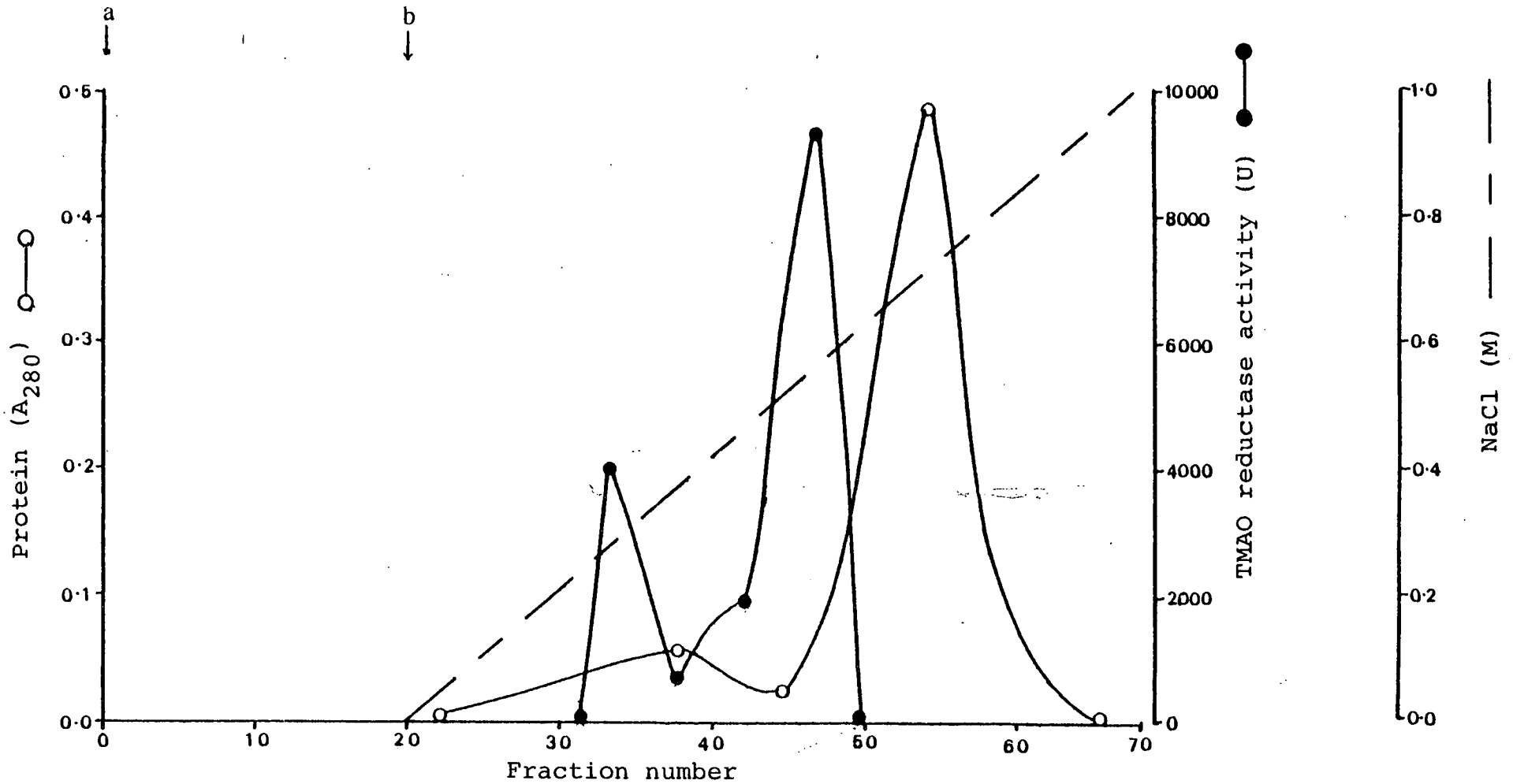


Figure 3.11 Ion Exchange Chromatography of Periplasm on DEAE Sepharose CL 6B

Sample Periplasm (5 ml 1.5 mg protein ml^{-1})
Buffer 50mM Tris HCl pH7.0
Gradient 0.0-1.0 M NaCl
Flow rate 12 ml h^{-1}
Fraction volume 6 ml
a, Wash; b, Elution

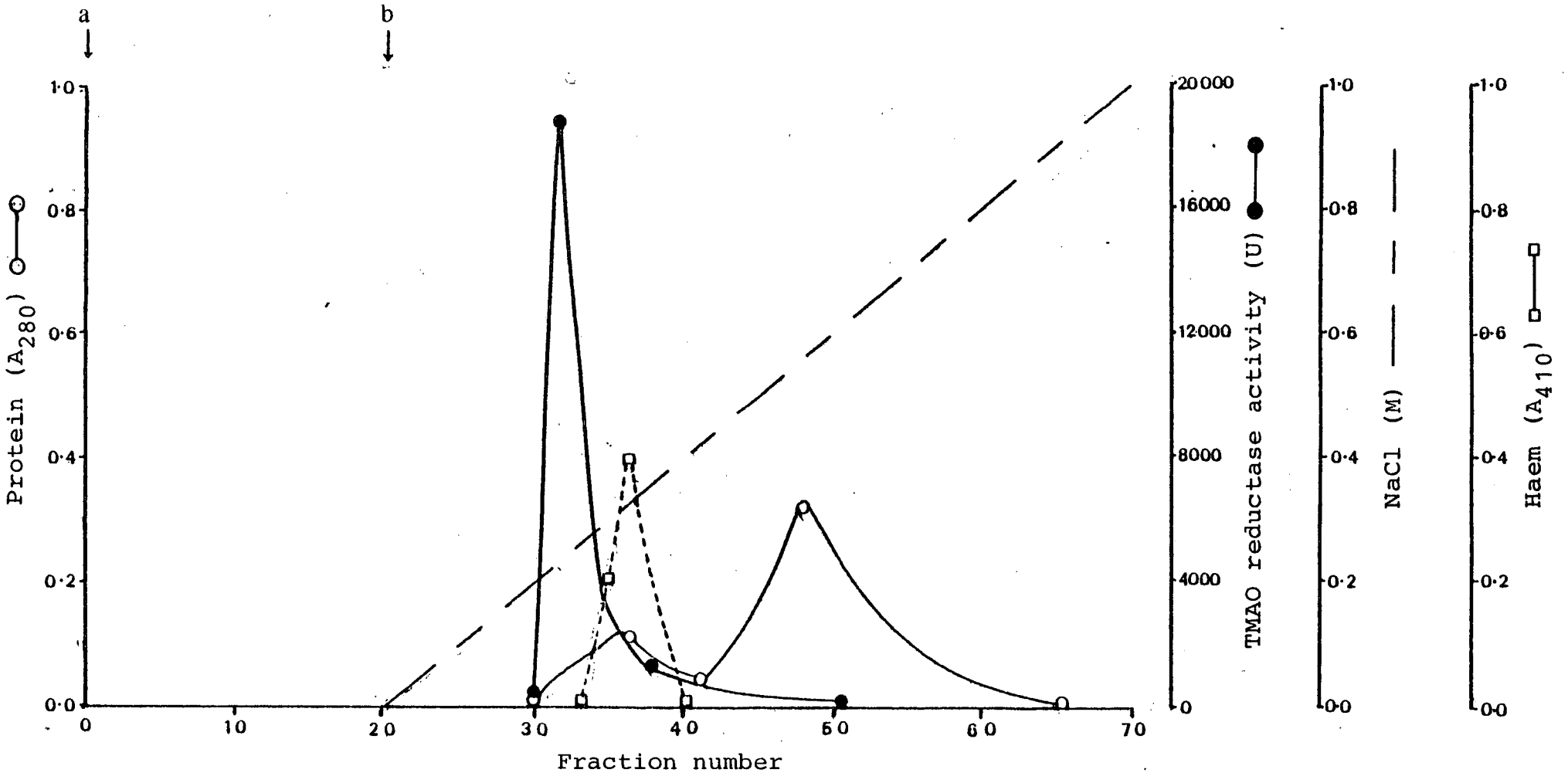


Figure 3.12 Ion Exchange Chromatography of 60-80% $(NH_4)_2SO_4$ Precipitated Periplasm (I)

Sample 60-80% $(NH_4)_2SO_4$ precipitated periplasm (5 ml, 1.1 mg protein ml^{-1})
Buffer 50mM Tris HCl pH7.0
Gradient 0.0-1.0M NaCl
Flow rate 12 ml h^{-1}
Fraction volume 6 ml
a, Wash; b, Elution

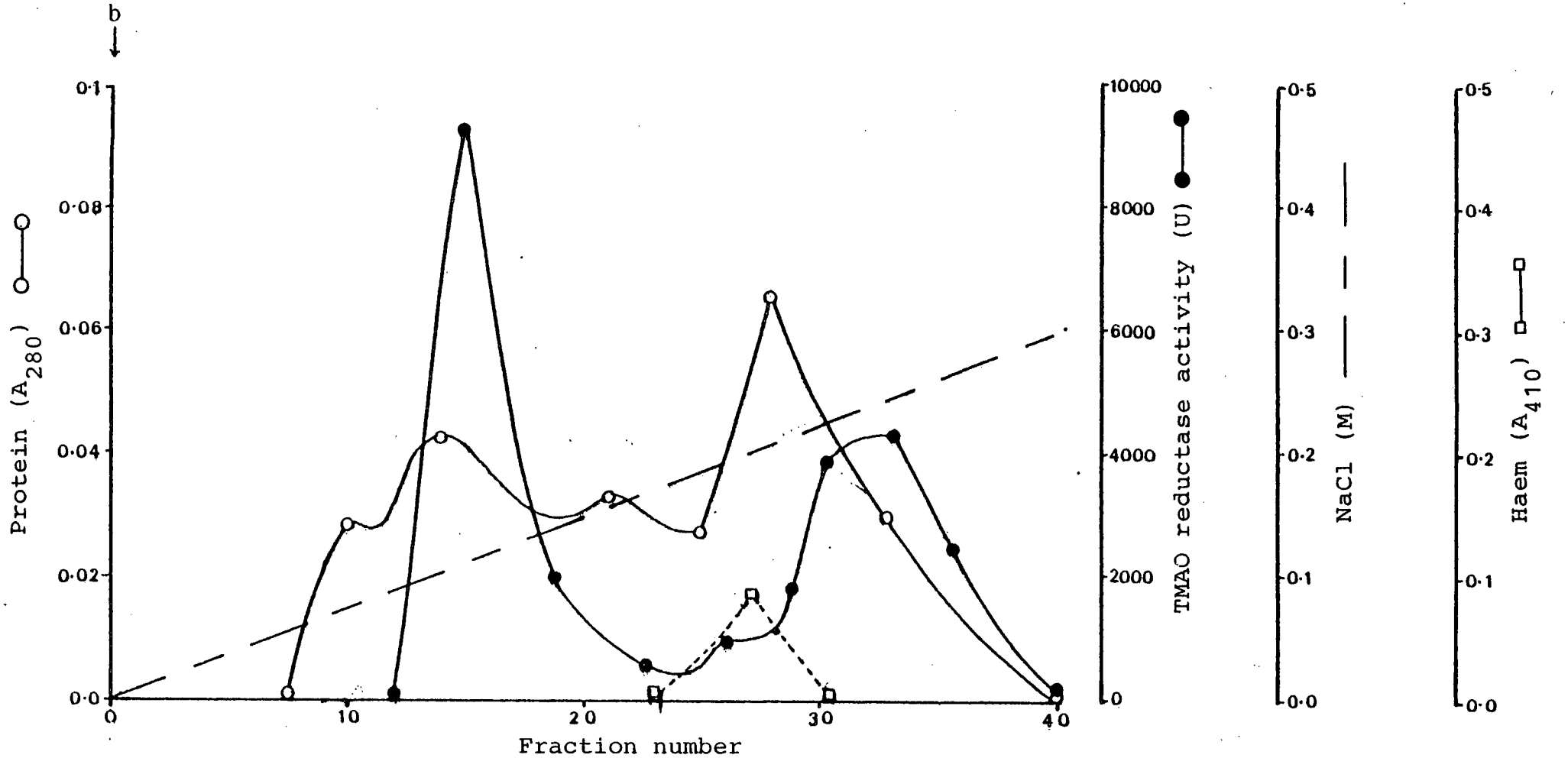


Figure 3.13 Ion Exchange Chromatography of 60-80% $(NH_4)_2SO_4$ Precipitated Periplasm (II)

Sample	60-80% $(NH_4)_2SO_4$ precipitated periplasm (1.1 mg protein ml^{-1})
Buffer	50mM Tris HCl, pH7.0
Gradient	0.0-0.3 M NaCl
Flow rate	12 ml h^{-1}
Fraction volume	7.5 ml
b,	Elution

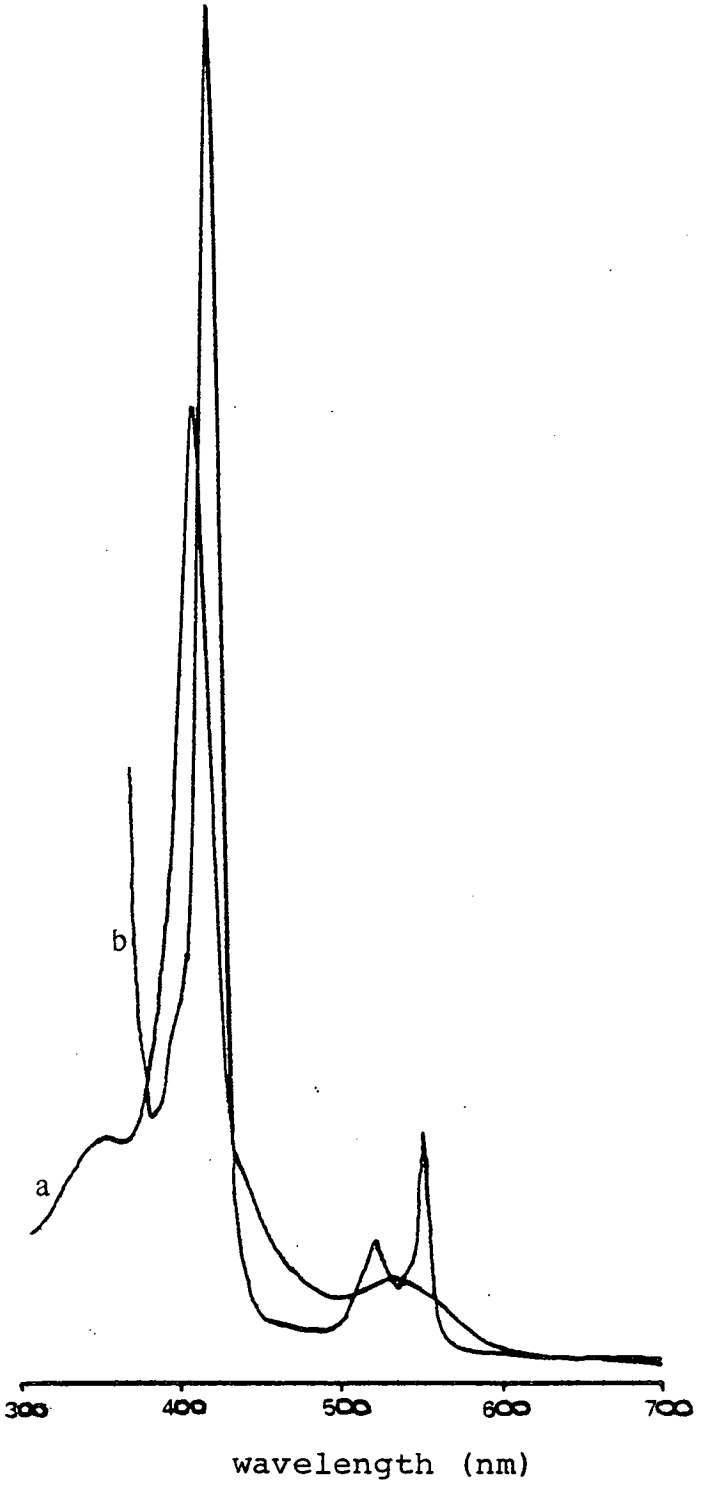


Figure 3.14 Oxidized and Reduced Spectra of a Cytochrome Eluted after Ion Exchange Chromatography.

a, Oxidized Spectrum

b, Reduced Spectrum

to a shoulder of a protein peak indicating contamination of the enzyme fractions with other protein. This was confirmed by SDS-PAGE and protein staining of fractions from the active peak, revealing approximately 12-15 bands, the high-M_r TMAO reductase being the most prominent (Plate 3.7). The bulk of non-enzymic protein remained bound to the column at 0.3M NaCl and was removed during re-equilibration of the column with buffer containing 1.0M NaCl. The fractions from the major peak of activity were pooled and the yield and purification obtained after ion exchange chromatography of the 60-80% $(NH_4)_2SO_4$ precipitate is given in Table 3.7. A drop in yield to approximately 18% occurred after ion exchange chromatography of the precipitate, little activity being lost during the dialysis prior to loading. An approximate 6-fold increase in purification was obtained.

3.2.3 Hydroxyapatite Chromatography

Hydroxyapatite chromatography of dialysed periplasm was performed as described in section 2.6.3. A single broad peak of protein was eluted at approximately 0.25M K_2HPO_4 (Figure 3.15), with little resolution of proteins into individual peaks. TMAO reductase activity eluted as a similar broad peak, following the peak of protein, at approximately 0.35M K_2HPO_4. The resolution of TMAO reductase from the major amount of protein was considered useful for purification purposes.

To assess the use of hydroxyapatite chromatography at a later stage of TMAO reductase purification, the dialysed pooled activities from ion exchange chromatography were used as samples (Figure 3.16). TMAO reductase activity was coincident with the major peak of protein at 0.35M K_2HPO_4 and was well separated from two minor peaks of protein eluting before and after that of the enzyme. The purification obtained and the yield of activity remaining after chromatography of the pooled peak of TMAO reductase is given in Table 3.8. The index of purification increased approximately two fold to 35 with the yield dropping to about 8%.

Table 3.7 Ion Exchange Chromatography of the TMAO Reductase Activity Pooled from the 60-80% $(NH_4)_2SO_4$ Precipitate of Periplasm

Sample	Purification (fold)	Yield (%)
Periplasm	1.0	100
60-80% $(NH_4)_2SO_4$ Precipitate	3.7±0.9 (1.8)	30.5±4.8 (9.3)
Ion exchange pool	16.8±3.8 (7.5)	18.0±5.5 (10.8)

n = 5

For Legend see Table 3.6

Table 3.8 Effect of Hydroxyapatite Chromatography on Pooled TMAO Reductase Fractions Obtained after Ion Exchange Chromatography

Sample	Purification (fold)	Yield (%)
Ion exchange pool	16.8±3.8 (7.5)	18.0±5.5 (10.8)
Hydroxyapatite pool	34.5±8.0 (15.8)	7.9±1.3 (2.6)

n = 4

For Legend see Table 3.6

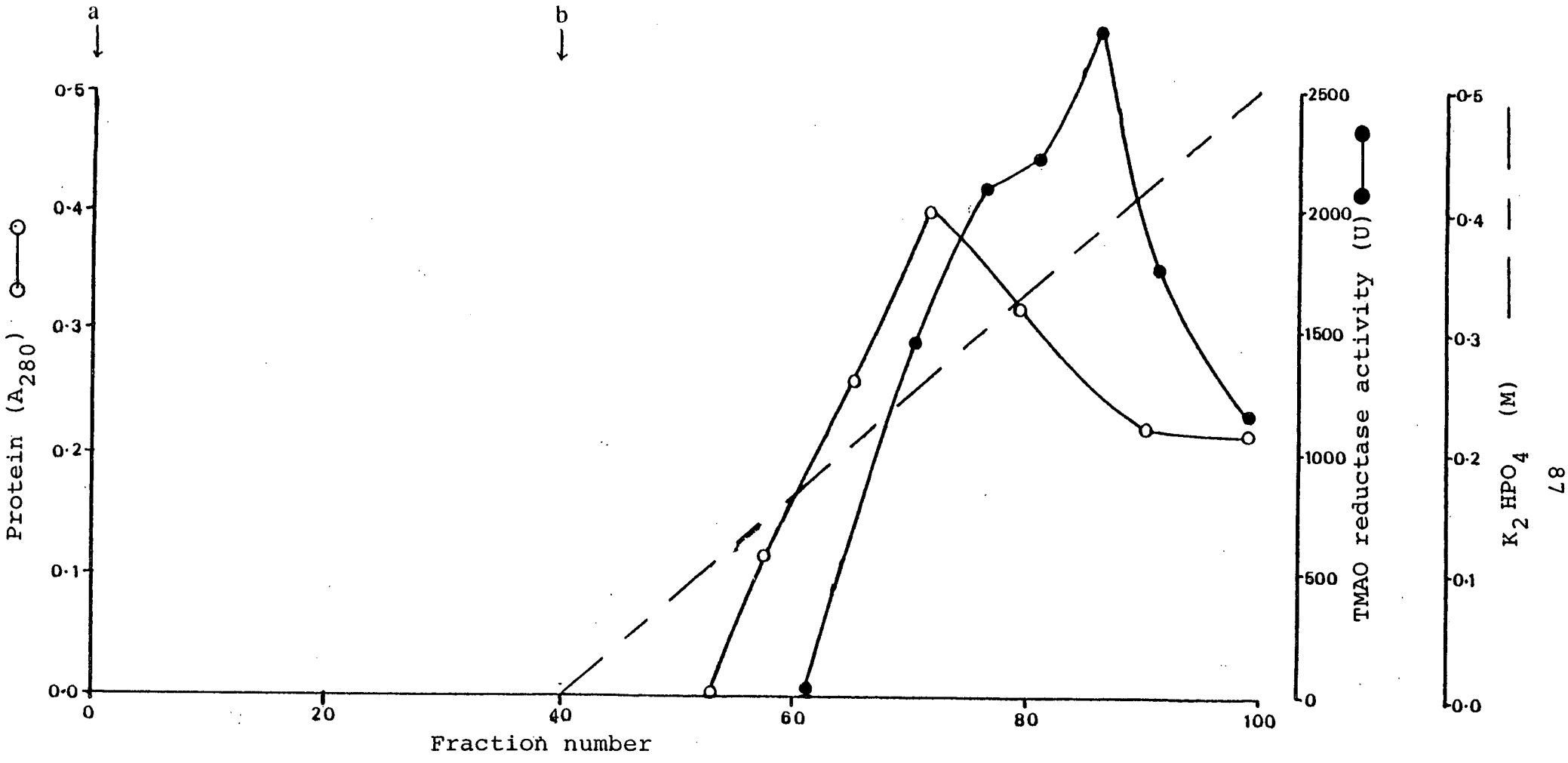


Figure 3.15 Hydroxyapatite Chromatography of Periplasm

Sample	Periplasm (10 ml, 1.9 mg protein ml^{-1})
Buffer	a, Wash, 10mM K_2HPO_4
	b, Elution 10-500mM K_2HPO_4
Flow rate	12 ml h^{-1}
Fraction volume	5 ml

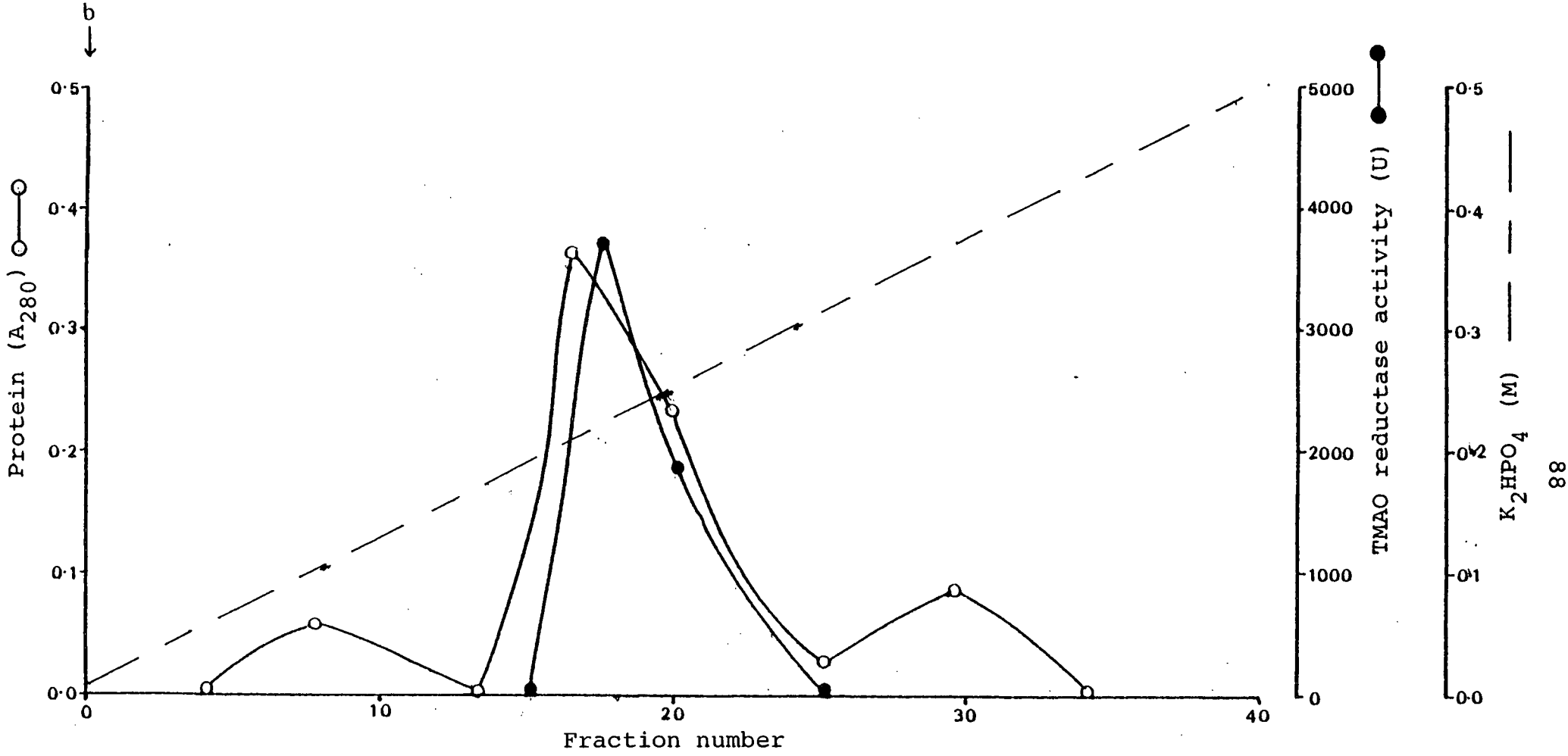


Figure 3.16 Hydroxyapatite Chromatography of Pooled TMAO Reductase Active Fractions from Ion Exchange Chromatography

Sample TMAO reductase active pool from ion exchange chromatography (30 ml, 0.19 mg protein ml^{-1})
Buffer b, Elution 10-500mM K_2HPO_4
Flow rate 12 ml h^{-1}
Fraction volume 7.5 ml

Before SDS-PAGE of the active fractions from hydroxyapatite chromatography, samples were dialysed against ion exchange buffer to prevent SDS precipitation by PO_4^{3-}. Gels stained for protein (Plate 3.7) revealed 2-3 bands, of which the high M_r TMAO reductase was the most prominent.

3.2.4 Affinity Chromatography

As an alternative approach to the methods of purification dependent upon the physical properties of TMAO reductase, affinity chromatography was attempted in order to make use of the biological activity of the enzyme. Experiments by Easter (1982) identified betaine as a suitable ligand, that when immobilized onto an inert support would selectively adsorb TMAO reductase from periplasm. Columns of betaine linked AH-Sepharose 4B were prepared (2.6.4) to repeat and improve the results obtained. As with previous experiments, periplasm was used as an initial sample in order to assess the use of the technique as an initial step in purification. Dialysed samples were loaded, washed and eluted with fractions monitored for protein content and TMAO reductase activity (Figure 3.17). Approximately 30% of the loaded protein did not bind to the column and was eluted during the wash phase. Bound protein was eluted in two peaks at 0.2M and 0.7M NaCl with TMAO reductase present in fractions at about 0.4M NaCl. No resolution was made between the two TMAO reductase activities.

Experiments were undertaken to evaluate the merit of this technique as a later stage method for the purification of TMAO reductase. Dialysed samples from ion exchange chromatography of periplasm containing TMAO reductase, were loaded onto the column and the column washed with buffer containing 0.2M NaCl in an attempt to remove some non-enzymic bound protein. Figure 3.18 presents the results obtained, with protein and TMAO reductase activity being eluted during the wash and gradient phases. At this concentration of NaCl, TMAO reductase appeared weakly bound and was eluted continuously, rather than remaining attached to the column.

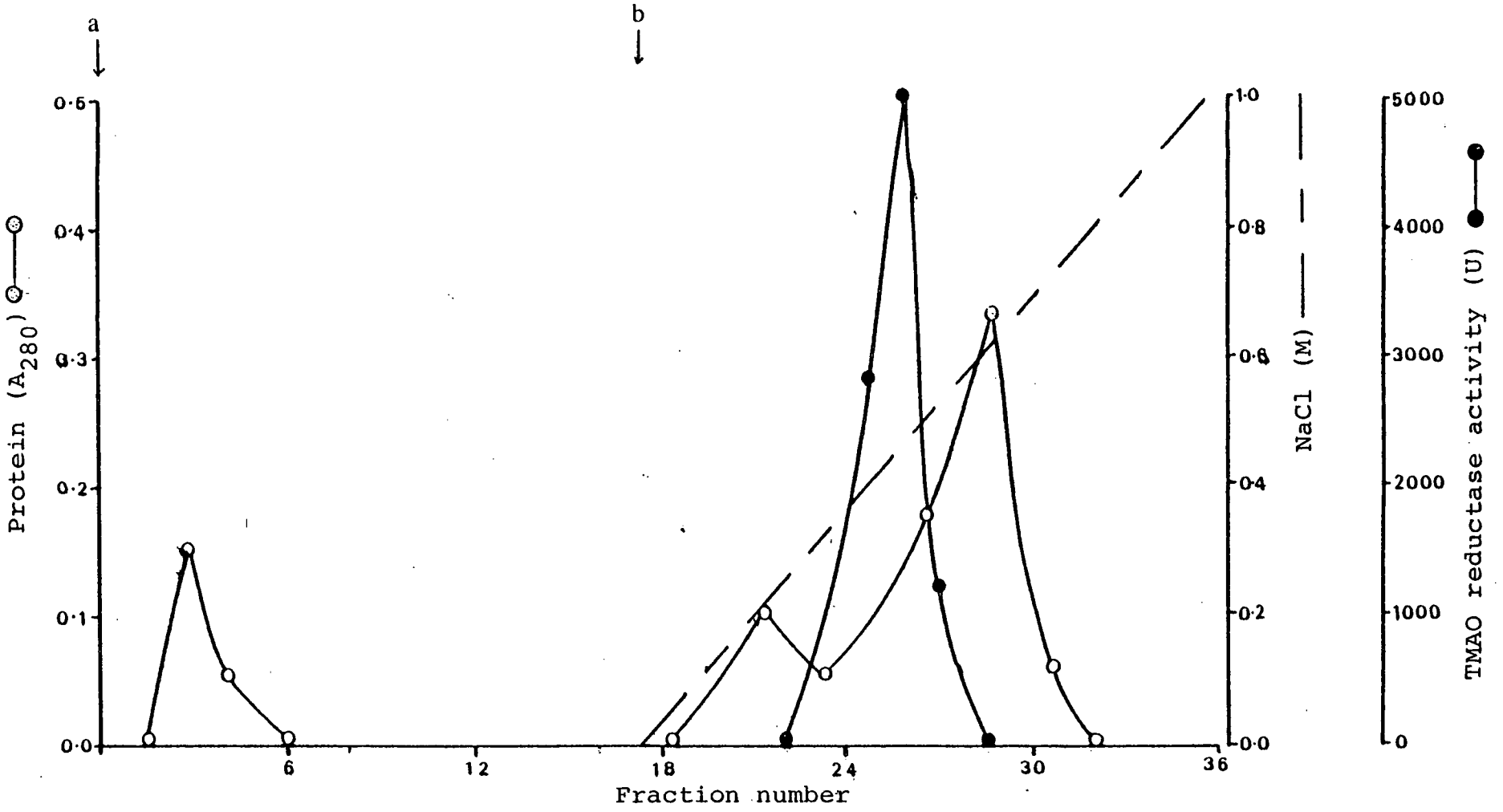


Figure 3.17 Affinity Chromatography of Periplasm

Sample	Periplasm (5 ml, 1.5 mg protein ml^{-1})
Buffer	10mM Tris HCl, pH7.0
Gradient	0.0-1.0 M NaCl
Flow rate	8 ml h^{-1}
Fraction volume	8 ml

a, Wash; b, Elution

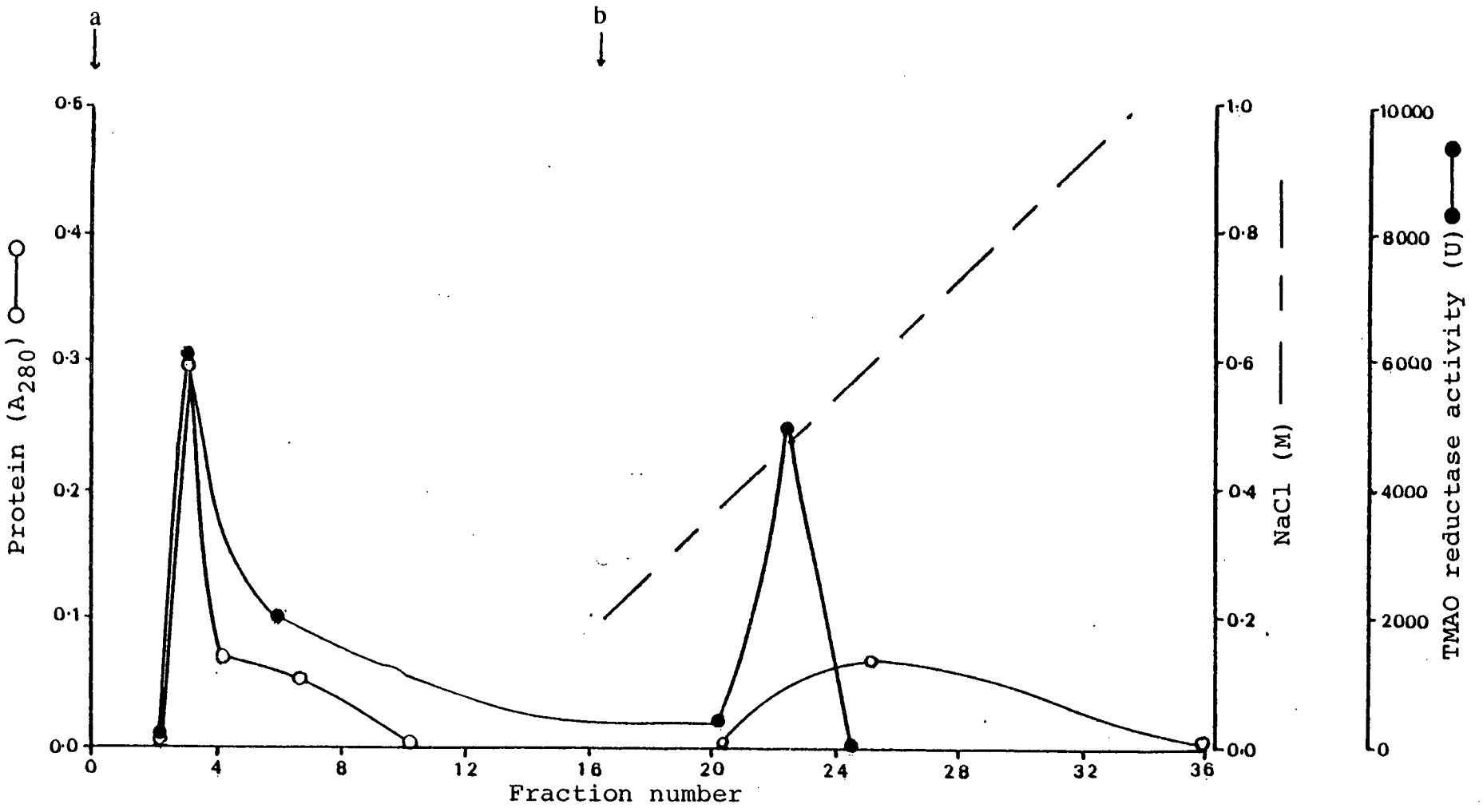


Figure 3.18 Affinity Chromatography of TMAO Reductase Active Fractions from Ion Exchange Chromatography of 60-80% Precipitated Periplasm.

Sample	Pooled fraction (7 ml, 0.8 mg protein ml^{-1})
Buffer	10mM Tris HCl pH 7.0
Gradient	0.2-1.0M NaCl
Flow rate	8 ml h^{-1}
Fraction volume	8 ml

a, Wash; b, Elution

3.2.5 Gel Filtration

The separation of proteins in periplasm on the basis of their molecular weight was carried out using gel filtration (2.6.1). Salt was present in the elution buffer to increase the ionic strength of the solvent as a safeguard against possible ionic interactions with the gel matrix. Samples of periplasm were loaded onto a column of Sepharose 4B, having a molecular weight fractionation range of $3x10^4$-$5x10^6$ daltons. Approximately four distinct peaks of protein were eluted (Figure 3.19) with TMAO reductase activity associated with the first and third peak. SDS-PAGE followed by zymogram staining of samples from the enzyme peaks indicated the presence of both the high- and low-M_r TMAO reductases of periplasm in each peak, resolution between the two proteins not being achieved. To assign a molecular weight to each of the eluted TMAO reductase peaks, the column was calibrated with a series of molecular weight standard proteins (2.6.1, Figure 3.20). The first peak, containing activity, eluted in the void volume of the column indicating a molecular weight for the proteins within the peak of approximately $5x10^6$ daltons or greater. The third peak also containing activity had an estimated molecular weight between 43 000 and 158 000 daltons.

Gel filtration was considered unsatisfactory as an early step in the purification of TMAO reductase because of poor column capacity and sample resolution. However the applicability of gel filtration for a later stage of purification was assessed. Sephacryl S-300 was chosen as the gel filtration matrix having a narrower fractionation range of $1x10^4$-$1.5x10^6$ daltons. The pooled activity from hydroxyapatite chromatography was used as a sample for gel filtration at a flow rate of 12 ml h^{-1}. A single peak of protein was eluted from the column and was coincident with TMAO reductase activity (Figure 3.21). Fractions from this peak were analysed by SDS-PAGE followed by protein staining revealing 2-3 protein bands of Rf 0.32, 0.37 and 0.61. The band of Rf 0.32 was the most prominent band and corresponded to TMAO reductase activity after zymogram staining. The bands

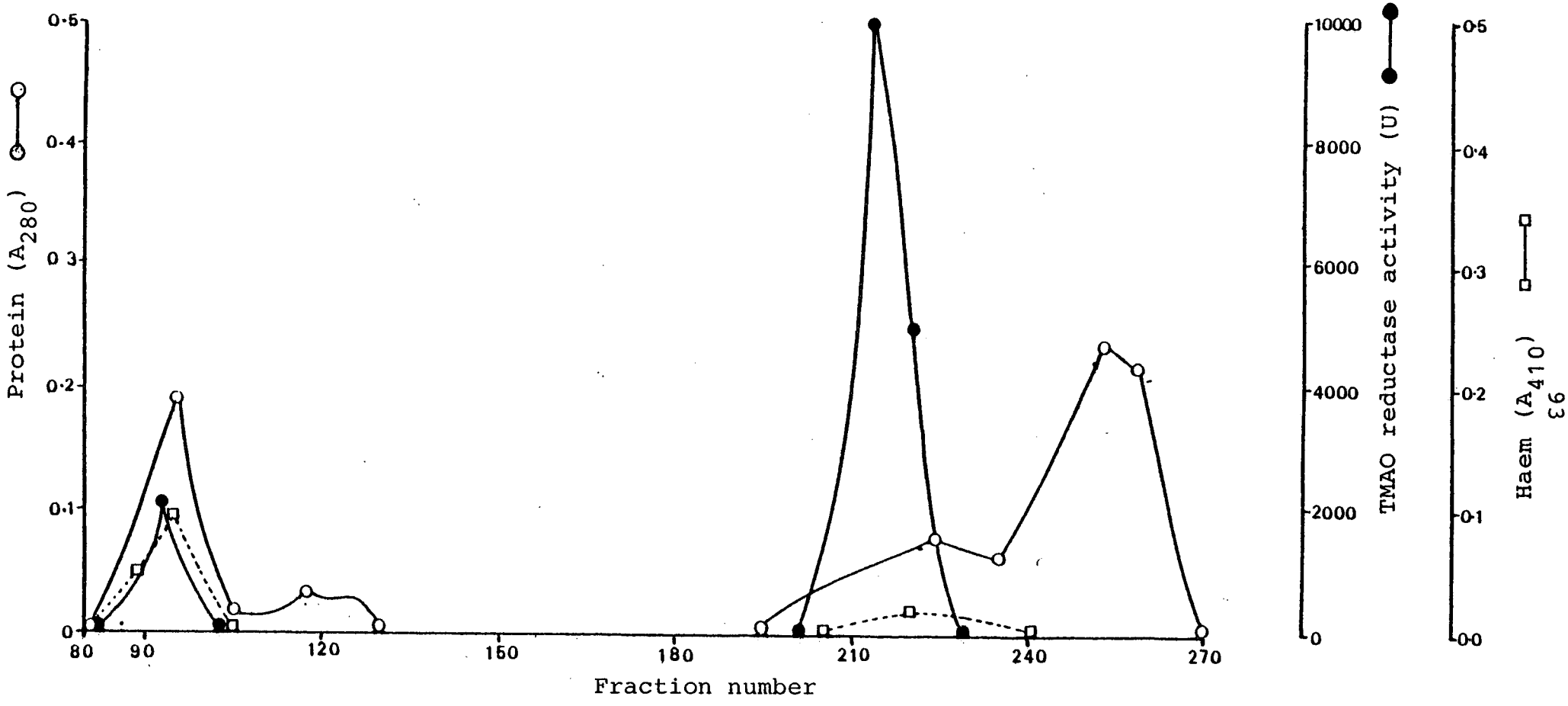


Figure 3.19 Gel Filtration of Periplasm on Sepharose 4B

Sample	Periplasm (5 ml, 1.5 mg protein ml^{-1})
Buffer	10mM Tris HCl, pH7.2 + 0.2M NaCl
Flow rate	15 ml h^{-1}
Fraction volume	1 ml

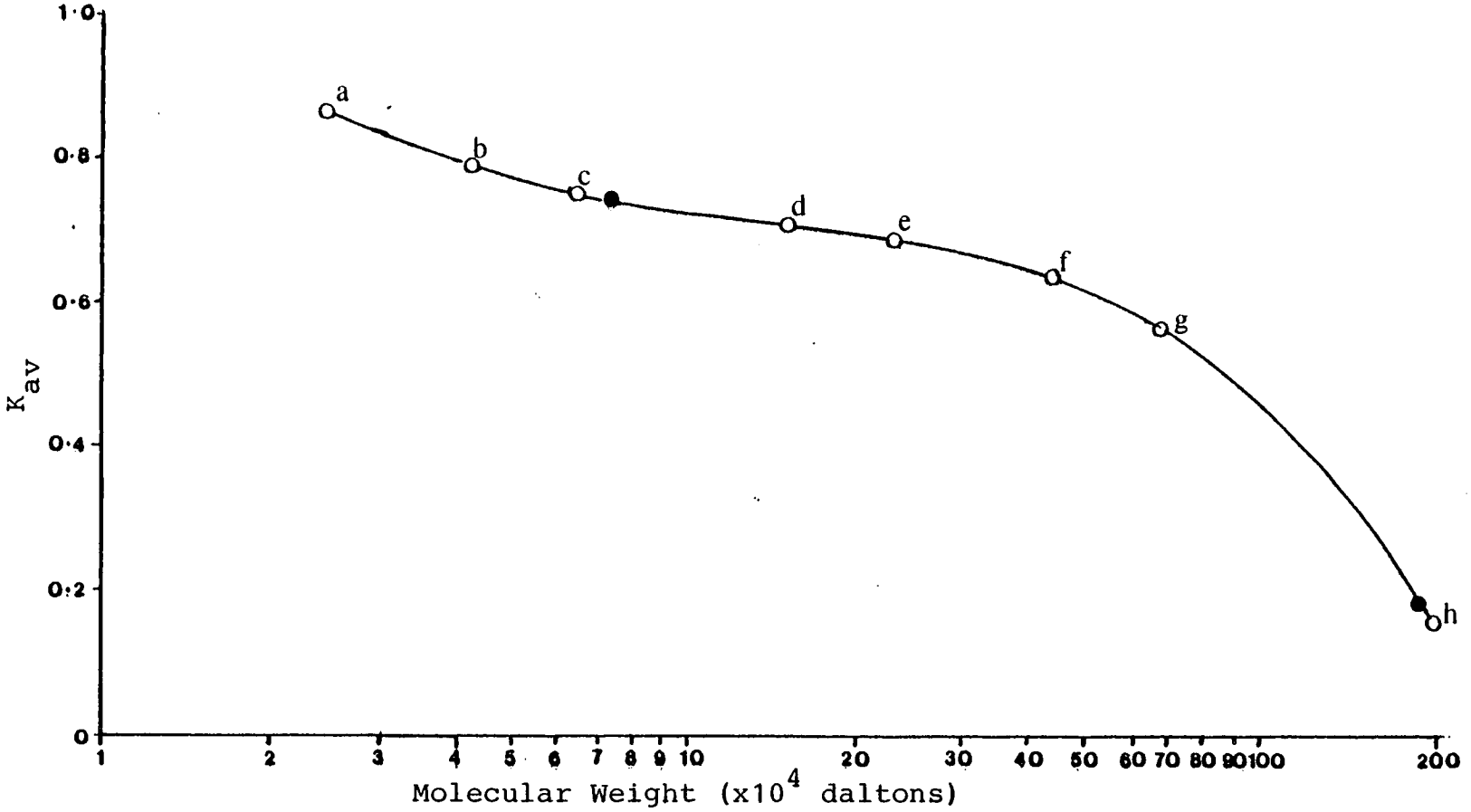


Figure 3.20 Molecular Weight Calibration Curve for Sepharose 4B Gel Filtration

Buffer	10mM Tris HCl, pH7.2 + 0.2M NaCl
Flow rate	15 ml h^{-1}
K_{av}	Partition coefficient

a, Chymotrypsinogen A (23 k daltons); b, Ovalbumin (44 k daltons); (c) Bovine serum albumin (67 k daltons); d, Aldolase (158 k daltons); e, Catalase (232 k daltons); f, Ferritin (440 k daltons); g, Thyroglobulin (660 k daltons); h, Blue dextran (2x10^6 daltons); ● TMAO reductase

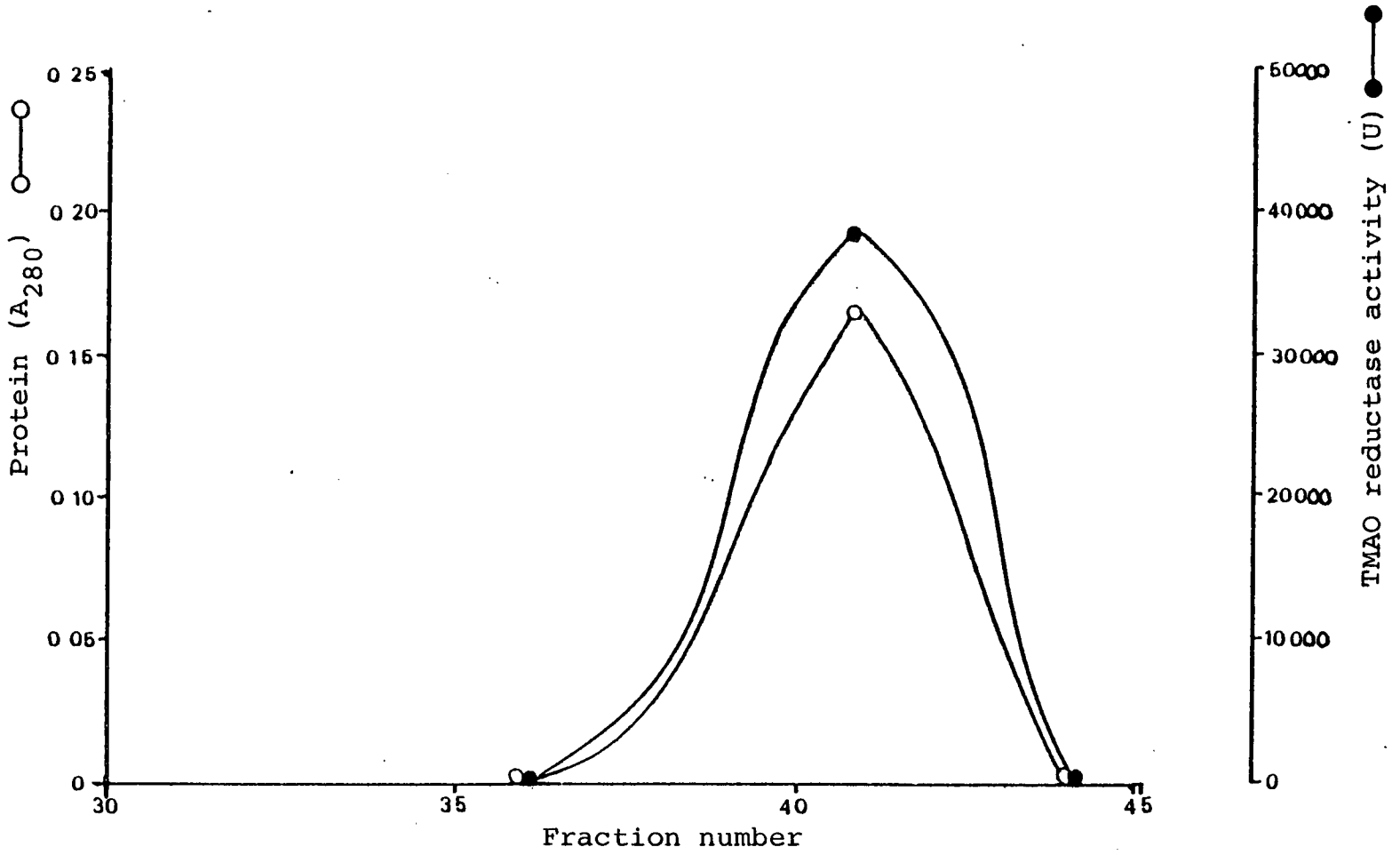


Figure 3.21 Gel Filtration of Hydroxyapatite TMAO Reductase Active Fractions on Sephacryl S-300

Sample	Pooled Fraction (5 ml, 0.6mg protein ml^{-1})
Buffer	10mM Tris HCl, pH 7.2 + 0.2M NaCl
Flow rate	12 ml h^{-1}
Fraction volume	3 ml

of Rf 0.37 and 0.61 were faint and without activity. These three proteins were also present in the sample before gel filtration and it was considered that no further purification had been achieved. The fractions containing TMAO reductase activity were concentrated by ultrafiltration, the protein concentrate appearing faintly brown in colour. Samples from this concentrate were subjected to reduced minus oxidized spectroscopy (2.14) to determine the presence of cytochrome. The results (Figure 3.22) indicated the presence of a c-type cytochrome with an α-band absorption maximum at 552 nm. This absorption peak was evident only after reduction using dithionite, but not with a reductant of higher potential like phenazine methosulphate (PMS) - ascorbate indicating the cytochrome to be of low redox potential. Using the α-band peak height and an extinction coefficient for cytochrome *c* of 24.1$mM^{-1}cm^{-1}$ (Fugita, 1966), the concentration of cytochrome was estimated to be 0.2 μM. To assign one of the bands of protein visible after SDS-PAGE, with the cytochrome, SDS-PAGE was repeated and followed by the peroxidase stain of Thomas *et al.* (1976, 2.12.4). A faint, blue staining band of Rf 0.37 was visible and identified this component as the cytochrome. By comparison of the Rf value with those obtained for standard proteins after SDS-PAGE (3.3.1, Figure 3.26) the estimated molecular weight of this cytochrome was 80 000 daltons.

In an attempt to improve the resolution of the column to obtain pure TMAO reductase, the elution flow rate for gel filtration was reduced to 8 ml h^{-1}. The same samples as used previously were loaded, with the elution of a large peak of protein coincident with TMAO reductase activity. Two minor peaks of protein, prior to and following the large peak, were also eluted from the column, both lacking enzyme activity (Figure 3.23). SDS-PAGE of the colourless concentrated peak (ultrafiltration, 2.16) gave a single band of protein, after staining for protein, using the silver and Coomassie blue methods (Plate 3.6). Reduced minus oxidized spectra were recorded for this sample and are presented in Figure 3.24. A shoulder of absorbance was

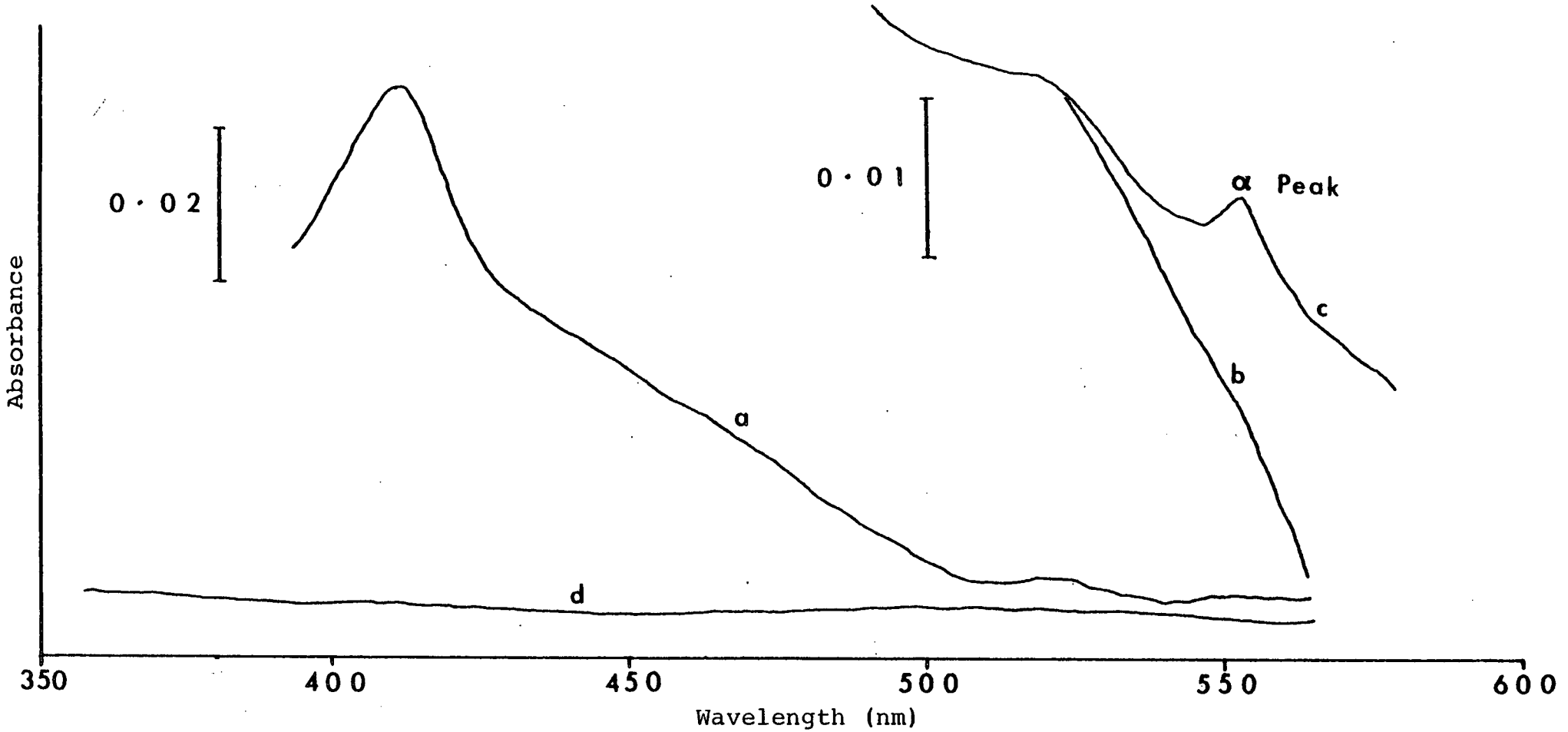


Figure 3.22 Absorption Spectrum of Concentrated TMAO Reductase Activity after Gel Filtration (impure)

a, Oxidized spectrum
b, Ascorbate-PMS reduced spectrum
c, Dithionite reduced spectrum
d, Baseline

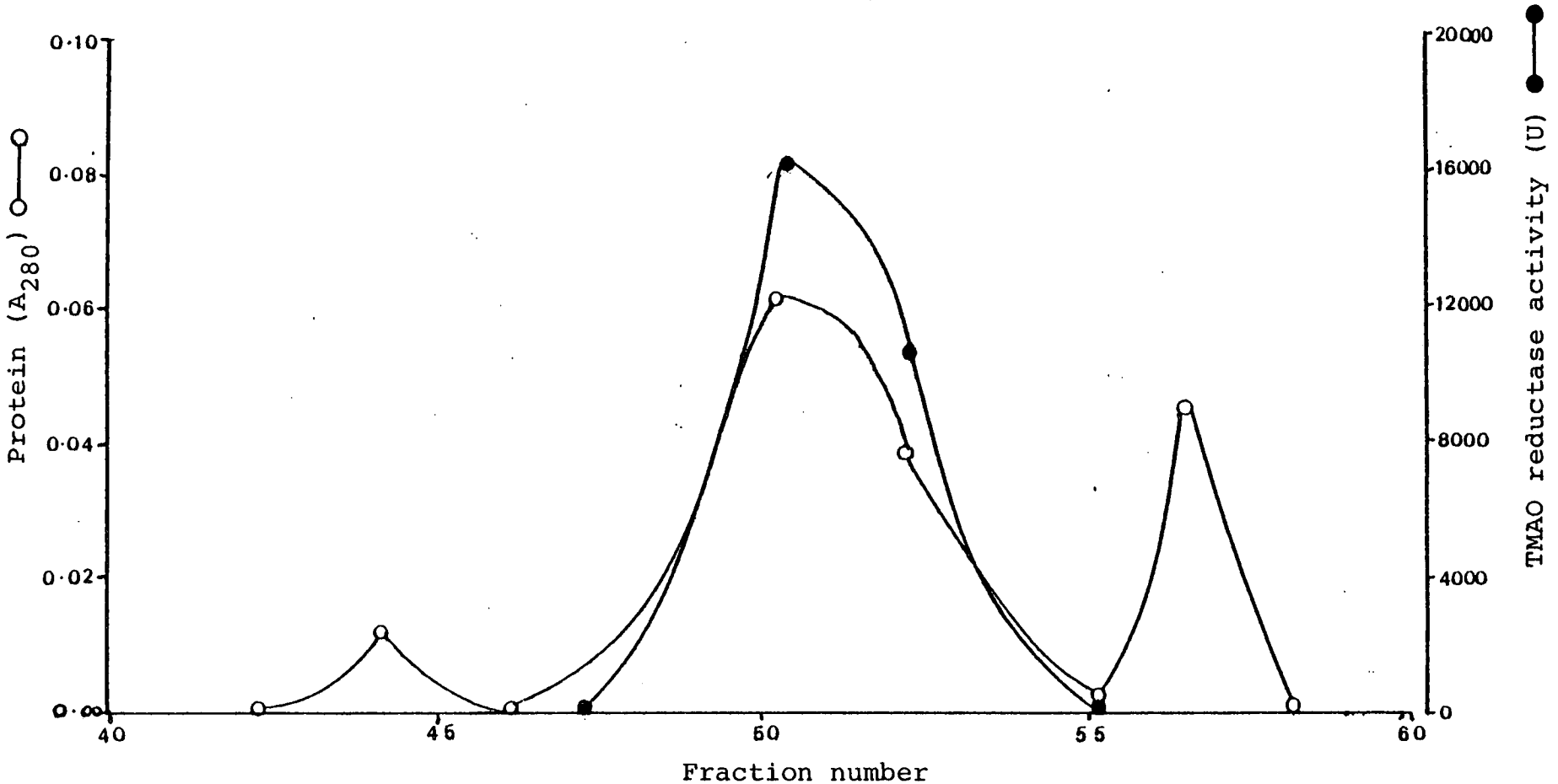


Figure 3.23 Sephacryl S-300 Gel Filtration of TMAO Reductase Active Fractions from Hydroxyapatite Chromatography

Sample	Pooled fraction (5 ml, 0.6 mg protein ml^{-1})
Buffer	10mM Tris HCl, pH7.2 + 0.2M NaCl
Flow rate	8 ml h^{-1}
Fraction volume	3 ml

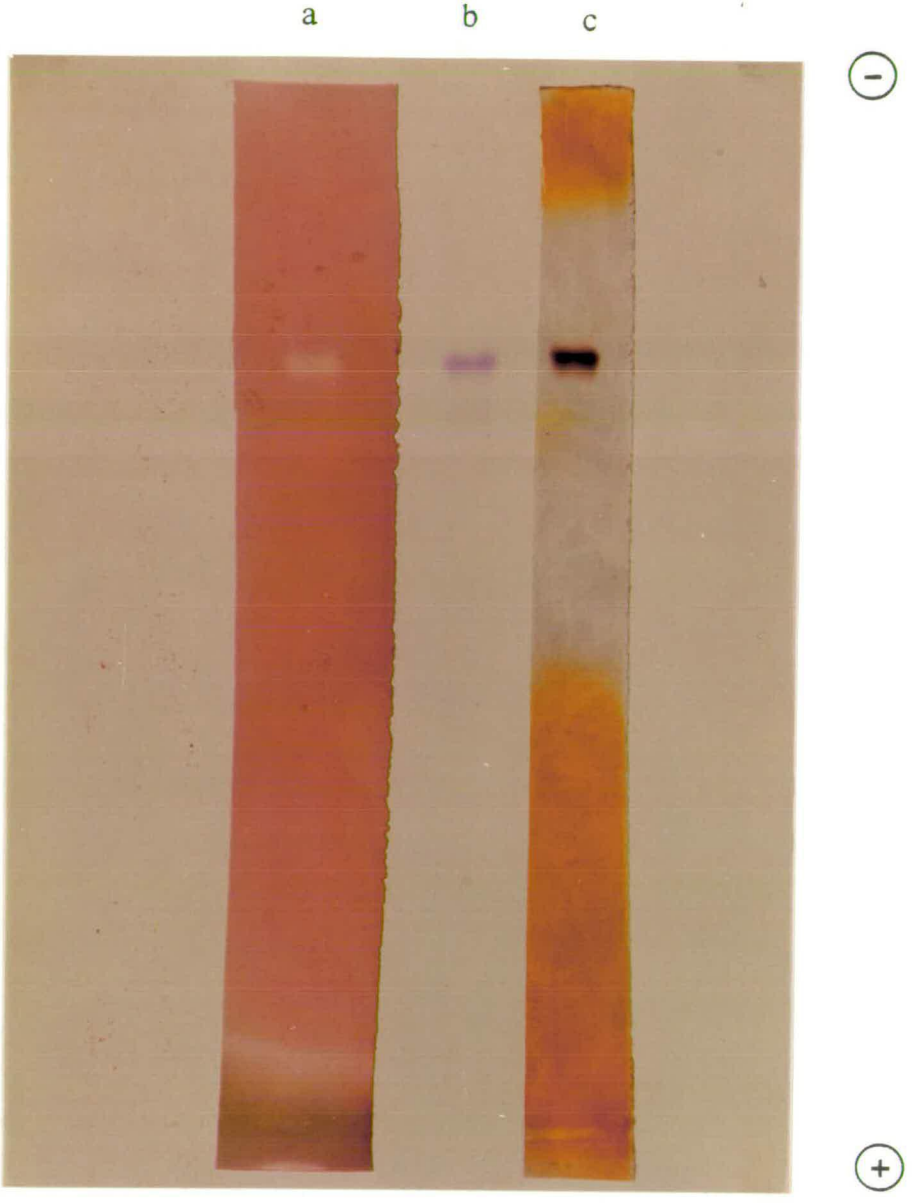


Plate 3.6 SDS-PAGE of Purified TMAO Reductase (Zymogram and Protein Stains)

		Protein load (µg)
a	Zymogram stain	2
b	Coomassie blue protein stain	10
c	Silver protein stain	2

(7.5-15% acrylamide)

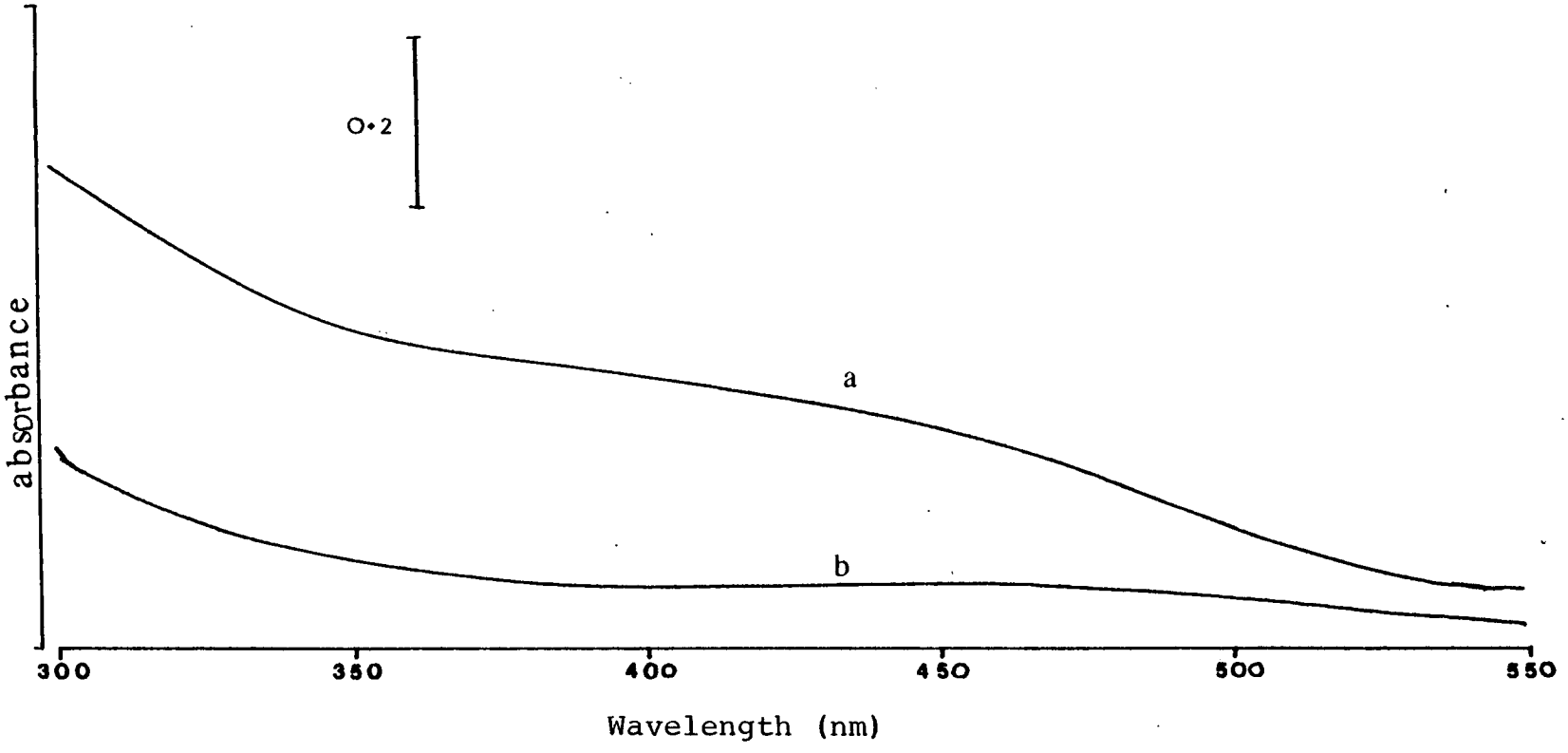


Figure 3.24 Oxidized and Reduced Spectra for Purified TMAO Reductase

a, Oxidized spectrum

b, Dithionite reduced spectrum

present between 350 and 450 nm, this being lost in the presence of dithionite. A molecular weight calibration curve was prepared for the Sephacryl S-300 column in the same way as for Sepharose 4B, using Blue Dextran as void volume marker (Figure 3.25). By comparison of the partition coefficient for the purified enzyme with those for the standard proteins, the molecular weight of native TMAO reductase was estimated to be 84 000 daltons. The purification and yield obtained after gel filtration of the hydroxyapatite pool are given in Table 3.9. Purification was increased by approximately 50% with little loss in total activity.

3.2.6 Protocol for Purification of TMAO Reductase

From the results obtained by precipitation and liquid chromatography of periplasm and partially purified samples, the techniques were combined to produce a protocol for the purification of TMAO reductase. The sequence listed in Table 3.10 resulted in a purified preparation of the high-M_r TMAO reductase with a moderate yield of activity. The Table presents the results of a typical purification experiment beginning with the periplasm obtained from a 7.5 l. culture of *Alteromonas* sp. NCMB 400, through to the purified enzyme. This protocol was reproducible, giving similar results with four replicates. Samples from each stage of the purification procedure were analysed by SDS-PAGE and protein staining so as to monitor the resolution of TMAO reductase from other proteins (Plate 3.7).

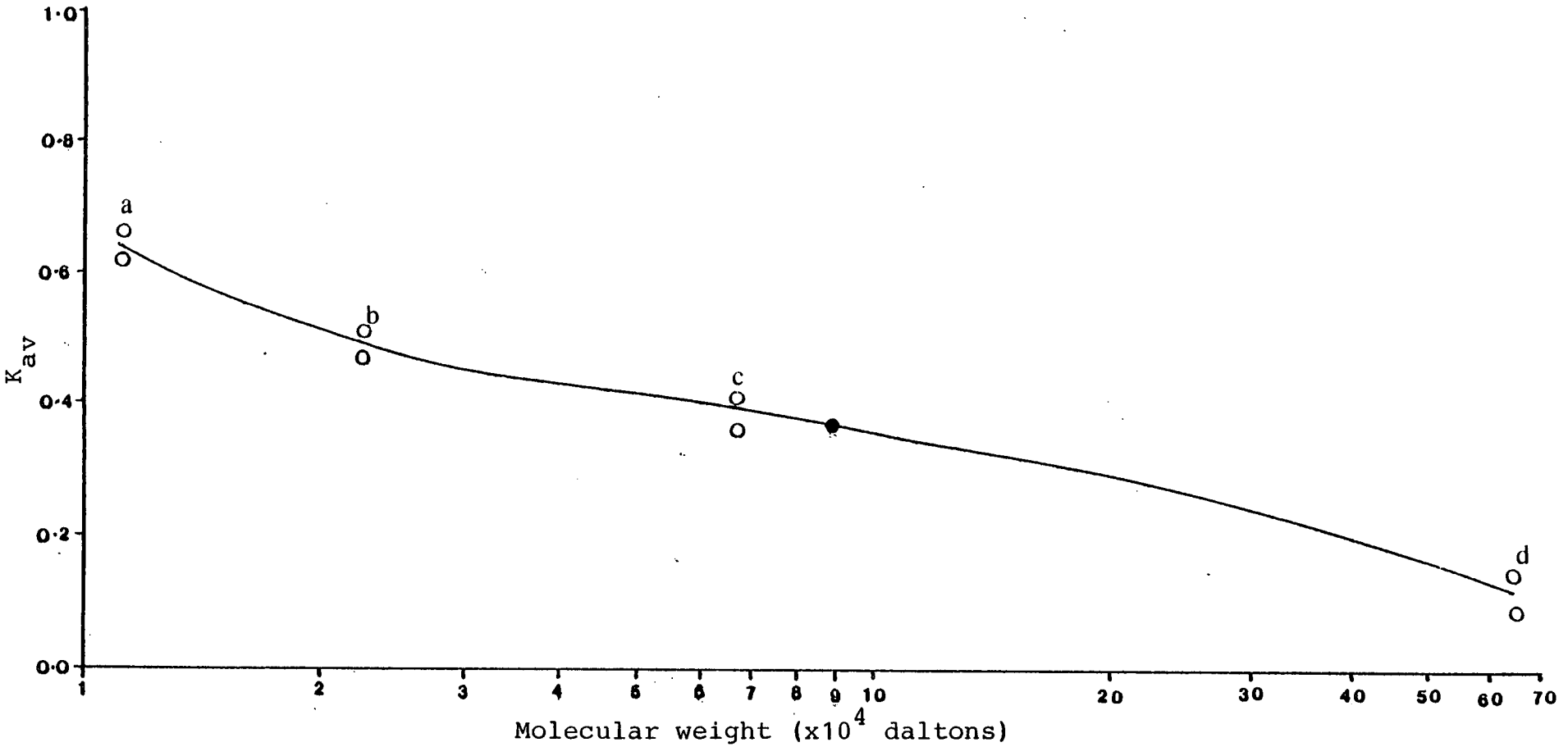


Figure 3.25 Molecular Weight Calibration Curve for Gel Filtration on Sephacryl S-300

Buffer 10mM Tris HCl, pH7.2 + 0.2M NaCl
Flow rate 8 ml h^{-1}
K_{av} Partition coefficient

a, Cytochrome *c* (12 k daltons)
b, Chymotrypsinogen A (23 k daltons)
c, Bovine serum albumin (67 k daltons)
d, Thyroglobulin (660 k daltons)
●, TMAO reductase

Table 3.9 Effect of Gel Filtration on Pooled TMAO Reductase Activity Following Hydroxyapatite Chromatography

Sample	Purification (fold)	Yield (%)
Hydroxyapatite pool	34.5±8.0 (15.8)	7.9±1.3 (2.6)
Gel filtration pool	56.3±0.9 (1.8)	6.9±0.1 (0.2)

n = 4

For Legend see Table 3.6

Table 3.10 Complete Purification Scheme for Periplasmic TMAO Reductase

Sample	Volume (ml)	Protein (mg ml^{-1})	Specific Activity (U)	Purification (fold)	Total Protein (mg)	Total Activity (U)	Yield (%)
Periplasm	180	1.91	3 846	1.0	343.38	1 320 639	100
$(NH_4)_2SO_4$ 60-80% precipitate	36	1.36	10 671	2.8	48.96	522 452	39
Ion exchange pool	36	0.13	72 525	18.8	4.68	339 417	26
Hydroxyapatite pool	7	0.13	121 591	31.6	0.92	112 257	9
Gel filtration pool	1	0.44	211 530	55.9	0.44	92 447	7

a b c d e

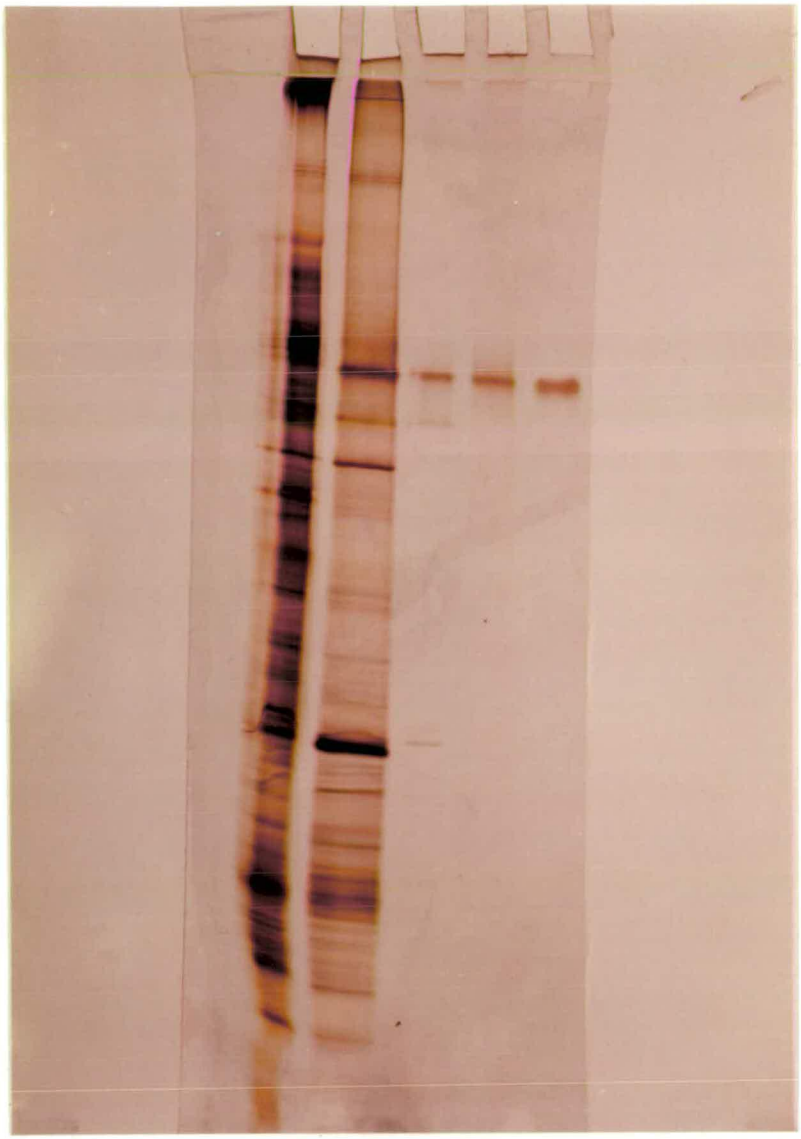


Plate 3.7 SDS-PAGE of Samples from the Purification Procedure (Protein Stain)

a Periplasm
b 60-80% $(NH_4)_2SO_4$ precipitate
c Ion exchange pool
d Hydroxyapatite pool
e Purified TMAO reductase

(7.5-15% acrylamide)

3.3 Characterization of Purified TMAO Reductase

3.3.1 Determination of TMAO Reductase Molecular Weight

An estimate of the molecular weight of the purified TMAO reductase was made by comparison with molecular weight standard proteins after SDS-PAGE (Table 3.11). All standard proteins were prepared in SDS sample buffer in the presence of dithiothreitol and with boiling (100°C). The purified TMAO reductase was prepared in SDS sample buffer with and without the reducing conditions. Electrophoresis was carried out using gels of 7.5-15% (Plate 3.8), 8-12% and 12% polyacrylamide concentration and the molecular weight of TMAO reductase was determined using the calibration curves obtained from each gel (Figure 3.26). Each of the gels of different polyacrylamide concentration gave the same molecular weight values. Under non-reducing conditions, TMAO reductase had a molecular weight of 90 000 daltons whereas under reducing conditions a value of 86 000 daltons was obtained. After electrophoresis under reducing conditions a smeared band was occasionally evident at the dye front, possibly a component of the enzyme.

3.3.2 Determination of TMAO Reductase Isoelectric Points

Flat-bed isoelectric focusing was used to determine the pI of TMAO reductase in periplasm and of the purified enzyme. Untreated samples of periplasm and purified enzyme were applied to separate lanes of a laboratory prepared gel (2.8) followed by electrofocusing. The gel was stained for protein and TMAO reductase activity together with a determination of the pH gradient across the gel. The zymogram stain revealed a series of irregular bands of activity from the periplasm sample, with estimated pI values of 4.0, 4.6, 5.1, 5.2 and 5.25. Purified enzyme gave a single irregular band of activity with an approximate pI of 5.6, distinct from the activities present in periplasm.

As NaCl, present in the periplasm and purified preparations of TMAO reductase is known to be a cause of band

Table 3.11 Estimated Molecular Weight of Purified TMAO Reductase by SDS-PAGE

Sample	Molecular Weight
TMAO reductase + SDS - dithiothreitol-100°C	90 000±816[1] (1600)[2]
TMAO reductase +SDS + dithiothreitol + 100°C	86 333±471[1] (923)[2]

n = 3

1 Standard error

2 Value with 95% confidence limits

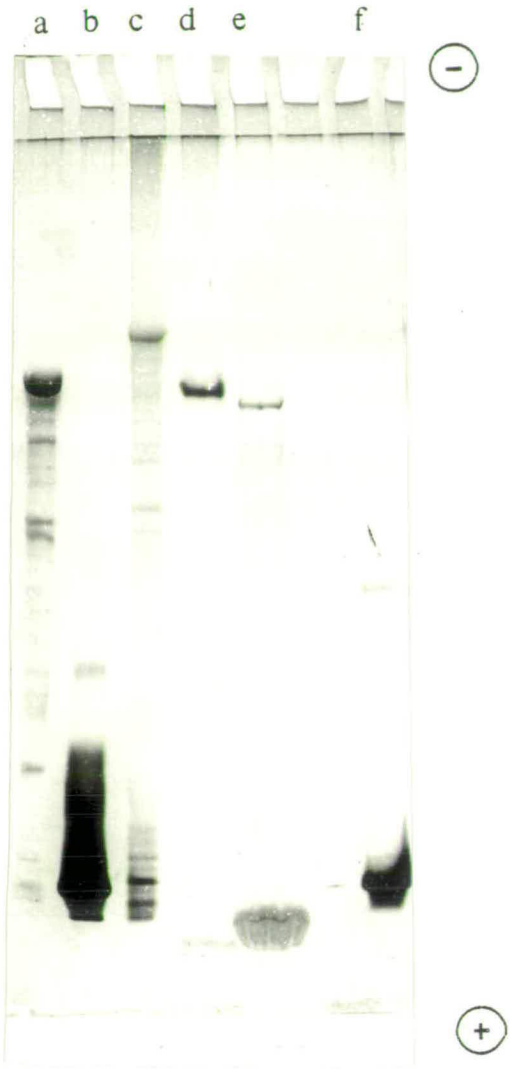


Plate 3.8 Molecular Weight Determination of Purified TMAO Reductase by SDS-PAGE

a Phosphorylase b (92.5 k daltons)
b Lysozyme (14 k daltons)
c β-Galactosidase (114 k daltons)
d Purified TMAO reductase (without reducing conditions)
e Purified TMAO reductase (with reducing conditions)
f Cytochrome *c* (12.5 k daltons)

(7.5-15% acrylamide, silver protein stain)

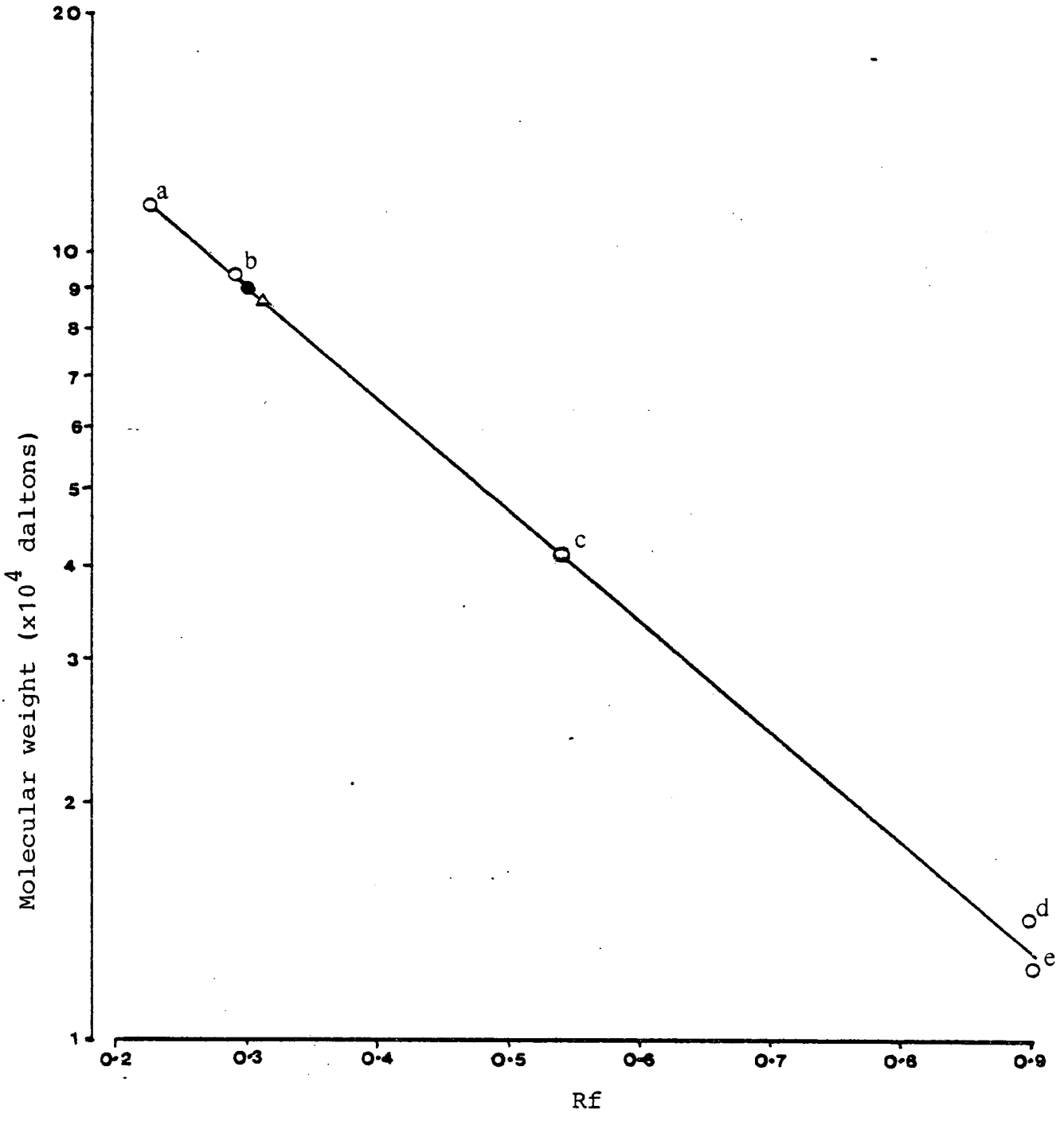


Figure 3.26 SDS-PAGE Molecular Weight Calibration Curve

a, β-Galactosidase (114 k daltons); b, phosphorylase b (92.5 k daltons); c, ovalbumin (43 k daltons); d, lysozyme (14 k daltons); e, cytochrome *c* (12.5 k daltons)

● TMAO reductase in the absence of reducing conditions

△ TMAO reductase in the presence of reducing conditions

(7.5-15% acrylamide)

perturbation during electrofocusing, each sample was dialysed overnight against 10mM Tris-HCl pH7.2 to remove salt. The dialysed samples of periplasm and purified enzyme were subjected to electrofocusing on an LKB-PAG plate, pH3.5-9.5 and stained for protein and enzyme activity (Plate 3.9) together with a determination of the pH gradient (Figure 3.27). The periplasm sample contained a series of regular bands of activity allowing accurate pI determinations of 4.4, 4.9 and 5.2. Bands of activity were also present at the sample application site. Purified TMAO reductase produced two bands of activity with pI values of 5.1 and 5.2. The protein stain of the periplasm sample revealed a series of bands at the acidic end of the gel with pI values between 3.5 and approximately 6.0. No protein was visible for the purified enzyme sample.

3.3.3 Determination of TMAO Reductase K_m and V_{max} for TMAO

To determine the half maximal substrate concentration ($K_{m\ TMAO}$) and the maximum velocity of enzyme catalysis with TMAO ($V_{max\ TMAO}$) purified TMAO reductase was assayed for activity in the presence of varying concentrations of the substrate (s). The electron donor for the enzyme in the assay system, methyl viologen, was maintained at saturating concentration (0.3mM) such that the Michaelis-Menten equation for a single substrate was obeyed. All other components of the system remained constant. Enzyme rates (v) were determined for each concentration of substrate and by use of the Michaelis-Menten equation a Lineweaver-Burk plot of 1/v (ordinate) against 1/s (abscissa) was constructed using statistical regression analysis (Figure 3.28). The $K_{m\ TMAO}$ and $V_{max\ TMAO}$ of the enzyme were calculated from the 1/s and 1/v intercepts respectively. Repeated determination of the parameters gave the values presented in Table 3.12.

Plate 3.9 Isoelectric Focusing of Periplasm and Purified TMAO Reductase (Zymogram Stain)

		Protein load (µg)	TMAO reductase Activity Load (U)
a	Periplasm	28	227
b	Purified TMAO reductase	2	106

→ sample application points

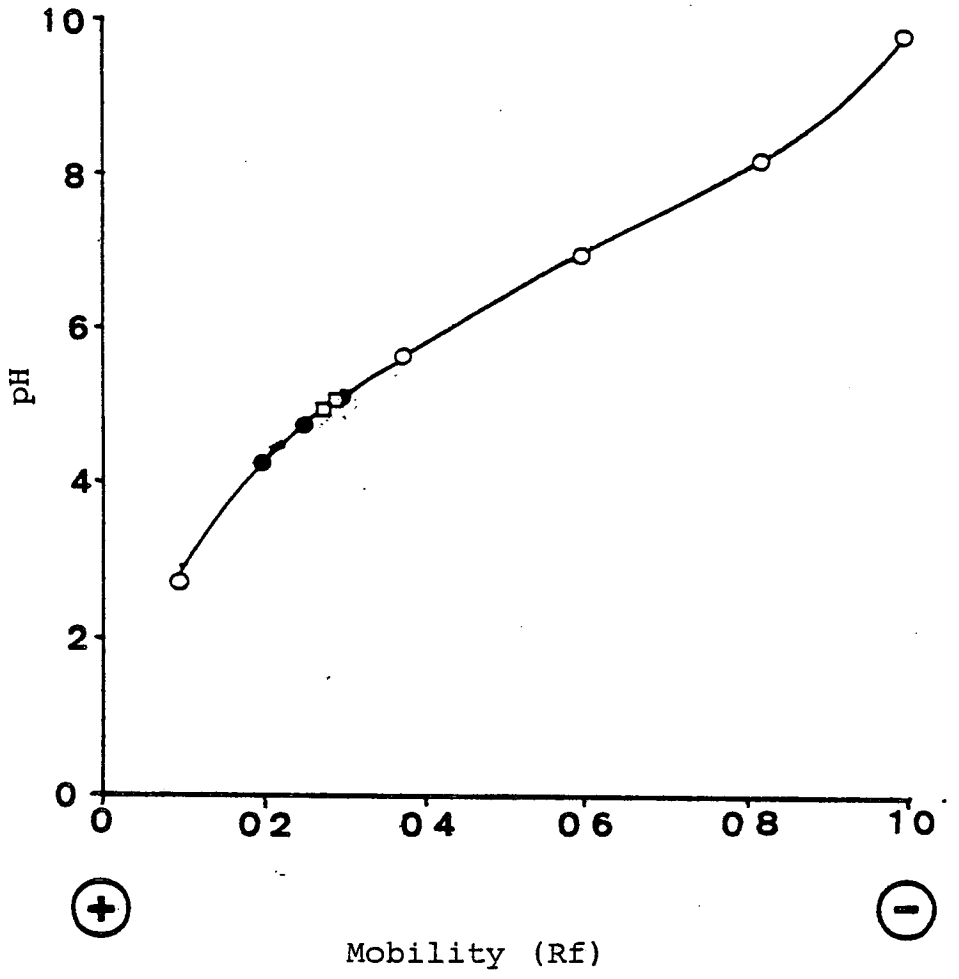


Figure 3.27 Isoelectric Focusing pH Gradient for TMAO Reductase after Dialysis

○, Standards

●, Periplasmic TMAO reductase

□, Purified TMAO reductase

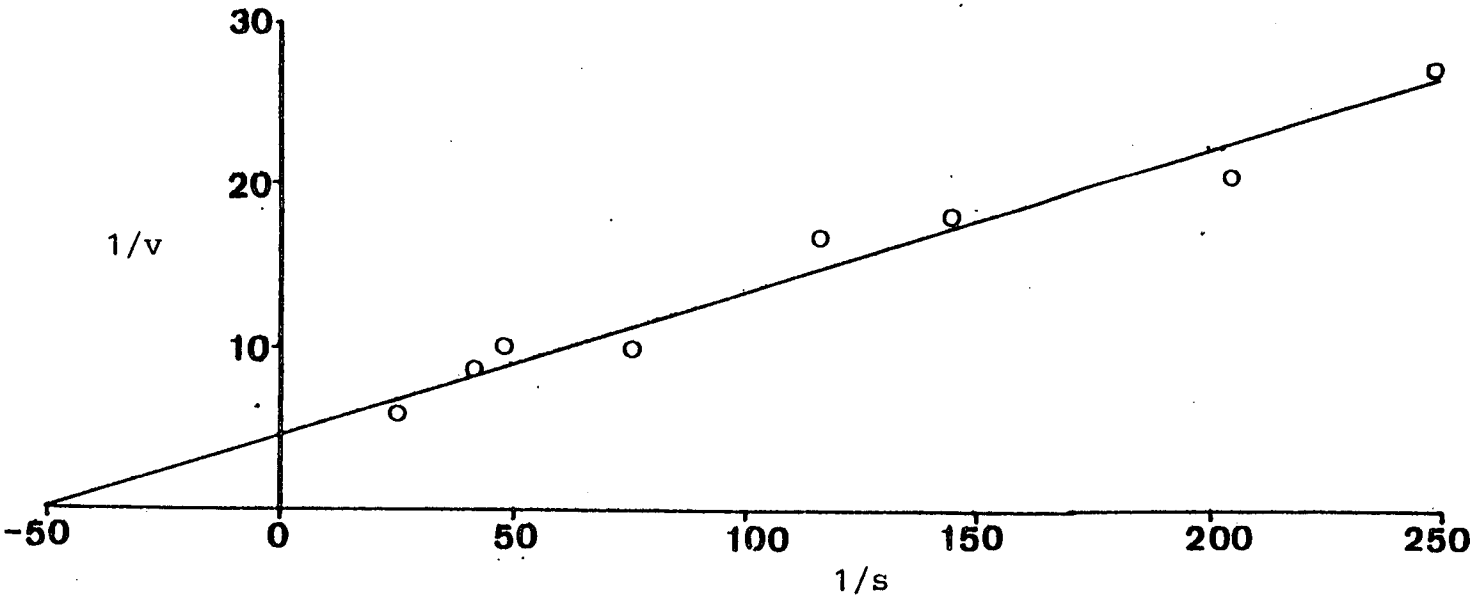


Figure 3.28 Lineweaver-Burk Plot of Purified TMAO Reductase Activity with TMAO as Substrate

s Substrate concentration (mM)

v Enzyme velocity (mM TMAO reduced min^{-1} $mg\ protein^{-1}$)

Correlation coefficient 0.98

Table 3.12 Determination of $K_{m\ TMAO}$ and $V_{max\ TMAO}$ for Purified TMAO Reductase

$K_{m\ TMAO}$ (μM)	19.0 ± 0.55 (1.1)
$V_{max\ TMAO}$ (μMol TMAO min^{-1} $mg\ protein^{-1}$)	19.5 ± 16.0 (3.0)

3.3.4 Alternative Electron Donors to TMAO Reductase

Periplasmic and purified TMAO reductase were examined for their ability to utilize potential alternative electron donors to TMAO. Benzyl viologen (BV, 0.3mM) was assayed as for MV by monitoring the oxidation of the dithionite reduced radical by the enzyme, at 600 nm. Dithionite-reduced FAD (0.1mM) to TMAO reductase activity was assayed for by the decrease in absorption of dithionite on re-oxidation, at 317 nm. Oxidation of the reduced pyridine nucleotides nicotinamide adenine dinucleotide (NADH, 0.1mM) and nicotinamide adenine dinucleotide phosphate (NADPH, 0.3mM) by TMAO reductase was monitored by the decrease in absorption at 340 nm, the assay not containing dithionite. PMS (5.0mM) and methylene blue (1.0mM) were partially reduced by dithionite and assayed for oxidation by the enzyme with the increase in absorbance at 450 nm and 600 nm respectively. The results (Table 3.13) indicated that of the compounds tested, BV was the only effective electron donor to either purified or periplasmic TMAO reductase, the activity being approximately 50% of that obtained using MV.

The effect of including FAD as a possible cofactor for the enzyme in the MV-TMAO reductase assay was investigated. Enzyme activity was not enhanced by the presence of FAD, the activity decreasing at 0.01mM concentration (Table 3.14).

3.3.5 Alternative Electron Acceptor Use by TMAO Reductase

TMAO reductase was analysed for the ability to utilize various potential alternative electron acceptors to TMAO. Compounds were prepared in 1M aqueous solution and the pH adjusted to 7.0, except for nicotinamide N-oxide which was prepared in 1M HCl. Each electron acceptor replaced TMAO in the enzyme assay at the concentration indicated (Table 3.15). A control for the nicotinamide N-oxide substrate assay was made by addition of 1M HCl to the appropriate final concentration in the standard enzyme-TMAO assay. The most effective compound as alternative substrate was LDAO, providing 63% and 41% of the normal activity, with periplasmic

Table 3.13 Alternative Electron Donors to TMAO Reductase

Electron donor (reduced)	Concentration (mM)	% of MV-TMAO Reductase Specific Activity	
		Periplasm	Pure enzyme
MV	0.30	100	100
BV	0.30	52	58
FAD	0.10	0	0
	0.01	0	0
NADH	0.10	0	0
NADPH	0.30	0	0
Methylene Blue	1.00	0	0
PMS	5.00	0	0

Table 3.14 Effect of Added Flavin Adenine Dinucleotide (FAD) on TMAO Reductase Activity

Addition	Concentration (mM)	% TMAO Reductase Specific Activity	
		Periplasm	Purified enzyme
None	-	100	100
FAD	0.010	89	76
FAD	0.001	100	100

Table 3.15 Alternative Electron Acceptor Specificity of Purified TMAO Reductase

Acceptor	Concentration (mM)	% of Spectific Activity with TMAO	
		Periplasm	Pure enzyme
TMAO	6.6	100	100
KNO_3	6.6	0	0
*KNO_2	6.6	-	-
Sodium fumarate	6.6	0	0
Betaine	6.6	0	0
	2.2	0	0
Carnitine	6.6	0	0
	2.2	0	0
γ Picoline N-oxide	6.6	11	12
	1.1	4	4
Pyridine N-oxide	6.6	6	6
	1.1	4	2
Hydroxylamine	6.6	19	3
LDAO	6.6	63	41
Adenosine N-oxide	6.6	40	35
Nicotinamide N-oxide	6.6	0	0
DMSO	6.6	11	0
Sodium chlorate	6.6	0	0

* KNO_2 autooxidized MV in TMAO reductase buffer

and purified enzyme respectively. Compounds also capable of being reduced by the enzyme were the N-oxides of adenosine, γ picoline and pyridine, together with hydroxylamine. The remaining compounds were ineffective as enzyme substrates.

The K_m and V_{max} for the substrates LDAO, picoline N-oxide and pyridine N-oxide were determined by Lineweaver-Burk plot, in the same way as for TMAO (3.3.3). The values for each substrate are presented in Table 3.16.

3.3.6 Effect of Metal Ions on TMAO Reductase Activity

A range of metal ions were examined for their effect on TMAO reductase activity by incubation of samples of periplasm or pure enzyme with the metal salt. Cations were prepared as aqueous solutions of the chloride salts, and the anions MoO_4^{2-} and WO_4^{2-} which were prepared from their sodium salts. The solutions were adjusted to pH7.0 and preincubated with the enzyme for approximately 5 min at the concentration indicated, before assaying for TMAO reductase activity (Table 3.17). The presence of Ca^{2+} and Mn^{2+} had little effect on enzyme activity, the activity of purified enzyme being slightly reduced in comparison with periplasm. In the presence of WO_4^{2-}, purified enzyme activity was reduced to 36% of that present in the control, with little reduction in the periplasmic activity. A problem with some of the metal ions tested was the autooxidation of reduced MV by the metal, and in some cases, precipitation. These effects were relieved to some extent by using MacLeod Buffer B (Martin and MacLeod, 1971) (50mM Tris-HCl, pH7.0 containing; 300mM NaCl; 50mM $MgSO_4$; 10mM KCl) and BV (0.3mM) with 30%-60% of TMAO reductase activity remained after incubation with MoO_4^{2-}. The ions Cu^{2+}, Fe^{2+} and Fe^{3+} also caused autooxidation of reduced BV.

3.3.7 Inhibition of TMAO Reductase Activity

Various potential inhibitory compounds were tested for their effect on TMAO reductase activity. The classes of inhibitor used were: metal chelating compounds; KSCN,

Table 3.16 The K_m values of Various Alternative Electron Acceptors for TMAO Reductase

Acceptor	K_m (mM)	[1] Correlation coefficient
TMAO	0.02	-
LDAO	2.4	0.99
Pyridine N-oxide	6.8	0.99
γ Picoline N-oxide	6.9	0.94

[1] Correlation coefficient for Lineweaver-Burk plot

Table 3.17 Effect of Metal Ions on TMAO Reductase Activity

Metal ion	Concentration (mM)	% of Specific Activity in absence of ion	
		Periplasm	Pure enzyme
None	-	100	100
Ca^{2+}	1	97	78
Mn^{2+}	1	100	78
WO_4^{2-}	1	93	36
[1] MoO_4^{2-}	1	39	28
	0.1	63	59

[2] Cu^{2+}, Fe^{2+}, Fe^{3+}, [3] Zn^{2+}

[1] Assayed in MacLeod buffer B pH7.0

[2] Autooxidized MV and BV in either TMAO reductase buffer or MacLeod buffer B.

[3] Prepared as $ZnSO_4$

thiourea; 1,10-phenanthroline; 2,2-dipyridyl; thiol group modifiers; iodoacetate, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), p-hydroxymercuribenzene sulphonate (PHMB) and general oxido-reductase inhibitors; NaN_3, sodium chlorate and KCN. These compounds were prepared at pH7.0 and preincubated with aliquots of periplasm or purified enzyme for approximately 5 min, before assaying for activity (Table 3.18). Each of the thiol-group modifying compounds caused inhibition of activity to some extent. Greatest inhibition was obtained with PHMB in both periplasm and pure enzyme, the inhibition being relieved by incubation in the presence of dithiothreitol (1mM). Less relief of inhibition by dithiothreitol was obtained with DTNB. Enzyme activity was not inhibited by dithiothreitol at 1mM. Of the other compounds tested, only sodium chlorate with the periplasmic enzyme and KCN with the enzyme from both sources caused any signficant inhibition.

Some of the compounds tested for their ability to act as electron acceptors from TMAO reductase (3.3.5) were analysed for their effect on enzyme activity in the presence of TMAO. Pyridine N-oxide, γ picoline N-oxide, betaine and LDAO were added to separate aliquots of purified TMAO reductase and incubated for 3 min prior to beginning the assay. The endogenous rate of activity in the absence of TMAO, where present, was recorded and subtracted from the rate after addition of TMAO, to give the rate due to TMAO alone. The results obtained are given in Table 3.19. Pyridine N-oxide, a poor substrate for the enzyme showed little inhibition of TMAO reductase activity, whereas picoline N-oxide and to a greater extent LDAO, both capable of being reduced by the enzyme, inhibited the reduction of TMAO. Betaine ineffective as an enzyme substrate gave rise to an approximate 50% reduction in activity.

3.3.8 Metal Content of Purified TMAO Reductase

Purified TMAO reductase was analysed for the presence of non-haem iron and molybdenum (sections 2.17 and 2.18). Using samples from concentrated preparations of enzyme

Table 3.18 Inhibition of TMAO Reductase Activity

Inhibitor	Concentration (mM)	% of Specific Activity in absence of inhibitor	
		Periplasm	Pure enzyme
None	-	100	100
NaN_3	5.0	86	83
KCN	5.0	62	69
	1.0	88	98
KSCN	5.0	101	91
1,10-Phenanthroline	1.0	97	84
2,2-Dipyridyl	2.5	96	85
Sodium chlorate	5.0	67	91
Iodoacetate	5.0	53	76
	1.0	90	100
DTNB	1.0	15	33
DTNB + 1.0mM dithiothreitol	1.0	26	35
PHMB	0.3	12	10
PHMB + 1.0mM dithiothreitol	0.3	87	93
Dithiothreitol	1.0	103	100

DTNB 5,5'-dithiobis-(2-nitrobenzoic acid)

PHMB *p*-hydroxymercuribenzene sulphonate

Table 3.19 Inhibitory Effect of Alternative Acceptors on TMAO Reductase Activity

Acceptor	Concentration (mM)	% Specific Activity in absence of acceptor
None	-	100
Pyridine N-oxide	6.6	95
	13.2	91
Picoline N-oxide	1.1	79
	6.6	31
	13.2	37
Betaine	1.1	70
	6.6	48
LDAO	6.6	29
	13.2	19

(1 nmol), sufficient to allow the detection of 2 nmol of non-haem iron (within the working sensitivity of the assay method), no non-haem iron was found.

Samples of enzyme (41 µg protein ml^{-1}) were subjected to molybdenum determination by furnace atomic absorption spectroscopy and found to contain 57.95 ng $Mo.ml^{-1}$ (Figure 3.29). On the basis of a molecular weight for TMAO reductase of 90 000 daltons, the measured molybdenum was equivalent to 1.32 g atoms mol TMAO $reductase^{-1}$.

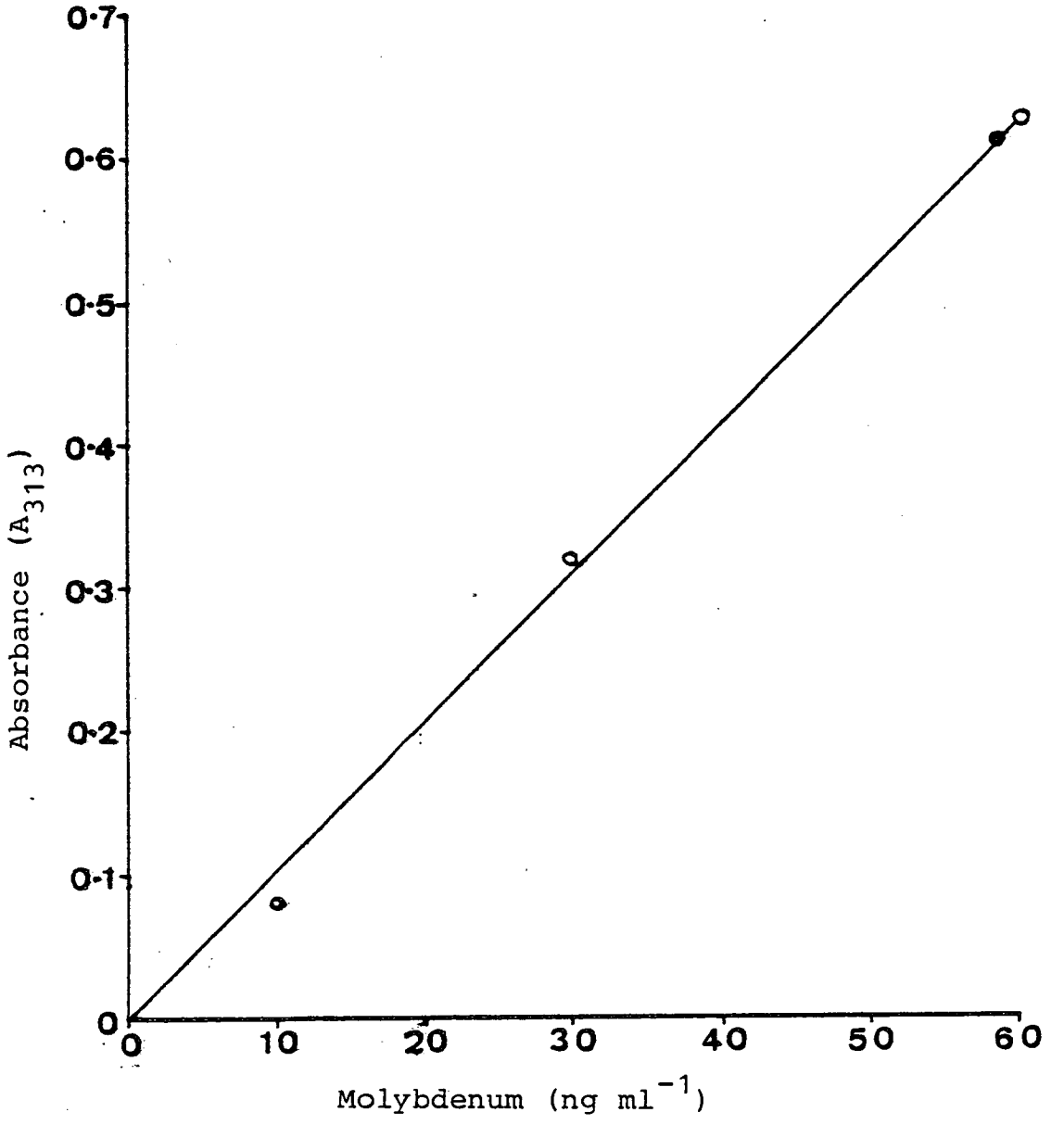


Figure 3.29 Determination of Molybdenum in Purified TMAO Reductase

○, Standards

●, TMAO reductase

3.4 Immunological Characterization of TMAO Reductase

Antiserum to purified TMAO reductase was raised in mice as detailed in 2.11 and from each bleed of the mice approximately 1.5 ml antiserum was obtained after removal of whole red blood cells. The titre of the antiserum was estimated by the double diffusion and immunoprecipitation method of Ouchterlony. Agarose plates (1%, 10x10 cm) were prepared as in Figure 3.30, with four sets of diffusion test per plate. Each plate was incubated overnight in a humid atmosphere, at room temperature, before being washed, pressed and stained (2.12). An immunoprecipitate was formed down to an antiserum dilution of 1 in 64 using an antigen loading of 50 µg protein. No immunoprecipitate was formed using a pre-immune serum taken from the mice (2.11) and TMAO reductase antigen.

The behaviour of TMAO reductase on agarose gels was assessed by one-dimensional electrophoresis on 5x5 cm plates using samples of periplasm and purified enzyme. Following electrophoresis, the plates were stained for protein (Coomassie blue) and enzyme activity (Plate 3.10). Two zones of activity were observed in the periplasm sample, one of low and the other of high mobility, the latter having a greater activity than the former. Purified TMAO reductase produced a single zone of activity coincident with the low mobility zone from periplasm. A series of protein bands were visible across the gel from the periplasm sample, a single band present from the purified enzyme sample.

Reactivity of the antiserum to TMAO reductase was investigated by C.I.E. (2.9.2) of periplasm and purified enzyme samples. Two immunoprecipitation peaks were observed in the second-dimension gel of the periplasm sample, corresponding to the position of the two activities after the first dimension (Plate 3.11). The low mobility enzyme produced a greater precipitation than the high mobility enzyme. The purified enzyme gave rise to a single immunoprecipitate peak corresponding to the low mobility activity. To determine the specificity of the antiserum to either one or both of

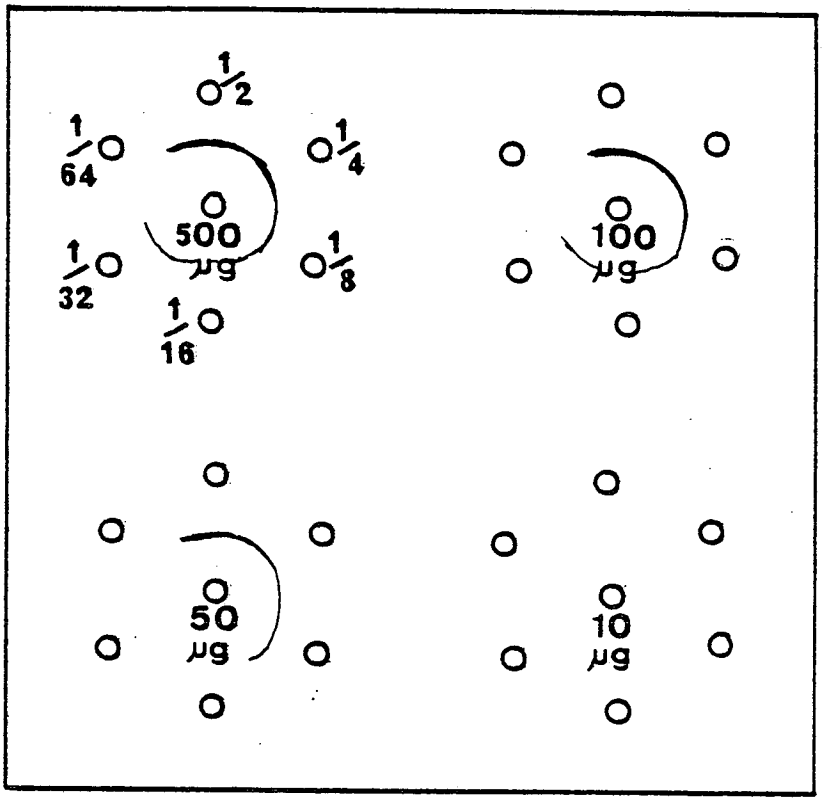


Figure 3.30 Estimation of Antibody Titre by Ouchterlony Plate Technique

Centre wells antigen (purified TMAO reductase)

Outside wells dilution of antiserum

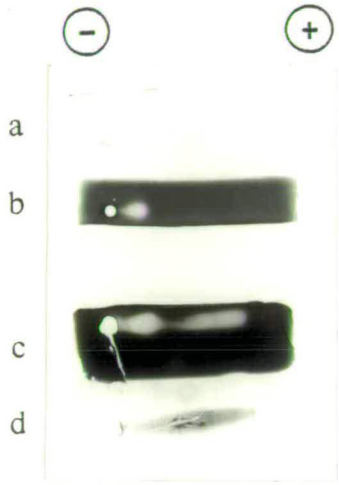


Plate 3.10 Agarose Gel Electrophoresis of Periplasm and Purified TMAO Reductase

Purified TMAO reductase (2 µg protein, 110 U activity)	a	Coomassie blue protein stain
	b	zymogram stain
Periplasm (28 µg protein 230 U activity)	c	zymogram stain
	d	Coomassie blue stain

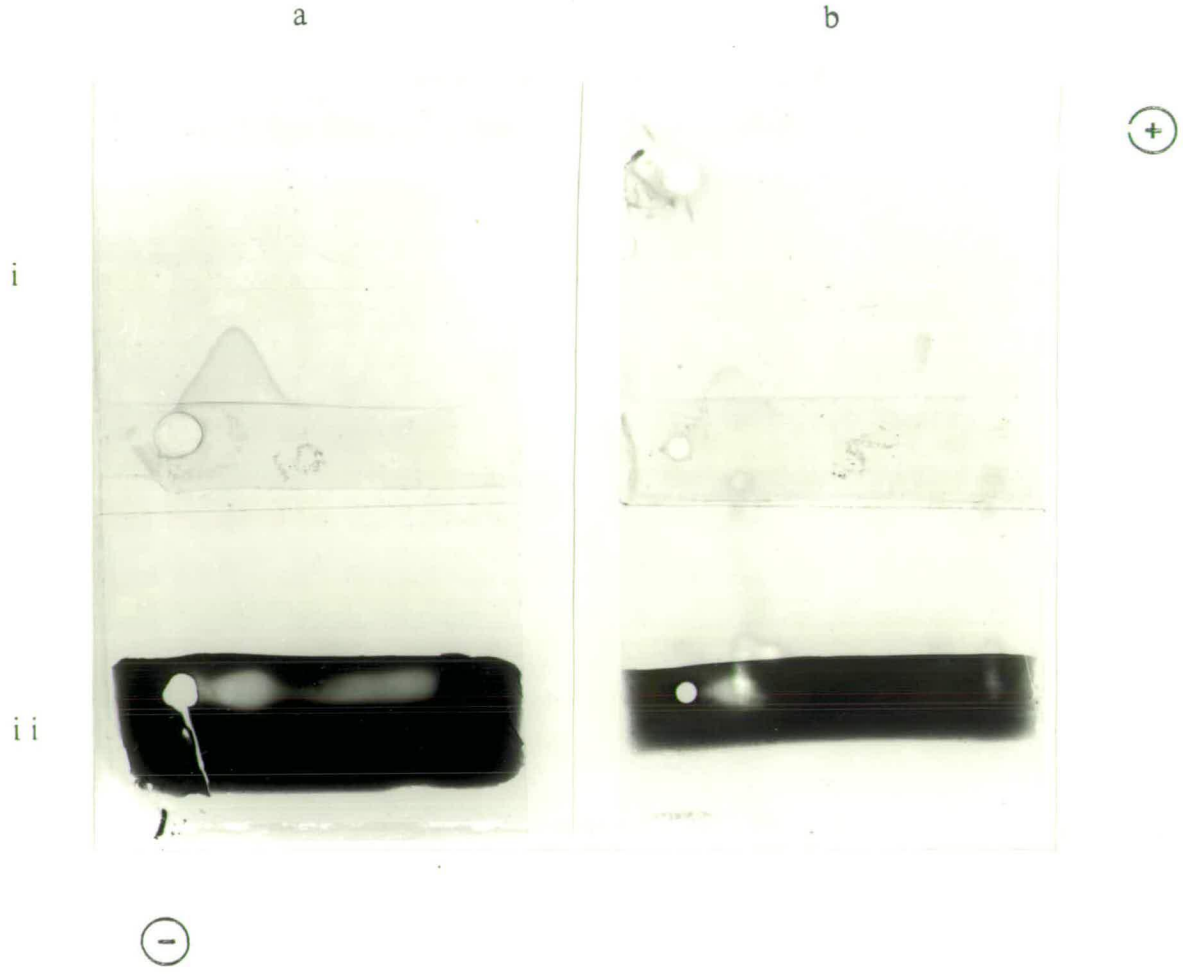


Plate 3.11 Crossed Immunoelectrophoresis (CIE) of Periplasm and Purified TMAO Reductase

5 µl antiserum in 2nd dimension of CIE plates

a	Periplasm (15 µg protein)	i	CIE (Coomassie blue stain)
		ii	Agarose electrophoresis (zymogram stain)
b	Purified TMAO reductase (2 µg protein)	i	CIE (Coomassie blue stain)
		ii	Agarose electrophoresis (zymogram stain)

the TMAO reductase enzymes, the two isolated enzymes from semi-preparative PAGE (3.1.4) were subjected to separate single dimension agarose electrophoresis. The results of the zymogram stained gel, with a comparison between these activities and those in periplasm and purified form is given in (Figure 3.31). The isolated low mobility TMAO reductase from PAGE gave two zones of activity after agarose electrophoresis. Major activity was of low mobility but some activity was also present at the high mobility position. The isolated high mobility enzyme gave rise only to an activity of high mobility in agarose. R.I.E. (2.9.1) was performed using the two isolated enzymes as samples. The low mobility enzyme gave a series of immunoprecipitate rockets whereas the high mobility enzyme was not precipitated.

The electroblotting-immunodetection/western blotting technique (2.10) was used to confirm the specificity of the antiserum to the high-M_r TMAO reductase. Samples of periplasm containing 5-150 µg protein per well and purified TMAO reductase, 6 µg per well were subjected to SDS-PAGE followed by electroblotting of the proteins onto nitrocellulose and immunodetection using antiserum directed against the high-M_r enzyme (Plate 3.12). A single stained protein band corresponding to the high-M_r enzyme was evident in both the purified TMAO reductase and periplasm samples. No other proteins, including the low-M_r enzyme in the periplasm sample were stained.

A comparison between the purified TMAO reductase of *Alteromonas* sp. NCMB 400 and similar enzyme activities in *E.coli* and *S.typhimirium* was made on the basis of their immunological relatedness. Microaerobic cultures of *E.coli* and *S.typhimurium* were prepared in the presence of TMAO (2.2.2). The periplasmic fraction from *E.coli* was obtained in the same way as for *Alteromonas* sp. NCMB 400, in the presence of the protease inhibitor PMSF (1mM). The spheroplasts were disrupted by passage through a French pressure cell at 27.6 MPa and following the removal of whole cells by centrifugation (10 000 g), the membrane fraction was

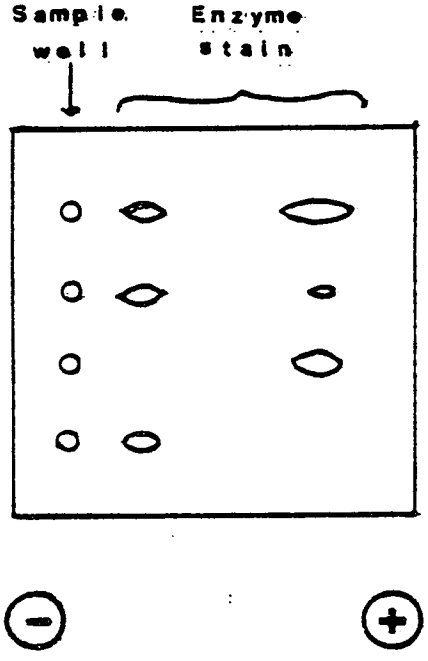


Figure 3.31 Agarose Electrophoresis and Zymogram Stains of Various TMAO Reductase Preparations

a, Periplasm (10 µl, 1.5 mg protein ml^{-1})

b, Isolated high-M_r TMAO reductase (10 µl, 0.2 mg protein ml^{-1})

c, Isolated low-M_r TMAO reductase (10 µl 0.1 mg protein ml^{-1})

d, Purified TMAO reductase (2 µl 0.2 mg protein ml^{-1})

Plate 3.12 Electroblotting and Immunodetection of Periplasm and Purified TMAO Reductase

			Protein load (µg)
a	Purified TMAO Reductase		6
b	Periplasm	i	153
		ii	75
		iii	37
		iv	18
		v	9
		vi	5

(7.5-15% acrylamide)

harvested by centrifugation at 160 000 g (2 h) and retained together with the cytoplasmic fraction. The membrane fraction was treated with 4% Triton X100 at 4°C overnight followed by centrifugation at 160 000 g (2 h) to prepare detergent soluble (supernatant) and insoluble (pellet) fractions. The harvested culture of *S.typhimurium* was disrupted by sonication in the presence of PMSF (1mM) followed by removal of whole cells and preparation of the membrane and cytoplasmic fractions as for *E.coli*. Each of the isolated fractions from *E.coli* and *S.typhimurium*, together with a sample of periplasm from *Alteromonas* sp. NCMB 400 were analysed by PAGE and SDS-PAGE followed by zymogram staining, so that a comparison could be made between the resolved TMAO reductase activities. A total of three activities were visualized in the *E.coli* cell fractions after PAGE, all being present in the periplasm sample. The high mobility band was not present after SDS-PAGE. Two activities were present in the *S.typhimurium* fractions after PAGE, the high mobility band being lost after SDS-PAGE (shown schematically in Figure 3.32). None of the activities present in the various cell fractions of *E.coli* or *S.typhimurium* corresponded to the two activities of *Alteromonas* sp. NCMB 400 periplasm in terms of relative mobility.

To study the immunological cross-reactivity between the *E.coli* and *S.typhimurium* TMAO reductase activities and the high-M_r enzyme of *Alteromonas* sp. NCMB 400, R.I.E. was performed using *E.coli* periplasmic and *S.typhimurium* sonicate fractions against *Alteromonas* sp. NCMB 400 high-M_r TMAO reductase antiserum. No rocket immunoprecipitates were produced from either the *E.coli* or *S.typhimurium* samples. A second comparison was made using the western blotting technique. Following blotting of electrophoresed proteins from *E.coli* periplasm, *S.typhimurium* sonicate and purified TMAO reductase onto nitrocellulose, cross-reacting antigens were visualized by immunodetection with the pure enzyme antiserum (Plate 3.13). The purified TMAO reductase from *Alteromonas* sp. NCMB 400 was heavily

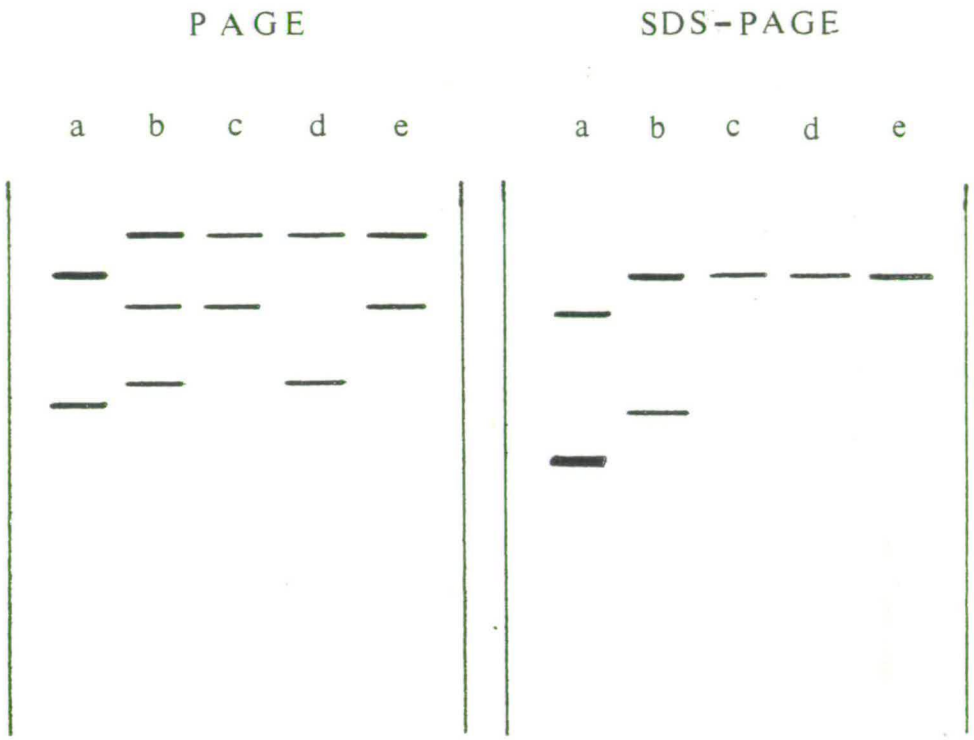


Figure 3.32 Schematic Representation of Bacterial TMAO Reductase Activity after PAGE and SDS-PAGE

a *Alteromonas* sp. NCMB 400 periplasm
b *Escherichia coli* periplasm
c " " cytoplasm
d " " membrane extract
e *Salmonella typhimurium* crude extract (sonicate)

(7.5-15% acrylamide, zymogram stain)

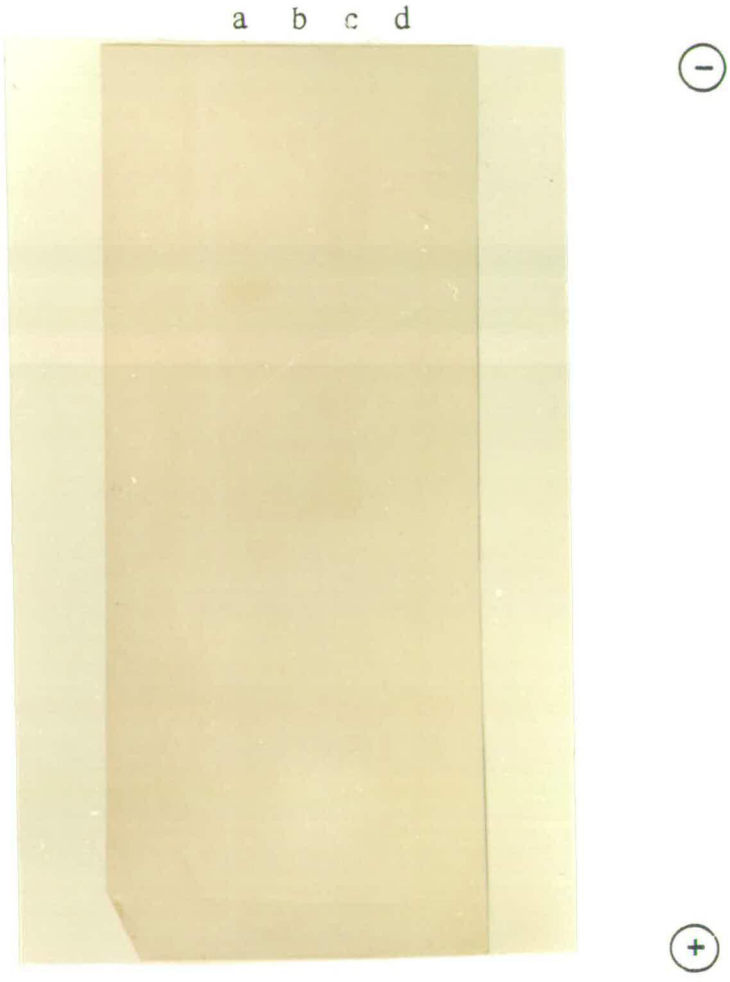


Plate 3.13 Electroblotting and Immunodetection of *Escherichia coli* and *Salmonella typhimurium* Cell Fractions

			TMAO reductase activity load (U)
a	*Alteromonas* sp. NCMB 400	periplasm	156
b	*Escherichia coli*	periplasm	169
c	*Escherichia coli*	cytoplasm	151
d	*Salmonella typhimurium*	crude extract (sonicate)	194

(7.5-15% acrylamide)

stained whereas no staining of *E.coli* or *S.typhimurium* proteins was present.

Groups of mutants lacking TMAO reductase activity have been isolated from *Alteromonas* sp. NCMB 400 by Nasser (1983) and been tentatively characterized as to their genetic lesion (Table 3.20). Each group of mutants was suspected to contain a different lesion and typical strains from some of the groups were taken for further study: group 3, strain 292; group 4, strain 322 and group 6, strain I58.

Strains were cultured for the induction of TMAO reductase as for the wild-type and the periplasm from each was isolated. TMAO reductase activity was assayed for in each of the mutant strains and compared with that present in the wild-type (Table 3.21). Some activity was present in strains 292 and 322, whereas strain I58 lacked almost all activity. The periplasmic and cytoplasmic fractions from the mutant strains were analysed by SDS-PAGE and zymogram staining to observe the effect of the genetic lesions on the separate TMAO reductase proteins (Plate 3.14). The periplasm of strain 292 contained a minor third band of activity present between the low- and high-M_r activities, with predominant activity associated with the low-M_r TMAO reductase as in the wild-type. The converse was true for the cytoplasm of this strain, less activity associated with the low-M_r enzyme than with the high-M_r enzyme. The periplasm from strain 322 had approximately equal activities associated with the two TMAO reductase enzymes, whereas the high-M_r activity was more prominant in the cytoplasm. Again, a faint third periplasmic band of activity was observed of intermediate molecular weight. Mutant I58 had a very low level of activity in comparison with the other strains for approximately the same load of protein. The high-M_r activity in both the periplasmic and cytoplasmic samples being less intense than the low-M_r activity. A third band of activity was present between the two normal bands of activity.

Table 3.20 Characterization of *Alteromonas* sp.NCMB 400 Mutants Defective in TMAO Reductase (from Nasser, 1983)

Group	Character	Strain Number
1	Low Mo-cofactor, low TMAO reductase activity	281, 341, 342, 343
2	High Mo-cofactor, low TMAO reductase activity	282, 283, 361, 401
3	High Mo-factor, undetectable TMAO reductase activity	291, 292, 301, 302, 403, 405
4	No Mo-factor, undetectable TMAO reductase activity	321, 322, 331
5	No anaerobic growth, moderate TMAO reductase activity	I4, I31, I66, I79, I106, I119, I123
6	Only defective in TMAO reductase	I58

Table 3.21 TMAO Reductase Activity in Cell Fractions of Strains of *Alteromonas* sp. NCMB 400

Strain	Cell Fraction	Specific Activity (U)	% of Wild-type Specific Activity
Wild-type	periplasm	30 604	100
Wild-type	cytoplasm	2 203	7
292	periplasm	5 709	19
292	cytoplasm	1 980	7
322	periplasm	11 313	37
322	cytoplasm	5 660	18
I58	periplasm	277	1
I58	cytoplasm	336	1

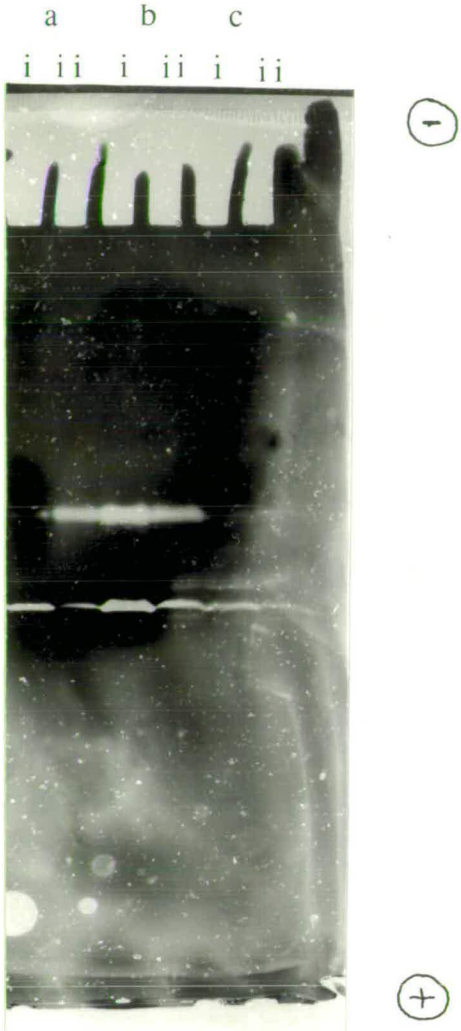


Plate 3.14 SDS-PAGE of Cell Fractions from *Alteromonas* sp. NCMB 400 Mutants Defective in TMAO Reductase Activity (Zymogram Stain)

				Protein load (µg)	TMAO Reductase Activity (U)
a	mutant 292	i	periplasm	64	183
		ii	cytoplasm	58	128
b	mutant 332	i	periplasm	16	437
		ii	cytoplasm	84	145
c	mutant I58	i	periplasm	104	47
		ii	cytoplasm	75	35

(7.5-15% acrylamide)

The mutant strains were analysed immunologically using antiserum against purified TMAO reductase, to determine the effect of the genetic lesion in each strain on the level of the high-M_r enzyme. Equal amounts of total protein from the periplasm fractions of the three mutants strains were loaded onto SDS-polyacrylamide gels, together with serial doubling dilutions of periplasm from the wild-type. Following electroblotting and immunodetection (Plate 3.15) a semi-quantitative titre of high M_r TMAO reductase stain was produced from the wild-type periplasm. Other bands of protein were detected by the antiserum probe, not at the position of the low M_r enzyme. In each of the mutant strain samples, the staining of the high-M_r TMAO reductase was equal to or greater than the st ain from the sample of wild-type periplasm containing an equal amount of protein. In addition to the high-M_r band, a second heavily staining band of protein was present at a mobility approximately equal to the third TMAO reductase activity seen after SDS-PAGE of the mutant samples.

a b c d

i i i iii iv v

Plate 3.15 Electroblotting and Immunodetection of *Alteromonas* sp. NCMB 400 Wild-type and Mutant Periplasmic Fractions

		Protein Load (µg)	TMAO Reductase Activity Load (U)
a	Wild-type i	22	674
	ii	11	335
	iii	5	161
	iv	3	92
	v	1	42
b	mutant 292	22	270
c	mutant 322	22	143
d	mutant I58	22	11

(7.5-15% acrylamide)

Chapter 4

Discussion

The major emphasis on research into bacterial anaerobic respiration has been directed towards an understanding and elucidation of respiratory chains with nitrate and fumarate as terminal electron acceptors. These have been fully characterized at a biochemical, biophysical and molecular genetic level such that detailed knowledge on the respective mechanisms of each system is now available. Although studies on the bacterial reduction of TMAO as part of a respiratory mechanism began in 1945 with the work of Neilands, a thorough biochemical investigation of the process was not undertaken until the 1970's with the studies of the Sakaguchi and Kawai group. As a result, knowledge of the mechanisms involved in TMAO reduction has not been elucidated as fully. Moreover, the work that has been done on TMAO reduction has largely been restricted to enterobacterial species, in particular *E.coli* probably because of the depth of knowledge on the physiology, biochemistry and genetics of these bacteria. However, the relevance of this process to the enterobacteria is questionable as the major ecological location of TMAO is within marine fish tissue, not a typical habitat of enterobacterial organisms. The association of marine bacteria with fish spoilage prompted an investigation into the involvement of TMAO reduction. Easter (1982), identified several strains of marine *Alteromonas* spp. capable of TMAO reduction and began physiological studies on the organisms and an initial characterization of the enzyme(s) specifically concerned with the process. Further detailed studies on a particular bacterium *Alteromonas* sp. NCMB 400, demonstrated a variety of anaerobic respiratory pathways and initiated a genetic approach to the investigation (Nasser, 1983). Purification and subsequent detailed analysis of the TMAO reductase from *Alteromonas* sp. NCMB 400 was considered necessary in order to extend the knowledge of the process in a bacterium associated with TMAO in its natural environment and to compare and contrast this with other organisms.

4.1 Location and Stability of TMAO Reductase

Before commencing the purification and characterization of TMAO reductase from *Alteromonas* sp. NCMB 400 it was necessary to investigate and assess various aspects of the enzyme within the cell. Without taking account of features such as the location and stability of TMAO reductase, losses in yield and activity would have resulted in an irreproducible purification protocol and variation in the characterized physiochemical properties of the enzyme.

The location of TMAO reductase in *Alteromonas* sp. NCMB 400 has been investigated by Easter (1982), after development of methods for cell fractionation. Using a spheroplasting technique based on the lysozyme-osmotic shock-EDTA method of Birdsell and Cota-Robles (1967), Easter *et al.* (1983) estimated 67% of the total TMAO reductase activity to be associated with the periplasmic cell fraction, the remaining 37% of activity residing with the intact spheroplasts. The work presented in this thesis indicates a non cytoplasmic cell location for TMAO reductase with approximately 7% of total enzyme activity associated with the cytoplasm (Table 3.21). According to the guidelines of Beecham (1979) and his study on periplasmic enzymes this classes the TMAO reductase of *Alteromonas* sp. NCMB 400 as a periplasmic enzyme in that the protein was released from the cell during spheroplasting. However, maximal release of the enzyme was obtained only after treatment with EDTA (Easter, 1982). Added EDTA is known to cause some disruption of cell membranes and often results in the release of membrane associated enzymes such as alkaline phosphatase (Beecham, 1979). Thus TMAO reductase may alternatively be considered as a membrane associated enzyme that is easily released into the soluble fraction of the cell on disruption. This view is supported by the work of Stenberg *et al.* (1984), using a spheroplasting method based on penicillin without the addition of EDTA. This procedure was considered to be less disruptive to the inner membrane of the cell and resulted in TMAO reductase activity being associated with the membrane, rather than the soluble periplasmic fraction. The findings from

these studies tend to support the view of TMAO reductase in alteromonads as being a loosely associated, membrane bound enzyme that is easily lost during the fractionation of the cell. A similar situation to that in *Alteromonas* spp. appears to be evident in members of the *Enterobacteriaceae* possessing TMAO reductase. In *E.coli* (Sagai and Ishimoto, 1973; Shimokawa and Ishimoto, 1979) and *S.typhimurium* (Kwan and Barrett, 1983b) TMAO reductases have been found to be both membrane bound and soluble after cell disruption by sonication. Using the same protocol for spheroplasting as applied to *Alteromonas* sp. NCMB 400, 3 of the 4 TMAO reductase enzymes from *E.coli* and 2 of the 3 enzymes from *S.typhimurium* were released into the periplasmic cell fraction (Figure 3.32), again indicating a loose membrane association. In general therefore, bacterial TMAO reduction appears to take place by enzymes that are loosely bound to the membrane, are easily released upon disruption of the cell and may be termed peripheral membrane enzymes (Beecham, 1979).

The location of TMAO reductase within the cell is in contrast to other terminal reductases such as those for fumarate and nitrate reduction that have an intrinsic membrane location in many bacteria (see Introduction). Rigorous treatments such as detergent solubilization are required for the release of these enzymes from the cytoplasmic membrane. In *Alteromonas* sp. NCMB 400 however, both nitrate reductase and fumarate reductase are released from the membrane upon spheroplasting (Nasser, 1983). *Alteromonas* sp. NCMB 400 therefore presents a different situation from that in other bacteria, particularly the enterobacterial species, with the possession of the terminal reductases in a peripheral membrane location.

The implication of TMAO reductase being released into the periplasm is that the enzyme is located on the outer aspect of the plasma membrane. This is in contrast to the nitrate and fumarate reductases of *E.coli* and the fumarate reductase of *V.parahaemolyticus*, that have their catalytic sites on the cytoplasmic aspect (Haddock and Jones, 1977).

The topography of TMAO reductase in *Alteromonas* spp. resembles that of dissimilatory nitrite reductases in a number of bacteria. In *P. denitrificans* and *Ps. aeruginosa*, nitrite reductase is a periplasmic enzyme released into the soluble fraction upon spheroplast preparation (Meijer *et al.* 1979; Alefounder and Ferguson, 1980; Wood, 1978). Reduction of nitrite is coupled to oxidative phosphorylation (Kell *et al.*, 1978), with electron transport to the soluble terminal enzyme being mediated by membrane bound and soluble cytochromes *b* and *c* (Wood, 1978; Meijer *et al.*, 1979). Periplasmic and membrane associated cytochromes c_{552} have been identified in *Alteromonas* sp.NCMB 400 and their functioning in electron transport to TMAO reductase is currently under investigation (Morris, unpublished data). The resemblance between the two enzymes cannot be extended to any structural features; nitrite reductase is a multi-haem enzyme whereas no haem was detected in the TMAO reductase purified in this study (Figure 3.24).

Reduction of TMAO during anaerobic respiration leads to the production of TMA which is not further metabolized and accumulates in the growth medium (Easter, 1982). TMA is a strongly basic compound and may be toxic to the cell, therefore a periplasmic location for TMAO reductase may be advantageous in allowing this end product to diffuse out of the cell.

In addition to determining the cellular location of TMAO reductase it was important to determine factors affecting the activity of this enzyme in order to optimize conditions for purification. Enzymes when released from their natural environment, particularly if intracellular may be denatured or inactivated. A number of the biological, chemical and physical parameters that can affect enzyme stability were determined for TMAO reductase. The work of Easter (1982) had identified an optimum pH for enzyme activity between pH 6.5 and 7.5 and that no dialysable cofactor was required for activity.

The release of a relatively concentrated solution of proteins such as present in the periplasm into a more dilute solution upon spheroplasting can lead to protein denaturation

and enzyme inactivation (Scopes, 1982). As further dilution of TMAO reductase periplasm was likely to take place during the purification of the enzyme, the periplasm was diluted 10-fold for the stability studies (3.1.2). The effect of this dilution on enzyme activity was analysed by the addition of glycerol to the diluted periplasm in order to mimic the conditions of relatively low water activity in undiluted periplasm. Glycerol forms strong hydrogen bonds with water so restricting the available water and reducing the water activity (Scopes, 1982). An increase in the activity of TMAO reductase was observed after inclusion of glycerol in the periplasm, suggesting a deleterious effect of dilution. However this increase in activity was only small, so the inclusion of glycerol (or alternatively BSA) during purification was considered unnecessary. No added stability of TMAO reductase was obtained by the inclusion of glycerol in periplasm, obviating the need for its presence during long term storage. An alternative, though not mutually exclusive explanation for the effect of glycerol is that it may interact hydrophobically with the enzyme because of its partially non-polar character. This would correlate with the presumed membrane association of the enzyme, glycerol providing an amphipathic environment for activity. To confirm the presence of hydrophobic regions within the enzyme, several approaches could be employed. Determination of the primary structure would identify hydrophobic regions and the retention of the enzyme during reversed phase or hydrophobic interaction chromatography would suggest the presence of exposed hydrophobic sites on the enzyme.

In addition to non-specific protective agents like glycerol, the effect of substrates on the stability of TMAO reductase was tested. The presence of the substrate in enzyme preparations may serve to protect the enzyme from inactivation as with ATP protecting ATPase against loss of activity during incubation at elevated temperatures or dialysis (Pullman *et al.*, 1960; Dixon and Webb, 1979). The inclusion of TMAO during the storage of periplasm was without

any marked effect on the enzyme, inferring either that the active site for TMAO reduction is no less stable than the whole enzyme during storage or that TMAO was an ineffective storage agent.

As TMAO reductase is synthesized predominantly under microaerobic conditions in *Alteromonas* sp. NCMB 400 (Easter *et al.*, 1983) the effect of oxygen on the enzyme was determined. The control sample of periplasm without any additions maintained activity over a 6 month incubation in an aerobic environment, suggesting a lack of lability. However minor losses of activity due to oxygen can arise from modifications to particular residues. Exposure of sulphydryl groups to oxygen can lead to disulphide group formation, partial oxidation to sulphinic acid or irreversible oxidation to sulphonic acid. Addition of sulphydryl-containing reagents can offer protection from such damage. On inclusion of dithiothreitol or glutathione as sulphydryl reagents to periplasm, Easter (1982) observed an increase in activity of TMAO reductase which was attributed to the possession of one or more thiol groups in the enzyme. The repetition of these experiments in this study gave results contrary to those of Easter (1982) in that a sharp decrease in activity was observed with periplasmic TMAO reductase, particularly with glutathione which occasionally caused protein precipitation. The presence of these reagents appeared to progressively denature the enzyme during the period of incubation. It is doubtful that the reducing conditions imposed by these reagents affected TMAO reductase activity as the enzyme was routinely assayed under reducing conditions in the presence of dithionite.

The temperature of storage was shown to be important in minimizing enzyme denaturation during storage. In general, the lower the storage temperature of periplasm, the better preserved was TMAO reductase activity. After an initial drop during the first 2 d of incubation at 4°C, enzyme activity was stable for up to two weeks which was considered a sufficient period for purification. An unusual feature of TMAO reductase activity during storage was the restoration of activity after the initial decrease. At -18°C and-80°C activity was observed

to drop over the first 2 weeks of incubation, followed by a rise to the initial level or greater over weeks 3-10, in the samples not containing dithiothreitol or glutathione. As a similar drop in activity over 2 d was observed at 4°C, there is some evidence for denaturation of the enzyme. The rise in activity following this decrease suggests a reactivation of the enzyme but the mechanism of this is unclear. These features were shown to be due to the enzyme itself rather than the assay system in that the results presented were from repeated experiments, each assay carried out under identical conditions with freshly prepared reagents. Repeated freezing and thawing of stored periplasm was shown to have a deleterious effect on TMAO reductase activity and was therefore avoided where possible.

The presence of proteolytic enzymes during the purification and characterization of TMAO reductase could have posed serious problems and led to invalid results. Proteolysis during purification can lead simply to a small loss of activity as observed for alcohol dehydrogenase (Bühner and Sund, 1969; Pringle, 1975) or be so great as to make purification impossible. A low degree of proteolysis can result in reduced yields and unstable preparations. From the evidence of the long term storage experiments with periplasm, complete inactivation by protease activity did not appear to be a problem. However, the initial drop in activity during the first period of incubation may have been the result of limited proteolysis. In addition to inactivation effects caused by proteolysis, modification of the native protein to a partially degraded but still fully active product may occur. In this case, a variation in other properties of the enzyme may be apparent, such as the appearance of isoenzymes with different molecular weights. To determine the possibility of this, experiments were carried out using SDS-PAGE of periplasm over a time course and observing any change in the mobility of the enzyme (zymogram stain) or general proteins (protein stain). As no variation in band position or intensity was observed, this was taken as evidence for lack of protease activity in periplasm. This apparent absence of proteolytic enzymes from the periplasm of

Alteromonas sp. NCMB 400 was unexpected in view of the evidence for proteases in similar bacteria. Two amino-peptidases have been identified in the periplasmic cell fraction of *Alteromonas* B-207, each with sensitivity to EDTA (Lee and Merkel, 1981; Merkel *et al.*, 1981). Recent interest in the processing and localization of cytoplasmically synthesized proteins through the cell membrane to the peri-plasmic or extracellular fractions has identified an endo-peptidase termed leader peptidase (Chang *et al.*, 1978), processing the precursor forms of proteins into the mature molecules (Wolfe *et al.*, 1982), located on the periplasmic face of the cytoplasmic membrane (Silhary *et al.*, 1983). However, as these proteases are very specific in their cleavage of only the signal peptide of the precursor proteins, their functioning in the further proteolysis of a protein appears doubtful. To determine the presence or absence of these various proteolytic activities from the periplasm of *Alteromonas* sp. NCMB 400, the neutral protease assay, PMSF inhibition sensitivity studies and the casein-agar plate test were employed. Each method provided no evidence for proteolytic activity in periplasm, confirming the conclusions of SDS-PAGE analysis.

Although proteolysis was apparently absent from periplasm, certain precautions were maintained to minimize the chance of proteolysis. Cells were grown in rich medium and harvested in mid exponential phase, as cells are known to contain less protease activity during this mode of growth and the turnover of proteins *in vivo* is reduced (Pringle, 1975). The harvesting of cells and spheroplasts was carried out as rapidly as possible at 4°C with the periplasm produced being immediately processed further, or stored at -20°C. As PMSF sensitive proteolysis was not detected, this inhibitor was not included during the preparation of periplasm. EDTA present as part of the spheroplasting procedure was not chelated with added $MgSO_4$ as in the original method of Easter (1982), in order to maintain inhibition of any divalent cation requiring proteases.

The results obtained from the study of the stability and proteolytic effects of periplasm therefore had a number of

implications for the successful purification of TMAO reductase. Given that the enzyme was located predominantly in the periplasm (Easter *et al.*, 1983) conditions were optimized for the preparation of this cell fraction without fear of proteolysis. Due to the initial loss of activity during short term storage at 4°C, the periplasm was either fractionated immediately or stored at -20°C which maintained a level of activity that was little improved by the inclusion of stabilizing reagents.

4.2 The Enzymes of TMAO Reduction

SDS-PAGE under denaturing conditions was carried out on samples of periplasm and the gels stained for protein so as to determine the complexity and approximate molecular weights of the constituent polypeptides (3.1.5). Greater than 50 polypeptideswere visible ranging in molecular weight from approximately 10 000 to 200 000 daltons. In common with the enzymes of TMAO reduction in other bacteria, multiple enzymes in *Alteromonas* sp. NCMB 400 were detected by gel electrophoresis. Two enzymes were resolved by PAGE and SDS-PAGE, indicating a lack of complete denaturation of either enzyme with the inclusion of the dissociating agent SDS. However, an observation made during the comparison of the two enzymes was the change in relative stain intensity after PAGE and SDS-PAGE. The high-M_r enzyme after PAGE had the greater stain intensity but following SDS-PAGE, this was reduced, so that the low-M_r enzyme was the most prominant. This suggested some slight selective denaturation of the high-M_r enzyme.

The fact that two enzymes were detected after both PAGE and SDS-PAGE was taken as preliminary evidence that the two enzymes were distinct rather than one being a subunit of the other. This was confirmed by the isolation of the high- and low-M_r enzymes after PAGE and their separate re-electrophoresis in an SDS containing gel, each enzyme migrating to the same relative position as previously, without dissociation.

Conclusive proof required the purification of the high-M_r enzyme and its electrophoresis under complete denaturing conditions so as to observe any possible subunit formation.

The presence of more than one TMAO reductase prompted the question as to whether they were isoenzymes or not. The definitions of isoenzymes are varied but it is generally accepted that primary isoenzymes are genetically distinct proteins or allelic variants, whereas secondary isoenzymes may arise from protein modifications such as conjugation with other groups, subunit associations or conformational variants (Dixon and Webb, 1979). Genetic analysis of TMAO reductase in *Alteromonas* sp. NCMB 400 (Nasser, 1983) has proved difficult due to the number of different genes required for the synthesis of a functional enzyme. Mutants have been isolated that were tentatively characterized as being defective in molybdenum cofactor synthesis or the processing of the cofactor into the enzyme and also a possible structural enzyme mutant. This latter mutant (I58) appeared the most promising for further study and was used as part of the immunological characterization of TMAO reductase in *Alteromonas* sp. NCMB 400 (3.4). Although the mutant was not reisolated after storage, only 1% of the wild-type specific activity remained, with a zymogram stain of the periplasm and cytoplasm from this mutant giving a very faint stain of both high- and low-M_r enzymes. This would tend to suggest a non-isoenzyme relationship between the two enzymes as defined by the genetic independence requirement but this is tentative and would require further genetic analysis. The demonstration of the lack of subunit relationship between the two enzymes of TMAO reduction goes some way to discounting them as secondary isoenzymes but further characterization is needed to establish their relatedness. The relationship between the multiple TMAO reductases reported in other bacteria has not been discussed in terms of possible isoenzymes but only in terms of their relative induction patterns (see 1.7.1).

4.3 Purification of TMAO Reductase

In order to study in depth the properties of a particular enzyme it is usually necessary to isolate the enzyme from other cellular components by purification. Other proteins present in crude extracts may interfere with the characterization of the enzyme of interest making it difficult to relate unequivocally the determined parameters to the particular enzyme. For these reasons and also because preliminary work identified two TMAO reductase proteins, purification was attempted.

The enzyme chosen for purification was the high-M_r TMAO reductase from *Alteromonas* sp. NCMB 400 because analysis of this enzyme would be more likely to provide information on the relationship between the two TMAO reductase enzymes, particularly in terms of subunit structure. The large volume of periplasm produced from a culture of *Alteromonas* sp. NCMB 400 presented a problem in the choice of method for the initial stage of purification (3.2.1). Ultrafiltration of periplasm was investigated but was not considered useful due to the long processing time during concentration and the lack of any protein fractionation. Salting out of protein was chosen as a suitable alternative step because of its speed, concentrative effect and adaptability for protein fractionation. The salt employed was high purity $(NH_4)_2SO_4$ on account of its large solubility, absence of toxicity and stabilizing effect on enzymes. These features are normally absent from a technique such as organic solvent precipitation which was not therefore used. Between 60% and 80% saturated $(NH_4)_2SO_4$ resolved the high-M_r enzyme from the low-M_r enzyme with the total recovered yield from each fraction approximately by 84%, indicating a lack of inhibition by $(NH_4)_2SO_4$ and small losses during handling. Approximately 30% of the total activity was present in the 60-80% precipitate, almost wholly the high-M_r enzyme as judged by SDS-PAGE. The remaining activity (due to the low-M_r enzyme) was predominantly present in the 0-60% precipitate. These $(NH_4)_2SO_4$ cuts were not refined any further, as the gains in purity would have led to greater losses of high-M_r

TMAO reductase total activity. The activity present in the 80% supernatant was probably composed of the high-M_r enzyme, but recovery of this was not attempted because of problems in handling the increased volumes required to precipitate the enzyme and the expense of the reagent required.

The 3-fold purification obtained after $(NH_4)_2SO_4$ precipitation suggested a reasonable increase in the purity of high-M_r TMAO reductase, which was confirmed by SDS-PAGE analysis with a protein band corresponding to TMAO reductase activity being prominent. However, many proteins were still present in the sample and a high resolution method was sought to further purify the enzyme. Ion exchange chromatography was examined as a suitable next stage in the purification protocol (3.2.2). The power of the resolution obtainable from ion exchange chromatography was demonstrated after chromatography of periplasm on a column of DEAE Sepharose CL 6B, two peaks of TMAO reductase activity eluting separately from the bulk of protein (Figure 3.11). When the technique was applied to the dialysed 60-80% $(NH_4)_2SO_4$ precipitate containing principally the high-M_r enzyme, a single peak of activity with an occasional second peak were eluted, both comprising the high-M_r enzyme. The appearance of two high-M_r enzymes suggested a difference in ionic charge of the two species or a change in the secondary structure, there being no change in the molecular weight as judged by SDS-PAGE. As the second peak of activity was not regularly present, only the first peak of activity was used in subsequent purification methods so as to avoid any possible variation in characterized properties. The loss of activity by discarding the second peak was in part responsible for the approximate 40% reduction in recovered activity (Table 3.7). Dialysis was not detremental to the enzyme at this stage (or any other) so it is probable some inactivation of the enzyme occurred during the ion exchange chromatography.

A 6-fold increase in TMAO reductase purification was obtained with the sample reduced to 12-15 polypeptides. For the further purification of high-M_r TMAO reductase, hydroxyapatite was investigated as a potentially suitable method (3.2.3). The adsorption of acidic proteins onto hydroxyapatite

is believed to be due mainly to the interaction of acidic groups on a protein with calcium sites at the surface of the hydroxyapatite crystals. Elution of bound material is achieved with competing ions for the calcium sites, particularly those with a high affinity for Ca^{2+} such as PO_4^{3-} rather than for example Cl^- (Bernardi, 1971 and Bernardi *et al.*, 1972). Since the basic mechanism of hydroxyapatite fractionation is quite different from that underlying ion exchange or gel filtration, it can provide a useful complement to other methods in purification (Gorbunoff, 1984a,b; Gorbunoff and Timasheff, 1984). The chromatographic behaviour of periplasm on hydroxyapatite gave an indication of the resolution that could be obtained, TMAO reductase activity eluting separately from the main peak of protein (Figure 3.15). When the partially purified fraction from ion exchange chromatography was applied to the column of hydroxyapatite a further degree of purification was observed by the elution of a peak of enzyme activity separate from inactive peaks of protein (Figure 3.16). When analysed by SDS-PAGE, this active peak contained predominantly TMAO reductase protein in comparison with a zymogram stained similar gel, with a reduction in contaminating polypeptides from approximately 20 in the initial sample to 2-3. The use of hydroxyapatite at this stage of purification therefore increased the level of purification by a further 2-fold with the eluted sample approaching homogeneity. The yield obtained after chromatography was about 8% of that initially present in the periplasm, representing a drop of approximately 60% between ion exchange chromatography and hydroxyapatite (Table 3.8). This reduction was surprising in view of the fact that most of the eluted peak from hydroxyapatite was separated from other protein peaks, allowing each of the active fractions to be recovered. It therefore appears that some inactivation of the enzyme had occurred during hydroxyapatite chromatography and assuming no alteration in the molecular weight of the protein, the active peak probably contained some inactive enzyme.

Gel filtration of various samples containing TMAO reductase activity enabled some simultaneous characterization

and purification of the enzyme to be carried out (3.2.5). Using periplasm as a sample, two peaks of TMAO reductase activity were eluted, both containing the two different molecular weight enzymes (Figure 3.19). Thus the resolution of this technique, fractionating proteins on the basis of their molecular weight, was not sufficient to separate low-M_r TMAO reductase from the high-M_r enzyme. The first peak of activity eluted at the void volume of the column, indicating a molecular weight for the enzyme at or above the exclusion limit of the gel matrix. As this represented a molecular weight of approximately $5x10^6$ daltons and because of the presence of both TMAO reductase enzymes it was considered that this peak was a large aggregate. Because an association of TMAO reductase enzymes alone appeared unlikely, at least to the extent suggested by the molecular weight of the aggregate, it was assumed that this was either a multiprotein aggregate or a lipid-protein association, formed during the spheroplasting procedure. This suggested lipid-TMAO reductase association would appear attractive in view of the proposed loosely membrane bound location for the enzyme.

Because of the lack of resolution obtained and poor loading capacity of the technique, gel filtration was not suitable at an early stage of TMAO reductase purification. Using a gel matrix with a narrower range of fractionation and a larger column to give increased resolution, gel filtration was applied for the further purification of the hydroxyapatite TMAO reductase fraction. Elution of this fraction at 12 ml h^{-1} was ineffective at purifying TMAO reductase from the remaining polypeptides (Figure 3.21). One such contaminant was characterized as a cytochrome c_{552} present at an estimated 0.2μM concentration or approximately 1% of the total protein in the sample, assuming a molecular weight of 80 000 daltons. This cytochrome may be identical with the cytochrome c_{552} identified after ion exchange chromatography and the presence of this cytochrome in almost pure fractions of TMAO reductase may implicate this cytochrome in electron donation to the enzyme. Redox potentiometry (Figure 3.22) however, determined the cytochrome to be of low potential, so it is an unlikely

donor to TMAO reductase because the potential of the TMAO/TMA couple is +130mV (Castell, 1950). Soluble and membrane bound cytochromes c_{552} of *Alteromonas* sp. NCMB 400 were identified by Easter (1982) but not further studied. Work is being undertaken at present to characterize these and other compounds of the respiratory chain to TMAO reductase (Morris, unpublished data).

The resolution of gel filtration was improved by reducing the flow rate of the eluting buffer to 8 ml h^{-1} as suggested by the manufacturers (Pharmacia, Gel Filtration, theory and practice). This led to the separation of two minor peaks of protein from the major peak of enzyme activity (Figure 3.23). Analysis of this active peak showed all fractions to be homogeneous for the high-M_r TMAO reductase as judged by the single band of silver stained protein after SDS-PAGE. As there are reports of the silver stain not detecting some proteins after SDS-PAGE (BioRad Bulletin 1089) a similar gel was loaded with a greater amount of sample and stained with Coomassie blue. This also gave a single band of protein, confirming the purity of the sample (Plate 3.6).

The *Alteromonas* sp. NCMB 400 TMAO reductase purification protocol differed in a number of respects from that of the enzymes from *E.coli* (Shimokawa and Ishimoto, 1979) and *S.typhimurium* (Kwan and Barrett, 1983b). The TMAO reductases purified from the latter two organisms were membrane bound enzymes requiring detergent solubilization at least in the case of *E.coli*. In each protocol, a crude cell extract was used as an initial sample of enzyme with no reported precautions against proteolysis. DNA present in each crude extract was eliminated by either digestion with DNAase (*E.coli*) or precipitation with polyethyleneimine (*S.typhimurium*). The purification of TMAO reductase from *E.coli* and *S.typhimurium* required a greater number of separate stages than did the purification of the enzyme from *Alteromonas* sp. NCMB 400. It is therefore probable that a better optimization of techniques was achieved in this study although the general approach to purification was the same in all three organisms. Ammonium sulphate fractionation followed by ion exchange

chromatography was commonly used but in *E.coli* the enzyme was then further purified by gel filtration, two ion exchange procedures and hydroxyapatite chromatography whereas in *S.typhimurium*, chromatofocusing and gel filtration replaced the ion exchange and hydroxyapatite stages. The yield and degree of purification obtained in each organism showed a remarkable similarity: 1% yield, 78-fold purification in *E.coli* (Shimokawa and Ishimoto, 1979); 3% yield, 76-fold purification in *S.typhimurium* (Kwan and Barrett, 1983b) and 7% yield, 55-fold purification in *Alteromonas* sp. NCMB 400 (Table 3.10).

The reproducibility of the purification protocol as judged by the statistical analysis of the increase in purification and yield was generally good. The yield from the 60-80% $(NH_4)_2SO_4$ fractionation showed some variation, attributable to the problems of recovering the whole amount of activity after precipitation (Table 3.6). Ion exchange chromatography showed variation in both the purification and yield (Table 3.7) due in part to the occasional appearance of the second peak of high-M_r enzyme activity that was not included for further purification. Hydroxyapatite chromatography gave some variability in the level of purification obtained (Table 3.9) due possibly to the variable degree to which some of the minor proteins were resolved from the active fractions. Gel filtration gave a high degree of reproducibility both in yield and purification (Table 3.9).

As affinity chromatography exploits the unique biological activity of an enzyme, it was investigated as an alternative step in the purification of TMAO reductase. The resolution obtained by this technique may lead to a one-step purification (Scopes, 1982) as with the isolation of α-chymotrypsinogen (Cuatrecasas and Afinsen, 1971). Affinity chromatography may be arbitrarily divided into two forms: group specific adsorptions as exemplified by the Cibacron Blue F3GA and Procion Red HE-3B triazine dye ligands, selective particularly for NAD- and NADP-dependent enzymes respectively (Scopes, 1982; Subramanian, 1984); and the specific adsorptions which bind little other than the proteins with a specificity for the immobilized ligand (Scopes, 1982). This latter type of

separation was employed by Easter (1982) for the purification of *Alteromonas* sp. NCMB 400 TMAO reductase, using the analogue of TMAO, betaine as ligand, attached to AH-Sepharose 4B. An 18-fold purification of the enzyme from periplasm was obtained in a single step with a yield of 47%. However, the fractions obtained were not pure in that they contained at least two contaminating proteins on SDS-PAGE. With these results in mind, the same technique was investigated as a rapid method of purifying TMAO reductase (3.4). Using the same procedure however, these results could not be repeated, the TMAO reductases in periplasm eluting within fractions containing non-enzyme protein (Figure 3.17). The lack of purification obtained and the poor loading capacity of the system prompted its use in a later stage of the protocol using samples further purified in TMAO reductase. Under the conditions employed, the enzyme was weakly bound to the column (Figure 3.18) suggesting only a slight interaction with the ligand. The reasons for the failure of the technique were not clear but several explanations suggested themselves. The lack of binding obtained with this experiment in comparison with that of Easter (1982) may have reflected a poor absorption capacity of the column through a lack of immobilized ligand. Although the amount of betaine bound to the matrix was not specifically assayed, the column was prepared in the same way as that of Easter and should have had an approximate equal capacity. Easter obtained the best results with loaded periplasm, the partially purified preparation containing both the TMAO reductase enzymes. This was the case in this study periplasmic TMAO reductases binding to the column rather than eluting. However a buffer with a concentration of salt sufficient for optimum purification resulted in the continuous elution of TMAO reductase from the partially purified sample, implying a difference in the reactivity of the two enzymes towards betaine. Further work on the application of this technique in the light of results obtained on the substrate use and inhibition of TMAO reductase with various alternative ligands is discussed in section 4.8.

4.4 Characterization of the Purified TMAO Reductase

The molecular weight of the purified high-M_r TMAO reductase was 90 000 daltons as determined by SDS-PAGE (3.3.1) and 86 000 daltons by gel filtration (Figure 3.25). This suggested that under native conditions, the protein did not form intermolecular aggregates resulting in a multimeric protein. In the partially purified preparation obtained by Easter (1982), polypeptides between 53 000 and 98 000 were recorded with the suggestion that these were subunits of a native protein of molecular weight 400 000-450 000 daltons by gel filtration. In this study, gel filtration of periplasm was carried out in the presence of NaCl to prevent random aggregation of proteins and interaction with the gel matrix, a precaution which was not observed by Easter. The major amount of activity eluted in a peak between 43 000-158 000 daltons and apart from a small amount of high molecular weight aggregate eluting in the void volume, no evidence for oligomer formation was apparent for either the high- or low-M_r enzymes. The molecular weight of the high-M_r enzyme dropped to 86 000 daltons under reducing conditions together with the appearance of a very low molecular weight band at the dye front; this may be indicative of the loss of a small component of the enzyme possibly a molybdenum cofactor (see 4.6). The high-M_r enzyme did not however dissociate into recognisable subunits and there was no evidence that the 47 000 dalton low-M_r enzyme was a subunit of the high-M_r enzyme. The molecular weight and the non subunit association of the purified enzyme in *Alteromonas* sp. NCMB 400 differs from the enzymes purified from either *S.typhimurium* (Kwan and Barrett, 1983b) or *E.coli* (Shimokawa and Ishimoto, 1979), though a direct comparison between these enzymes must be treated with caution due to the multiplicity of the enzymes in each organism. A native molecular weight of 332 000 daltons was obtained for the purified *S.typhimurium* TMAO reductase after gel filtration, being a tetramer of an 84 500 dalton subunit. The inducible TMAO reductase of *E.coli* was also an oligomer of molecular weight 222 000 daltons with non-identical 60 000 and 80 000

dalton subunits.

The pI of purified *Alteromonas* sp. NCMB 400 TMAO reductase was shown to be markedly affected by the presence of salt in the sample (3.3.2). When salt was included (0.2M), a single irregular band of activity with pI of approximately pH 5.6 was detected by zymogram staining, no similar band being present in the periplasm. On removal of salt, two separate bands of activity were detected with pI values of pH 5.1 and 5.2, corresponding to activity present in the periplasm. These values differ slightly from those obtained by Easter (1982) for the partially purified preparation of *Alteromonas* sp.NCMB 400 TMAO reductase at pH 5.45 and 5.50 whereas those for the purified enzymes of *E.coli* and *S.typhimurium* were pH 5.2 and 5.65 respectively (Shimokawa and Ishimoto, 1979; Kwan and Barrett, 1983b).

For a single protein to produce two zones of activity after electrofocusing suggests a partial modification of the enzyme into two separate ionic forms. This was confirmed by the observation that only a single active band was observed if the ionic strength was high. It is not known if the two peaks occasionally observed during ion exchange chromatography of the high-M_r enzyme are related to the forms of different pI.

The $K_{m\ TMAO}$ value obtained for the purified enzyme of *Alteromonas* sp. NCMB 400 (19µM) was significantly lower than that obtained for the same enzyme by Easter (1982) at 93-150µM; *S.typhimurium*, 890µM; *E.coli*, 1.5mM and *V.parahaemolyticus*, 1.7mM (Kwan and Barrett, 1983b; Shimokawa and Ishomoto, 1979; Unemoto *et al.*, 1965). This 50-100-fold higher affinity of the high-M_r enzyme of *Alteromonas* sp. NCMB 400 for TMAO than in other organisms may be taken as evidence of a greater importance of TMAO respiration in this bacterium, particularly as it is non-fermentative. The value obtained for $K_{m\ TMAO}$ of *Alteromonas* sp. NCMB 400 is of the same order of magnitude as the nitrate reductase of various bacteria; *Azotobacter chroococcum*, 2µM; *Bacillus licheniformis*, 57µM (Guerrero *et al.*, 1973; van't Riet *et al.*, 1979), but not that from *E.coli* at 0.64mM (Azoulay *et al.*, 1972).

4.5 Regulation and Induction of TMAO Reductase

The regulation of most anaerobic respiratory systems appears to be under a two step control; induction by the appropriate alternative terminal electron acceptor and repression under aerobic conditions. The results of Easter (1982; Easter *et al.*, 1983) demonstrated the induction of TMAO reductase under microaerobic growth conditions; both in the presence and absence of TMAO, oxygen severely repressing TMAO reductase activity. These results were confirmed in this study with 11% of the activity present under microaerobic conditions being present under aerobic conditions (Table 3.4). The level of activity observable under aerobic conditions may have been due to the demand for oxygen by the culture exceeding the oxygen supply, such that localized microaerobic conditions were present. The importance of TMAO in the full induction of TMAO reductase was demonstrated by the fact that in the absence of TMAO, only 1% of the normal induced activity was present (Table 3.4). Thus the two-step regulatory model is applicable in that both microaerobic conditions and the terminal electron acceptor are required for the full induction of TMAO reductase in *Alteromonas* sp. NCMB 400, as is the case in *E.coli* (Sakaguchi and Kawai, 1975a, 1977b).

The biochemical basis of this regulatory phenomenon has been best studied with the fumarate reductase of *E.coli* by use of genetic analyses. Pleiotropic mutants have been isolated that are simultaneously defective in nitrate and fumarate utilization, designated *fnr*$^-$, and are characterized by their reduced content of nitrate and fumarate respiratory chain components, although still remaining sensitive to chlorate (Lambden and Guest, 1976). The product of the *fnr* gene is seen as acting as a positive regulator of respiratory enzyme synthesis such that under anaerobic conditions, the *fnr* product is active and able to function as a positive regulator whereas under aerobic conditions, the product is inactive and transcription of the relevant operon does not proceed.

Although the *fnr* product has a role in the regulation of anaerobic respiration, the product does not appear to control all anaerobic metabolic processes, nor is it universally distributed. In the metabolism of tripeptides under anaerobic conditions, the *tpp* B gene product is induced as a permease that is not under the control of the *fnr* product (Higgins, personal communication). Alternative models have been proposed to that of a direct involvement of the *fnr* product to take account of the role of the alternative electron acceptor in regulation and to make it more generally applicable to other bacterial respiratory systems. In the model developed by Stouthamer for the regulation of nitrate reductase in *Proteus mirabilis*, electron flow from primary donor through the respiratory chain to terminal acceptor is envisaged as the regulatory factor (De Groot and Stouthamer, 1970a,b). In the model developed by Cole (personal communication) using the nitrate reductase system of *E.coli*, the regulatory gene products of *nar L* and *nar K* (Stewart, 1982) are seen to exert autogenous control of the nitrate reductase in the presence of an appropriate terminal electron acceptor (nitrate), transcription of the nitrate reductase genes proceeds whereas other terminal reductase operons are repressed. In this case, the *fnr* product may exert control by interacting with the regulatory product before its interaction with a terminal electron acceptor to prevent transcription of the reductase operon in the acceptors absence.

For the hydrogenase and formate hydrogen lyase of *E.coli*, Pecher *et al.* (1983) described a repressive effect of various terminal electron acceptors on the induction of appropriate terminal enzymes that was dependent on the size of the corresponding redox potential; the greater the redox span between primary electron donor and terminal electron acceptor, then the more repressive the effect on respiratory chains using narrower redox couples. However, contrary evidence for this redox control is beginning to appear with more emphasis being placed on the control exerted by oxygen. Sulphate reducing bacteria have been shown to utilize sulphate simultaneously with, or in preference to, nitrate as terminal electron

acceptor, even though the redox span to nitrate is greater (Cole, personal communication). A similar situation may exist with TMAO reduction in *Alteromonas* sp. NCMB 400, nitrate not repressing TMAO reduction even though nitrate possesses a more positive redox potential than TMAO (Nasser, 1983).

To clarify further the regulation of TMAO reductase in *Alteromonas* sp. NCMB 400, the effect of alternative electron acceptors on the induction of the enzyme was investigated. Nitrate or fumarate included in the growth medium in place of TMAO were ineffective as inducers of TMAO reductase, the levels of activity being equal to or lower than the control without added terminal electron acceptor (Table 3.3). As some activity was present in each case, it was possible to visualize and compare the effects of growth in the absence of TMAO on the two TMAO reductase activities by zymogram staining (Plate 3.3). Two features were evident from these results; the different controls for induction of the two enzymes and the differing effects of the alternative electron acceptors as inducers. With nitrate in the growth medium, only the low-M_r enzyme was evident; this could indicate a difference in regulation with inductive synthesis of the high-M_r enzyme and constitutive synthesis of the low-M_r enzyme. In the absence of added terminal electron acceptor, both enzymes of TMAO reduction were present, an observation that supports the proposal that nitrate affects the high-M_r enzyme through repressive control. In contrast, in the presence of fumarate, low levels of 4 enzymes capable of TMAO reduction were detected; the high- and low-M_r enzymes and two other enzymes not previously detected. Thus the low-M_r enzyme was present under each growth condition, implying a constitutive synthesis of the enzyme, but the qualitative increase in the activity of this enzyme with TMAO, as judged by zymogram staining, also suggests an element of inductive regulation. The high-M_r enzyme was present at three levels of activity: high in the presence of TMAO, low in the presence of fumarate or no alternative electron acceptor and absent with nitrate. This implies an inductive synthesis of the enzyme by TMAO from a low residual level that is completely repressed by nitrate.

The inclusion of nitrate or fumarate in conjunction with TMAO in microaerobic culture did not lead to the repression of TMAO reductase synthesis (Nasser, 1983), possibly indicative of the methods of regulation described earlier. It has been proposed that TMAO is the preferential electron acceptor under microaerobic conditions in *Alteromonas* sp. NCMB 400 (Nasser, 1983) unlike in *E.coli* where the further formation of TMAO reductase is repressed by the presence of nitrate or fumarate in conjunction with TMAO (Sakaguchi and Kawai, 1975a). It is unfortunate that a similar analysis of the effect growth conditions have on the multiple TMAO reductase enzymes of *E.coli* has not been conducted, so precluding a comparison with the results presented here.

DMSO and pyridine N-oxide were shown to be effective inducers of TMAO reductase from the alternative electron acceptors added to microaerobic cultures in the absence of TMAO (Table 3.3). In the presence of DMSO, both the enzymes of TMAO reduction were induced as judged by SDS-PAGE analysis (Plate 3.3) but preliminary results (Kennedy, unpublished data) using a zymogram stain for DMSO reductase activity have indicated that only the low-M_r TMAO reductase has any DMSO reductase activity. DMSO was shown to be a poor substrate for TMAO reductase: there was no activity with the purified high-M_r enzyme and only 11% of the TMAO induced activity in the periplasm (presumably due to the low-M_r enzyme). DMSO may therefore be considered a gratuitous inducer of the high-M_r TMAO reductase in that full activity of the enzyme is induced, but the compound does not act as a substrate for the enzyme.

This apparent gratuitous induction of TMAO reductase by DMSO resembles that which occurs with various substrate analogues of the aliphatic amidases of *Ps.aeruginosa*. The analogue substrate lactamide and N-acetyl acetamide are both poor substrates for the amidase but fully induce the enzyme activity (Clarke, 1978). Similar induction, substrate specificity and inhibition experiments were conducted with periplasmic TMAO reductase and the purified enzyme using analogues of TMAO (Tables 3.2, 3.15 and 3.19). Pyridine

N-oxide was a poor substrate for the high-M_r TMAO reductase or the periplasmic enzymes and did not greatly inhibit the activity of the purified enzyme with TMAO. This substrate did however induce TMAO reductase activity in the periplasm to 85% of that induced by TMAO, making this substrate a gratuitous inducer of TMAO reductase. LDAO was the best alternative substrate for TMAO reductase giving 63% and 41% of the activity obtained with TMAO from the periplasmic and purified enzymes respectively. LDAO inhibited enzyme activity with TMAO as substrate to 29% of that obtained in its absence, with the probability that inhibition was greater due to LDAO acting as a substrate, and contributing to the observed enzyme activity. The LDAO added to cultures of *Alteromonas* sp. NCMB 400 caused lysis of the cells leading to only 3% of the normally induced activity being present. Betaine, used as an immobilized ligand during affinity chromatography was ineffective as a substrate and an inducer of TMAO reductase. The compound did show some interaction with the enzyme in that activity was reduced to 50% of that obtained in the absence of betaine. This poor interaction with the enzymes of TMAO reduction may explain the lack of binding of the enzymes to the affinity column. A possible better affinity ligand would be LDAO, but this compound did not possess suitable reactive groups with which to couple it to the gel matrix support.

On the basis of our present knowledge of TMAO reduction in *Alteromonas* sp. NCMB 400 it is possible to propose a scheme for the regulation of enzyme synthesis. The presence of a specific repressor protein would prevent transcription of the genes coding for the high- and low-M_r TMAO reductases under aerobic conditions by negative regulation. With the onset of microaerobic conditions, the repression would in part be relieved either directly or indirectly by the redox state of the cell leading to the partial induction of the two enzymes. Further control would be imposed by the alternative electron acceptors available, nitrate specifically interacting with the repressor to re-establish repression of high-M_r TMAO reductase but not the low-M_r enzyme, fumarate causing further but only partial induction of the high-M_r enzyme and secondary

enzymes capable of TMAO reduction, and TMAO, DMSO and pyridine N-oxide fully interacting with the repressor protein so relieving inhibition of transcription and allowing full induction.

4.6 TMAO Reductase Catalysis

The catalytic mechanism of TMAO reduction in *Alteromonas* sp. NCMB 400 was investigated by analysis of the substrate requirements of the periplasmic and purified TMAO reductases, the presence of redox centres in the purified enzyme and the involvement of other chemical groups in catalysis.

The electron acceptor substrate specificity (Table 3.15) provided information on the nature of the components required to accept electrons from the enzyme. As expected, the enzyme showed a high degree of subtlety in catalysis. Compounds lacking an oxide moiety were in general ineffective as substrates, with the suggestion that this was the reactive group in catalysis. The nature of the oxygen component was also shown to be important, with the carboxyl groups of carnitine or betaine and the hydroxyl group of hydroxylamine being unable to accept electrons from TMAO reductase. The specificity of reduction was towards N-oxide groups such as TMAO, pyridine N-oxide and picoline N-oxide rather than the S-oxide group of DMSO. Further specificity was imparted by the molecular structure supporting the reactive N-oxide, the methyl groups in TMAO and LDAO allowing a greater activity than the aromatic groups of pyridine, picoline and nicotinamide N-oxides. Adenosine N-oxide provided an exception to this in that it was a moderate substrate even though the N-oxide was bound to an aromatic ring.

The specificity of the enzyme was reflected in the K_m values for various substrates (Table 3.16) with a 100-fold lower affinity of the enzyme for LDAO and a 300-fold lower affinity for pyridine N-oxide and picoline N-oxide than for TMAO. The periplasmic enzymes had a similar restricted range of substrate specificity, with additional utilization of

DMSO and in general, a greater activity towards the substrates than the purified enzyme. This compares with the broader substrate utilization range for the enzyme of TMAO reduction from *E.coli* (Sagai and Ishimoto, 1973; Shimokawa and Ishimoto, 1979), leading to the description of the enzyme as an amine N-oxide reductase. On the basis of these results therefore, the enzyme from *Alteromonas* sp. NCMB 400 has a greater specificity for TMAO and is best described as a TMAO reductase.

Only the artificial bipyridylium compounds were effective as immediate electron donors to either periplasmic or purified TMAO reductase, other potential donors such as NADH, NADPH or FAD being ineffective (Table 3.13). This contrasts with the results presented for most other TMAO reductases, there seemingly being a variety of electron transfer reactions between the enzymes in different organisms. The TMAO reductase from *Alteromonas* sp. NCMB 400 appears similar to that purified from *S.typhimurium* in that in those organisms, FAD, FMN, cytochrome *c*, NAD(P)H and ascorbate were all ineffective as electron donors (Kwan and Barrett, 1983b). In *E.coli* however, FAD, FMN (Shimokawa and Ishimoto, 1979) and cytochrome *c* (Sagai and Ishimoto, 1973) were utilized as electron donors by TMAO reductase.

The presence of flavin in the enzyme was tentatively suggested from absorption spectrophotometry results, a broad peak of absorption present between 350nm and 450nm lost upon reduction with dithionite. These two features of absorption and bleaching are indicative of flavin content, but they were not observed to a great extent, particularly in comparison with that of free FAD or FMN (Dixon and Webb, 1979). Although the properties of the flavin may be modified by combination with the enzyme protein, the results were not as convincing as has been obtained by similar studies with other enzymes, for example the NADH dehydrogenase from *B.subtilis* as purified by Bergsma *et al.* (1982). To confirm the presence or absence of flavin in the enzyme further studies are required. Alternative methods for the detection of flavin include thin layer chromatography or fluorescence studies on acid extracted material but again, the results obtained are dependent on

the properties of the initial flavoprotein complex. EPR studies provide perhaps the least ambiguous results since the paramagnetic species involved tend to exhibit diagnostic and well resolved features (Palmer and Olsen, 1980). From the lack of stimulation of activity provided by incubation of the assay reactants in the presence of FAD (Table 3.14) any enzyme bound FAD must be tightly associated with the enzyme or not function in the MV mediated reduction of TMAO.

In contrast to the purified TMAO reductase from *E.coli*, shown to possess 1.8 mol Fe mol enzyme^{-1} (Shimokawa and Ishimoto, 1979) neither haem nor non-haem iron was detected in the purified enzyme from *Alteromonas* sp. NCMB 400 (Figure 3.24, 3.3.8). The iron present in the enzyme of *E.coli* was believed to be involved in catalysis from the effect iron chelators had in reducing activity (Shimokawa and Ishimoto, 1979). The fact that the *Alteromonas* sp. NCMB 400 enzyme was active even in the presence of SDS, known to be disruptive to iron-sulphur centres (Boxer, personal communication), supports the data on the absence of iron or suggests that it is not involved in the MV linked reduction of TMAO. Further methods for the detection of iron in the enzyme include EPR measurements on iron-sulphur clusters and the quantitative labelling of the enzyme with ^{59}Fe.

Molybdenum has been detected and subsequently implicated in the catalysis of a number of metabolic and respiratory enzymes including the formate dehydrogenase of *E.coli* (Enoch and Lester, 1974, 1975) and the nitrate reductases of many bacteria (see Hewitt and Notton, 1980). Molybdenum has also been implicated in the functioning of *E.coli* TMAO reductase, by stimulation and inhibition studies with MoO_4^{2-} and WO_4^{2-} respectively, (Shimokawa and Ishimoto, 1979) and in *Alteromonas* sp NCMB 400, by the identification of pleiotropic mutants resistant to the enzyme inhibitor chlorate and their characterization as being defective in one or more aspects of molybdenum cofactor processing (Nasser, 1983). The presence of molybdenum in the high-M_r TMAO reductase of *Alteromonas* sp. NCMB 400 was confirmed with the finding of 1.32g atoms Mo mol enzyme^{-1} (3.3.8). This figure could be

interpreted as either an underestimate, the actual value approaching 2g atoms Mo mol enzyme^{-1} suggesting a loss of molybdenum during purification, or an overestimate the real value being close to 1. Evidence on the stability of molybdenum in molybdoenzymes is rather contradictory perhaps reflecting the dependence of stability on the enzyme under study. Results from a number of sources with a variety of molybdoenzymes indicate that molybdenum is only freely exchangeable when in the form of an unassociated cofactor and that when the cofactor is incorporated into the enzyme it is effectively locked, making replacement impossible (Lee, 1978; Alikulov, 1980; Nasser, 1983). Thus the loss of molybdenum during the purification of the enzyme appears unlikely and a value of approximately 1g atom Mo mol enzyme^{-1} would be in more agreement with the estimates obtained for the nitrate reductase and associated formate dehydrogenase of *E.coli* at 1.0 and 0.95g atoms Mo mol enzyme^{-1} respectively (Forget, 1974; Bosma *et al.*, 1978; Enoch and Lester, 1974, 1975). Losses in molybdenum have however been reported and explained, particularly during the purification of spinach nitrate reductase with a variety of affinity chromatography techniques (Hewitt and Notton, 1980).

In addition to the respiratory molybdoenzymes previously described, a number of other enzymes have been characterized as containing molybdenum including xanthine oxidase, sulphite oxidase and aldehyde oxidase (Pope *et al.*, 1980). These enzymes together with the respiratory enzymes appear to possess a very similar cofactor whereas that in nitrogenase is an iron-molybdenum cofactor and is unable to reconstitute the nitrate reductase of *N.crassa nit* 1 mutant defective in molybdenum cofactor (Shah and Brill, 1977; Pieknos *et al.*, 1977).

The molybdenum of *Alteromonas* sp. NCMB 400 was shown to be of the non-nitrogenase type in being able to reconstitute nitrate reductase activity in the *nit* 1 mutant of *N.crassa* (Nasser, 1983).

It is generally believed that the electron acceptor substrate interacts initially at the molybdenum cofactor in

molybdoenzymes (Spence, 1980) as do the non physiological electron donors MV and BV (Hewitt and Notton, 1980). All the investigated molybdoenzymes contain at least one other redox centre in addition to molybdenum, as FAD, Fe/S or haem Fe (Spence, 1980). The *E.coli* nitrate reductase has the various redox centres located on separate subunits of the holoenzyme (see introduction). The molybdenum and Fe/S centres are situated on the same subunit (A), subunit B is involved in the membrane association of the enzyme and the haem *b* is located in subunit c (Enoch and Lester, 1974; MacGregor, 1975b; De Moss, 1977; Chaudhry and MacGregor, 1983a). Thus, the finding of only molybdenum in the purified TMAO reductase of *Alteromonas* sp. NCMB 400, given that the presence of flavin was tentative, is unusual in the respect that no other redox centres were present and that no subunits containing such centres were identified. Further characterization of the redox centre(s) in the enzyme is obviously required to clarify the catalytic mechanism, but a possible interpretation of these results is that the enzyme as purified is a subunit of a larger enzyme containing other subunits with separate redox centres. As the enzyme was purified from the soluble fraction with no evidence of a larger molecular weight enzyme, the additional subunits may be membrane bound and not released upon spheroplasting, so providing a site of attachment of the purified subunit to the membrane. Further research is required to test this hypothesis, possibly along the lines of a less disruptive spheroplasting technique, a membrane purification protocol and the use of physiological *in vivo* electron donors to the enzyme, to characterize the electron transport sequence. Preliminary results (Morris, unpublished data) with a soluble c-type cytochrome from the periplasm of *Alteromonas* sp. NCMB 400 has shown that this is incapable of electron donation to the purified enzyme, possibly supporting the presence of other components of the enzyme required for *in vivo* catalysis. This is supported by the finding that in the presence but not absence of membranes, formate reduces c-type cytochromes that can be subsequently reoxidized by TMAO (Morris, unpublished data).

The sensitivity of purified TMAO reductase activity to thiol group modifying reagents such as DTNB and in particular PHMB (Table 3.18) and the relief of inhibition by added dithiothreitol suggests the presence of a sulphydryl group associated with activity. A similar inhibition of the partially purified enzyme was found by Easter (1982), together with a stabilizing effect from dithiothreitol and glutathione that was not found in this study. Inhibition by PHMB was obtained with the TMAO reductase of *V.parahaemolyticus* (Unemoto *et al.*, 1965) as with the fumarate reductase of *E.coli* (Robinson and Weiner, 1982) and *P.denitrificans* nitrate reductase (Lam and Nicholas, 1969). The inhibition of *Alteromonas* sp. NCMB 400 TMAO reductase suggests that an SH-group is accessible to inhibition and either directly or indirectly part of the active site of the enzyme. The number of such SH-groups was not determined but a titration of activity against added concentration of inhibitor would determine the groups involved in catalysis. One group was shown to be directly involved in the reduction of fumarate by *E.coli* fumarate reductase as well as other non-essential groups (Robinson and Weiner, 1982).

Cyanide inhibition is mediated through iron complexing, such that enzymes containing haem are particularly sensitive to inhibition (Lehninger, 1975). Some inhibition of periplasmic and purified TMAO reductase was observed (>30%) possibly indicating the presence of iron but the lack of haem as judged by the absorption spectrum and the lack of non-haem iron is in apparent contradiction to this. A similar low level of cyanide inhibition was obtained with the partially pure preparation of TMAO reductase (Easter, 1982) while inhibition was absent from the TMAO reductases of *S.typhimurium* (Kwan and Barrett, 1983b) and *V.parahaemolyticus* (Unemoto *et al.*, 1965). However the *E.coli* nitrate reductase subunit A, containing an iron-sulphur centre was inhibited by cyanide (Forget, 1974). The respiratory inhibitor sodium azide caused only a slight reduction in the activity of periplasmic or purified TMAO reductase as was found in the enzymes from *V.parahaemolyticus* (Unemoto *et al.*, 1965). *E.coli* (Sagai

and Ishimoto, 1973; Shimokawa and Ishimoto, 1977) and *S.typhimurium* (Kwan and Barrett, 1983b). Again, the nitrate reductase subunit A of *E.coli* was inhibited by azide (Forget, 1974). Interaction of nitrate reductase with an inhibitor such as azide *in vivo* prevents electron transport and when included in the growth medium at appropriate concentrations, azide has been shown to cause an increased induction of nitrate reductase during anaerobic growth, cells trying to escape inhibition of nitrate respiration by overproduction of the terminal enzyme (see introduction, De Groot and Stouthamer, 1970a). Similar experiments were carried out with 1mM azide included in the microaerobic incubation of *Alteromonas* sp. NCMB 400, with the aim of obtaining increased levels of TMAO reductase. This proved ineffective with a decrease in culture turbidity indicating toxicity of azide at this concentration.

Metal chelating agents including the molybdenum chelator KSCN (Lam and Nicholas, 1969) were without effect on the activity of TMAO reductase, indicating that molybdenum is tightly associated with the enzyme and is not easily exchangeable with other metals, as suggested earlier. The chemical similarity between tungsten and molybdenum, as group VI B elements accounts for the observed inhibition of many molybdoenzymes by tungsten (Pope *et al.*, 1982). Tungstate was shown to partially inhibit TMAO reductase (Table 3.17) particularly the purified enzyme (to 36% of the original activity) in comparison to the enzymes in periplasm (to 93% of the original activity). This is in agreement with the results obtained by Nasser (1983) with a lesser degree of inhibition (85% of the original at the same concentration as used here) and the *E.coli* TMAO reductase (Shimokawa and Ishimoto, 1979). The extent of this inhibition with the purified enzyme from *Alteromonas* sp. NCMB 400 was surprising in that a competitive type of inhibition is believed to account for the loss of activity, the tungstate replacing molybdate in the cofactor which does not then function as a redox centre. However the absence of any molybdenum chelator-induced loss of activity was explained by a tight association of the metal in the

cofactor. The demonstration that molybdate also inhibited enzyme activity in periplasmic and purified forms may imply a general toxic effect of the metals rather than a specific redox centre replacement.

The lack of chlorate reduction, particularly by the purified TMAO reductase is unusual in that the TMAO reductase purified from *E.coli* has significant chlorate reductase activity (Shimokawa and Ishimoto, 1979), as do many nitrate reductase enzymes from different sources (Hewitt and Notton, 1980). The terminal moiety of the enzyme (subunit A) is believed to be the reactive centre of the enzyme for nitrate or chlorate reduction as it contains the iron-sulphur and molybdenum centres (Chaudhry and MacGregor, 1983a). However, when purified separately from the enzyme (Taniguchi and Itagaki, 1960; Clegg, 1976), although maintaining activity with MV, the subunit is incapable of reducing chlorate, indicating the requirement for the additional structural features of the native enzyme (Hewitt and Notton, 1980). In this respect the purified TMAO reductase from *Alteromonas* sp. NCMB 400 resembles subunit A of *E.coli* nitrate reductase in being unable to reduce chlorate.

All the inhibition studies were carried out using the artificial electron donor MV and the effect of the various inhibitions should be interpreted in the knowledge that any inhibition is related to the electron transfer between MV, the molybdenum centre and TMAO. More information on catalysis might be obtained by the use of physiological donors to TMAO and extended inhibition studies. As it has been suggested that the purified enzyme is a component of large enzyme with additional redox centres, this holoenzyme would have to be characterized and isolated before further inhibition studies could be carried out.

4.7 Immunological Relatedness of TMAO Reductases

Antiserum from mice containing antibodies against the purified high-M_r TMAO reductase of *Alteromonas* sp. NCMB 400 was used to compare the two activities in the organism in terms of their immunological identity. The preliminary results from C.I.E. experiments indicated a slight cross reaction between the two enzymes, with the formation of immunoprecipitate corresponding to the position of each activity, from a periplasm sample resolved in an agarose gel (Plate 3.11). The immunoprecipitate formed from the low-M_r enzyme was shown to be due to a contamination of this activity with the high-M_r enzyme. The electrophoretic behaviour of the two enzymes on agarose was shown to be different from that on polyacrylamide in that no contamination of the low-M_r activity by the high-M_r activity was evident with the latter (Figure 3.31). The reasons for this discrepancy are not clear, but as PAGE and particularly SDS-PAGE separate proteins on the basis of their molecular weight, unlike agarose electrophoresis, where separation is more on the basis of their mobility in an electric field, a weak association between the high- and low-M_r enzyme might have remained during agarose electrophoresis and been dissociated by PAGE or SDS-PAGE. Using RIE of the two separate enzymes isolated by elution from a polyacrylamide gel, the antiserum was shown to be specific for the high-M_r enzyme rather than the low-M_r enzyme with the development of rocket immunoprecipitates only with the former. These results were confirmed by use of the electroblotting-immunodetection or western blotting procedure of Towbin *et al.* (1979). Stained bands corresponding to the high-M_r enzyme were the only proteins detected with the antiserum probe after SDS-PAGE of periplasm and blotting onto nitrocellulose (Plate 3.12). This assumes that, as with the high-M_r TMAO reductase, the native low-M_r enzyme is not altered in tertiary structure by blotting onto nitrocellulose. The conclusion from these experiments was that there is no immunological identity between the high- and low-M_r TMAO reductase enzymes of *Alteromonas* sp. NCMB 400.

A similar immunological comparison was made between the high-M_r enzyme of *Alteromonas* sp. NCMB 400 and TMAO reductase activities in *E.coli* and *S.typhimurium*. After growth under conditions for the induction of TMAO reductase, the periplasmic, cytoplasmic and solubilized membrane fractions of *E.coli* and the membrane and sonicate fractions of *S.typhimurium* were analysed for the location of TMAO reductase by PAGE, SDS-PAGE and zymogram staining. Using the same method of spheroplast preparation for *E.coli* as used for *Alteromonas* sp. NCMB 400, 3 of the previously determined 4 activities (Shimokawa and Ishimoto, 1979) were released into the periplasm fraction (Figure 3.32). As only 2 activities have been reported to be loosely membrane bound, the other 2 being more strongly membrane bound, this suggests a rather disruptive effect of the spheroplast technique used as discussed earlier (4.1). The fourth enzyme activity was absent from both the periplasmic and cytoplasmic fractions, the reason for this being unknown. The 3 activities detected after PAGE were reduced to 2 after SDS-PAGE due probably to denaturation of the third enzyme. A similar situation to that of *E.coli* TMAO reductases was evident with the enzymes from *S.typhimurium*. Two of the three characterized enzymes (Kwan and Barrett, 1983a) were present in the sonicate cell fraction, one being lost after SDS-PAGE (Figure 3.32). Each of the enzymes from either *E.coli* or *S.typhimurium* had a different mobility to those present in *Alteromonas* sp. NCMB 400 after PAGE or SDS-PAGE indicating a superficial difference between the enzymes, whereas the two activities in *S.typhimurium* after PAGE corresponded in mobility to two enzymes in *E.coli* suggesting a possible relationship.

When analysed by R.I.E., no immunoprecipitate was formed between either the *S.typhimurium* sonicate fraction or the *E.coli* fraction and the antiserum raised against the purified high-M_r enzyme from *Alteromonas* sp. NCMB 400. This was confirmed by electroblotting and immunodetection (Plate 3.13). A similar immunological comparison of different bacteria has been carried out to determine the relatedness of fumarate reductase (Unden and Cole, 1983) and nitrate reductase

(van't Riet *et al.*, 1979) with the outcome that within a bacterial family there is a degree of relatedness, not present between different families. The fumarate reductases of various genera were also compared on the basis of DNA hydridization to confirm the results but this was not attempted here.

Various mutant strains of *Alteromonas* sp. NCMB 400 defective in TMAO reductase activity have been isolated and partially characterized (Table 3.20) as lacking both molybdenum cofactor and enzyme activity (strain 322) or activity only (strains 292 and I58, Nasser, 1983). An attempt was made to characterize these mutants in terms of the effect of the mutation on the TMAO reductase protein rather than on the activity by using the antiserum against purified TMAO reductase.

Preliminary experiments showed that the mutants possessed a very low level of induced TMAO reductase activity (Table 3.21). This indicated either that the lesions were due to point rather than deletion mutations leaving some residual activity or that the mutants partially reverted. No reisolation of mutants was carried out. In all three mutant strains a third enzyme activity was present after zymogram staining of an SDS-PAGE gel which was not apparent in the wild-type (Plate 3.14). The origin of this additional activity is not clear but it may have been produced by, or in response to the mutation in these strains. Electroblotting and immunodetection was used to determine the relative amounts of the high-M_r TMAO reductase in the mutant strains in comparison to that in the wild-type (Plate 3.15). Each sample of periplasm from the mutants produced a stain equal to or greater than that of the wild-type of equal protein loading, when challenged with antiserum to the high-M_r enzyme, even though their activity was very much reduced. From this it can be concluded that the high-M_r enzyme is synthesized at a level equal to or greater than that in the wild-type but that it is only partially active. This further suggests that these mutant strains were not defective in the synthesis of the enzyme protein or its translocation across the membrane into the cytoplasm, probably confirming the defect in molybdenum co-

factor processing. The semi-quantitative estimate that the mutant strains produced equal or greater amounts of high-M_r TMAO reductase protein may have regulatory implications. The cell was able to synthesize the enzyme even though the enzyme was largely inactive, resulting in some cases in the overproduction of the enzyme, perhaps as part of an escape synthesis. This in part resembles the regulatory model proposed by De Groot and Stouthamer (1970a) for nitrate reductase, in which interuption of the electron flow from donor to substrate either by inhibition (azide with nitrate reductase) or mutational loss of activity (mutant strains of *Alteromonas* sp. NCMB 400 in TMAO reductase) leads to the overproduction of the terminal enzyme. This further implies an aspect of regulatory control which is at the level of DNA transcription rather than translation, assuming a uniformity in the time of translation and life of the message. Further characterization requires the description in more depth of the various genotypic and phenotypic features of these and other mutants, particularly as applied to the regulatory control of the enzyme.

In the three mutants the third band of activity was detected by western blotting as a heavily stained band that was absent in the wild-type. In addition several other faint bands were detected in the wild-type and mutant samples, possibly due to an impure sample of TMAO reductase being used to produce the antiserum used in this set of experiments. The enzyme activity of the third bands as judged by zymogram staining was low but gave an immunodetection stain equal to if not greater than the high-M_r enzyme immunostain. This band therefore bears a strong immunological identity with the high-M_r enzyme, prompting the suggestion that it may be a proteolytic product or an incorrectly processed precursor of the same enzyme. As this band was present in each of the mutants of supposedly different defect in TMAO reduction, the implication is that it is the general phenomenon of lack of TMAO reduction that is responsible for the appearance of the third activity. A more detailed genetic analysis is required to clarify the origin of this protein and the application of a series of monoclonal antibodies in comparison studies would aid in characterizing the specific regions or epitopes the two enzymes share in common.

4.8 Future Work

The purification of high-M_r TMAO reductase from *Alteromonas* sp. NCMB 400 allowed a biochemical, biophysical and immunochemical characterization of the enzyme and a subsequent comparison with other enzymes of TMAO reduction. The techniques used in purification were optimized at each stage, for each application and then combined to produce an efficient protocol. A problem associated with the purification was the low yield of enzyme, so preventing subsequent analytical techniques requiring relatively large amounts of protein. Various approaches could be undertaken to improve the yield of enzyme, either by rapid methods to allow the purification or more enzyme in unit time, or by increasing the initial concentration of enzyme in the cell.

Modern high performance and fast protein liquid chromatography techniques (HPLC and FPLC) provide versatile and rapid methods that can be applied to the purification of TMAO reductase. In the light of the results obtained from gel filtration and ion exchange chromatography the same HPLC and FPLC methods could be employed and optimized for a rapid and reproducible purification protocol. Although affinity chromatography was unsuccessful in TMAO reductase purification, various alternative approaches using this method might be of greater benefit in the light of some of the results obtained in this study. Although certain alternative substrates were shown to interact with TMAO reductase, their application in affinity chromatography was limited by a lack of functional sites with which to couple the potential ligand to the gel support. A wider range of either commercially available or artificially synthesized N-oxide substrates might be investigated for their suitability for this technique. Immunoaffinity chromatography provides a similarly specific purification method and the generation of antiserum to the purified enzyme provides an opportunity to develop such a technique. Alternatively, monoclonal antibodies could be raised and by the selection of clones with a reduced affinity for the enzyme, the technique could be adapted to prevent problems associated

with the tight binding of the enzyme to polyclonal antibodies (Scopes, 1982),

Other affinity chromatography techniques utilize more general characteristics of enzymes for their method of action and are therefore less specific. The PHMB sensitivity of the enzyme could be exploited by use of columns with immobilized *p*-chloromercuribenzoate, as supplied commercially (Pierce). Although no metal interactions were determined for the purified TMAO reductase, analysis of a wider range, particularly heavy metals, might reveal suitable applications for metal chelate chromatography.

The alternative approach to increasing the yield of purified material is to increase the initial amount of enzyme in the cell, either by modifying the regulatory control as investigated in the induction studies undertaken with TMAO reductase, or by genetic manipulation. The molecular genetic techniques applied to *E.coli* fumarate reductase resulted in upwards of a 30-fold increase in levels of the enzyme (Lemire *et al.*, 1983; Weiner *et al.*, 1984). The application of this technique to the TMAO reductase of *Alteromonas* sp. NCMB 400 therefore appears attractive from a purification standpoint and almost essential for the further characterization of TMAO respiration in the organism as discussed later. The lack of development along this line of research has been due to a number of potentially large practical problems. The basic genetics of the organism is understood only superficially with as yet no account of the number of genes concerned with TMAO respiration or the terminal enzyme. Several approaches might be employed in order to clone the gene(s) for *Alteromonas* NCMB 400 TMAO reductase. The cloning of the relevant gene(s) in *E.coli* as host has practical advantages, with the depth of knowledge on the molecular genetics of this organism. However expression of heterologous DNA is not always obtained, as, for example, with the genes for the degradative enzymes of *Pseudomonas* spp. when cloned into *E.coli* (Jakoby *et al.*, 1978) and problems might be encountered with the expression of DNA from *Alteromonas* sp. NCMB 400. The purification of the *Alteromonas* sp. NCMB 400 TMAO reductase from *E.coli* would

presumably be complicated by the native TMAO reductases of *E.coli* unless differential expression was obtained.

Cloning of the *Alteromonas* sp. NCMB 400 TMAO reductase gene(s) within *Alteromonas* sp. NCMB 400 is the most attractive in terms of proper expression and appears feasible due to presence of plasmids within the organism (Ward, personal communication). To date, these plasmids remain uncharacterized and cryptic, with no transformation system yet available (Ward, personal communication). A possible alternative approach is to use broad host range plasmids to develop *in vitro* hybrid plasmids containing the *Alteromonas* sp. NCMB 400 gene(s) for TMAO reductase, to transfer these into *E.coli* followed by conjugation into *Alteromonas* sp. NCMB 400, preferably a strain defective in TMAO reductase, for expression.

Two other enzymes capable of TMAO reduction were identified in *Alteromonas* sp. NCMB 400, one present constitutively and the second evident only in mutants with reduced TMAO reductase activity. Further analysis is required to extend the immunological relationship of these enzymes as previously discussed and to understand their role in TMAO respiration. A molecular genetic approach to the study of these enzymes would clarify their isoenzyme status and sequence analysis would allow a high degree of structural comparison.

The various catalytic studies on the purified enzyme revealed many differences in comparison with the purified and characterized enzymes of *E.coli* and *S.typhimurium*. The enzyme from *Alteromonas* sp. NCMB 400 showed a greater specificity for TMAO utilization with fewer analogue N-oxides being reduced and a higher affinity of the enzyme for TMAO. The differences in redox centre content, inhibitor sensitivity and restricted range of electron donor utilization prompted the hypothesis of this study that the purified enzyme was in fact a component of a large TMAO reductase holoenzyme that was easily released into the soluble fraction from a membrane association with the remaining component(s). A similarity with the subunit A of nitrate reductase was suggested. This might be tested by a purification of the holoenzyme, but the ease with which the

catalytic component is lost might make such a purification difficult. A similar approach might be taken to that of nitrate reductase in *E.coli* by the use of membrane solubilization procedures with various detergents (Forget, 1974; Lund and De Moss, 1976). An alternative approach might involve chemical cross-linking of proteins *in vivo* so as to maintain the holoenzyme, though too great a degree of cross-linking could result in invalid interpretation of the results.

Further characterization of the catalytic mechanism of TMAO reduction requires the analysis of the *in vivo* electron donors to the enzyme and a better understanding of the mechanisms of electron transfer within the enzyme, in addition to the clarification of possible other subunit involvement. The components of the respiratory chain to TMAO are currently under investigation (Morris, unpublished data) but preliminary data suggest the presence of c-type and b-type cytochromes, which are also present in the *E.coli* respiratory chain (Sakaguchi and Kawai, 1978b; Bragg and Hackett, 1983a). With the development of a cloning strategy for the TMAO reductase of *Alteromonas* sp. NCMB 400, the base and amino acid sequences could be determined followed by a comparison with similar TMAO reductases or well characterized redox centre enzymes such as nitrate and fumarate reductases. Such studies might reveal homology between the enzymes, particularly between the redox centres sites and the binding or active sites for substrate oxidation and reduction. EPR spectroscopy could be employed to implicate redox components in the enzyme and by subsequent trapping-techniques investigate intramolecular electron transport.

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