Trimethylamine-N-oxide reduction by alteromonas spp.

EASTER, M.C.

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M. C. Easter B.Sc.

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Trimethylamine-N-oxide (TMAO) is a nitrogenous compound widely distributed in marine fish and invertebrates. It has long been known that TMAO is reduced by bacteria during the post mortem spoilage of iced marine fish, but the mechanism involved and the physiological role have remained unknown. Studies with *Escherichia coli* suggested that TMAO acted as a terminal electron acceptor supporting anaerobic growth, but few studies have been performed with fish spoilage organisms. The work presented here describes some of the properties of TMAO reduction in typical fish spoilage bacteria, *Alteromonas* spp., and comparisons made with that of *E. coli*.

TMAO reduction was shown to be a property of several Gram negative bacteria and it supported anaerobic growth. It was not confined to marine bacteria and only certain marine species could reduce TMAO. In *Alteromonas* sp., NCMB 400, the enzyme TMAO reductase was induced by TMAO, and repressed by oxygen and fumarate but not nitrate. Membrane-bound cytochrome c_{552} and TMAO reductase were simultaneously induced. TMAO reductase was located predominantly in the periplasm, but difficulties were encountered with cell fractionation and the results are discussed with reference to the cell wall structure. The enzyme was partially purified using affinity chromatography and some biophysical characteristics determined. The kinetic properties of the enzyme were determined using resting cell suspensions and partially purified preparations.

Similarities and differences were observed in the TMAO reductases of *Alteromonas* sp. and *E. coli*. TMAO reductase has the properties of an anaerobic respiratory enzyme, and appears to be similar to nitrite reductase. A possible mechanism for energy conservation coupled to TMAO reduction is proposed in terms of the chemiosmotic hypothesis. TMAO reduction has significance in fish spoilage; it probably confers a competitive advantage to certain spoilage organisms, and thus the accumulation of trimethylamine in spoiling fish is not a fortuitous association as has been suggested.

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To my parents, and to my wife for her patience

and support.

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CHAPTER 1

INTRODUCTION

1.1 Trimethylamine-N-oxide - its structure and distribution

Trimethylamine-N-oxide (TMAO), $(CH_3)_3NO$, is a characteristic constituent of marine fish and invertebrates. It was first synthesized chemically in 1894 and was found in the muscle of dogfish by Suwa in 1909a. Its distribution, shown in Table 1, is well documented and in general the elasmobranchs (or cartilagenous fish) contain more than the teleosts (or bony fish) (for reviews see Shewan, 1951; Groninger, 1959; Yamada, 1967; Bickel, 1969; Harada, 1972). Deriha & Yamada It is virtually absent in fresh water fish and terrestrial animals. Many factors contribute to the variation in the TMAO content of fish including geographical location, the type of environment, seasonal changes, species differences, size of the specimen, tissue sample, handling and storage conditions after death, and the condition of the specimen under test (Shewan, 1951; Groninger, 1959; Tokunaga, 1980). Some of the variation is thought to be due to the decomposition of TMAO to trimethylamine (TMA).

N-oxides other than TMAO have not yet been unequivocally detected as body constituents of animals, but they frequently occur in plants and microorganisms (Bickel, 1969). In plants they are thought to be involved in the formation of alkaloids, and those produced by certain microorganisms have antibiotic properties or are pigments. N-oxides are thought to be biological oxidants and many have been prepared synthetically which are pharmacologically active e.g. psychrotropic drugs such as tranquillizers, and carcinogenic (or oncogenic) agents.

1.2 Origin and physiological function of TMAO in fish

Little is known of the origin and biosynthesis of TMAO. Benoit & Norris (1945) observed that TMAO is virtually absent in young salmon

TABLE 1 : The distribution and concentration of TMAO in some aquatic organisms (modified from Groninger, 1959).

Organism	No. of species	Concentration of TMAO (mM)		
		Range	Mean	Standard deviation
Molluscs*	35	0-139	25	37
Crustacea*	18	2-75	31	20
Elasmobranchs*	19	21-196	96	46
Marine teleost	72	0-102	39	19
Fresh water teleost	27	0-12	5	4

* Marine species

dwelling in fresh water, whereas considerable amounts are found in the adult animal living in sea water. They also showed from feeding experiments that TMAO was of dietary origin. Groninger (1959) inferred that zooplankton must constitute the major producer of TMAO for marine food chains, since plants do not contain it. Strøm (1979) showed that TMAO was synthesised from TMA by the marine zooplankton, Calanus spp., and the enzyme catalysing the reaction was described as a TMA monooxygenase. It has been suggested that TMA, betaine, choline and possibly lecithin and carnitine may be precursors for the formation of TMAO in animals. However, the question of animal TMAO being of exogenous, endogenous or of bacterial origin has yet to be resolved (Bickel, 1969; Watts & Watts 1974).

The high TMAO content of many marine fish has stimulated much speculation as to its physiological function, but knowledge of its role is largely hypothetical. TMAO is almost completely reabsorbed by the kidney of some elasmobranchs and several workers have suggested that it has an osmoregulatory function. It is not known whether TMAO has a similar role in teleosts (Shewan, 1951; Groninger, 1959).

The excretion of TMAO by certain fish has lead other workers to suggest that it is also an end product of nitrogen metabolism (Bickel, 1969; Yamada, 1967). Since N-oxidation of TMA occurs in addition to N-oxide reduction, it is possible that TMAO functions as a regulatory system of redox processes. The TMA/TMAO quotient in urine has been postulated as an index of oxidation processes in the body e.g. the ratio of oxidative metabolism to glycolysis (Bickel, 1969).

1.3 Microbial spoilage of iced marine fish

An historical review of fish microbiology is given by Liston (1979). At the turn of the century, interest in fish bacteriology was medically

oriented, when the involvement of shellfish as carriers of human enteric disease was recognised. But, as early as 1907, fish spoilage was clearly shown to be the result of bacterial proliferation, and the normal bacterial flora of fish was later shown to be responsible for spoilage. The microbiology of fish spoilage was investigated thoroughly by many workers from 1920-1950, and was reviewed by Reay & Shewan (1949).

Shewan (1962) described the chemical changes that occur in fresh and spoiling fish. The muscle tissue of fish contains up to 5% soluble nitrogenous constitutents including free amino acids, carnosine, anserine, creatine, TMAO, amines, ribose, various sugar phosphates and lactic acid. In elasmobranch fishes, large amounts of urea are also present. Thus fish tissue provides an ideal nitrogenous substrate for the growth of proteolytic and putrefactive organisms which are normally associated with them (Tarr, 1954). The nature and amount of these extractives vary markedly with species, although within limits related species have rather similar compositions. However, even within a single species, individual components fluctuate greatly depending on such factors as size, season, environment (temperature and salinity), nutritional status, amount of struggling in the net and even on the type of muscle sampled.

After the death of the fish, the regulatory mechanisms preventing invasion of the tissues by bacteria cease to function and in a short time, some microorganisms can be detected in some tissues. The main routes of attack appear to be from the gills and the kidney into the flesh via the vascular system (Reay & Shewan, 1949; Shewan, 1962). Immediately beneath the skin there are only a small number of bacteria which possibly gain entry to the tissue through the pores along the lateral line. Bacteria appear to be confined to the surface layers of fish until spoilage is advanced and proteolysis occurs (Shewan, 1977). At storage temperatures above 5°C, bacterial penetration of the flesh occurs with the subsequent degradation of muscle constituents (Shewan & Murray, 1979). The chemical and biochemical changes occurring during

fish spoilage have been studied in detail and the major changes produced by the action of bacteria are shown in Table 2 (Reay & Shewan, 1949; Shewan, 1962; Shewan, 1977).

One of the characteristic odours associated with fish spoilage is that of TMA, which is normally produced in increasing amounts during the process of putrefaction, and its measurement has been used as a means of following the microbiological deterioration of iced marine fish. Tarr (1954) reported that this property is unreliable, as spoilage can occur without the formation of appreciable amounts of TMA. He also suggested that it was "probably impossible to evolve a single objective test which will prove a universal panacea in detecting fish spoilage." Nevertheless, Shewan (1962) considers that since the TMA content increases in a sufficiently regular manner, it provides a useful correlation with odour and flavour changes, albeit an indirect or fortuitous relationship, so as to furnish a reasonably useful index of freshness in iced fish.

The spoilage flora of marine fish from cold waters is composed of Gram negative bacteria (Tarr, 1954; Reay & Shewan, 1949; Shewan, 1962). The bacteria residing in the surface slime of these fish belong to the genera Pseudomonas, Achromobacter, Flavobacterium. However, some of these organisms have been reclassified as a consequence of recent advances in bacterial taxonomy. Some pseudomonads are now identified as Alteromonas spp. because the mol % G & C of their DNA is too low for that of the Pseudomonas genus. Lee, Gibson & Shewan (1977) have allocated Alteromonas spp. to 3 phenons, C, D and E, of which only strains in phenons D and E can reduce TMAO. Strains in phenon E have been named Alteromonas putrefaciens, and are considered to be of great importance in the spoilage of proteinaceous foods (Herbert, Hendrie, Gibson & Shewan, 1971; Lee, 1979). The Achromobacter strains have been reclassified as Moraxella spp. and Acinetobacter spp. (Gibson/

TABLE 2 : Some of the chemical changes that occur during fish spoilage (from Shewan, 1977).

Substrate	Compound produced by bacterial action.
Inosine	Hypoxanthine
Carbohydrates, lactate	Acetic acid, CO_2 and H_2O
Methionine, cysteine	H_2S, methyl mercaptan, dimethyl sulphide.
Glycine, leucine, serine	Esters of acetic, propionic, butyric and hexanoic acid.
TMAO	TMA
Urea	NH_4 and CO_2

Hendrie, Houston & Hobbs, 1977). The bacterial flora of the fish intestine vary according to the food being consumed, but it normally contains facultative anaerobes and some aerobes and anaerobes e.g. Vibrio spp., Aeromonas spp., Pseudomonas spp., and Clostridia spp. (Liston, 1979).

The flora of fish from warmer waters consists mainly of Gram positive bacteria such as micrococci, coryneforms and bacilli (Liston, 1979), and the shelf life of these fish on ice is longer than that of cold water fish, possibly due to the lack of an intrinsic psychrophilic flora (Shewan, 1977; Liston, 1979).

When fish such as haddock and cod are stored in ice, the numbers of bacteria remain low for 4 days, then increase exponentially. The increase is accompanied by the predominance of the Pseudomonas spp. and Alteromonas spp., whilst the Moraxella/Acinetobacter spp. and Flavobacterium persist but at a decreasing level (Reay & Shewan, 1949; Shewan, 1962; Liston, 1979). Shewan (1977) has suggested that the dominance of the spoilage flora was almost certainly due to the shorter generation times of the Pseudomonas spp. and Alteromonas spp. at chilled temperatures. This may be a consequence of their temperature coefficient of growth (Q_{10}) being greater at -4° to $5^{\circ}C$ than at higher temperatures (Tarr, 1954).

TMAO reduction is not confined to fish spoilage bacteria since many species of both marine and non-marine origin which have fermentative and non-fermentative metabolism can reduce TMAO. It is a property mainly associated with Gram negative bacteria, but a few Gram positive organisms can perform the reduction (Castell, 1946; Castell, 1949a; Baird & Wood, 1944). TMAO reducing bacteria include Pseudomonas spp. and/or Alteromonas spp. (Shewan, 1962; Castell, 1949a; Lee et al, 1977; Gillespie, 1981), some Moraxella spp. (Chai, 1981), most of the species of the Enterobacteriaceae (Wood & Baird, 1943; Wood, Baird & Keeping, 1943; Castell, 1949a; Kim & Chang, 1974; Sakaguchi & Kawai, 1976; Strøm & Larsen, 1979), Vibrio parahaemolyticus (Castell, 1949a; Unemoto,

Hayashi, Miyaki & Hayashi, 1965), certain Campylobacter spp. (Park, Razi & Skirrow, 1980), Rhodopseudomonas capsulata (Madigan & Gest, 1978) and some Clostridium spp. (Castell, 1946; Ando & Inoue, 1957).

1.4 Bacterial TMAO reduction

There are 2 major routes for the catabolism of TMAO by heterotrophic microorganisms, (a) TMAO reduction to TMA as occurs during fish spoilage and (b) TMAO demethylation to dimethylamine and formaldehyde, a property of certain methylotrophic bacteria able to use TMAO as a carbon source (Colby, Dalton & Whittenbury, 1979). TMAO can also be demethylated by fish tissue enzymes and by heating (Tokunaga, 1980) but during fish spoilage it is primarily reduced to TMA.

Suwa (1909b) and Beatty (1938) showed that the production of TMA during fish spoilage was the result of bacterial action on TMAO, and the latter suggested that TMAO may have a respiratory function. Watson (1939a) showed that TMAO reduction was not a function of the total bacterial population of spoiling fish, but was due to the "reducing Achromobacters" (probably now regarded as Pseudomonas-like bacteria), which produced TMA under conditions of low oxygen tensions. This activity was inhibited by intense aeration which was referred to as the "sparing effect"; it was suggested that facultative anaerobes can effect their oxidations more profitably from molecular oxygen than through anaerobic oxidation by hydrogen acceptors. It was also shown that the reduction of nitrate by the "reducing Achromobacters" was comparable to the reduction of TMAO, and these organisms were capable of reducing both nitrate and TMAO, whilst the flavobacteria could only reduce nitrate (Watson, 1939a,b). Achromobacters were also shown to grow anaerobically on fumarate (Watson, 1939b). Kim & Chang (1974) obtained similar

results with Salmonella typhimurium, and suggested that the TMAO and nitrate reductase systems had similar genetic loci and/or common components, since chlorate resistant mutants defective in nitrate reductase, also lost the ability to reduce TMAO.

Tarr (1939) and Watson (1939b) showed that a variety of oxidisable substrates e.g. lactate, pyruvate, glucose and glycogen could support TMA production in resting cell suspensions.

In the spoilage of cod muscle press juice, Watson (1939b) reported that lactate disappeared with the concomitant production of TMA, and he suggested that TMA production was the result of an energy yielding reaction between TMAO and the appropriate hydrogen donors, especially carbohydrates and their derivatives. He examined the oxidation of lactate and reduction of TMAO and derived the relationship shown in equation 1;

$$AH_2 + TMAO \longrightarrow A + TMA + H_2O \qquad (1)$$

where AH_2 is the hydrogen donor e.g. lactate, and A is the oxidised substrate. Both Watson (1939b) and Collins(1941) showed that lactate oxidation and the production of acetate and TMA were closely related, and it was estimated that 2 mol TMAO were reduced per mol acetate formed (equation 2).

$$\text{Lactate} + 2\ TMAO + H_2O \longrightarrow \text{Acetate} + 2\ TMA + CO_2 + 2H_2O \qquad (2)$$

Strøm & Larsen (1979) have studied the spoilage of herring stored at 15°C under anaerobic conditions, and found that an Enterobacter sp., Proteus sp. and an Aeromonas sp. had the highest growth rates. Ribose, hexoses and lactate were the main substrates used and TMAO was concomitantly reduced. Strøm, Olafsen, Refsnes & Larsen (1979) showed that ribose was used preferentially and that lactate was oxidised to acetate with the same stoicheiometry as shown in equation 2. Although, the growth yields of Proteus sp. on pyruvate and lactate were similar, twice as much TMA was produced during growth on lactate. They

suggested that their strain contained 2 methods for TMAO reduction, one of which was linked to energy production (possibly via the oxidation of formate and oxidative phosphorylation), whilst the other mechanism involved the initial oxidation of lactate to pyruvate. A similar explanation may account for the results of Watson (1939b) and Collins (1941).

Accordingly, by 1940, it was understood that TMA production during fish spoilage was a consequence of bacterial growth and it was thought that TMAO could support anaerobic growth. This prompted several workers to investigate the enzymology of TMAO reduction in resting cell populations of typical spoilage bacteria. Tarr (1939) first described the enzyme as trimethylamine oxidase, and showed it to be a heat labile enzyme that was not removed from the bacterial cell by autolysis. However, he also showed that the enzyme could reduce other N-oxides to their respective bases, and termed the enzyme a "triamineoxidase" (Tarr, 1940). Its properties were investigated by several workers who showed that:

(i) its pH optimum was ca. pH 7.5;

(ii) nitrite inhibited its activity but had a very slight effect on bacterial growth, and nitrite was most inhibitory between pH 6-7, the inhibition by nitrite varied between species;

(iii) it was inhibited by NaCl, and by low temperatures, and,

(iv) several respiratory inhibitors e.g. cyanide and azide, and heavy metals and chelating agents were also inhibitory (Neilands, 1945; Castell & Snow 1949; Castell 1949b; Dyer, 1949; Tomizawa, 1951; Castell & Snow, 1951; Tsuchiya & Endo 1952).

It was suggested that TMAO reduction required not only an oxidisable substrate with its specific dehydrogenase, but also a second carrier or series of carriers between the dehydrogenase and the oxide (Neilands

1945). Tomizawa (1951) showed the triamine oxidase to be "adaptable" i.e. in whole cell suspensions it was either induced or stabilized by TMAO. He suggested that the enzyme contained a heavy metal e.g. iron, and he was also the first to obtain a cell free extract of the enzyme, by using ultrasonic vibration at low temperatures.

There was little published work on bacterial TMAO reduction between 1950 - 1964, possibly due to the limited understanding of bacterial metabolism. At this time, the existence of the tricarboxylic acid cycle was known, the major steps in the biosynthetic and degradative pathways were elucidated, and aerobic respiration was explained in terms of successive electron transfer reactions occurring in the cell membrane (see Lehninger, 1975). A mechanism of coupling energy production to respiration i.e. oxidative phosphorylation was postulated by Mitchell in 1961, and by 1973 his chemiosmotic hypothesis had become widely accepted (Mitchell, 1976). The details of anaerobic respiration coupled to nitrate reduction were investigated in the late 1960's (Wimpenny & Cole, 1967). Therefore, by the 1970's, the conceptual framework of bacterial metabolism and respiration was laid down and interest in TMAO reduction was revived. Another reason for the lack of interest in TMAO reduction may be due to the relatively tedious assay methods involved e.g. TMA production cannot be determined directly using spectroscopy, but had to be measured by the formation of its picrate salt, or some other chemical assay. However, since the early 1970's, the use of viologen dyes has made such studies easier (Thorneley, 1974).

Unemoto et al. (1964 and 1965) investigated the properties of TMAO reduction in Escherichia coli, Pr. morganii and V. parahaemolyticus. Their results from donor and inhibitor studies confirmed the observations made by previous workers (as summarised above), who used Achromobacter spp. and mixed cultures of spoilage bacteria. Unemoto et al. (1964 and 1965) also showed that formate and NADH, but not NADPH acted as hydrogen donors for TMAO reduction, and that NADH oxidation and TMA

production were equimolar (equation 3);

$$NADH + TMAO + H^+ \longrightarrow NAD^+ + TMA + H_2O \qquad (3)$$

the enzyme was called trimethylamine-N-oxide reductase and is classified as NADH:TMAO oxidoreductase by the International Union of Biochemistry (1978). A cell free extract from V. parahaemolyticus was prepared and the enzyme was found in the membrane fraction.

From the mid 1970's onwards, detailed studies on bacterial TMAO reductase have been carried out mainly by Japanese workers using E. coli. Sakaguchi & Kawai (1975a) and Madigan & Gest (1978) showed that TMAO reduction can support anaerobic bacterial growth and TMAO was not used as a carbon and/or nitrogen source. Kim & Chang (1974) and Castell (1946) showed that TMAO (50-130mM) was not toxic to Gram negative bacteria, and suggested that its reduction was not a detoxification.

From anaerobic growth yield experiments and theoretical thermodynamics, it was proposed that energy production could be coupled to TMAO reduction via oxidative phosphorylation, thus providing a method of anaerobic respiration, which is analogous to that of nitrate and fumarate reduction (Yamamoto & Ishimoto, 1977; Ishimoto & Shimokawa, 1978; Strøm, Olafsen & Larsen, 1979). Yamamoto & Ishimoto (1977) showed that the anaerobic growth yield of E. coli was the same when nitrate, fumarate or TMAO were used as the terminal electron acceptor (TEA) and formate was the donor. They suggested that there existed a common site of ATP production in the electron transfer systems from formate to the 3 TEA, possibly between formate dehydrogenase and a cytochrome.

Sakaguchi & Kawai (1977 - 1979) have shown that the TMAO reductase of E. coli has the following properties (i) formate, NADH and NADPH were effective electron donors that supported TMAO reduction, (ii) it was a membrane bound enzyme, and was induced by TMAO, (iii) it was repressed by nitrate and oxygen and its activity was inhibited by oxygen but not by nitrate, and (iv) it was inhibited by respiratory inhibitors

including cyanide and 2-N-heptyl-4-hydroxy-quinoline-N-oxide (HOQNO). Sakaguchi, Kan & Kawai (1979) showed that TMAO reductase and haem c were both induced under similar conditions, and Ishimoto & Shimokawa (1978) showed that cytochrome c_{552} was oxidised by TMAO. Sakaguchi et al. (1979) have proposed a mechanism for TMAO reduction in E. coli (Fig. 1);

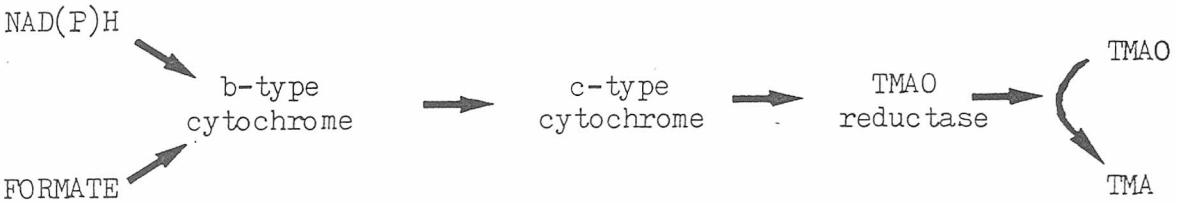


Figure 1 : Proposed electron transport chain for TMAO reduction in E. coli (after Sakaguchi et al., 1979).

There are no reports in the literature on the purification of TMAO reductase, but Sagai & Ishimoto (1973) and Shimokawa & Ishimoto (1979) have purified a tertiary amine N-oxide reductase that has the ability to reduce TMAO, adenosine-N-oxide and several other amine-N-oxides. It was induced by TMAO but not by adenosine-N-oxide, and the authors consider TMAO to be the natural substrate. It has other properties in common with TMAO reductase e.g. it is associated with the cytoplasmic membrane, the Km_{TMAO} is 1 - 2mM (see Unemoto et al., 1965) and its activity is affected by similar inhibitors. Therefore, this purified enzyme may be regarded as a TMAO reductase, or TMAO reduction is the property of a non-specific N-oxide reductase as suggested by Tarr (1940).

1.5 Anaerobic bacterial growth.

There are two basic mechanisms for the regeneration of reducing equivalents, i.e. NAD^+, which allow the growth of bacteria under anaerobic

conditions. They are fermentation reactions and electron acceptor dependent growth.

During fermentation the electron acceptor is an organic molecule generated as an intermediate product in the breakdown of the fermentable substrate, and redox balance is achieved without additional exogenous oxidants and reductants. Energy is obtained by substrate level phosphorylation and NAD^+ is regenerated in the terminal steps of fermentation. During electron acceptor dependent growth, energy is produced in a similar manner except that electrons are transferred to exogenous electron acceptors, either inorganic or organic, and further energy may be obtained from oxidative phosphorylation (Cole, 1981).

The electron acceptor is said to be acting as an electron sink if its reduction cannot be coupled to oxidative phosphorylation e.g. NADH: nitrite reductase (Pope & Cole, 1981; Cole, 1981). If electron transport is coupled to oxidative phosphorylation the process can be termed anaerobic respiration, and examples include nitrate and fumarate reduction using formate as the electron donor (Haddock & Jones, 1977). TMAO reduction supports anaerobic growth either by acting as an electron sink or by providing a means of anaerobic respiration. The evidence reviewed in section 1.4 is more in favour of the latter.

A respiratory system is composed of an electron transport chain and a terminal reductase, both of which are generally bound to the cytoplasmic membrane. The electron transport chain usually contains some but not necessarily all of the following components - dehydrogenases, cytochromes, iron-sulphur (Fe/S) proteins and quinones. The number and variety of electron transport components present is characteristic of individual respiratory systems, and they operate in a particular sequence. The anaerobic respiratory systems to nitrate and fumarate have been studied extensively; both contain formate dehydrogenase, a quinone (probably menaquinone) and b-type cytochromes, and all components are

membrane bound (Haddock & Jones, 1977; Kröger, 1978).

It is generally thought that energy is produced by oxidative phosphorylation at the cytoplasmic membrane as a consequence of electron transport and the generation of proton gradients (see later). The membrane location of a terminal reductase is considered to be evidence that the respiratory system is coupled to energy conservation. The reduction of nitrite can support anaerobic growth, but nitrite reductase is present as a soluble enzyme in the periplasmic fraction produced by sphaeroplast formation (Wood, 1978). Pope & Cole (1981) suggested that nitrite reduction can be coupled to oxidative phosphorylation using formate as the electron donor, but it was not energy linked when NADH was the donor.

The reduction of nitrite is thought to involve c-type cytochromes and the electron transport systems for nitrite and nitrate reduction are compared in Fig. 2. From the available literature, the TMAO reductase system appears to have properties similar to both nitrate and nitrite reductase.

The organization of electron transport components in the membrane and the location of the terminal reductase are important in the explanation of the mechanism of oxidative phosphorylation. The most widely accepted theory for the coupling of oxidative phosphorylation to electron transport is the chemiosmotic hypothesis (Mitchell, 1976). Haddock & Jones (1977) have described it in simple terms:

> "the chemiosmotic hypothesis requires that a proton-translocating electron transport chain and a proton-translocating adenosine triphosphate (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 (Δ pH) 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 and, although these components are not identical, they are all related and interconvertible as described by the expression:-
>
> $$\Delta_P = \Delta\psi - Z\Delta_{pH}$$ "

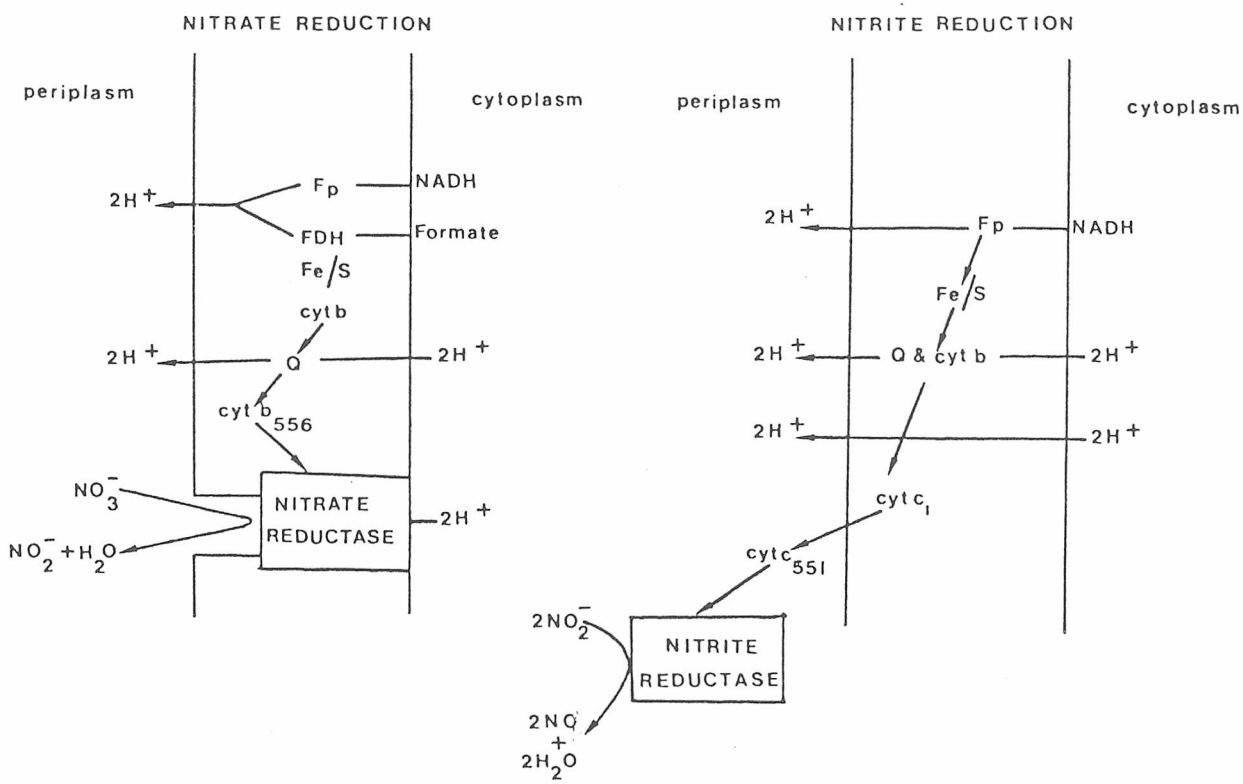


Figure 2 : Electron transport systems for nitrate and nitrite reduction . (See Haddock & Jones, 1977; Wood, 1978).

Fp, flavoprotein; Fe/S, non-haem iron sulphur protein; FDH, formate dehydrogenase; Q, quinone; cyt, cytochrome.

Under suitable conditions, ΔP can be used to drive a variety of cellular functions such as ATP synthesis i.e. oxidative phosphorylation, and solute transport. Protons are translocated out of bacteria during aerobic respiration and anaerobic respiration using nitrate (Haddock & Jones, 1977), fumarate (Kröger, 1978) or nitrite (Pope & Cole, 1981) as the TEA. Proton translocation during TMAO reduction has not yet been reported.

1.6 *Alteromonas* spp. - typical fish spoilage organism.

The most recent studies on bacterial TMAO reduction have involved members of the *Enterobacteriaceae* which are rarely associated with the spoilage of iced marine fish. TMAO reduction by typical fish spoilage bacteria such as *Alteromonas* spp. has not been studied for 30 y. *Alteromonas* spp. are non-fermentative, Gram negative bacteria. They have polar flagella, mol % G+C of their DNA is 40-55%, and possess catalase, oxidase and DNAase activity. Their ability to reduce TMAO is important because it is the only method by which such non-fermentative organisms can grow anaerobically. Most alteromonads require Na^+ for growth and their metabolic versatility is less than that of *Pseudomonas* spp. i.e. they grow on fewer single carbon sources (Lee *et al.*, 1977). Glucose is metabolised via 6-phosphogluconate and the Entner-Doudoroff pathway, although it is not always used as the principal energy forming route, and can provide precursors for biosynthesis by way of the pentose phosphate pathway. The components of the tricarboxylic acid cycle and substrates closely allied to it e.g. some amino acids, were rapidly utilized by these organisms (Gibson *et al.*, 1977). *A. putrefaciens* has been isolated from a wide range of environments and

is considered to be one of the most important organisms encountered in the spoilage of proteinaceous foods kept at chill-room temperatures (Lee, 1979).

A. haloplanktis, NCMB 19, (formerly Pseudomonas sp.,B16) is the only alteromonad to have been studied in detail. Its properties have been investigated by the Macleod/Costerton group and are reviewed by MacLeod (1965, 1979). Marine bacteria require inorganic ions for their nutrition and metabolism, and also for (a) the maintenance of the cell wall structure and integrity, (b) the transport of many metabolites into the cell, and (c) the retention of intracellular solutes by the cell. The cell envelope of A. haloplanktis consists of 5 layers (Fig. 3), and Mg^{++} ions were required for its stabilisation, probably because they increased the extent of cross-linking in the peptidoglycan layer and the outer membrane. Martin & MacLeod (1971) and Nelson & MacLeod (1977) examined the chemical composition of the cell wall components of A. haloplanktis using modifications of the method of sphaeroplast formation described by Costerton, Forsberg, Matula, Buckmire & MacLeod (1967). Sphaeroplasts are spheres devoid of the outer membrane and are composed of the cytoplasm bound by the inner membrane. Cell fractionation involving the formation of sphaeroplasts is often used to determine the distribution of enzymes within the cell e.g. alkaline phosphatase was found in the periplasmic fraction of A. haloplanktis (Thompson & MacLeod, 1974b).

Mitchell (1961) coined the term "periplasm" to describe an area of the cell wall of Gram negative bacteria, between the cytoplasmic membrane and the outer envelope in which certain enzymes can be retained while some of their substrates are free to pass through one or both of these bounding structures. In a recent review on periplasmic enzymes of Gram negative bacteria,Beacham (1979) defines periplasmic enzymes as:

> "those enzymes between the inner and outer membranes, whose association with either of these membranes, if any, is so tenuous that they are released during sphaeroplasting"

Layer	% Dry Weight
outer loosely bound layer	4.7
outer double track layer or outer membrane	7.9
Periplasmic layer	6.1
Peptidoglycan layer	1.0
inner or cytoplasmic membrane	12.0
cytoplasm.	

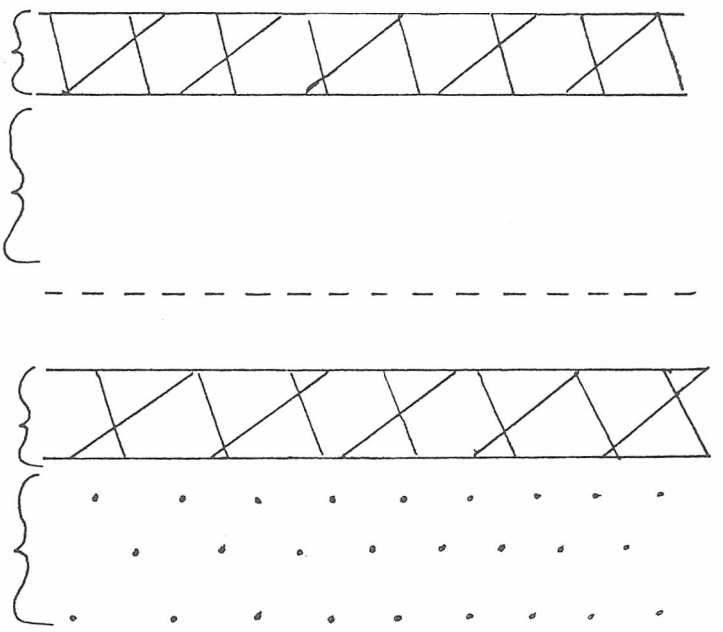


Fig. 3 : Structure of the cell wall of A. haloplanktis, NCMB 19, (after MacLeod, 1979).

The periplasm occupies 20-40% of the total cell volume and contains several degrad ative enzymes and some binding proteins (Heppel, 1971; Beacham, 1979; Costerton, Ingram & Cheng, 1974). Periplasmic enzymes of Gram negative bacteria are similar to the extracellular enzymes of Gram positive bacteria and to the enzymes present in lysosomes of eukaryotic cells. This probably reflects the adaptation of Gram negative cells to life in a dilute aqueous environment, since the degradative enzymes are retained in association with the cell wall, such that the products of digestion are immediately available to the transport system of the cell, and they are not lost to the environment. Similarly, the large number of periplasmic enzymes including phosphoglucomutase and phosphoglucose isomerase, permits bacterial growth on a wide variety of nutrients with only a limited number of specific permeases (Costerton et al., 1974). Periplasmic enzymes also inactivate certain antibiotics e.g. penicillins and cephalosporins (Costerton, 1977) and so confer resistance to the cell. Thus the multilayered cell wall of Gram negative bacteria constitutes a metabolically important cell organelle, since it confers rigidity, excludes toxic molecules and antibiotics and aids metabolic versatility (Costerton et al.,1974).

1.7 Summary

The reduction of TMAO to TMA by bacteria during the spoilage of fish has been recognised since the turn of the century,and the TMA content of fish has long been used as an index of fish freshness and quality. The significance of the reaction was not realised because of the lack of knowledge of bacterial metabolism and respiration. Recent studies of TMAO reduction have involved bacteria whose physiology and biochemistry are better understood, e.g. E. coli. It is currently

believed that in such organisms TMAO acts as a TEA and provides a method of anaerobic respiration. If this were so in the bacteria associated with fish spoilage, then such bacteria would have a competitive advantage over the rest of the flora,especially the non-fermentative strains.

The purpose of the work described here is to examine the reduction of TMAO by typical fish spoilage bacteria. This study describes the distribution of TMAO reduction and the properties of the system in Alteromonas spp.

CHAPTER 2

MATERIALS AND METHODS

2.1 Chemicals

Chemicals were generally obtained from British Drug Houses Ltd., (Macfarlane Robson, Glasgow), Sigma London Chemical Company Ltd. (Poole) or Koch Light Laboratories Ltd. (Colnbrook) and were all 'Analar' quality.

Media were obtained from Oxoid Ltd., (Basingstoke) or Difco Ltd., (West Molesey).

2.2 Bacteria and Microscopy

2.2.1 Bacteria

Cultures were obtained from the National Collection of Marine Bacteria (NCMB) and the National Collection of Industrial Bacteria (NCIB) held at the Torry Research Station (TRS), Aberdeen (Table 3). Marine bacteria were maintained on sea water agar and other bacteria on nutrient agar, and all organisms were subcultured at intervals of 6-8 weeks.

2.2.2 Microscopy

The microscopic examination of bacteria was carried out using a photomicroscope (Carl Zeiss, Carl Zeiss House, London) fitted with a x 10 eyepiece and a x 100 Neofluar objective.

The solutions used for Gram-staining were: (a) crystal violet, 0.5% (w/v) aqueous solution; (b) Lugol's iodine containing 1% (w/v) iodine and 2% (w/v) KI in distilled water, or Gram's iodine, a 1:3 dilution of Lugol's iodine; (c) safranin red, 1% (w/v) aqueous solution and (d) dilute fuchsin containing 0.1g basic fuchsin, 0.5g phenol, 10ml ethanol and 100ml distilled water. Bacteria were heat fixed to a glass slide and treated

TABLE 3 : List of bacterial strains

Organism	Strain designation
Alteromonas spp.	NCMB 130, 224, 301, 399, 400, 1516, 1520, 1521, 1731, 1788, 1789.
A. haloplanktis	NCMB 19
A. putrefaciens	NCMB 1733, 1735 NCIB 8615, 8768, 10471, 10472, 10473, 10761, 11156, 11157.
Pseudomonas spp.	NCMB 320, 1790
P. taetrolens	NCIB 11024
P. fluorescens	NCMB 9326, 9327, 9396.
Vibrio parahaemolyticus	16 TRS isolates
V. alginolyticus	17 TRS isolates
Acinetobacter calcoaceticus	NCIB 9019, 9020, 9021, 9016, 9211, 9213, 9292, 9294, 9296, 9298, 9300, 9302, 9304, 10487.
Moraxella spp.	NCIB 9871
M. osloensis	NCIB 10693
Moraxella-like bacteria	NCMB 27, 98, 100, 103, 124, 135, 196 201, 206, 214, 307, 132.
Escherichia coli	NCIB 86, 9132 and 5 isolates *
Klebsiella aerogenes	1 TRS isolate
Citrobacter spp.	1 TRS isolate

* City Hospital, Aberdeen.

with crystal violet for 30 sec. The slide was then flooded with Gram's or Lugol's iodine for 30-40 sec , drained and decolourized using an ethanol wash (15 sec) followed by an acetone wash (30-45 sec). It was rinsed with distilled water and counterstained with either safranin red or dilute fuchsin for approximately 60-90 sec at room temperature. The slide was drained, washed with distilled water, dried at room temperature and viewed under the microscope through immersion oil.

The phase contrast facility of the microscope was used to observe sphaeroplast formation, and photographs were taken using the camera attachment with automatic exposure.

2.3 Media

Sea water agar contained (g l^{-1}): Lab Lemco (Oxoid), 10; Bactopeptone (Difco), 10; Bacto-Agar (Difco), 15; 750ml filtered sea water and 250ml distilled water. The medium was adjusted to pH 7.3 and autoclaved at 103 x 10^3 Pa for 15 min.

Nutrient agar (Oxoid) contained (g l^{-1}): Lab Lemco powder, 1; yeast extract, 2; peptone, 5; NaCl, 5 and agar, 15. The medium was adjusted to pH 7.4 and autoclaved at 103 x 10^3 Pa for 15 min.

The medium of Wood and Baird (1943) contained (g l^{-1}): Bacto-peptone, 5; glucose, 2.5; TMAO.HCl, 1; $MgSO_4.7H_2O$, 1; K_2HPO_4, 1 and NaCl, 5. This medium was adjusted to pH 6.5, supplemented with NH_4Cl (0.5 g l^{-1}), and autoclaved at 103 x 10^3 Pa for 15 min. For the growth of NCMB strains, NaCl was increased to 20 g l^{-1}. In some experiments, the TMAO.HCl concentration ranged from 0-20 mM (0-2.23 g l^{-1}), or was replaced with sodium fumarate, 10 mM (1.6 g l^{-1}), KNO_3, 5 mM (0.5 g l^{-1}) or adenosine-N-oxide, 10 mM. Adenosine-N-oxide was filter sterilized (Millipore (UK) Ltd., London, model Swinnex 25, pore size 0.2 μm) and aliquots of the stock solution (100 mM, 23.3 g l^{-1}) were added to the medium, to give a concentration range of 1-10 mM.

2.4 Growth

2.4.1 Culture conditions

Cultures were incubated at their optimum growth temperature i.e. 20°C for *Pseudomonas* spp., *Alteromonas* spp., *Acinetobacter* spp., and *Moraxella*-like bacteria, and 37°C for *Vibrio* spp., and strains of *E. coli*. Aerobic culture conditions were achieved by incubating 50-100 ml of medium in a 250 ml Erlenmeyer flask in an orbital incubator (Gallenkamp, model IH-465) at 150 rev. min^{-1}. For micro-aerobic growth conditions a culture vessel completely filled with the appropriate medium was used and was not agitated. Anaerobic growth conditions were obtained in several ways depending on the culture vessel used;

(i) anaerobic jars evacuated and flushed three times with a gas mixture of H_2/CO_2, or $N_2/H_2/CO_2$ (BOC Ltd., Glasgow) in the presence of a catalyst;

(ii) anaerobic jars containing a Gas-Kit (Oxoid) and a catalyst;

(iii) anaerobic culture tube with a side arm containing alkaline pyrogallol, as described by Pankhurst (1967), and

(iv) test tube cultures, including the conductance electrode cell described by Richards, Jason, Hobbs, Gibson & Christie (1978), overlaid with sterile liquid paraffin.

2.4.2 Determination of bacterial growth

Bacterial growth was monitored as the increase in optical density at 660 nm ($O.D._{660}^{1cm}$) using a single beam spectrophotometer (Cecil instruments Ltd., Cambridge, model CE 373). The number of viable bacteria was determined as the number of colony forming units per ml, using the plate count method described by Bousfield, Smith & Trueman (1973), or the Spiral Plater (Don Whitley Scientific Ltd., Shipley)

2.4.3 Harvesting and washing bacteria

Bacteria were harvested by centrifugation at 10000 g for 15 min at 4°C , using an automatic refrigerated centrifuge (Du Pont (UK) Ltd., Hitchin, Sorval model RC2-B). Except when stated otherwise, the cells were washed once by centrifugation after resuspension in one tenth original volume in phosphate-salts buffer (100 mM phosphate buffer (Na_2HPO_4 + KH_2PO_4) pH 7.0, containing 100 mM NaCl and 1 mM $MgSO_4.7H_2O$).

2.5 Conductance measurements

The equipment described by Richards et al.(1978) was used. The samples were placed in standard bacteriological test tubes fitted with platinum wire electrodes 10 mm long and 50 mm apart. The tubes were held in a well-stirred, well insulated waterbath thermostatted to ± 0.002°C of the selected temperature by means of a proportional temperature controller, since conductance and impedance are greatly affected by temperature. The instrument took up to 128 samples at a time,and at intervals of 5, 10, 20 and 60 min it measured the resistance component (R) of the impedance of each sample by means of a phase sensitive detector. The output was fed through a data logging system and appeared as punch paper tape suitable for a computer. The data were converted to conductance (1/R) and the change in the conductance (G_B) calculated. The computer presented plots of G_B against time, and also the rate of change of G_B ($\frac{dG_B}{dt}$) against G_B. The dG_B/dt versus G_B plots lead to the calculation of the rate of growth, the mean generation time. This apparatus was used for screening for bacterial TMAO reduction, and an 8 channel version connected to a strip chart recorder (displaying G_B) was used for whole cell enzyme assays of TMAO reduction (section 2.7.1b).

2.5.1 Screening for TMAO reduction

The Wood & Baird medium described in section 2.3 was dispensed in 13ml aliquot samples into sterile electrode tubes and equilibrated for 12 h either at 20°C for psychrophiles or 37°C for mesophiles. The inoculum was 10µl of an overnight culture of the test organism in the same medium. The conductance change (G_B) was recorded during growth for 48 h when the chemical spot test for TMA (section 2.8.2.b) was carried out. TMAO reduction under anaerobic conditions was determined using 10ml of the same medium overlaid with ca 3ml of sterile liquid paraffin.

2.6 Cell Fractionation

The location of TMAO reductase within the bacterial cell was investigated using Alteromonas sp., NCMB 400. The preparation of sphaeroplasts/protoplasts was an essential part of the cell fractionation procedure and several different methods were examined.

2.6.1 Preparation of sphaeroplasts/protoplasts.

Method A: The conventional method of sphaeroplast formation from Gram negative bacteria viz. E. coli is essentially that described by Kaback (1971), and involves the treatment of plasmolysed cells with lysozyme and ethylenediaminetetra - acetic acid (EDTA). Whole cells were washed in Tris-salts buffer [50mM Tris-(hydroxymethyl) aminomethane (Tris-HCl), 300mM NaCl, 50mM $MgSO_4.7H_2O$ and 10mM KCl, pH 7.2] and resuspended to a concentration of 3-5mg protein ml^{-1} in 50mM Tris-HCl, pH 8.0 containing either 500mM sucrose or 500mM sucrose and 10mM EDTA (as Na_4 salt). Lysozyme was added to give a final concentration of 150µg ml^{-1} and after incubation for 15 min at 30°C sphaeroplast formation was observed using phase contrast microscopy. EDTA was added stepwise in 100-250 µmol amounts

to those cell suspensions from which it had been previously omitted.

Method B: Sphaeroplast formation in the absence of EDTA was examined. The method used was essentially that of Cheng, Ingram & Costerton (1971). Aerobically grown cells (50ml overnight culture) were harvested, resuspended in 20ml 500mM sucrose, pH 6.5 and incubated at 30°C for 30 min in an orbital incubator at 150 rev. min^{-1}. The cells were again harvested, resuspended in 500mM sucrose and centrifuged immediately at 22000g for 15 min at 4°C. The cell pellet was resuspended in 10mM Tris-HCl, pH 8.4 containing 200mM $MgCl_2$, and lysozyme was added to a final concentration of 500 $\mu g\ ml^{-1}$: the suspension was incubated at 30°C for 30 min in an orbital incubator at 150 rev. min^{-1}. Cells were harvested and resuspended in 20ml 10 mM Tris-HCl, pH 8.4 containing 10mM $MgCl_2$ and sphaeroplast formation at room temperature was followed by phase contrast microscopy.

Method C: A method of sphaeroplast formation from A. haloplankis NCMB 19, was described by Costerton, Forsberg, Matula, Buckmire & MacLeod (1967). Sphaeroplasts were produced by the gradual removal of the outer layers of the cell wall using successive washes with NaCl and sucrose, followed by a lysozyme treatment. This method was modified slightly and used successfully to produce sphaeroplasts of Alteromonas spp., NCMB 400. Cells from a 2 L micro-aerobic culture were harvested and washed three times in volumes of 500mM NaCl equal to one half the volume of the growth medium. The washed cells were resuspended in the same volume of 500mM sucrose, (or 500mM sucrose containing 5 mM EDTA, pH 9.0), and the suspension was incubated at 25°C for 30 min in an orbital incubator at 100 rev. min^{-1}. The cells were harvested, resuspended in the same volume of 500mM sucrose and centrifuged immediately at 22000g for 15 min at 4°C. The supernatants from the sucrose treatment and sucrose wash were pooled and termed the periplasm. At this stage, the cells retained their rod shape and were termed mureinoplasts following

the nomenclature of DeVoe, Thompson, Costerton & MacLeod (1970). The mureinoplasts were resuspended in Tris-salts buffer, pH 7.5 and lysozyme was added to a final concentration of 150 $\mu g\ ml^{-1}$. The suspension was incubated for 30 min. at room temperature with gentle agitation, and sphaeroplast formation was followed using phase contrast microscopy.

Method D: Birdsell & Cota Robles (1967) described a method of sphaeroplast formation which involved osmotic shock and/or treatment with low concentrations of EDTA. Cells from a 2 l microaerobic culture were harvested, washed once with 200ml 10mM Tris-HCl, pH 8.0 containing 100mM NaCl, and resuspended in 30ml 10mM Tris-HCl, pH 8.0 containing 500mM sucrose. Lysozyme was added to a final concentration of 30 $\mu g\ ml^{-1}$ and the suspension was incubated at room temperature for 10 min. An equal volume of 10 mM Tris-HCl, pH 8.0 was added and sphaeroplast formation was followed by phase contrast microscopy. EDTA was added dropwise with stirring, to a final concentration of 1mM, and sphaeroplast formation was noted after 15 min. incubation at room temperature. When sphaeroplast formation was complete, $MgSO_4.7H_2O$ was added to a finalconcentration of 50mM, so as to counteract the effect of EDTA and prevent further losses of alkaline phosphatase activity (see section 3.4).

2.6.2 Preparation of the periplasm, cytoplasm and membrane fractions.

Sphaeroplasts were prepared using the modified method of Birdsell & Cota-Robles (1967), harvested by centrifugation at 16000g for 15 min at room temperature and the supernatant was retained; this was the periplasmic fraction or periplasm. The sphaeroplasts were gently resuspended in Tris-salts buffer at room temperature, and disrupted on ice by ultrasound using a Soniprobe (Dawe Instruments Ltd., London, model 7532B) at 70% output in 2-3 x 10 sec treatments with 45 sec cooling periods until the

suspension was transluscent. Whole cells and debris were removed by centrifugation at 10000g for 15 min at 4°C , and the supernatant was centrifuged at 100000g for 1 h at 4°C (MSE Ltd., Crawley, model Superspeed 50). The supernatant from the high speed centrifugation was decanted and retained as the cytoplasm, while the pink pellet, the membrane fraction, was resuspended in Tris-salts buffer (ca. 1mg protein ml^{-1}).

2.7 Enzyme Assays

All enzyme assays were performed at room temperature. Spectrophotometric assays were carried out in a single beam spectrophotometer (Cecil Instruments Ltd., Cambridge, model CE 373 or CE 212) linked to a chart recorder (Belmont Instruments Ltd., Glasgow, model Servoscribe IS).

A unit of enzyme activity (U) is defined as μ mol min^{-1} substrate utilized or product formed (1 U = 1000 mU). Specific activity is expressed as U mg $protein^{-1}$ or mU mg $protein^{-1}$.

2.7.1 TMAO reductase

(a) Whole cells assay

The activity of TMAO reductase was determined as the rate of TMA production by whole cells of *Alteromonas* sp., NCMB 400. The reaction mix (final volume 50ml) contained 500 μmol of the electron donor, and approximately 50mg of the washed cell suspension (as prepared in section 2.4.3) in the phosphate salts buffer. Anaerobic conditions were achieved by continuous gassing with oxygen-free nitrogen (OFN). The suspension was equilibrated at 20° ± 1°C. for 5-10 min , and the reaction was started by the addition of 50 μmol TMAO. At regular intervals, 5 ml aliquots were removed and added to 1 ml 25% trichloroacetic acid to terminate the reaction. The TMA produced was determined using either the picrate assay (section 2.8.2.a) or

the gas sensor (section 2.8.4).

Formate was used as the electron donor in all cases, except in experiments which compared the efficiency of other electron donors. Compounds used as electron donors included lactate, pyruvate, glucose, glycerol, alanine, peptone (0.05% w/v), acetate, citrate, isocitrate, α-oxaglutarate, succinate, fumarate, malate and oxaloacetate.

For the determination of the pH optimum of TMAO reductase, buffers to cover the range pH 5.0 - 9.5 were made up as described by McKenzie (1969) and supplemented with 100mM NaCl and 1mM $MgSO_4$. The buffers included citric acid-Na_2HPO_4 (100mM), pH 5.0 - 7.5; Clark and Lubs solution, K_2HPO_4-NaOH (100mM), pH 6.8 - 8.0, and Clark and Lubs solution KCl/H_3BO_3-NaOH (100mM), pH 8.0 - 9.5.

(b) Conductance assay

A 2 l micro-aerobic culture of Alteromonas sp., NCMB 400, was prepared, and cells were harvested and washed as described in section 2.4.3. Cells were resuspended in phosphate-salts buffer, to a density of ca. 10mg protein ml^{-1}, and were used as the source of the enzyme, TMAO reductase. The reaction mix contained 0.5ml washed cell suspension, an electron donor (100 µmol) and was made up to 9.5ml with the phosphate buffer in the electrode test tube. The mixture was flushed with OFN for 2-4 min, and the tube was made air tight with a rubber bung (Suba-Seal, William Freeman & Co. Ltd., Barnsley). The electrode tubes were placed in a water bath at 20° ± 0.002°C, and connected to the conductance meter; a stable base line was achieved within 10 min. The reaction was started by injecting 50 µmol of temperature equilibrated TMAO (0.5ml 100mM TMAO.HCl, pH 7.0) through the Suba-Seal, and G_B was monitored on a chart recorder. The results were calculated as µmol TMA produced $min.^{-1}$, since a conductance change of 1 µS was equivalent to the production of 1.3 µmol TMA. The electron donors and buffers used were as stated in section 2.7.1.a.

(c) Spectrophotometric assay

TMAO reductase activity was assayed by an adaption of the method of Jones and Garland (1977). The bipyridylium compound, methyl viologen (MV^{++}), was partially reduced by sodium dithionite to produce a radical ($MV^{\cdot+}$) with a violet colour, and in the presence of an appropriate electron acceptor e.g. TMAO/TMAO reductase, the dye was oxidized.

A 1 cm-light path glass cuvette (Chemlab Instruments, Hornchurch, type 21 stoppered cuvette) fitted with a wide Teflon stopper was used. A hole (1 mm diameter) had been drilled in the centre of the stopper, to provide a loose fitting for a micro-syringe needle. Some antibumping granules were placed in the cuvette, together with 50-100 µl of the enzyme source, and the cuvette was completely filled carefully, with OFN-saturated Tris- salts buffer, pH 7.2 containing 0.3 mM MV^{++}. The stopper was inserted, displacing the excess buffer, and the contents of the cuvette were mixed by inversion. The hole in the stopper was occluded by the operator's thumb which was protected from the toxic bipyridylium compound by a plastic glove. Partial reduction of the methyl viologen i.e. $MV^{++} \longrightarrow MV^{\cdot+}$, was achieved by adding small amounts (30-50 µl) of freshly prepared 25mM $Na_2S_2O_4$ in 10mM NaOH, with a micro-syringe, until the absorbance had risen to 1.0. The reaction was started with the addition of 4.2 µmol TMAO using a micro-syringe, and the enzyme activity was determined from the rate of decolourization at 600nm assuming the extinction coefficient for $MV^{\cdot+}$ 13 $M^{-1}cm^{-1}$ (Thorneley, 1974).

(d) NAD(P)H : TMAO oxidoreductase

NADH and NADPH linked TMAO reduction was determined using the method of Unemoto et al. (1965). The reaction mixture (2ml) contained phosphate buffer (section 2.4.3), 40-80 nmol NAD(P)H, 50-100 µl enzyme and 2-10 µmol TMAO in a wide necked quartz cuvette (Thermal Syndicate Ltd., Wallsend,

model RA5/5 N10) fitted with a Suba-Seal. The source of enzyme was a cell extract prepared either by sonication of cells in the Tris-salts buffer, or lysis of cells using lysozyme (150 μg ml^{-1}) and EDTA (5mM) in 10mM Tris-HCl, pH 8.0.

The reaction mixture, less enzyme and TMAO, was sparged with OFN for 2-4 min, the cell extract was added and mixed by inversion. There was an initial decrease in absorbance at 340 nm which was due to residual oxygen in solution supporting NADH oxidase activity. When no further activity was observed, the reaction was started by the addition of nitrogen-saturated TMAO. The decrease in absorbance at 340nm due to oxidation of NAD(P)H was measured and the rate calculated using the extinction coefficient 6.22 x 10^3 $M^{-1}cm^{-1}$.

2.7.2 Nitrite reductase and Adenosine-N-oxide reductase

Nitrite reductase and adenosine-N-oxide reductase were assayed using the viologen dye assay described in section 2.7.1c, except that TMAO was replaced by 10 μmol $NaNO_2$ or 4.2 μmol adenosine-N-oxide.

2.7.3 Alkaline phosphatase

Alkaline phosphatase was assayed as the rate of hydrolysis of p-nitrophenyl phosphate (Thompson & MacLeod, 1974a). The reaction mix (2 ml) contained 20 μmol p-nitrophenyl phosphate, Tris-salts buffer, pH 8.8, and the reaction was started by the addition of 50-100 μl of the enzyme source. The reaction was monitored as the increase in absorbance at 420 nm due to nitrophenol and the rate was calculated assuming that 1 OD unit = 1 μmol nitrophenol.

2.7.4 Isocitrate dehydrogenase

Isocitrate dehydrogenase was assayed by the formation of NADPH from NADP (Reeves, Rabin, Wegener & Ajl, 1971). The assay mix contained 20 μmol Tris-HCl buffer, pH 7.5, 2 μmol $MnCl_2$, 0.5 μmol $NADP^+$, 0.5 μmol isocitrate and 50 μl enzyme in a total volume of 1 ml. The reaction was started by the addition of isocitrate and the activity was determined from the increase in absorbance at 340 nm due to NADPH formation using the extinction coefficient 6.22 x 10^3 $M^{-1}cm^{-1}$.

2.7.5 Succinate dehydrogenase

Succinate dehydrogenase was assayed as the reduction of ferricyanide using the method described by Veeger, DerVartanian & Zeylemaker (1969). The reaction mix contained 300 μmol phosphate buffer, pH 7.6, 3 μmol EDTA, 3 μmol KCN, 120 μmol succinate, 0.3% w/v Bovine Serum Albumin, 3.75 μmol $K_3Fe(CN)_6$ in a total volume of 2.9ml. The reaction was started by the addition of 50 μl of enzyme, and the decrease in absorbance at 455nm was monitored. The activity was calculated as μmol succinate reduced min^{-1} assuming the extinction coefficient for ferricyanide was 150 $M^{-1}cm^{-1}$ and 1 mol succinate reduced 2 mol $K_3Fe(CN)_6$.

2.7.6 NADH oxidase

NADH oxidase was determined as the oxidation of NADH at 340nm. The reaction mix (1 ml) consisted of phosphate buffer (section 2.4.3), 80-160 nmol NADH and 50 μl cell extract (section 2.7.1.d). The reaction was started by the addition of the cell extract, and the activity was calculated using the extinction coefficient for NADH 6.22 x 10^3 $M^{-1}cm^{-1}$.

2.8 Determination of TMA

2.8.1 Gas liquid chromatography (glc).

The method used was essentially that of Keay & Hardy (1972) except that Chromosorb P and Igepal CO630 were used as the column packing materials, instead of Silocell C22 and Dowfax 9N9, which are no longer available.

Preparation of the column : Chromosorb P, mesh 60-80 (Chromatographic Services, Merseyside) 40g, was added to 200ml of 90mM KOH to form a free flowing slurry, which was taken to dryness in a rotary evapo rator at 50°C. The dried powder was added to 100-150ml methylene chloride containing 20g Igepal CO630 (Field Instruments, Richmond), and evaporated to dryness. This is packing material A. The same procedure was used to prepare packing material B, except that the KOH concentration was increased to 270mM. A clean 3 m glass column (4mm bore) was filled with packing material A to within 16 cm of the inlet end, and packing material B was added to the remainder of the column to within 1-2 cm of the inlet, which was then plugged with glass wool. The column was conditioned for 24 h at 150°C using OFN as the carrier gas (25 ml min^{-1}).

Operating conditions : A gas chromatograph with a flame ionisation detector (Perkin Elmer, Beaconsfield, model F11) linked to an automatic digital integrator (Infotronics Ltd., Co. Clare, Ireland, model CRS-204) was used. The oven temperature was 125°C and the injection temperature 133°C. The carrier gas was OFN (96.5×10^3 Pa), and the pressure of the hydrogen and oxygen supplies for the detector were 138×10^3 and 207×10^3 Pa, respectively. The internal standard was t-butylamine (final concentration of 0.025% w/v), and the injection volume used was 1 µl. The retention times of TMA and t-butylamine were 140 and 280 sec respectively.

2.8.2 Colorimetry

(a) Picrate assay

The method described by Murray & Gibson (1972a,b) was used. Primary and secondary amines were rendered non-volatile by treatment with formaldehyde, and the tertiary amine i.e. TMA, was displaced with alkali (45% KOH) into toluene. The non-miscible toluene layer was removed, dried and added to a solution of picric acid in toluene. TMA caused ionisation to the yellow picrate which was determined by its absorption at 410 nm. TMA concentrations were found with reference to a calibration curve, which was linear in the range 0.5 - 5.0 μmol TMA.

(b) Spot test

A spot test for detecting TMA in culture media was described by Laycock & Regier (1971). Lee (1973) modified this method by using as alkali 45% KOH instead of saturated K_2CO_3.

The method consists of displacing TMA from a formalized solution with alkali at ca. 50°C. TMA was detected with filtered paper treated with bromophenol blue indicator pH 3.0; it turned the indicator from yellow to blue.

2.8.3 Steam distillation

The method consisted of distilling a formalized amine solution withalkali, trapping the distillate in a boric acid indicator and back-titrating with HCl to determine the TMA content.

The boric acid indicator consisted of two solutions, A and B, mixed in the ratio of 90:1. Solution A contained 10g H_3BO_3 in 200ml ethanol and 700ml distilled water, and solution B contained 33mg bromocresol

green, 66mg methyl red and 66mg phenol red in 100ml ethanol.

The sample (2ml) containing 5-200 μmol TMA was distilled with 10 ml 20% (w/v) NaOH for 10 min using the steam distillation apparatus described by Hoskins (1944) (Gallenkamp, London). The distillate was trapped in the boric acid indicator solution, the colour of which changed from brown to blue. The mixture was then back titrated against 10mM HCl to the colourless/grey end point and the TMA concentration was calculated assuming that 1 mol HCl displaced 1 mol TMA.

2.8.4 Gas sensor

The gas sensor made by M. Storey and referred to by Connell & Shewan (1979) was used to monitor TMA production in culture media and whole cell enzyme assays. The method measures the change in conductivity of a charged sensor caused by the presence of a combustible gas and is analogous to the operation of domestic smoke detectors.

The apparatus consists of a heated Taguchi gas sensor (Watford Electronics, Watford, model 813) connected to a digital volt meter (The Solarton Electronics Group Ltd., Farnborough, model LM 1480.3). The volatile amine, TMA, was liberated from solution by 20% (w/v) NaOH and vigorous aeration, and the gas flow (150-200 ml $min.^{-1}$) was directed over the heated gas sensor. The response of the digital volt meter was linear in the range 1-6 nmol TMA which corresponded to a change of 0-300mV.

2.9 Determination of TMAO

TMAO was reduced chemically to TMA using $TiCl_3$ at 1-3% (w/v) final concentration for 10 min at room temperature. The TMA produced was determined using one of the assay methods described in section 2.8.

2.10 Other assays

2.10.1 Protein estimations

Protein concentrations were determined using the Folin-Ciocalteu reagent in the method of Lowry, Rosenbrough, Farr & Randall (1951). The reagents used were (a) 50 ml 2% (w/v) Na_2CO_3 in 100mM NaOH which was mixed with 0.5 ml 1% (w/v) $CuSO_4.5H_2O$ and 0.5 ml 2% (w/v) sodium potassium tartrate prior to use and (b) Folin-Ciocalteu phenol reagent (1.0 ml) diluted with 2.14 ml distilled water.

The sample (1 ml) containing 25-200 µg of protein was added to 5 ml of reagent (a), mixed and incubated at room temperature for 10 min. Reagent (b) (0.5 ml) was added with rapid mixing and the solution was incubated at room temperature for 30 min, when the absorbance was determined at 750 nm. The assay was calibrated with bovine serum albumin (Fraction V, Sigma) and was linear in the range 25-200 µg.

The presence of 50-200mM sucrose had no significant effect on this assay, but the method could not be used in the presence of ascorbate/glutathione as used in affinity chromatography (section 2.13.3). In such cases, protein was estimated using Peterson's (1977) modification of the method described by Lowry *et al.*(1951). Proteins (5-100 µg) were precipitated with trichloroacetic acid in the presence of sodium deoxycholate, and determined spectrophotometrically using the Folin-Ciocalteu phenol reagents containing sodium dodecylsulphate (SDS).

2.10.2 Determination of oxygen tension

The amount of dissolved oxygen in the culture medium was estimated as the percentage oxygen saturation, using a Mackereth-type oxygen electrode (E.I.L., Chertsey) and an oxygen controller (E.I.L., model 94B). The instrument was calibrated using aerated culture medium and 5% (w/v) Na_2SO_3 as 100% and 0% oxygen saturation, respectively.

2.10.3 Determination of nitrite

Nitrite was determined using the colo rimetric assay of Radcliffe & Nicholas (1968). The diazo compound formed in the presence of nitrite, sulphanilamide and naphthylethylenediamine, was determined from its absorbance at 540 nm.

The reagents used were : 1% (w/v) sulphanilamide in 1M HCl (reagent A), and 0.02% (w/v) aqueous N-1-naphthylethylenediamine dihydrochloride (reagent B).

The sample (0.2ml) containing 0-200 nmol nitrite were mixed with 8.8ml reagent A. Reagent B (1 ml) was added with rapid mixing, and the pink colour was allowed to develop for 20 min at room temperature. The absorbance at 540 nm was determined against a distilled water blank, and calibrated using $NaNO_2$. The assay was linear in the range 0-200 nmol $NaNO_2$.

2.11 Cytochrome spectra analysis

Reduced minus oxidised difference spectra of cell fractions (0.3 - 3.0mg protein ml^{-1}) were determined using a recording spectrophotometer, (i) Pye Unicam SP 1800 (Pye Unicam, Cambridge) and (ii) Gilford, model 2600 (Gilford Instruments Ltd., Teddington). Measurements were made at room temperature in 1cm pathlength quartz cuvettes. Samples were oxidized with $K_3Fe(CN)_6$ and reduced with $Na_2S_2O_4$. Cytochrome c was quantified using the extinction coefficient of 24.1 $M^{-1}cm^{-1}$ (Fujita, 1966).

2.12 Enzyme Concentration

An ultrafiltration apparatus (Standard Stirred Cell, Amicon Ltd., Woking, model 52) was used to concentrate the periplasmic fraction, and on one occasion was used to concentrate the purified enzyme. A filter with an exclusion limit of 10000 daltons was used, (Amicon Ltd., type PM10) so that only molecules with a molecular weight greater than 10000 daltons were retained. Filtration was carried out under pressure (207 x 10^3 Pa) using OFN, while the solution was stirred using a magnetic stirrer (Chemlab Instruments Ltd., Hornchurch, model SS3). The periplasm (65ml) was filtered over a period of 4-5h, and chilled conditions were maintained by placing an ice pack between the stirrer and the filtration unit.

The enzyme purified by affinity chromotography was also concentrated by centrifugation at 750g for 10 min at 4°C using the Centriflo Cones (Amicon Ltd.), with an exclusion limit of 25000 daltons.

2.13 Enzyme Purification

2.13.1 Ammonium sulphate precipitation.

The periplasm was treated with increasing volumes of saturated $(NH_4)_2SO_4$, so as to produce a range of concentrations from 0-60% saturation. The solutions were incubated at 4°C for 2h and centrifuged at 17000g for 15 min at 4°C.

2.13.2 Gel filtration

A chromatography column (Whatman Biochemicals Ltd., Maidstone, 19 x 3cm) was filled with 140ml Sephacryl S-300 Superfine (Pharmacia Fine Chemicals Ltd., Hounslow), a cross-linked dextran-acrylamide mixture. It was equilibrated for 1-2h by eluting with 250ml 50mM Tris-HCl, pH 7.5 at room temperature. The void volume was estimated to be 35ml by passing the calibration dye, Blue Dextran (2×10^6 daltons) through the column.

The concentrated periplasm (3ml) was applied to the column which was irrigated with 50mM Tris-HCl, pH 7.5 and the eluant was collected in 4ml volumes using a fraction collector (LKB Instruments Ltd., Croydon, model Ultrarac). Protein in each fraction was detected as the absorbance at 280 nm, and quantified using the Folin-Ciocalteu reagent (section 2.10.1).

TMAO reductase activity was determined using the assay described in section 2.7.1c.

2.13.3 Affinity chromatography

An affinity matrix was prepared by covalently linking betaine to a Sepharose support by means of a coupling agent such that the trimethylammonium group was the effective ligand.

AH-Sepharose 4B (Pharmacia Fine Chemicals, Hounslow) 4g , was swollen and washed in a total volume of 800ml 500mM NaCl, further washed in distilled water, collected in a sintered glass funnel, and resuspended in 60ml 300mM betaine hydrochloride, pH 4.5, and stirred gently with a glass rod. The coupling agent, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (Alwich Chemical Co. Ltd., Gillingham) was added as a solid to give a final concentration of 100mM, and the solution was held for 24 h at room temperature; the pH was maintained between pH 4.5-6.0 forthe first 2 h. The gel was then washed thoroughly by alternating between 100mM Tris-HCl, pH8.5 containing 1 M NaCl and 100mM sodium acetate/acetic acid buffer, pH 4.5 containing 1 M NaCl. The gel was washed with distilled water, resuspended in the running buffer and degassed. A chromatography column, 15 x 1 cm. (Whatman), was filled with the affinity gel and equilibrated with the running buffer at room temperature. The column was stored at room temperature in 50mM Tris-HCl, pH 8.5 containing 1 M NaCl, when not in use.

The column was equilibrated with 50mM phosphate buffer, pH 6.5 and a 3ml sample of the concentrated periplasm was applied in the same buffer. The column was eluted with 50mM Tris-HCl, pH 7.5 and a gradient of 0.2-0.8 M NaCl created by a gradient mixer, (LKB, model Ultragrad 11300). Fractions were collected in 4-5ml amounts and the protein content and TMAO reductase activity were determined as described in section 2.13.2.

On one occasion 5mM ascorbic acid and 5mM glutathione were added to both the running and the eluting buffers, and under these circumstances the protein content of the eluant was determined using the method of Peterson (1977) (section 2.10.1).

2.13.4 Polyacrylamide gel electrophoresis

The method of polyacrylamide gel electrophoresis (PAGE) was that of Ames (1974).

The acrylamide solution contained 28g acrylamide and 0.73g NN'-methylenebisacrylamide in 100ml distilled water and was filtered through Whatman No.1 filter paper. It was stored at 4°C for not more than 14 d.

The running gel, 8 x 14 x 0.15cm, contained 7% acrylamide. It was prepared from 17ml acrylamide solution, 6ml 1.5M Tris-HCl, pH 8.8, 0.6ml 0.2M EDTA, 0.03ml NNN'N'-tetramethylethylenediamine and 37ml distilled water, and was polymerised with $(NH_4)_2S_2O_8$ (1%(w/v) final concentration). The stacking gel (2 x 14 x 0.15cm) was prepared in the same way except that it contained 10.7ml acrylamide and 7.5ml 50mM Tris-HCl, pH 6.8. The electrophoresis buffer contained (g l^{-1}); Tris-HCl, 3; glycine, 14.4 and EDTA, 0.83.

The gel was prerun at 100V using a constant voltage power supply (Shandon Scientific Co. Ltd., London, model Vokam 2541) to remove $(NH_4)_2S_2O_8$. The samples were applied in 20-50µl volumes and the gel was run for 1.5-2.0 h at 1°C at 100V.

The gel was stained for TMAO reductase activity using the method described by Shimokawa & Ishimoto (1979). It was immersed in a solution containing 50mM Tris-HCl, pH 7.4, 2mM methyl viologen, 7.5mM $KHCO_3$, 40mM TMAO. HCl and 5mM $Na_2S_2O_4$ for 5 min at room temperature. Excess solution was drained off and zones of decolourisation against the violet background stain were observed in those areas containing TMAO reductase activity.

The distribution of protein bands within the gel was also determined by staining. The staining solution contained Brilliant Blue R (0.625g) dissolved in 227ml 50% methanol and 23ml glacial acetic acid. The solution was freshly prepared, filtered through a Whatman No.1 filter paper and used only once. The gel was incubated in the staining solution

at room temperature for 10-15 min with agitation. It was destained for 1-3d by washing with frequent changes in a solution containing 75ml glacial acetic acid, 50ml methanol and 875ml distilled water.

For SDS PAGE, samples were boiled for 2-3 min in 2.5% (w/v) SDS and 5mM dithiothreitol, and applied to the gel. The electrophoretic conditions were identical to those of PAGE except that SDS (0.1% w/v) was incorporated into both the gels and the buffer. Proteins of known molecular weight (electrophoresis calibration kits (Pharmacia)), were used for calibration of SDS PAGE.

2.13.5 Isoelectric focusing

A Multiphor unit (LKB, model 2117) linked to a DC power supply (LKB, model 3371E), and an Ampholine polyacrylamide plate (LKB) with a pH range of pH 3.5-9.5 were used. The samples (25-75µl) were applied on pieces of filter paper, and the running conditions were 1200V for 2 h at 6°C. The sample application papers were removed after 30 min. TMAO reductase activity was assayed using the method of Shimokawa & Ishimoto (1979), as described in section 2.13.4.

The protein bands were fixed by immersion for 1 h in a solution containing 17.25g sulphosalicyclic acid and 57.5g trichloroacetic acid, washed in destaining solution (500ml ethanol + 160ml glacial acetic acid + 1340ml distilled water) and treated for 10 min at 60°C with freshly prepared protein stain (0.115g Brilliant Blue R in 100ml destaining solution). The gel was destained for 1-2 d by washing with frequent changes in the destaining solution.

2.13.6 Ion exchange chromatography

A cation exchange resin, Dowex 50W-X8, mesh 20-50, was used. It was washed thoroughly with 2 l of 1 M HCl over 24 h, rinsed several times with distilled water and equilibrated with 50mM citrate phosphate buffer, pH 7.0. It was then degassed and poured into a chromatography column (Pharmacia, 13 x 1.6cm). Concentrated periplasm (3ml) was applied to the column in 50mM citrate phosphate buffer, pH 7.0 and eluted with 50mM glycine-NaOH buffer, pH 10.0. Fractions (4ml) were collected and assayed for protein and TMAO reductase activity as described in section 2.13.2.

2.13.7 High voltage paper electrophoresis

Filter paper sheets (60 x 15 x 0.1cm) were soaked in buffer and positioned on the electrophoresis apparatus (Locarte, London). The buffers used were either (i) pH 2.0, containing 8.7% (v/v) acetic acid and 2.5% (v/v) aqueous formic acid (89%), or (ii) pH 6.5, containing 2.5% (v/v) pyridine and 0.3% (v/v) acetic acid. The sample was applied as a streak across the filter paper and the apparatus was run 5000V for 30-40 min at 6°C. The paper was stained for protein and TMAO reductase activity as described in section 2.13.4.

CHAPTER 3

RESULTS

3.1 Comparison of assay methods for TMA and TMAO

The characteristics of the methods for the detection and quantitation of TMA and TMAO are summarised in Table 4. The most sensitive and specific method was glc. It detected TMA in the range 0.6 - 60 nmol, and after calibration, a result could be obtained within 5 min of the sample injection. Culture media and assay solutions containing TMA could be assayed directly without any adverse effects on the column packing material (Fig. 4). However, poor results were obtained with TMAO samples treated with $TiCl_3$ due to deterioration of the column packing material; the chromatograms are shown in Fig. 5a. Deterioration resulted in decreased sensitivity, and split and additional peaks appeared in the chromatograms. It was probably due to neutralization of the alkaline packing material. It is shown in Fig. 5b that the results can be improved slightly by extracting the TMA formed from TMAO into toluene, and acid washing the toluene with 60mM HCl to obtain a cleaner aqueous solution of TMA. However, full recovery of TMAO as TMA was not achieved probably due to losses incurred during extraction and washing. The best method for the quantitation of TMAO by glc was to steam distill the $TiCl_3$/TMA solution and assay the distillate for TMA.

The advantage of glc over the other assay methods for TMA/TMAO is its specificity, in that it separates TMA from other tertiary, secondary or primary amines. Chromatograms of spent culture media in which Alteromonas spp. had been grown showed only 1 peak, and its retention time was equivalent to that of TMA (Fig. 4b). These organisms did not convert TMAO to dimethylamine and formaldehyde, a property of certain methylotrophic bacteria (Colby et al., 1979). The disadvantage of the method was the eventual inactivation of the column packing material by

TABLE 4 : Comparison of assay methods for TMA and TMAO

Method	Detection of TMA	Detection of TMAO	Linear calibration range (nmol)	Speed	Apparatus
Gas liquid chromatography	+	± *	0.6 - 60	Fast for TMA slow for TMAO	Complicated and expensive
Picrate assay	+	+	500 - 5000	Moderate	Simple & cheap
Spot test	+	+	none †	Fast	" "
Steam distillation	+	+	$2 - 20 \times 10^3$	slow	" "
Gas sensor	+	+	1 - 10	Fast	simple ††

* Assay unreliable due to deterioration of the glc column caused by $TiCl_3$ - see text for details.

† Qualitative only $\geqslant$ 12000 nmol.

†† Not commercially available.

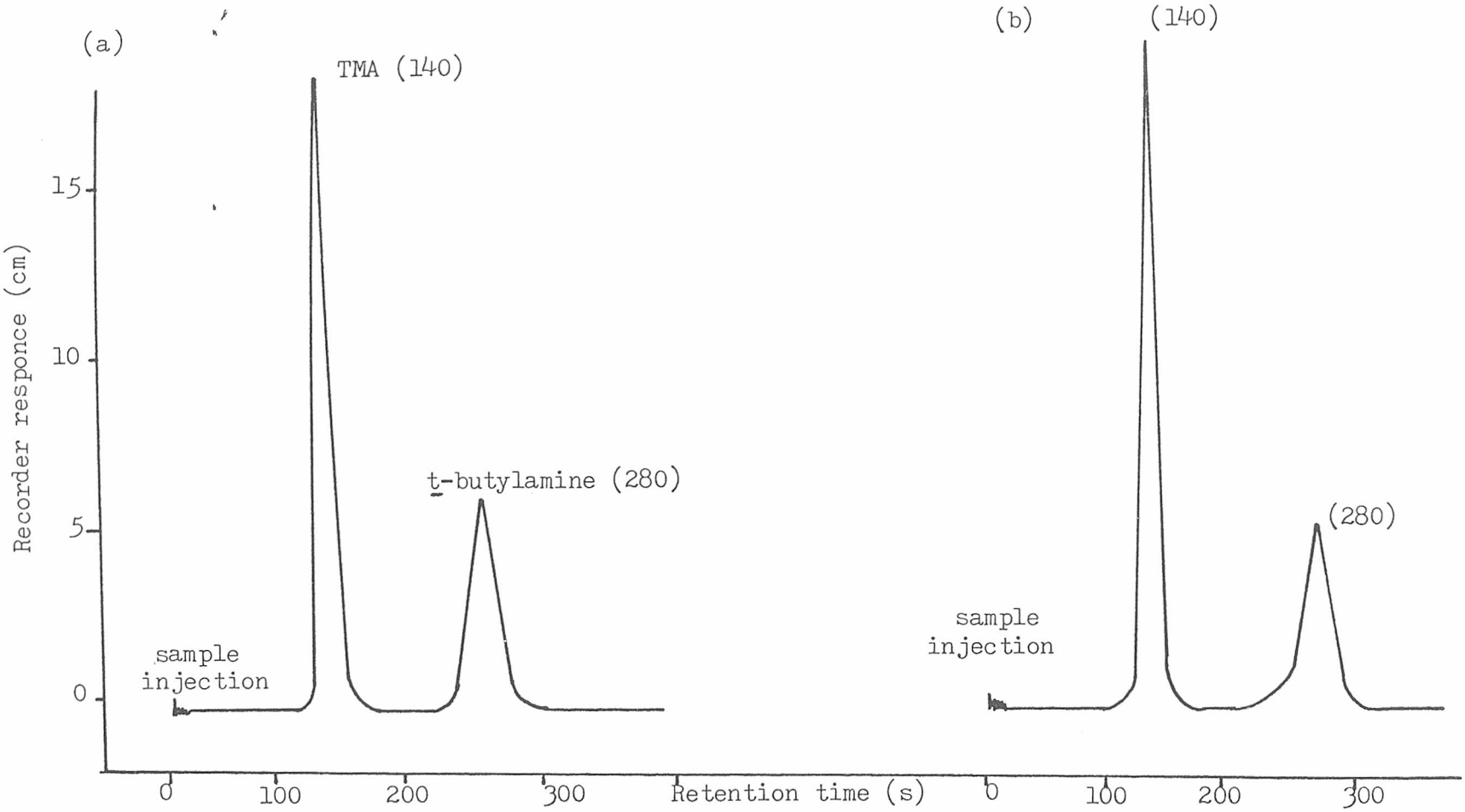


Figure 4 : TMA chromatograms (a) calibration with TMA (1 nmol)

(b) spent culture media of Alteromonas sp., NCMB 400, grown on the medium of Wood & Baird (1943).

(Figures in parentheses are retention times in seconds).

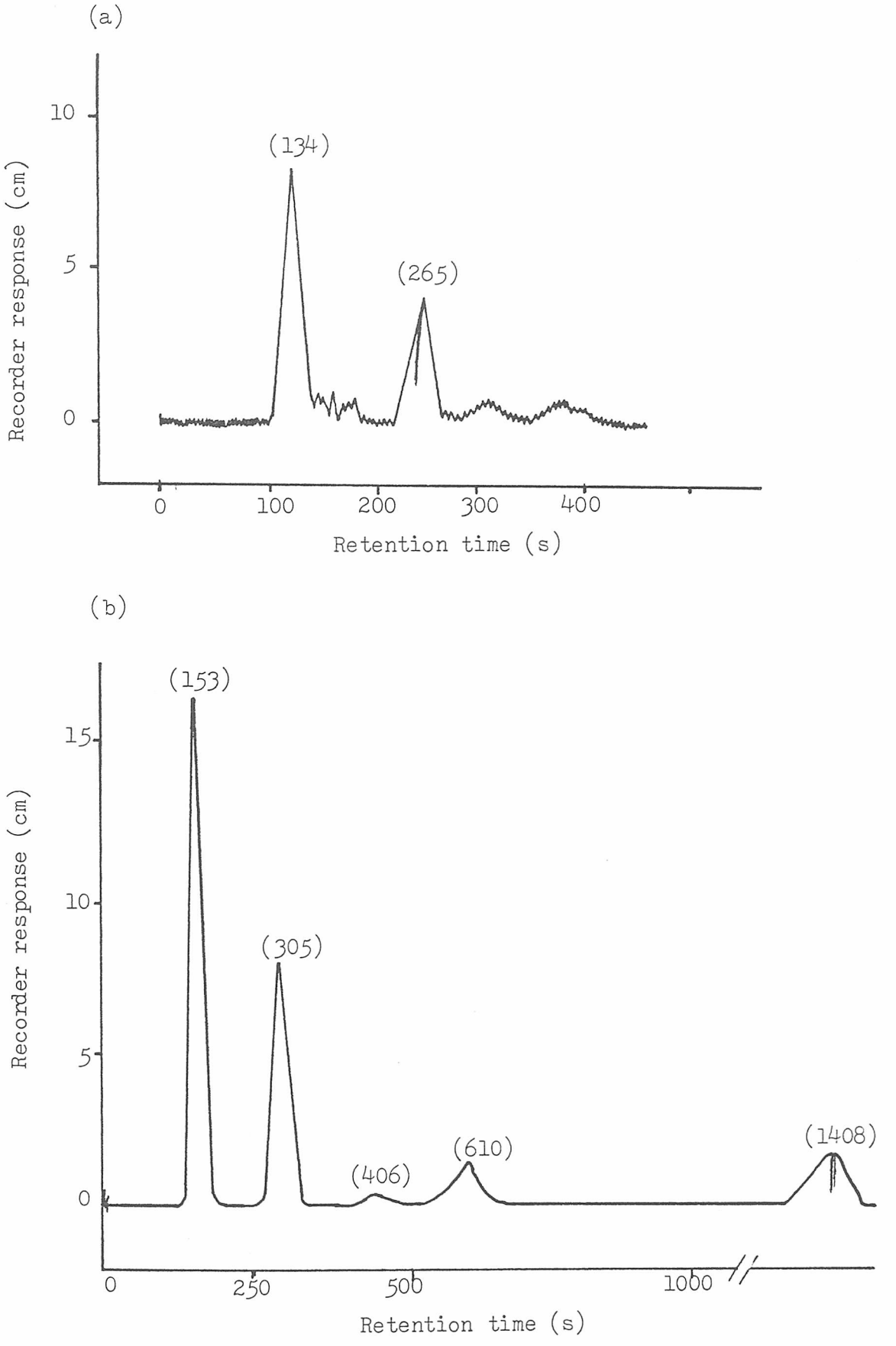


Figure 5 : Effect of $TiCl_3$ on chromatograms of $TiCl_3$/TMA solutions (a) before and (b) after extraction with toluene and acid washing. (Figures in parentheses are retention times in seconds).

the successive addition of acidic samples. Hence it was necessary to replace the packing material and calibrate the assay regularly.

The gas sensor was less sensitive than glc being able to detect 1-10 nmol TMA. Calibration took ca 2h, but thereafter TMA determinations could be made in 5-15 min depending on the number of replicates required and their TMA content. The more concentrated the TMA sample the greater the duration of the assay, since the voltmeter output had to show 0mV before the next sample could be injected.

The picrate assay was less sensitive than glc and the gas sensor, but it was generally used for TMA analysis because it was relatively sensitive, rapid, reproducible and easy to set up. Both the picrate assay and the gas sensor were used to determine the TMA content of culture media and enzyme reaction mixes, and the results obtained were in complete agreement. Both methods were non-specific for tertiary volatile amines and could not distinguish between TMA and dimethylamine. However, the results from glc showed that TMA was the only tertiary amine produced during the growth of Alteromonas spp. in the presence of TMAO. Therefore, the assays provided a valid measurement of TMA produced under such conditions.

The spot test for TMA was used to detect this amine in spent culture media, and it took 10 min to perform. Disadvantages were (i) a lack of sensitivity ($\geqslant$ 12 μmol), (ii) the test was non-quantitative and (iii) the use of concentrated alkali could lead to false positives if mishandled. The steam distillation technique was not used regularly for TMA estimations mainly because of its low sensitivity (2-20 μmol TMA). It was also a slow method, and continuous supervision of the apparatus was necessary.

Although $TiCl_3$ was shown to affect the glc assay of TMA, it had no such effect on any of the other assays. Thus the picrate assay was

a suitable method for the determination of both TMAO and TMA.

3.2 Anaerobic bacterial growth and TMAO reduction

3.2.1 Selection and some properties of TMAO reducing bacteria

Seven cultures of Alteromonas spp. were used - 4 strains of phenon C: NCMB 19, 130, 1516 and 1789; 1 strain of phenon D: NCMB 400; and 2 strains of phenon E: NCIB 11156 and 10471, as classified by Lee et al. (1977). The bacteria were all Gram-negative rods whose morphology appeared to vary with the age of the culture; uniform short rods were observed during rapid growth, but after 2-3 d incubation some cells (ca 10-20%) became curved and elongated (2-10 times). Elongation was particularly evident at growth temperatures above 25°C.

All the alteromonads grown on solid media (Sea water agar or nutrient agar) produced colonies that were white to grey-white in colour, except for NCMB 1520 which had 2 colony-types after prolonged storage at 4°C. These colonies were white or yellow in colour, and in subsequent spread plate cultures the yellow colony form reverted to the white form.

The optimum temperature for growth of the 7 alteromonads was 20°C but the temperature profiles of the marine isolates showed an additional peak at 5°C, most apparent after 4 d incubation (Fig. 6).

The ability of bacteria to reduce TMAO under micro-aerobic conditions was investigaged in test tube cultures using the medium of Wood & Baird (as described in section 2.3). After 2-3 d incubation at 20°C, growth was determined spectrophotometrically, and the presence of TMA determined using the spot test. It was found that only 3 strains showed a significant amount of growth and reduced TMAO to TMA viz. NCMB 400, NCIB 10471 and NCIB 11156. The results were consistent with those of Lee et al. (1977)

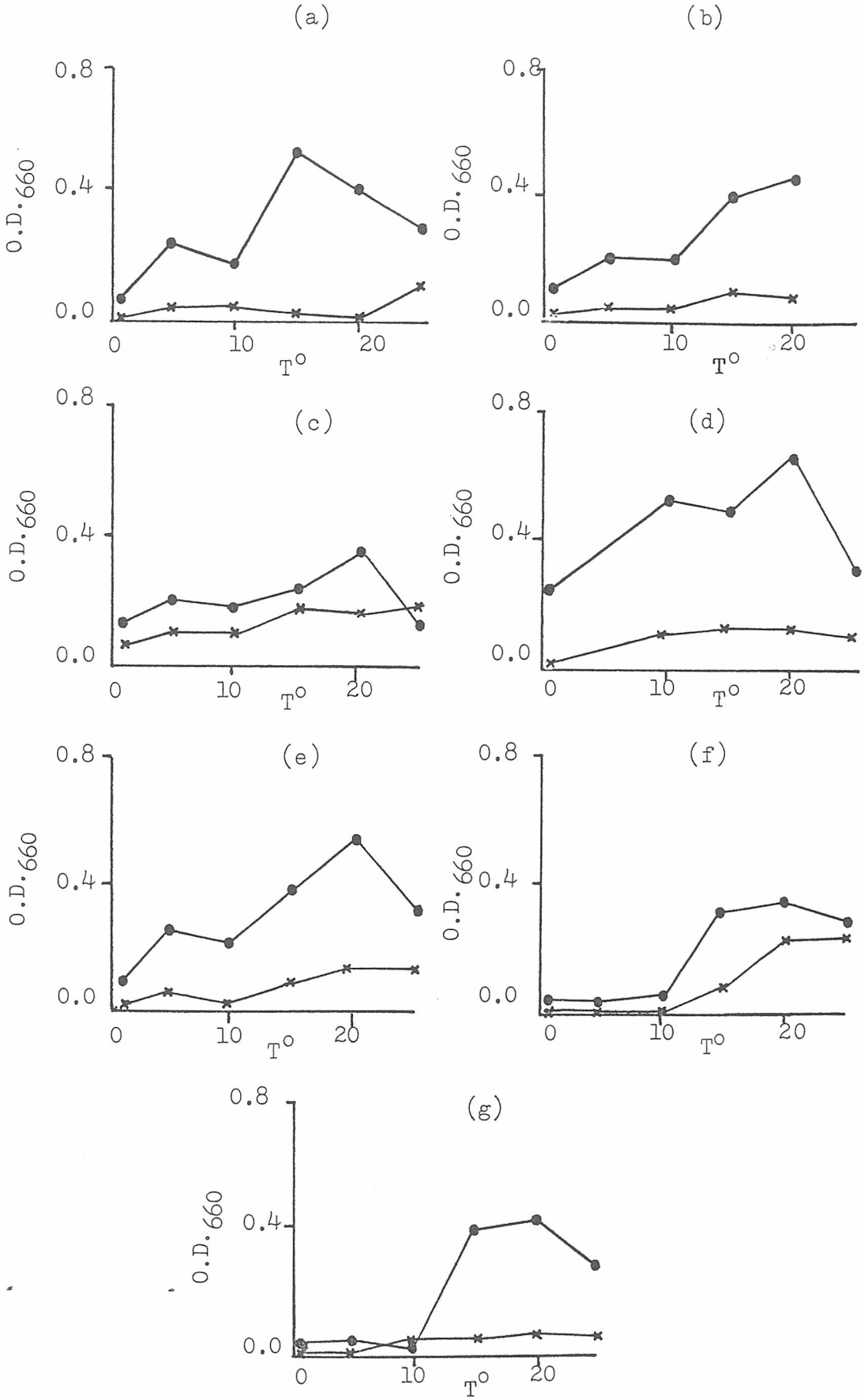


Figure 6 : Temperature (T^{o}) profiles of growth ($O.D._{660}$) of Alteromonas spp.

(a) NCMB 19; (b) NCMB 1516; (c) NCMB 130; (d) NCMB 400;
(e) NCMB 1789; (f) NCIB 11156; (g) NCIB 10471;
×, 1 d growth; ● 4 d growth.

and since NCMB 400 was the only organism of marine origin (isolated from sea water), it was chosen for further studies.

Further examination of the properties of these bacteria was not undertaken, since bacterial identification and classification were outwith the scope of this study. However, the purity of the stock culture of Alteromonas sp., NCMB 400, was checked regularly (ca 6-8 weeks) by examining (i) the microscopic appearance of both wet mounts and Gram stained preparations; (ii) growth on TMAO and the production of TMA and (iii) sensitivity to antibiotics using multidiscs. Alteromonas sp., NCMB 400, was resistant to penicillin G (4 units), Novobiocin (5 μg), sulphafurazole (100 μg), oleandomycin (5 μg), streptomycin (10 μg) and tetracycline (10 μg), but sensitive to streptomycin (25 μg), chloramphenicol (10 and 50 μg), tetracyclin (25 μg), polymyxin B (250 units) and erythromycin (10 μg).

3.2.2 Anaerobic growth of Alteromonas spp.

The ability of several alteromonads to grow anaerobically at the expense of TMAO, nitrate or fumarate was tested using the anaerobic test tube apparatus described by Pankhurst (1967) and the medium of Wood & Baird containing 10mM TMAO, 5mM KNO_3 or 10mM sodium fumarate. The results are shown in Table 5 but agreement between replicates was poor in some cases, possibly due to (a) contamination of the culture medium with alkaline pyrogallol or (b) leakage of oxygen into the culture vessel and failure to maintain anaerobiosis. Great care had to be taken when setting up the Pankhurst tube assay and the following precautions are recommended, (i) plug and bridging arm with non-absorbent cotton wool, and smear its edges with vaseline; (ii) use absorbent cotton wool to retain the alkaline pyrogallol in the side vessel, and (iii) plug the 2 main openings firmly

TABLE 5 : Anaerobic growth of Alteromonas spp., using TMAO, nitrate and fumarate as the terminal electron acceptor.

Phenon	Organism	Anaerobic Bacterial Growth ($O.D_{660}^{*}$) on the following Terminal Electron Acceptors			
		None	TMAO	Nitrate	Fumarate
C	NCMB 19	0.031	0.032	0.033	0.554
	NCMB 1516	0.210	0.212	0.230	0.307
	NCMB 130	0.111	0.137	0.142	0.139
D	NCMB 400	0.044	0.202	0.085	0.130
E	NCMB 1733	0.072	0.190	0.238	0.168
	NCIB 11156	0.066	0.236	0.061	0.172

* Mean of 3 replicates.

with a rubber bung or Suba-seal. During incubation agitation was avoided to reduce the risk of contaminating the culture medium with alkaline pyrogallol mixture.

Some growth was observed in the absence of a TEA probably due to residual oxygen dissolved in the culture medium. The growth of cultures, NCMB 1516 and 130 under such conditions was greater than the other alteromonads tested. It is possible that such growth was a consequence of their metabolism or that strict anaerobiosis was not achieved. Most of the alteromonads examined could grow anaerobically on fumarate, the only TEA to support the growth of strains in phenon C. Some of the bacteria could grow on nitrate and TMAO while others could only use TMAO.

Using test tube cultures in anaerobic jars, the growth of 9 TMAO reducing alteromonads on TMAO or nitrate was compared and the results are shown in Table 6. One third of the strains tested could grow on TMAO alone, whilst the remainder could be grown on both TMAO and nitrate. All the TMAO reducers were strains of phenons D and E, although the ability to reduce nitrate was not present in them all. The 5 NCMB strains could reduce both TMAO and nitrate. The same technique was used to examine the anaerobic growth of these alteromonads in the presence of TMAO or nitrate or both TEA's, and the results are shown in Figs. 7 and 8. When both TMAO and nitrate were present, the growth yield was either equal to or less than that of TMAO alone, but both TMA and nitrite were detected in the culture medium. It was not determined which TEA was used first, but since most of the TMAO had been reduced and only half the nitrate was reduced, it is possible that TMAO was used preferentially. Nitrite was shown to inhibit anaerobic growth and the reduction of TMAO and nitrate in all the alteromonads tested (Figs. 7 and 8). This may account for the decreased growth yield in the presence of TMAO and nitrate in that nitrite produced *in situ* from nitrate reduction was inhibitory.

TABLE 6 : The Anaerobic growth of *Alteromonas* spp. on TMAO and nitrate.

Phenon	Strain	Anaerobic Growth on	
		TMAO	Nitrate
D	NCMB 400	+	+
	NCMB 1520	+	+
	NCMB 1521	+	+
E	NCMB 1733	+	+
	NCMB 1735	+	+
	NCIB 11157	+	+
	NCIB 11156	+	-
	NCIB 10472	+	-
	NCIB 10473	+	-

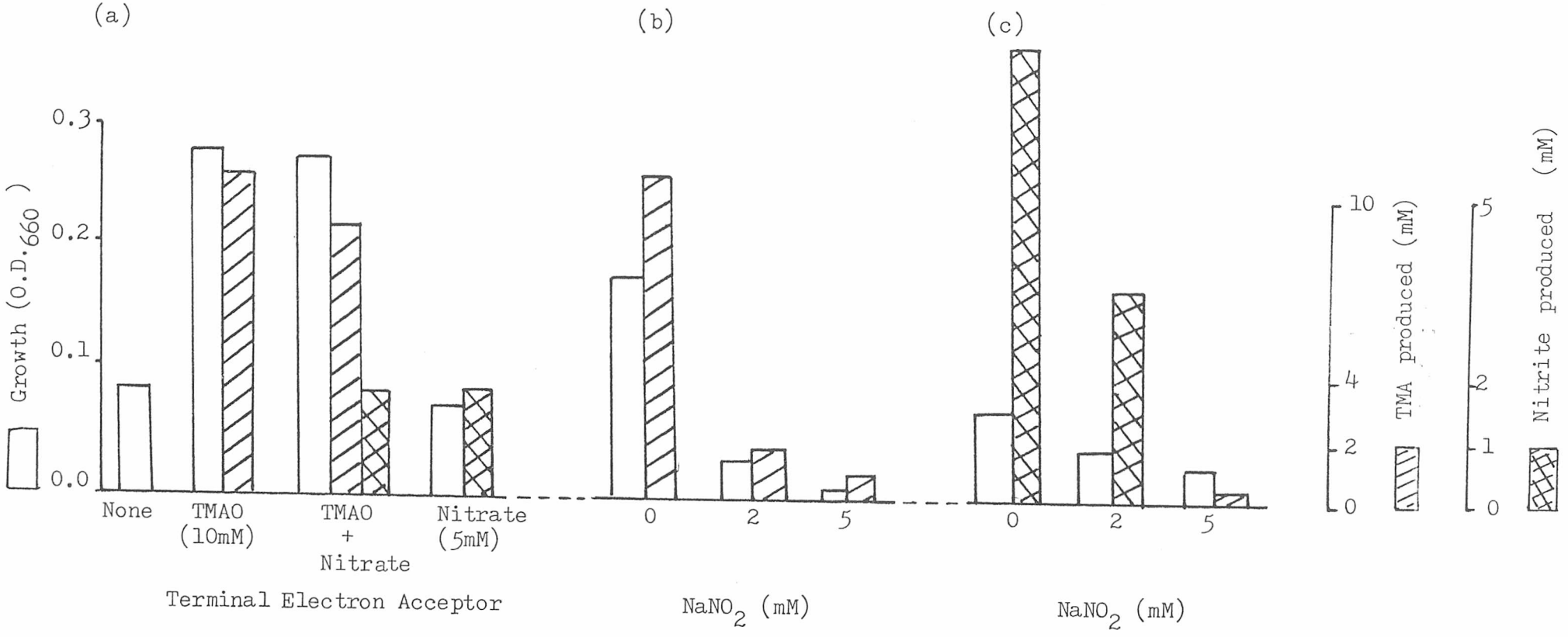


Figure 7 : Effect of nitrite on anaerobic growth of Alteromonas sp., NCMB 400,

(a) growth in the presence of TMAO and/or nitrate

(b) effect of nitrite on growth and TMAO reduction

(c) effect of nitrite on growth and nitrate reduction

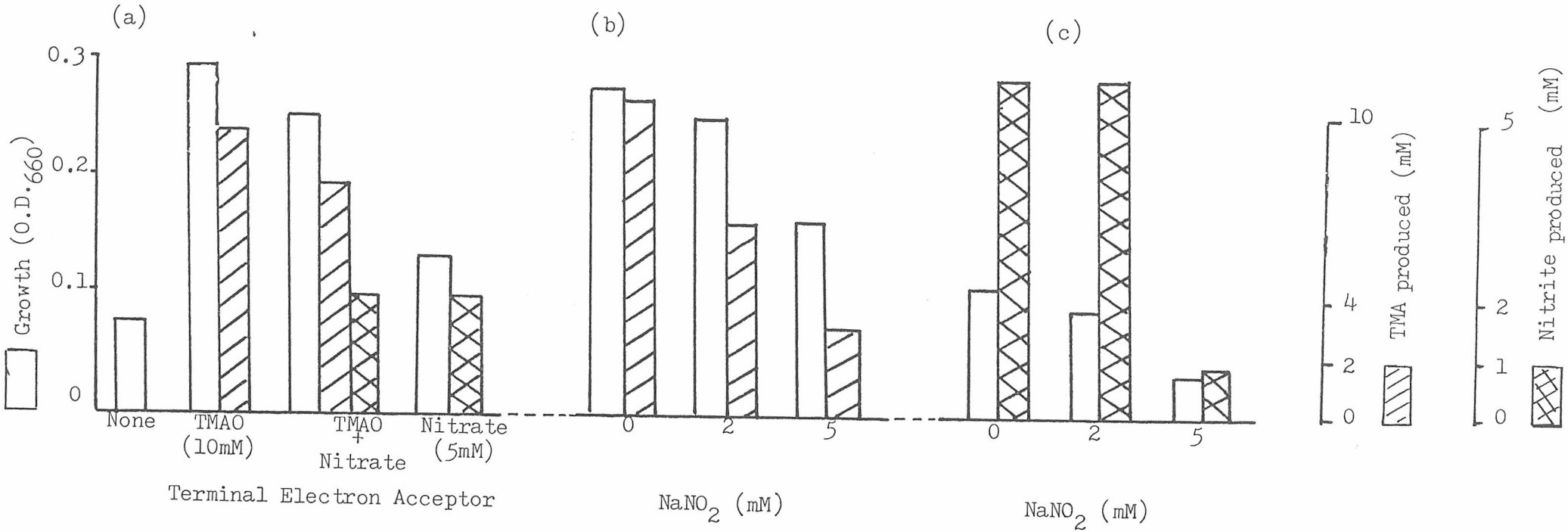


Figure 8 : Effect of nitrite on anaerobic growth of A. putrefaciens, NCMB 1735,

(a) growth in the presence of TMAO and/or nitrate

(b) effect of nitrite on growth and TMAO reduction

(c) effect of nitrite on growth and nitrate reduction.

3.2.3 Use of conductance for monitoring bacterial TMAO reduction.

Conductance measurements have been used as a method of following bacterial growth by monitoring the change in the charge carrying capacity of the medium (Richards et al., 1978). The reduction of TMAO to TMA was expected to produce a large conductance change since TMAO is neutral and TMA is very basic. Thus conductance measurements should provide a method of following bacterial TMAO reduction and an experiment was carried out to test this.

Alteromonas sp., NCMB 400, was grown under microaerobic conditions in the Wood & Baird medium containing TMAO and during the logarithmic phase of growth, the conductance change (G_B), bacterial numbers ($N-N_o$), $O.D_{660}$, TMAO and TMA concentrations were measured. The results are shown in Fig. 9 and several relationships were established (see equations 4-10).

$$G_B \propto \text{O.D.} \quad (4)$$

$$G_B \propto \text{TMA production} \quad (5)$$

$$G_B \propto \text{TMAO reduction } (TMAO_o - TMAO_t) \quad (6)$$

where $TMAO_o$ and $TMAO_t$ were the TMAO concentration at times zero and t respectively.

$$\log G_B \propto \log (N-N_o) \quad (7)$$

$$\therefore \quad G_B \propto (N-N_o)$$

where N = bacterial count and N_o the initial count.

$$\text{O.D.} \propto \log (N-N_o) \quad (8)$$

$$\text{O.D.} \propto \text{TMA production and TMAO reduction} \quad (9)$$

$$(N-N_o) \propto \text{TMA production and TMAO reduction} \quad (10)$$

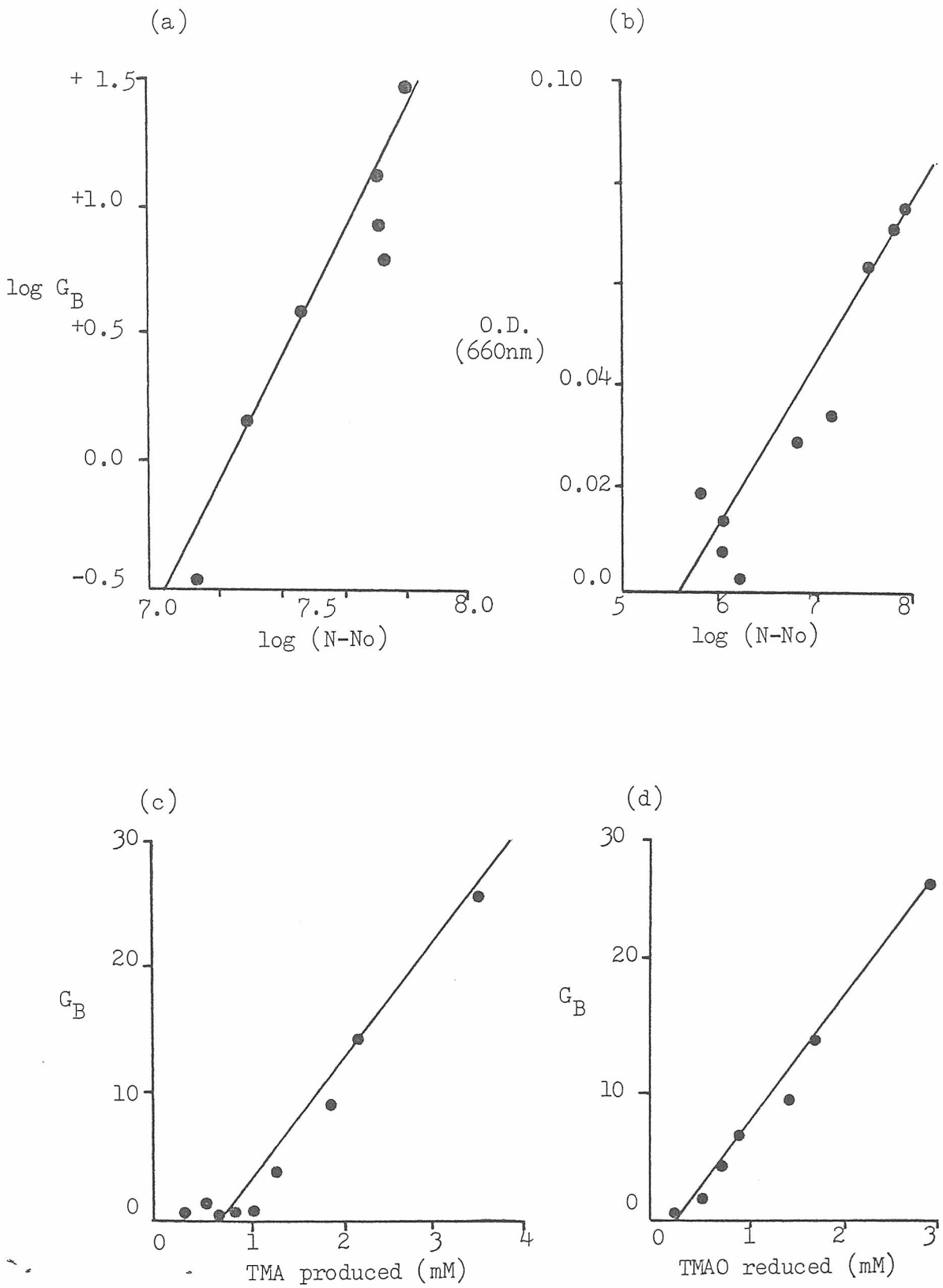


Figure 9 : Relationships between conductance change (G_B,μS), bacterial numbers (N-No), TMAO reduction and optical density (O.D.).

Thus, conductance measurements were shown to be a valid method for monitoring bacterial growth and TMAO reduction. Consequently, further measurements were undertaken to investigate TMAO reduction by Alteromonas sp., NCMB 400.

The effect of TMAO on bacterial growth was determined spectrophotometrically and using conductance measurements. Under micro-aerobic conditions, the presence of TMAO supported the growth of Alteromonas sp., NCMB 400, and the extent of growth was limited by the amount of TMAO available (Fig. 10). In the absence of TMAO, growth was poor and the conductance change was low, $G_B < 10\ \mu S$. The plots of G_B against time were sigmoidal, typical of a bacterial growth curve (Fig. 11a) and the G_B achieved when all the TMAO was utilized, was proportional to the initial TMAO concentration (Fig. 11b). Similar results were obtained under anaerobic conditions, and with another Alteromonas sp., NCIB 11156. All the TMAO supplied in the medium was recovered as TMA, and so it seems unlikely that any TMAO or TMA was used as a carbon or nitrogen source during anaerobic growth. The growth rate was independent of the initial TMAO concentration in the range 1-20mM, and the mean generation time was relatively constant at 1.64 ± 0.59 h.

The conductance apparatus was also used to test the ability of many Gram-negative bacteria to reduce TMAO. The test organisms(Table 3) were grown under micro-aerobic conditions in the Wood & Baird medium containing 10mM TMAO, and the conductance changes were monitored for 1-2 d, after which the medium was assayed for TMA using the chemical spot test. The results are shown in Fig. 12 and Table 7. The reduction of 10mM TMAO produced a final conductance change of 100-140 μS, with some strain variation. Using conductance alone, $G_B > 100\ \mu S$ with a rate of change $(dG_B/dt) > 2\ \mu s\ h^{-1}$ was considered to be a positive result for TMAO reducing organisms (see Fig. 12). Confirmation of TMAO reduction was

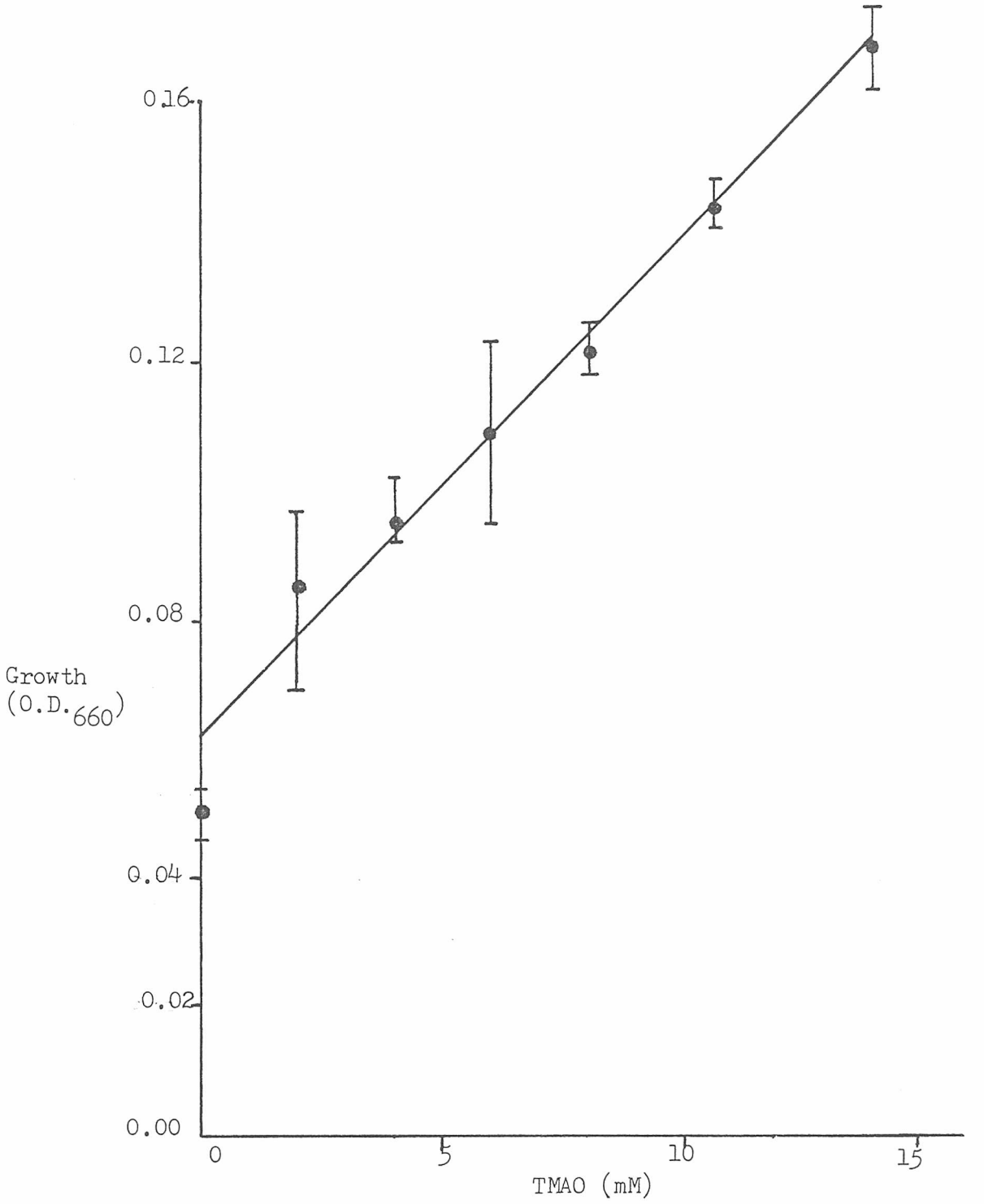


Figure 10 : Micro-aerobic growth of Alteromonas sp., NCMB 400, supported by TMAO.

The plot does not go through the origin since residual oxygen in the medium supported some aerobic growth.

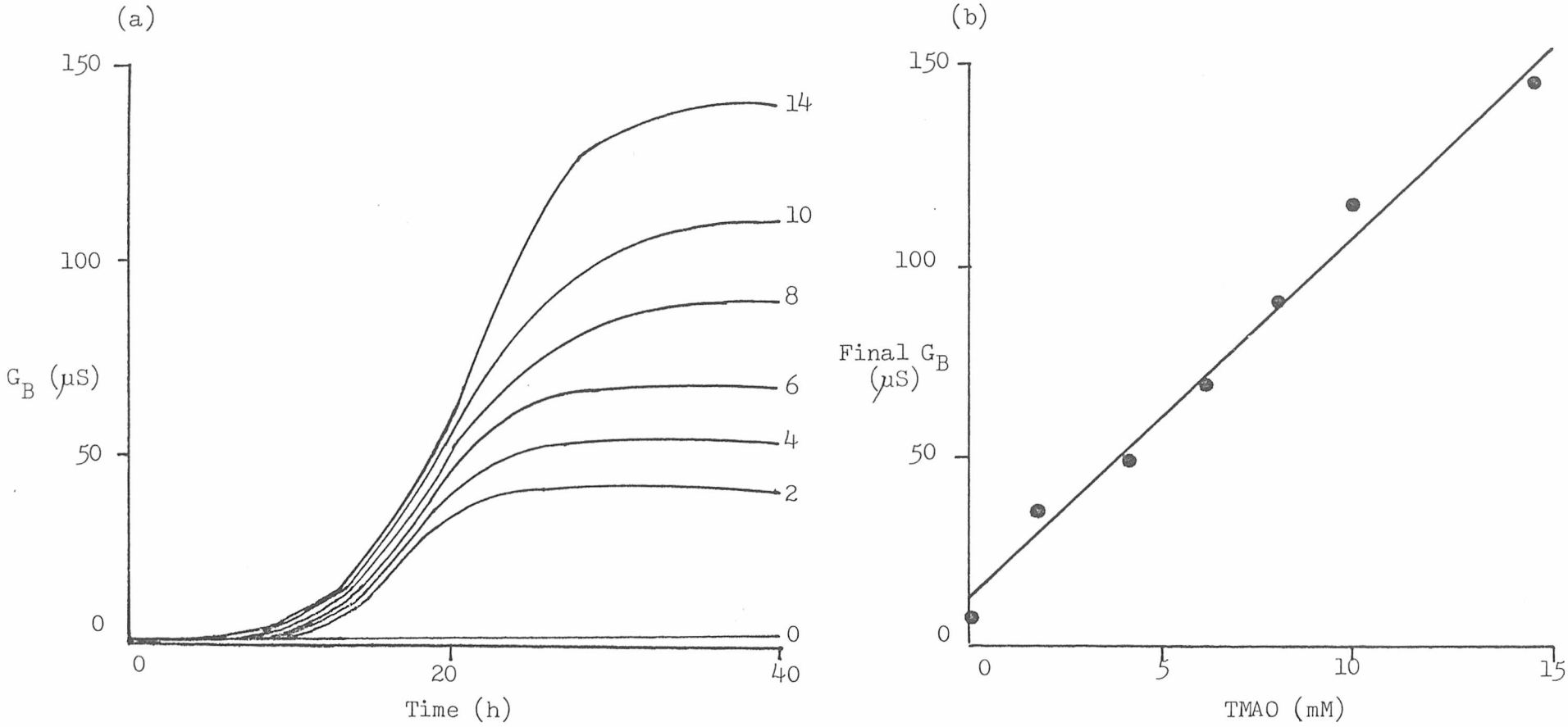


Figure 11 : Conductance change (G_B, μS) during micro-aerobic growth of Alteromonas sp., NCMB 400, on TMAO.

(a) Plot of G_B against time with 0, 2, 4, 6, 8, 10, and 14 mM TMAO.

(b) Plot of the final G_B against the initial TMAO (mM)

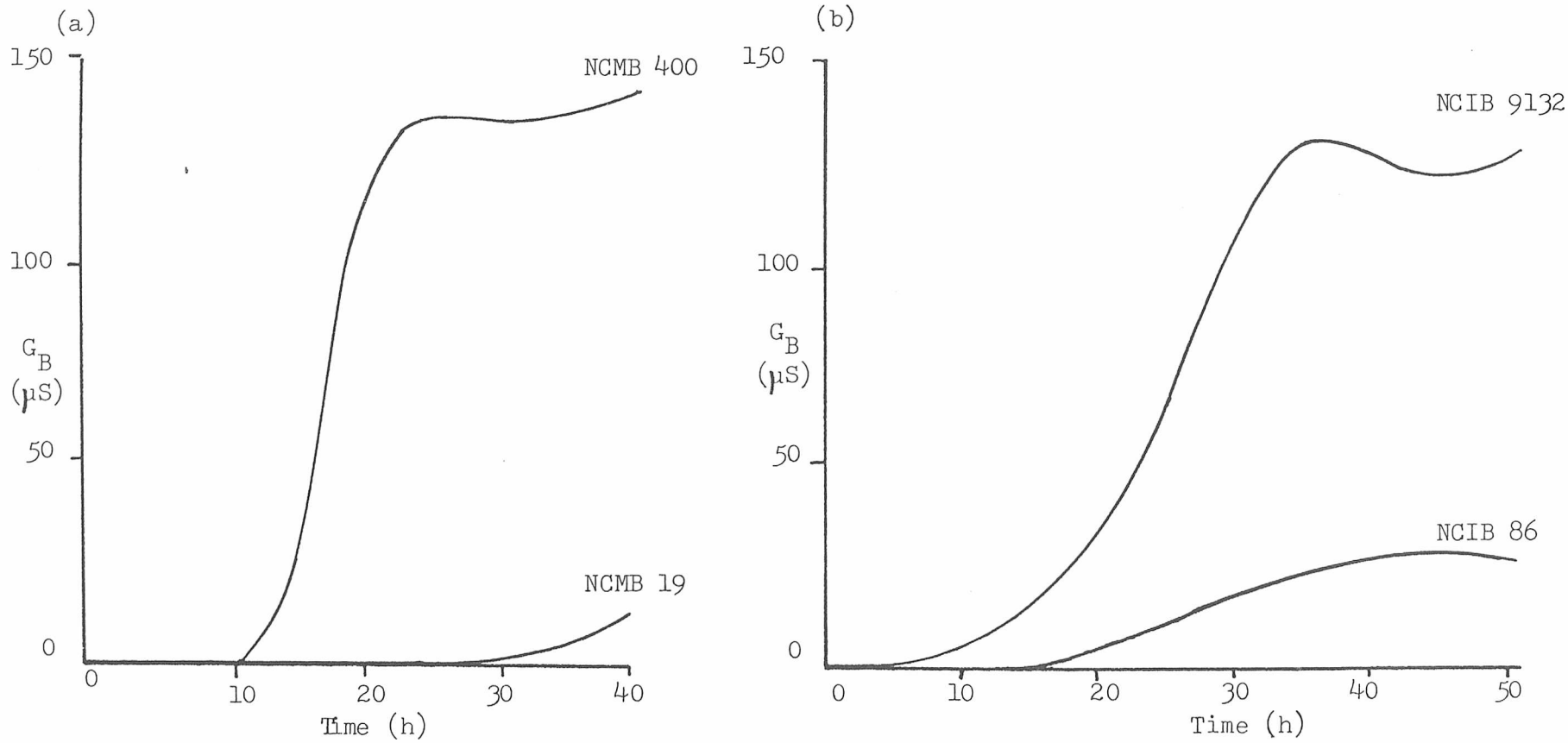


Figure 12 : Detection of TMAO reducing bacteria by conductance measurements

(a) Alteromonas spp., (b) E. coli strains.

TABLE 7 : The distribution of TMAO reduction in Gram negative bacteria.

Species	No. of strains tested	% reducing TMAO	Mean generation time (h)
Pseudomonas	6	0	1.09
Alteromonas	22	50	1.46
V. parahaemolyticus	16	94	0.24
V. alginolyticus	17	88	0.29
E. coli	7	57	0.33
Moraxella and Moraxella-like bacteria	14	0	N.D.
Acinetobacter	14	0	N.D.
K. aerogenes	1	negative	0.34
Citrobacter	1	positive	0.35

N.D. = not determined.

obtained using the spot test for TMA, and the combination of these two methods clearly distinguished TMAO reducing strains. The ability to reduce TMAO was found to be a widespread property of both marine and non-marine isolates. Only 50% of the alteromonads tested could reduce TMAO. All the marine isolates of A. putrefaciens were able to reduce it, but other strains of this organism varied.

3.3 Cell fractionation

The intracellular location of TMAO reductase in Alteromonas sp., NCMB 400, was determined by separating the major subcellular fractions of the bacterial cell i.e. the inner and outer membranes, the cytoplasm and the periplasm. A reproducible method of cell fractionation was therefore required for which it was necessary to include the formation of sphaeroplasts such that clean subcellular fractions were obtained. The preparation of sphaeroplasts caused some considerable difficulties and several methods were tried (section 2.6.1), the procedures of which are outlined in Table 8.

Method A was that of Kaback (1971) which produced sphaeroplasts of E. coli using a lysozyme/EDTA treatment of plasmolysed cells. Sphaeroplasts from aerobically grown cells of Alteromonas sp., NCMB 400, were produced but lysis occurred when they were subjected to cold shock (a drop in temperature from 20° to 0°C) or mild osmotic shock (by resuspension in Tris-salts buffer). Plasmolysed cells of the alteromonad grown to stationary phase under micro-aerobic conditions were found to lyse when lysozyme and EDTA were added. In the absence of EDTA, cells generally retained their rod-like shape although some lysis was evident, but sphaeroplast formation was not observed. If the cells were harvested during the log phase and washed in the Tris-salts buffer instead of Tris-HCl, they were more stable and no lysis was observed in sucrose/lysozyme buffer in

TABLE 8 : Summary of the methods used for sphaeroplast formation

Procedure	Method A	Method B	Method C	Method D
Cell Wash	x 1 in 10mM Tris-HCl	None	x 3 in 0.5M NaCl	x 1 in 10mM Tris-HCl containing 100mM NaCl
Treatments and Conditions	Sucrose, 500mM Tris-HCl, 10mM EDTA, 10mM + lysozyme, 150µg ml^{-1} pH 8.0, 15min, 30°C	(i) Sucrose, 500mM, pH6.5 30 min, 30°C + Sucrose wash (ii) $MgCl_2$, 200mM Tris-HCl, 10mM + lysozyme, 500µg ml^{-1} (iii) Harvest and resuspend in $MgCl_2$, 10mM Tris-HCl, 10mM pH 8.4, 30 min, 25°C	(i) Sucrose, 500mM, pH6.5 or Sucrose, 500mM EDTA, 5mM pH9-10, 30min, 25°C + Sucrose wash (ii) Harvest and resuspend in Tris-salts buffer + lysozyme 150µg ml^{-1} pH 7.5, 30min, 25°C	(i) Sucrose, 500mM Tris-HCl, 10mM + lysozyme, 30µg ml^{-1} pH 8.0, 10min, 25°C (ii) Dilute 1:1 with 10mM Tris-HCl pH 8.0 (iii) Stepwise addition of EDTA up to 1mM pH 8.0, 15 min, 25°C
Result	Lysis and some sphaeroplast formation.	No lysis or sphaeroplast formation	No lysis and slow sphaeroplast formation in the absence of EDTA. Fast sphaeroplast formation with EDTA at high pH only	Some lysis and rapid sphaeroplast formation

the absence of EDTA. Sphaeroplasts were produced from such cells by the slow addition of small amounts of EDTA, and ca. 70-80% of them were formed at 5mM EDTA (Fig. 13). They were also susceptible to cold and osmotic shock. EDTA was apparently necessary for sphaeroplast formation from both aerobically and micro-aerobically grown cells, but with the latter the effective concentration was critical to avoid lysis. Overall, the method was not reproducible since it was difficult to prevent lysis, especially with micro-aerobically grown cells, therefore it was not used further in the cell fractionation procedure.

Method B was essentially that of Cheng et al. (1971) who used it to produce sphaeroplasts of P. aeruginosa using a lysozyme/osmotic shock treatment in the absence of EDTA. It failed to yield sphaeroplasts from Alteromonas sp., NCMB 400, even after the outer membrane had been previously weakened by washing in sucrose (Costerton et al., 1967).

Costerton et al. (1967) described how to prepare sphaeroplasts from A. haloplanktis, NCMB 19, by washing with NaCl and treating with sucrose and then lysozyme in the Tris-salts buffer. With Alteromonas sp., NCMB 400, sphaeroplasts were formed slowly i.e. ca. 30% in 60 min. It was found that the addition of 5mM EDTA to the sucrose treatment resulted in more efficient sphaeroplast formation (i.e. Method C). However, this was dependent on the pH of the sucrose/EDTA treatment as shown in Table 9. Treatment at high pH (> pH 8.0) were required for > 50% sphaeroplast formation whereas at near neutrality fewer sphaeroplasts were produced, but they were less fragile. The results obtained from Methods A, B and C (section 2.6.1) suggested that it was necessary to treat cells with EDTA for sphaeroplast formation, but the contact time, concentration of EDTA and precise conditions employed were critical.

Birdsell & Cota Robles (1967) described a method of sphaeroplast formation from E.coli involving lysozyme treatment of plasmolysed cells and mild osmotic shock. Some sphaeroplasts of Alteromonas sp., NCMB 400,

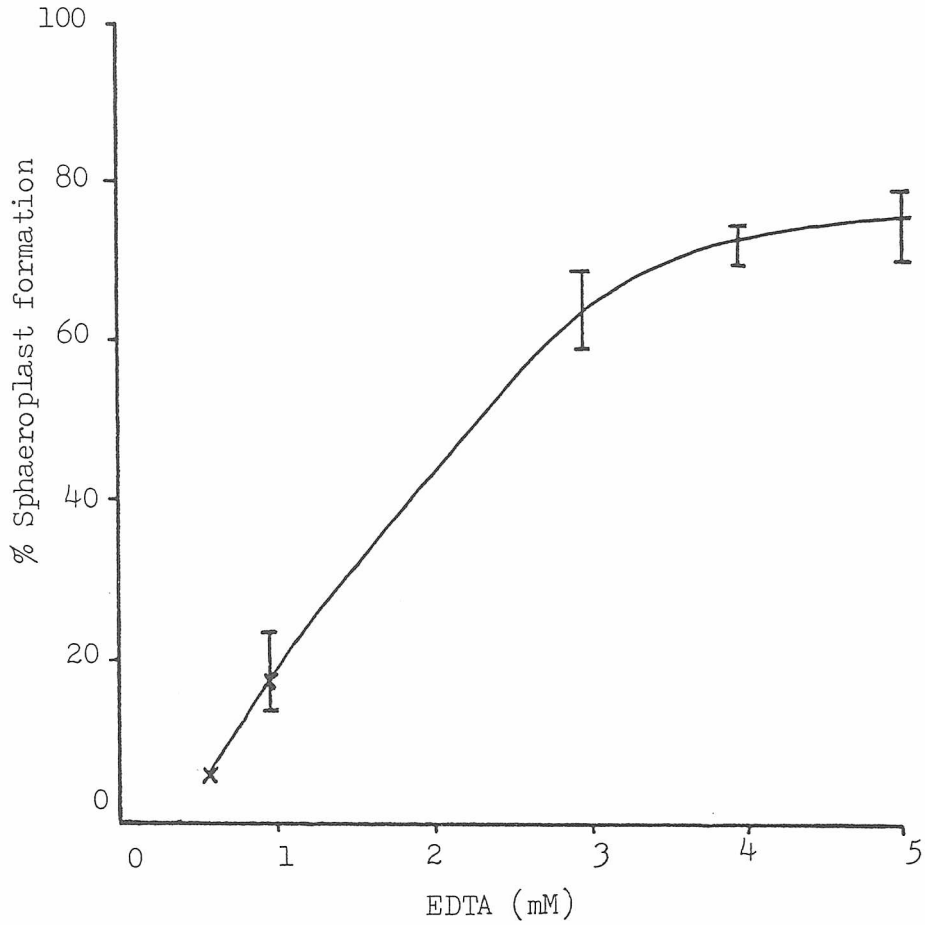


Figure 13 : Effect of EDTA on sphaeroplast formation from Alteromonas sp., NCMB 400, using the modified method of Kaback (1971).

TABLE 9 : The effect of pH on sphaeroplast formation from Alteromonas sp., NCMB 400, using method C.

pH of Sucrose/EDTA treatment	% sphaeroplast formation after 15 min at room temperature
6.5	10 - 20
8.0	40 - 50
8.5	60 - 70
9.0	90 - 95
9.5	100
10.0	100

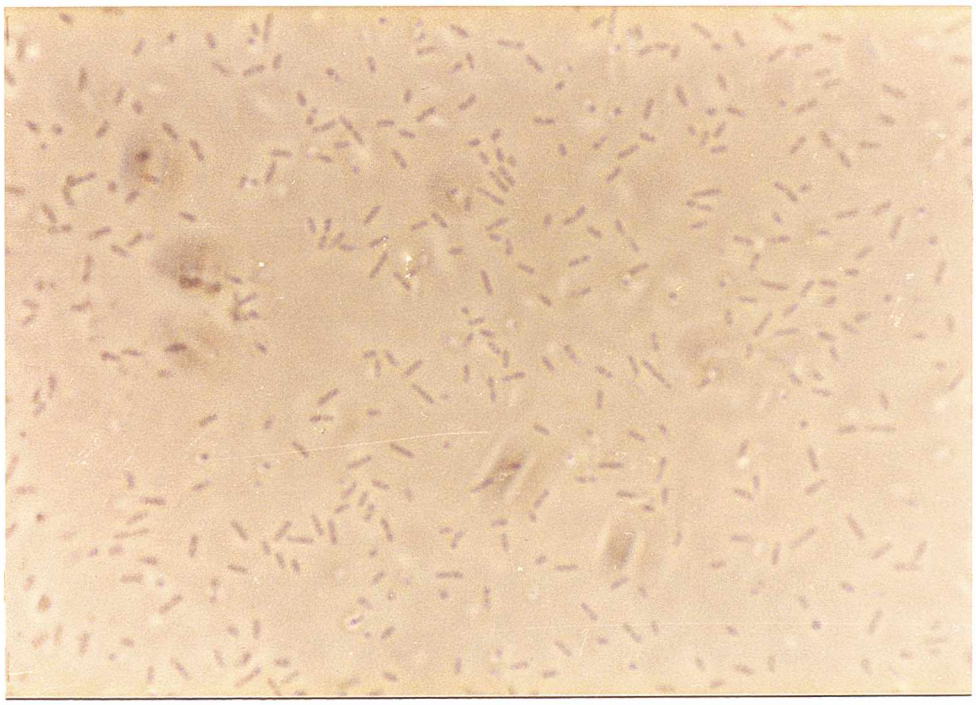
were produced by this method but further treatment with 1mM EDTA was necessary for complete sphaeroplast formation, although EDTA caused some lysis. The cells were washed before sphaeroplasting and the ionic composition of the washing solution was found to have a significant effect on both sphaeroplast formation and lysis. Magnesium ions decreased the lytic effect of EDTA but markedly decreased the amount of sphaeroplasts formed to $\leqslant$ 10%. Sodium chloride was less effective in reducing cell lysis but slightly improved sphaeroplast formation. Consequently, the method was modified (section 2.6.1, Method D) such that whole cells were washed in 10mM Tris-HCl containing 100mM NaCl and after treatment with lysozyme and EDTA, 90-100% sphaeroplasts were formed within 15 min (Plate 1). Accordingly, this method was chosen for the cell fractionation procedure, and was used in experiments to determine the cellular location of TMAO reductase because (i) it was reproducible, (ii) it produced a relatively concentrated periplasm in contrast to that obtained by method C, and (iii) sphaeroplasts could be obtained quicker and at less extremes of pH than those obtained from Method C, such that activity losses were reduced.

3.4 Cellular location of TMAO reductase

Alteromonas sp., NCMB 400, was grown in the presence of TMAO under micro-aerobic condition to induce TMAO reductase. Sphaeroplasts were prepared and the periplasmic fraction was obtained by centrifugation. The sphaeroplasts were disrupted by sonication and the cytoplasm and inner membrane separated by differential centrifugation as described in section 2.6.2. The distribution of TMAO reductase and the marker enzymes was examined in the various cell fractions and the results are shown in Table 10. The marker enzymes were alkaline phosphatase,

PLATE 1 : Sphaeroplast formation from Alteromonas sp., NCMB 400 (a) before and (b) after treatment using method D (section 2.6.1).

(a) Whole cells before treatment (Magnification x 1500)



(b) Sphaeroplasts formed after treatment (Magnification x 1500)

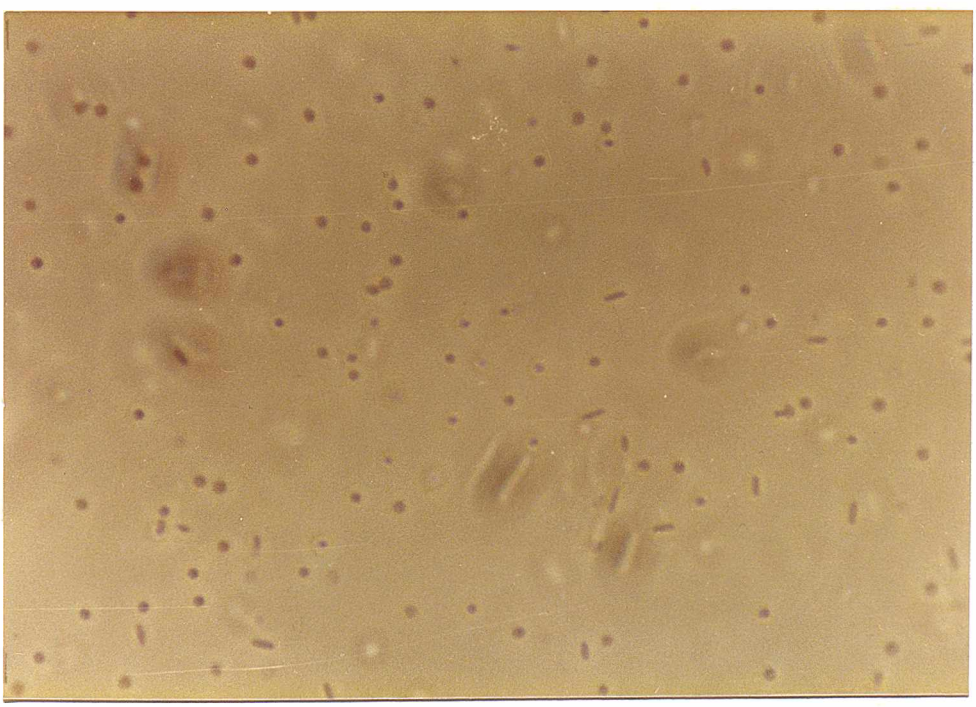


TABLE 10 : The distribution of TMAO reductase, cytochrome c_{552} and marker enzymes in cell fractions of Alteromonas sp., NCMB 400.

Cell fraction	Alkaline phosphatase			TMAO reductase			Isocitrate dehydrogenase			Succinate dehydrogenase			Cytochrome c_{552}		
	Total activity (U)	%	Specific activity (U mg protein^{-1})	Total activity (U)	%	Specific activity (U mg protein^{-1})	Total activity (U)	%	Specific activity (U mg protein^{-1})	Total activity (U)	%	Specific activity (mU mg protein^{-1})	Total (n mol)	%	n mol mg protein^{-1}
Sonicate	8.71	100	173	411	100	3.18	13.50	100	104	4.27	100	33	315	100	2.44
Periplasm	5.91	68	103	295	72	5.15	2.56	19	41	0.00	0	0	253	80	4.41
Sphaeroplast	2.80	32	15	143	35	0.78	9.86	73	54	4.55	106	249	81	25	0.44
Cytoplasmic membrane	0.17	2	18	9	2	0.92	0.24	2	16	3.42	80	352	46	15	4.79
Cytoplasm	1.92	22	51	78	19	2.07	9.28	69	245	0.00	0	0	46	15	1.20

succinate dehydrogenase and isocitrate dehydrogenase which were proteins representative of the periplasm, cytoplasmic membrane and cytoplasm, respectively. TMAO reductase was located predominantly in the supernatant fraction after sphaeroplast formation, and as alkaline phosphatase had a similar distribution, TMAO reductase can be regarded as a periplasmic enzyme. Cytochrome c_{552} was also found predominantly in the periplasm of the alteromonad (Table 10).

The activity of alkaline phosphatase was expressed in terms of its total recovered activity in Table 10, since sucrose and EDTA caused a 60% and 30% loss of enzyme activity, respectively (Table 11). The loss of activity due to EDTA could be partly restored by the addition of Mg^{++}, and accordingly, $MgSO_4.7H_2O$ was added as soon as sphaeroplast formation was complete (section 2.6.1, Method D). Thompson & MacLeod (1974a) found a similar effect with A.haloplanktis. When the results of the localization experiment were corrected for the inhibition of alkaline phosphatase by sucrose and EDTA (as shown in Table 11), it was found that osmotic shock alone i.e. the periplasm obtained prior to the addition of EDTA, was sufficient to release 99-100% of the alkaline phosphatase activity.

In a similar experiment, the distribution of TMAO reductase in the periplasm and sphaeroplast fractions of the alteromonad was determined before and after EDTA treatment, and the results are shown in Table 12. The recovery of TMAO reductase was not affected by sucrose or EDTA. Osmotic shock released 28% of the activity, and treatment with EDTA was required to facilitate the further release of the enzyme and for complete sphaeroplast formation. The release of periplasmic cytochrome c_{552} was similar to that of TMAO reductase (Table 12). When the sphaeroplasts were disrupted to yield the soluble cytoplasm and particulate membrane fractions, the remaining TMAO reductase was solubilized and found in the cytoplasmic fraction. This would suggest that TMAO reductase was held more firmly within the periplasm than alkaline phosphatase.

TABLE 11 : The effect of sucrose and EDTA on the activity of alkaline phosphatase.

Cell fraction	* Mg^{2+}	Alkaline phosphate activity (U)	%	corrected† activity (U)	specific activity (U mg protein^{-1})
Whole cells	+	8.28	N.D.	N.D.	2.36
Sonicate	+	10.62	100	10.62	3.71
Periplasm 1- released by mild osmotic shock	-	4.32	41	10.54	0.54
	+	4.36	41	10.62	0.55
Periplasm 2- released during sphaeroplast formation	-	0.61	6	10.20	0.05
	+	1.48	14	12.32	0.12

* - present (+), absent (-)

N.D.- Not determined.

† - Activity corrected for the inactivation by sucrose and EDTA.

TABLE 12 : The release of TMAO reductase and cytochrome c_{552} during sphaeroplast formation.

Cell Fraction	TMAO reductase					Cytochrome c_{552}		
	Activity (U)		%	Specific Activity (U mg protein^{-1})		nmol	%	nmol mg protein^{-1}
	$-Mg^{++}$	$+Mg^{++}$		$-Mg^{++}$	$+Mg^{++}$			
Whole cells	N.D.	293	93	N.D.	2.40	N.D.	N.D.	N.D.
Sonicate	N.D.	316	100	N.D.	3.08	195	100	1.90
Periplasm 1	51	39	28	6.90	5.49	52	27	3.63
Periplasm 2	113	116	73	8.98	9.67	102	52	4.25

N.D. = not determined.
Periplasm 1 = Periplasm released by mild osmotic shock.
Periplasm 2 = Periplasm released during sphaeroplast formation with EDTA

3.5 Induction of TMAO reductase

The induction of TMAO reductase in whole cells was examined in the presence and absence of TMAO, and under various conditions of aerobiosis. The results are shown in Table 13. The enzyme was induced by TMAO, and under conditions of low oxygen tensions, thus it is repressed by oxygen, and derepressed by anaerobiosis. This induction pattern and the significance of TMAO in respiration was demonstrated during micro-aerobic growth. A non-induced aerobic culture was used to inoculate a fermenter (1 l capacity) containing 1 l of Wood & Baird medium without TMAO. The suspension was incubated at 20°C without agitation, and the following parameters were monitored : growth ($O.D._{660}$), percentage oxygen ($\%O_2$) saturation, TMA production and TMAO reductase activity. The results are shown in Fig. 14. The residual oxygen dissolved in the medium supported an initial phase of aerobic growth, after which growth ceased and TMAO reductase was induced (or derepressed). The addition of TMAO resulted in the immediate resumption of growth, the production of TMA and the increased synthesis of TMAO reductase. When all the TMAO had been used and reduced to TMA, growth ceased and TMAO reductase activity decreased slightly. These results confirmed that (i) oxygen repressed and anaerobiosis derepressed TMAO reductase; (ii) TMAO induced TMAO reductase and (iii) TMAO reduction supported anaerobic bacterial growth.

In a similar experiment, the induction of TMAO reductase and the production of TMA were determined during aerobic growth. A non-induced aerobic culture grown in the Wood & Baird medium without TMAO, was used to inoculate (1% v/v) 500 ml of the medium containing TMAO in a 2 l Erlenmeyer flask. The culture was incubated aerobically at 20°C for 24 h and the following parameters were measured; growth ($O.D_{660}$), $\%O_2$ saturation, TMA production and TMAO reductase activity in washed whole cells.

TABLE 13 : The induction of TMAO reductase in whole cells of Alteromonas sp., NCMB 400.

Growth Conditions	Specific Activity of TMAO Reductase ($mU\ mg\ protein^{-1}$) in whole cells grown	
	without TMAO	with TMAO
Aerobic	0	250
Micro-Aerobic	163	560
Anaerobic	96	374

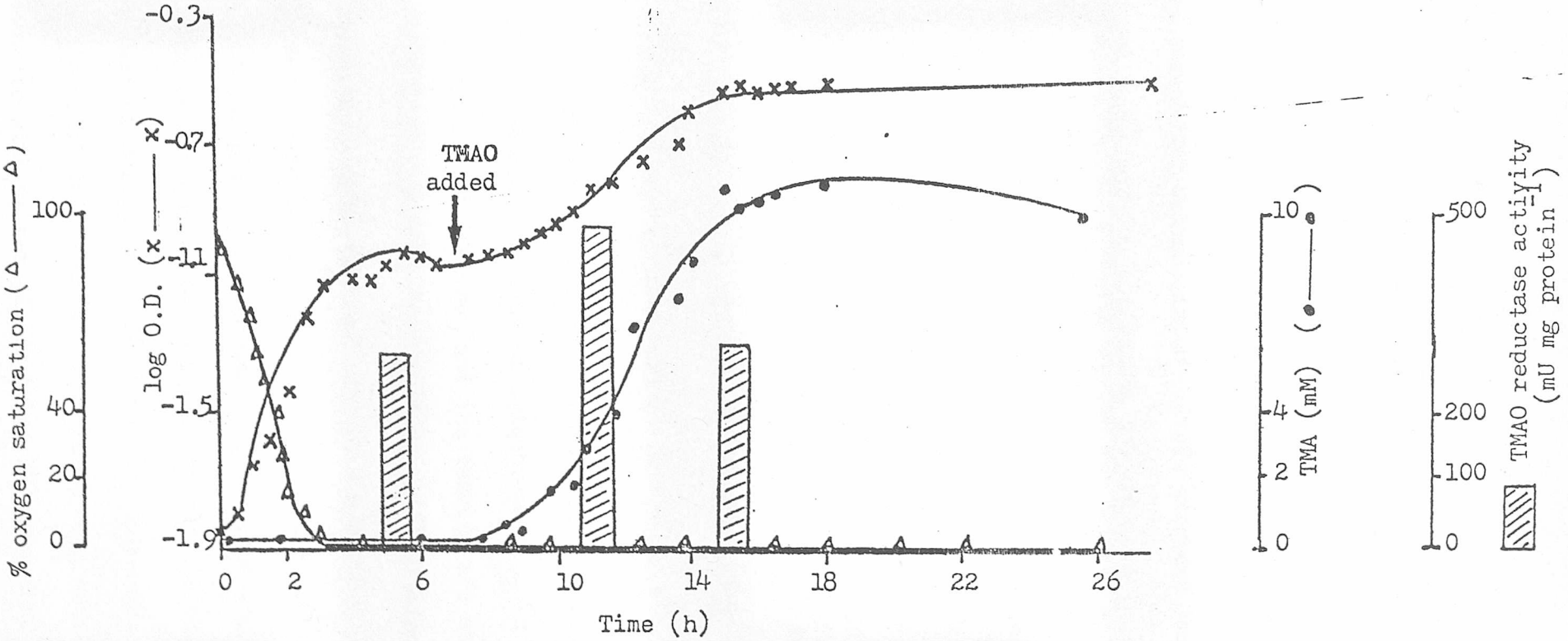


Figure 14 : Induction and activity of TMAO reductase during micro-aerobic growth of Alteromonas sp., NCMB 400.

The results in Fig. 15 show that TMAO reductase was induced within 6 h and TMA production was detected after ca 8 h, when the oxygen tension was ca 60% O_2 saturation. The rate of TMA production and the specific activity of the enzyme were both much lower in cells grown aerobically than micro-aerobically. This would suggest that under these growth conditions, the oxygen demand of the culture exceeded its supply, even though the medium was 50-60% saturated.

The effect of nitrate and fumarate on the induction of TMAO reductase in micro-aerobically grown cells was examined and the results are shown in Fig. 16. Nitrate had little effect on the induction, but fumarate appeared to repress the enzyme. In the presence of nitrate and TMAO, or fumarate and TMAO, the specific activity of TMAO reductase was higher than that induced by TMAO alone.

The periplasmic fraction of Alteromonas sp., NCMB 400, was isolated from whole cells grown under different conditions of aerobiosis and in the presence and absence of TMAO. The periplasm was assayed for TMAO reductase and nitrite reductase activities using the viologen dye assay (section 2.7). The concentration of cytochrome c_{552} in both sphaeroplasts and periplasm was also determined and the results are shown in Table 14. TMAO reductase was induced by TMAO and repressed by oxygen confirming the results obtained from whole cells (see Table 13). Cytochrome c_{552} from the sphaeroplasts had a similar induction pattern to that of TMAO reductase, but periplasmic cytochrome c_{552} was repressed by TMAO under aerobic growth conditions. The induction of nitrite reductase was similar to that of periplasmic cytochrome c. **Reduced minus oxidised** spectra were carried to quantitate cytochrome c_{552} and it was also apparent from the results with sphaeroplasts (Fig. 17) that TMAO induced membrane bound cytochrome c_{552}. In sphaeroplasts of non-induced cells, the difference spectrum had a main peak at 552 nm i.e. a c-type cytochrome,

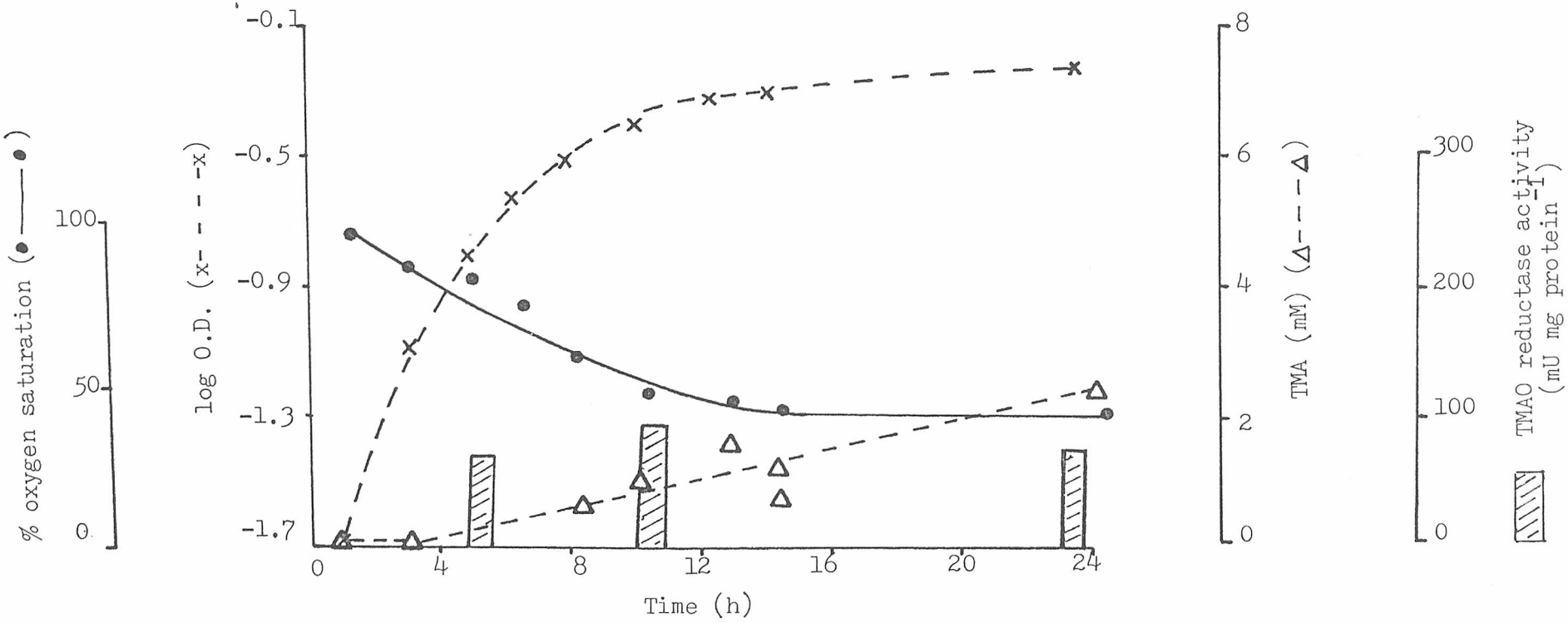


Figure 15 : Induction and activity of TMAO reductase during aerobic growth of Alteromonas sp., NCMB 400, in the presence of TMAO.

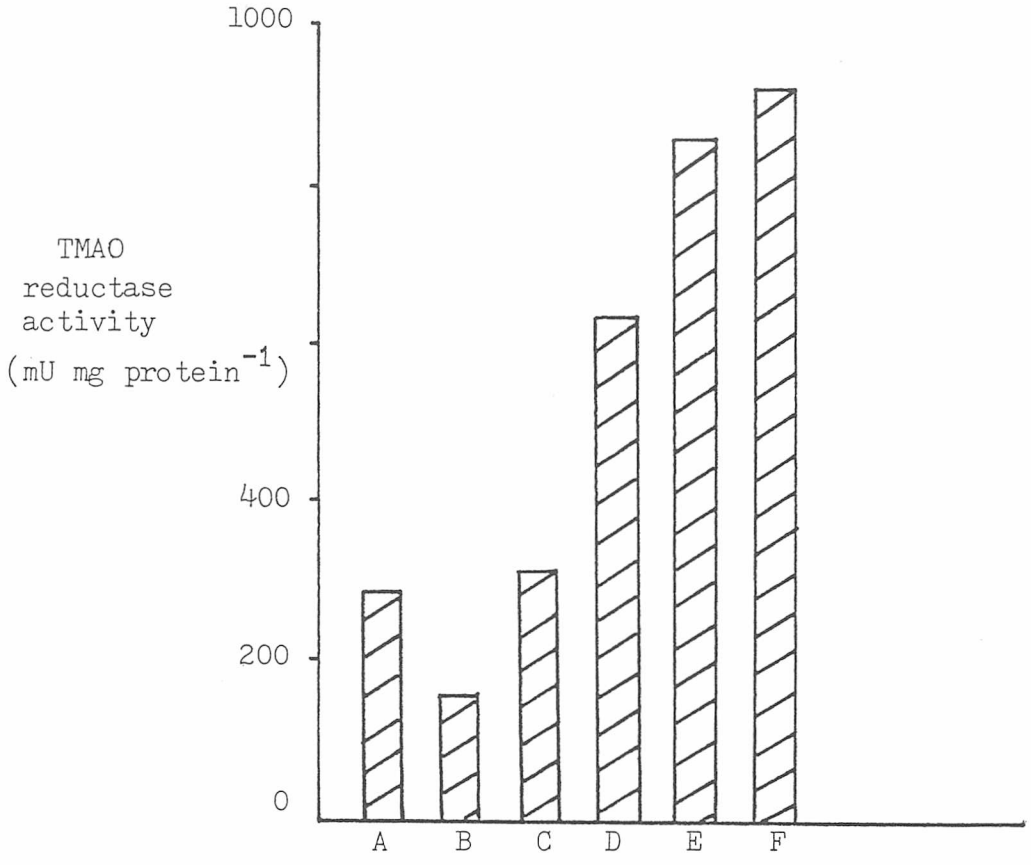


Figure 16 : Micro-aerobic induction of TMAO reductase by nitrate fumarate and TMAO in whole cells of Alteromonas sp., NCMB 400.

A, no electron acceptor
B, sodium fumarate (10mM)
C, KNO_3 (5mM)
D, TMAO (10mM)
E, TMAO + KNO_3
F, TMAO + sodium fumarate.

TABLE 14 : The induction of TMAO reductase, cytochrome c_{552} and nitrite reductase in Alteromonas sp., NCMB 400.

Growth Condition	TMAO *	TMAO reductase activity ($U\ mg\ protein^{-1}$)	Cytochrome c_{552} ($nmol\ mg\ protein^{-1}$) in Sphaeroplast	Cytochrome c_{552} ($nmol\ mg\ protein^{-1}$) in Periplasm	Nitrite reductase activity ($U\ mg\ protein^{-1}$)
Aerobic	-	0.00	0.21	0.94	0.94
	+	0.47	0.33	0.33	0.13
Micro-Aerobic	-	0.39	0.52	3.15	4.46
	+	6.74	1.31	6.40	17.31

* - (+), present.
(-), absent.

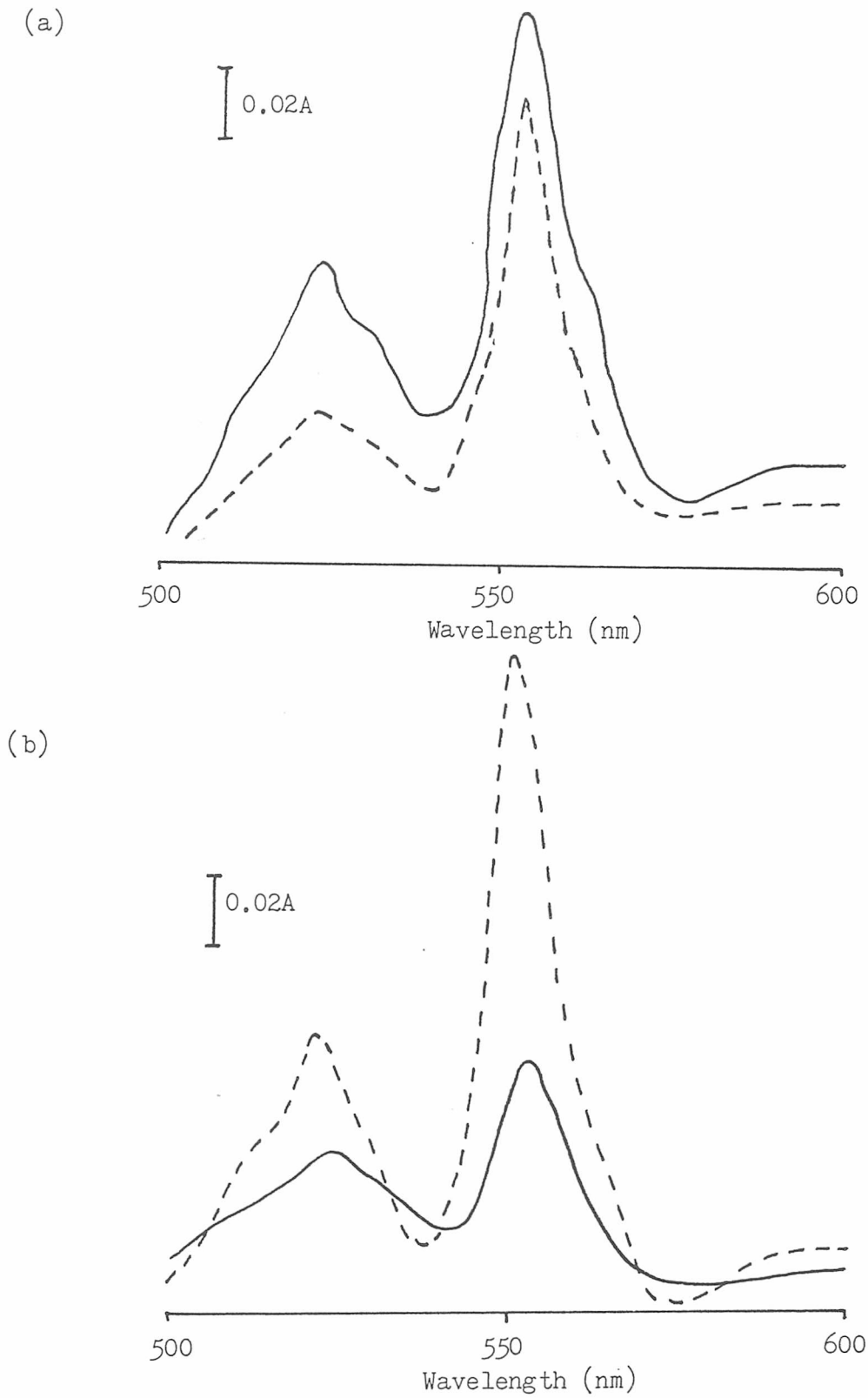


Figure 17 : **Reduced minus oxidised** difference spectra of sphaeroplasts from (a) aerobically grown cells, (b) micro-aerobically grown cells of Alteromonas sp., NCMB 400.

——— without TMAO

- - - - with TMAO

and a shoulder at 560 nm which corresponded to a b-type cytochrome. The shoulder was masked in induced cells due to the increase in the cytochrome c_{552} concentration.

3.6 Properties of TMAO reductase

3.6.1 Whole cell enzyme assays

TMAO reduction by whole cells of Alteromonas sp., NCMB 400, was measured by 2 methods, (a) assays of TMA production, using the picrate assay and the gas sensor, and (b) conductance measurements. The results obtained in both cases were in excellent agreement.

TMA production was absent under aerobic conditions obtained by sparging with air, and was therefore determined under anoxic conditions by sparging with OFN. The ability of various compounds to support TMAO reduction is shown in Table 15. Pyruvate, lactate and formate were the best electron donors for TMAO reduction, whereas glucose and glycerol were poor donors. All the tricarboxylic acid cycle intermediates tested, except oxaloacetate, were poor electron donors.

NADH and NADPH did not support TMAO reduction in whole cells. Crude extracts were prepared by (a) sonication (see section 2.6.2) or (b) lysis after treating whole cells in hypotonic buffer with lysozyme and EDTA. NADH was found to support TMAO reduction in both preparations, but no activity could be detected with NADPH.

The activity of NADH:TMAO oxidoreductase (0.3-0.4 mU mg protein^{-1}) was 14-18% of the NADH oxidase activity (2.2 mU mg protein^{-1}). Unemoto et al. (1965) found similar results for V.parahaemolyticus and also showed that NADH oxidation and TMA production were equimolar. Assuming this to be true of Alteromonas sp., then NADH would be classed as a poor

TABLE 15 : Electron donors supporting TMAO reduction by whole cells of Alteronomas sp., NCMB 400.

Donor	Good	Moderate	Poor
Efficiency* Ratio	4.3 - 8.1	2.6 - 3.1	< 2.2
Compound	Pyruvate	Succinate	∝-oxaglutarate
	Lactate	Fumarate	Citrate
	Formate	Malate	Isocitrate
	Peptone	Acetate	Glucose
	Alanine		Glycerol
	Oxaloacetate		

* Efficiency ratio = $\frac{\text{TMAO reduction rate supported by the donor}}{\text{TMAO reduction rate in the absence of a donor.}}$

donor. NADH:TMAO oxidoreductase could not be determined under aerobic conditions using cyanide to inhibit NADH oxidase. This was probably because NADH oxidase was found to be resistant to cyanide e.g. 1 and 5mM KCN caused ca 60% and 95% inhibition respectively. TMAO reduction was also inhibited by cyanide e.g. 5mM KCN caused 68% inhibition (see Table 17 and section 3.6.2).

The pH optimum of TMAO reductase was determined by assaying with several buffers ranging from pH 5.0 - 9.5. The results are shown in Fig. 18. The pH optimum for TMAO reductase was pH 6.8. Tris-HCl was not used at alkaline pH because it was found to be a weak inhibitor of TMAO reductase (Table 16).

The rate of TMA production was determined at different TMAO concentrations in the range 0.1-1.0mM, and the results are shown as a double reciprocal plot in Fig. 19. TMAO reductase was found to have a high affinity for its substrate TMAO; the Km_{TMAO} being 93 ± 16 μM. The fastest reaction rate (Vmax) occurred at ca. 1mM TMAO, and at higher concentrations there was no marked change in enzyme activity. At Vmax the presence of TMA (1-10mM) had no effect on the rate of TMAO reduction; therefore the enzyme was not subject to end product inhibition.

The effect of several compounds on TMAO reduction measured at its Vmax was determined and the results are shown in Table 17. Respiratory inhibitors such as cyanide, azide and HOQNO, decreased the rate of TMAO reduction. Several N-oxides including nitrate, nitrite and HOQNO, also inhibited TMAO reduction, but adenosine-N-oxide markedly increased the reaction rate.

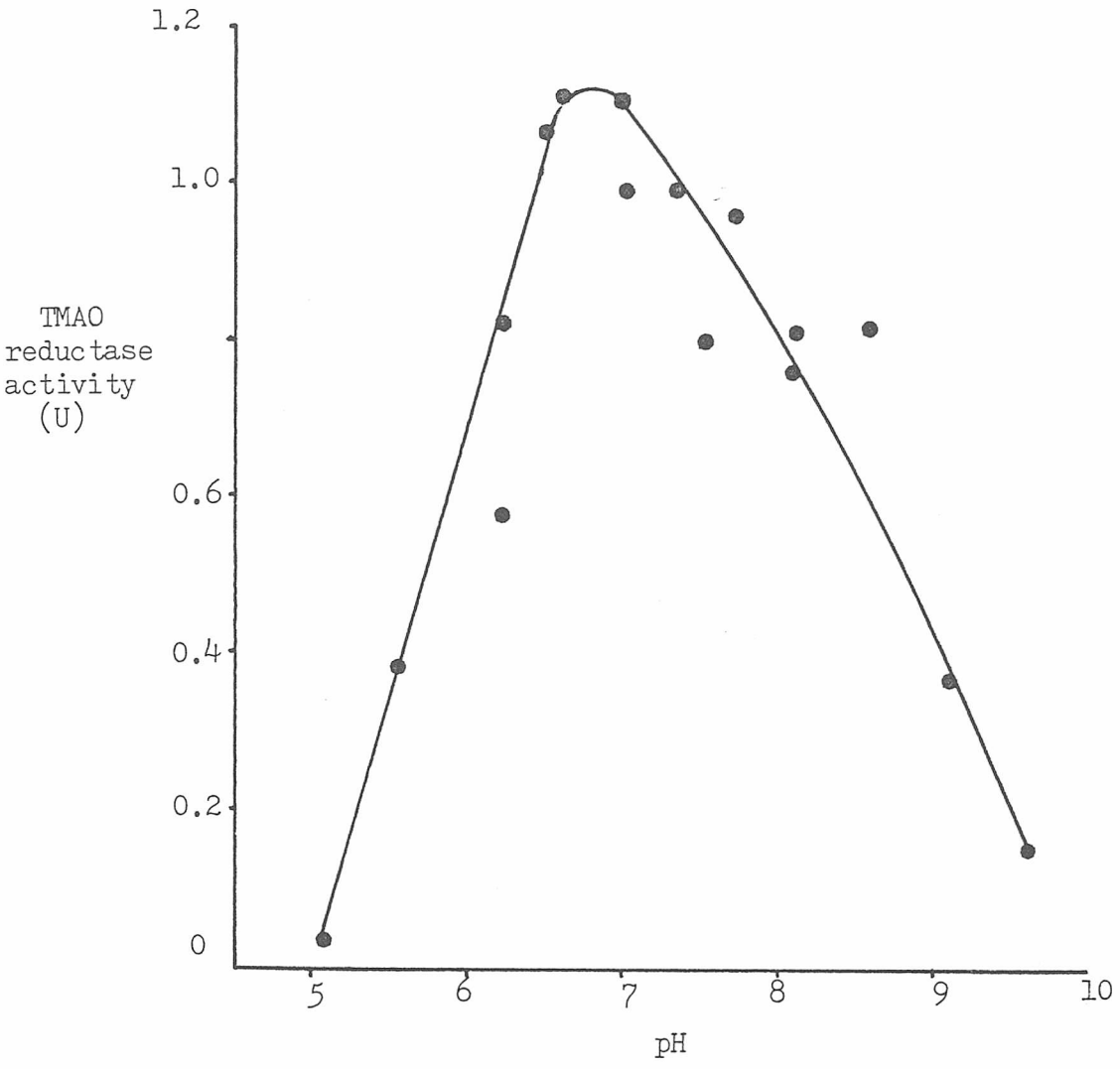


Figure 18 : Effect of pH on TMAO reductase activity in whole cells of Alteromonas sp., NCMB 400.

TABLE 16 : The inhibition of TMAO reduction by Tris-HCl in whole cells of Alteromonas sp., NCMB 400.

Tris-HCl (mM)	TMAO reductase activity (U)	Inhibition (%)
0	0.922	-
5	0.752	18
10	0.758	18
25	0.618	33
50	0.498	46
100	0.207	78

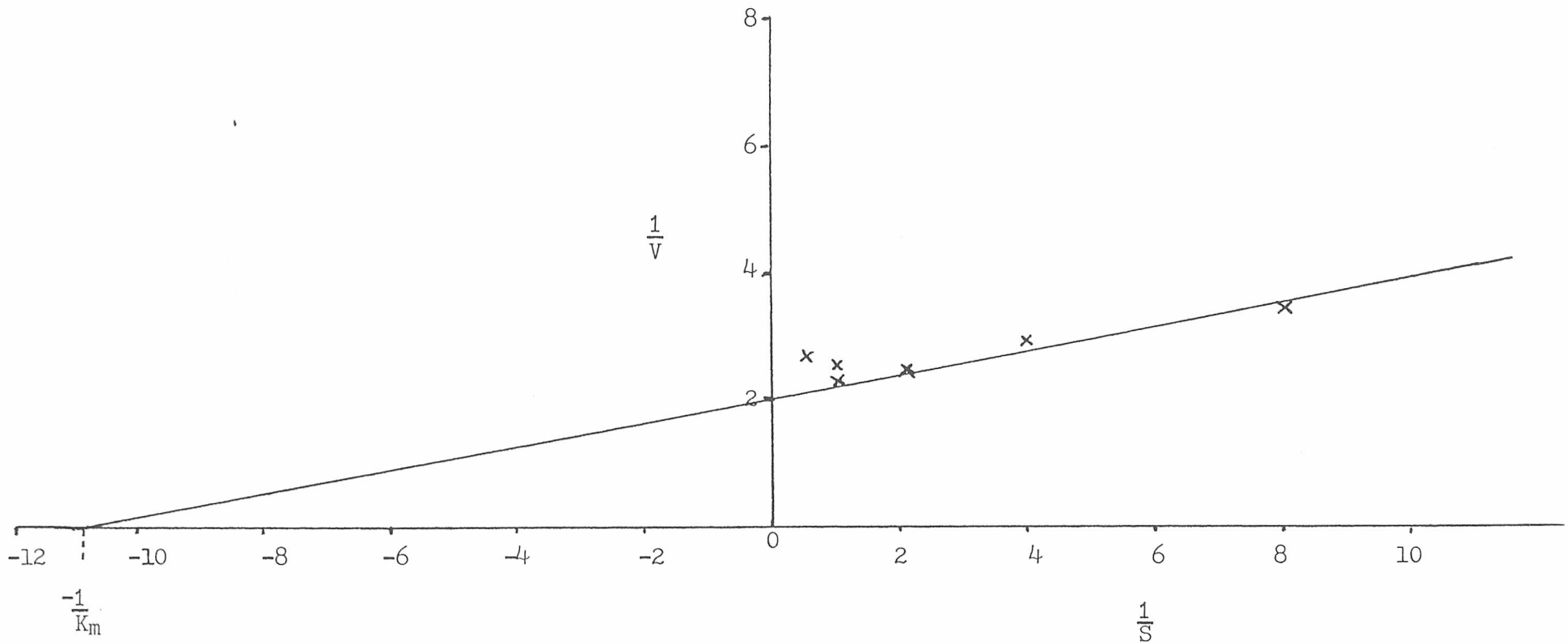


Figure 19 : Michaelis-Menten double reciprocal plot for TMAO reduction in whole cells of Alteromonas sp., NCMB 400.

TABLE 17 : Inhibitors of TMAO reductase in whole cells of Alteromonas sp., NCMB 400.

Compound	Concentration (mM)	Inhibition of TMAO Reduction (%)
KCN	1	15
	5	68
NaN_3	1	45
	5	61
KNO_3	5	52
$NaNO_2$	5	69
nitromethane	10	9
nitroethane	10	33
	50	45
2-nitropropane	10	27
	50	45
Pyridine-N-oxide	10	46
N-nitropyridine-N-oxide	10	58
Adenosine-N-oxide	10	+ 62*
Tris(hydroxymethyl)nitromethane	10	27
Tris(hydroxynethyl)amino glycine	10	33
Tris(hydroxymethyl)amino methane	10	18
2-N-heptyl-4-hydroxyquinoline-N-oxide	0.002	11
	0.020	27
	0.200	88

* Adenosine-N-oxide stimulated enzyme activity

3.6.2 Periplasmic TMAO reductase

The viologen dye assay was used to determine TMAO reductase activity in the periplasmic fraction. The rate of decolourization of the $MV^{\cdot +}$ radicle was not always linear, a typical result is shown in Fig. 20. The decolourization trace showed an initial activation phase, followed by a linear portion and a final deceleration phase. This was probably a function of the viologen dye. TMAO reductase activity was determined over the linear portion and was shown to be a valid measure of enzyme activity since reaction rate was proportional to both the enzyme concentration (Fig. 21) and substrate concentration (as shown for the determination of K_m, see later).

Using several buffers containing 0.3mM MV^{++}, the effect of pH on TMAO reductase was determined in the range pH 5.5-9.5, and the results are shown in Fig. 22. The pH optimum of the enzyme was pH 6.9, similar to that obtained in whole cells.

Some N-oxides caused chemical oxidation of $MV^{\cdot +}$ and therefore the effect of these compounds on TMAO reduction could not be determined. The N-oxides concerned were nitromethane, nitroethane, 2-nitropropane, N-nitropyridine-N-oxide and Tris-(hydroxymethyl)- nitromethane. Cyanide, azide, nitrate and Tris-HCl inhibited TMAO reduction (Table 18), but the extent of inhibition was less marked than in whole cells. Tetramethyl-ammonium chloride was also inhibitory to TMAO reduction.

The viologen dye assay was also used to determine the activity of nitrite reductase. Hence it was not possible to determine the effect of nitrite on TMAO reduction using this assay system, because in the presence of TMAO and nitrite, both activities were present and could not be separated.

Losses in the activity of TMAO reductase were apparent during storage and purification, and the factors affecting the stability of periplasmic

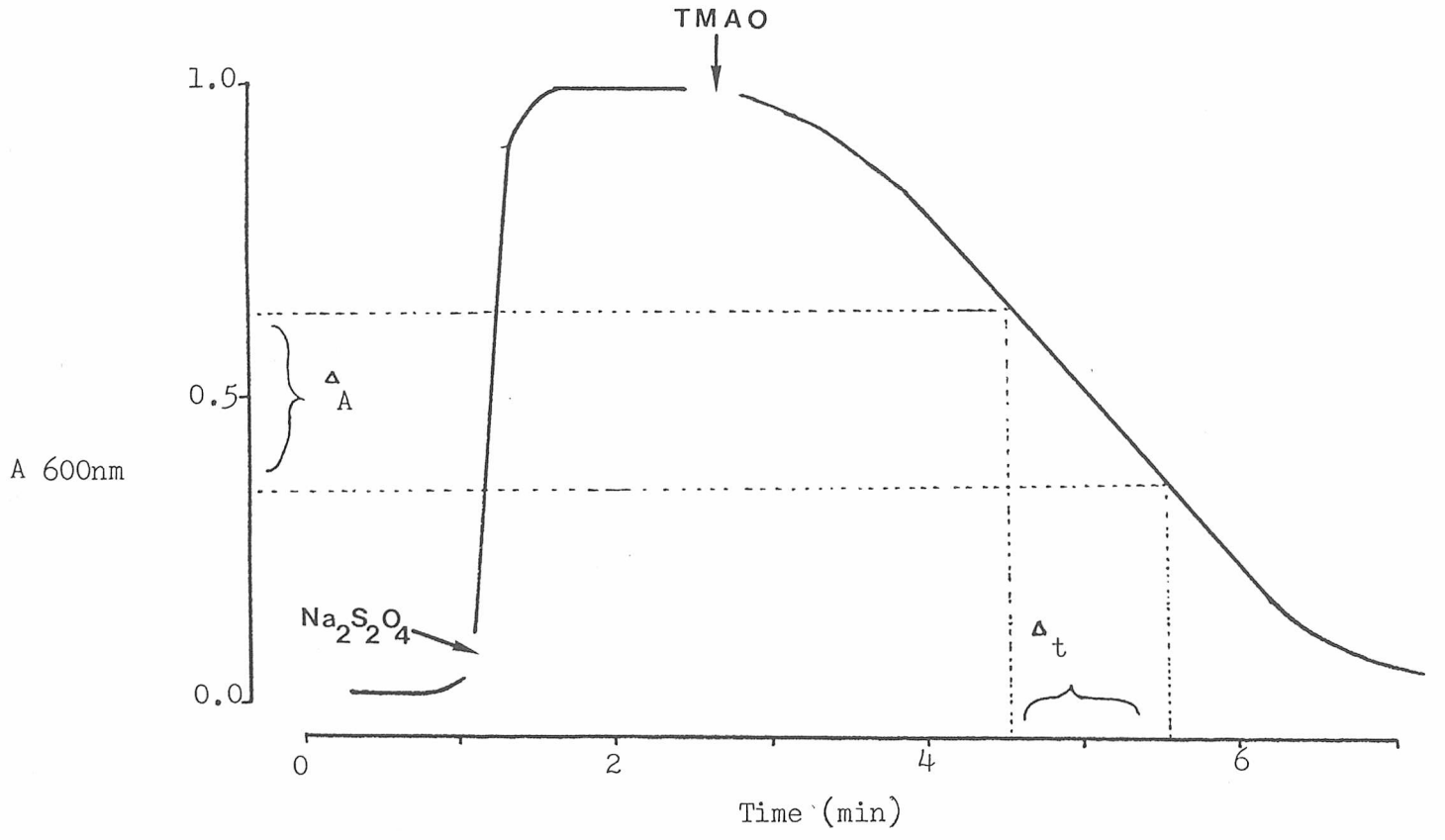


Figure 20 : Determination of TMAO reductase activity using the viologen dye assay.

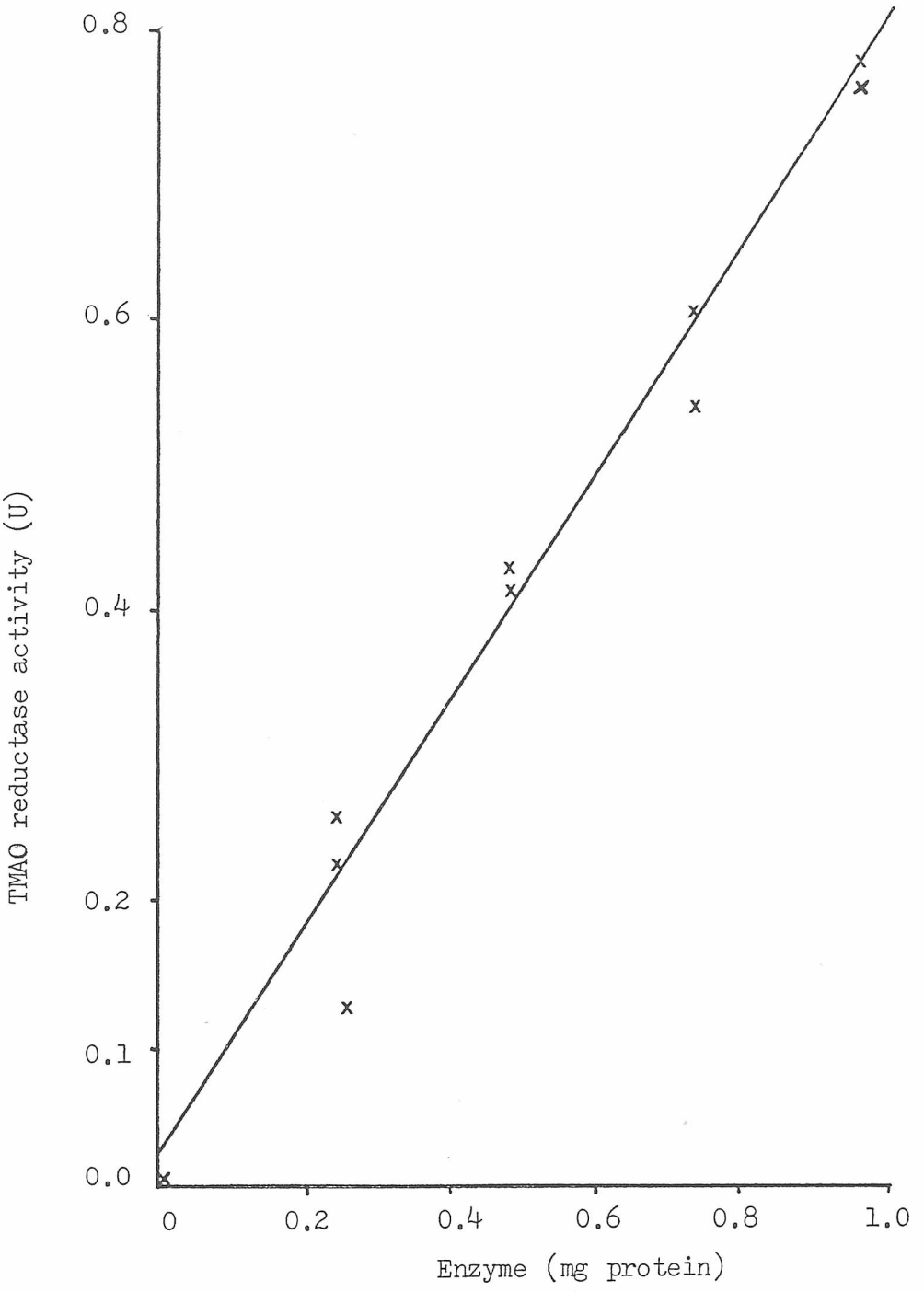


Figure 21 : Effect of enzyme concentration on the viologen dye assay.

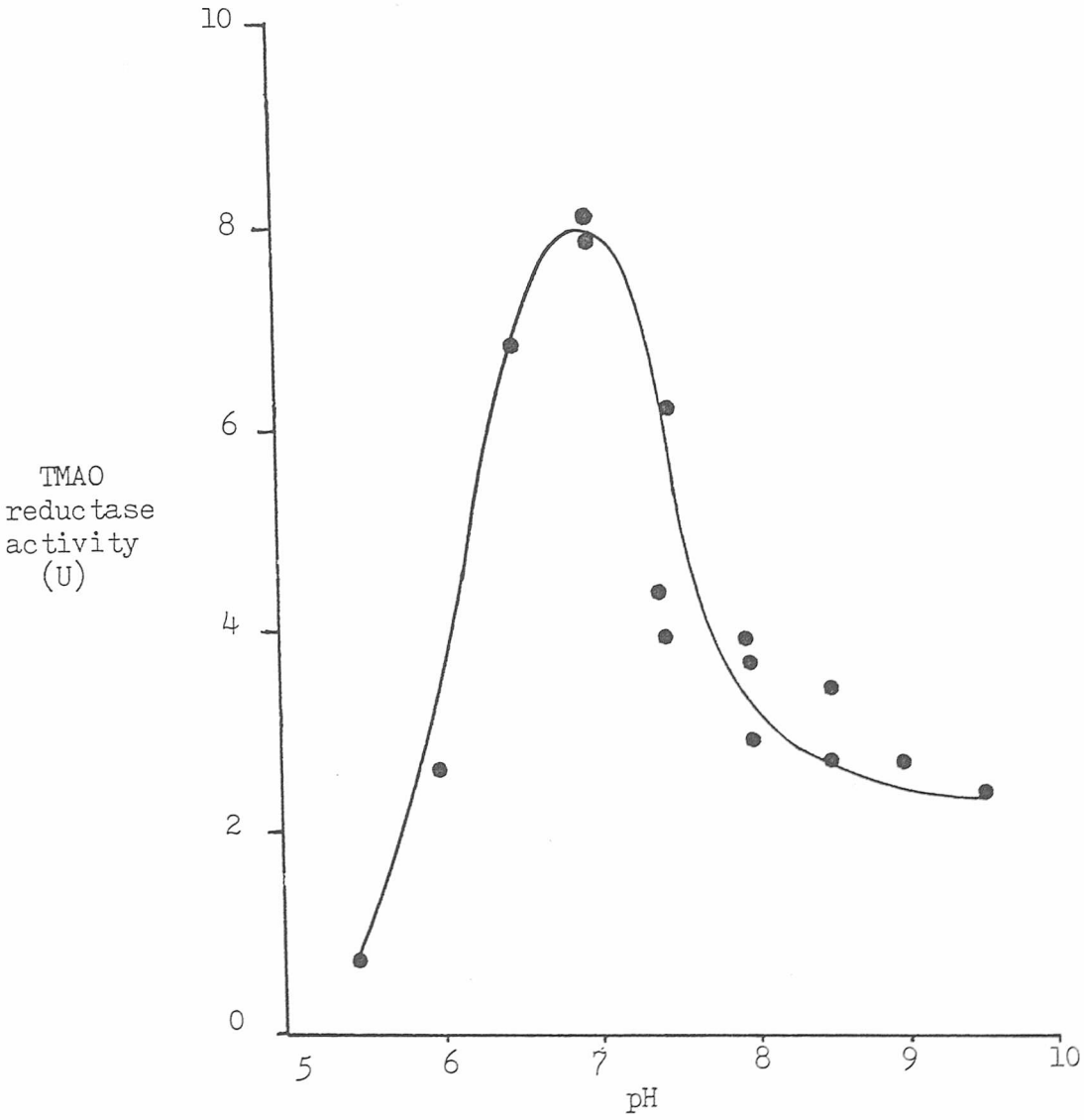


Figure 22 : Effect of pH on the activity of periplasmic TMAO reductase.

TABLE 18 : Inhibitors of periplasmic TMAO reductase

Compound	Concentration (mM)	TMAO Reductase Activity (U)	Inhibition (%)
Control - No Inhibitor	-	3.94	-
KCN	5	3.42	13
	10	1.70	57
NaN_3	5	3.88	2
	10	2.29	42
KNO_3	5	3.62	8
	10	2.04	48
Tetramethyl ammonium chloride	5	3.55	10
	10	1.85	53
Tris-HCl	10	3.15	20
	20	2.76	30
	50	2.36	40

TMAO reductase were investigated. The results are shown in Table 19 and 20. Low temperatures (-18° to 0°C) and neutral pH (ca pH 7.6) were particularly effective in preventing activity loss (Table 19). The rate of activity loss at pH 7.6 and 0-4°C was ca. 2% h^{-1}. Dialysis against 6 l of glass distilled water at 1°C for 18 h, caused an additional 6% loss of enzyme activity. Hence it was unlikely that the enzyme required a low molecular weight cofactor for activity. Sodium chloride did not have a marked effect on the reductase e.g. 1 M NaCl caused a 12% loss of enzyme activity compared with the control, when the periplasm was stored at 0-4°C for 20 h.

Several compounds were added to the periplasm which was then stored at 0-4°C for 20 h. The activity of TMAO reductase was determined before and after storage, and the results are shown in Table 20. Two groups of compounds were particularly effective in preventing the loss of enzyme activity; (i) substances protecting sulphydryl groups e.g. glutathione and dithiothreitol, and (ii) compounds decreasing the hydrophilic nature of the solution e.g. glycerol, ethylene glycol and dimethylsulphoxide. This would suggest that TMAO reductase contains a thiol group and was stabilized by a hydrophobic environment. Ascorbic acid also prevented activity loss; in another experiment the effect of ascorbic acid and glutathione was found to be additive. Compounds with a similar structure to TMAO also decreased the activity loss of TMAO reductase during storage e.g. TMAO, TMA, Tris-HCl and betaine, but they were not as effective as glutathione or ethylene glycol (Table 20).

TABLE 19 : The effect of storage temperature and pH on the activity loss of periplasmic TMAO reductase.

Storage temperature (°C)	TMAO reductase activity (U) after 20h at pH				
	2.6	4.4	6.1	7.6	9.1
-20	0	6.33	5.95	7.02	6.20
0	0	4.07	5.49	6.40	5.49
20	0	4.22	2.11	3.19	3.17
50	0	0	0	0	0

TABLE 20 : The effect of various compounds on the activity of periplasmic TMAO reductase during storage.

Compound added to Periplasm	Concentration (mM)	TMAO reductase activity (U) Before Storage	After† Storage	Activity* Change (%)
None	-	13.53	8.50	- 37
Inositol	10	11.94	9.24	- 23
Glutamate	10	12.18	9.81	- 20
Bovine serum albumin	1mg ml^{-1}	12.94	9.31	- 28
TMA	10	13.65	11.19	- 18
TMAO	10	17.58	15.80	- 10
Tris-HCl	50	13.36	11.5	- 14
Betaine	10	13.58	10.7	- 21
Ascorbic acid	10	10.52	10.52	0
Dithiothreitol	10	10.16	11.9	+ 17
Glutathione	10	9.38	11.8	+ 26
Dimethylsulph-oxide	100	10.52	12.5	+ 19
Glycerol	20% w/v	10.00	11.9	+ 19
Ethylene glycol	20% w/v	9.61	12.3	+ 28

† Storage conditions were 20h at 4°C

* Activity change: - = loss, + = gain.

3.7 Purification of TMAO reductase

The aim of enzyme purification was to obtain a preparation free of other proteins and/or other enzyme activities, such that the properties of TMAO reductase could be determined free from other periplasmic components.

3.7.1 Separation of the periplasmic proteins

The periplasmic fraction was concentrated ca 8 fold using the Amicon Standard Stirred Cell. In various preparations the concentrate contained 90-100% of the TMAO reductase activity, but the specific activity of the enzyme was only slightly increased. Therefore most of the periplasmic proteins had molecular weights $>$ 10000 (10k) daltons. The cytochrome c_{552} was also retained by filtration and no TMAO reductase or cytochrome c_{552} could be detected in the filtrate (Fig. 23).

The first step in the purification of TMAO reductase was the isolation of the periplasmic fraction from whole cells. This increased the specific activity ca 2 fold. Ammonium sulphate was not very effective in precipitating TMAO reductase from the periplasm as shown in Fig. 24, where at 60% saturation ca 50% TMAO reductase activity was precipitated. Very little protein and no TMAO reductase were salted out below 40% $(NH_4)_2SO_4$ saturation, and it was estimated that ca 80% saturation would be required for the total precipitation of TMAO reductase. The yield of the reductase was not increased significantly by $(NH_4)_2SO_4$ precipitation, and so the technique was not used in the purification procedure.

The concentrated periplasm was applied to a chromatography column packed with Sephacryl S-300 superfine, and during elution proteins were separated on the basis of their size in the range 10^4 - 10^6 daltons; large molecules ($>10^6$ daltons) being excluded from the gel pores were

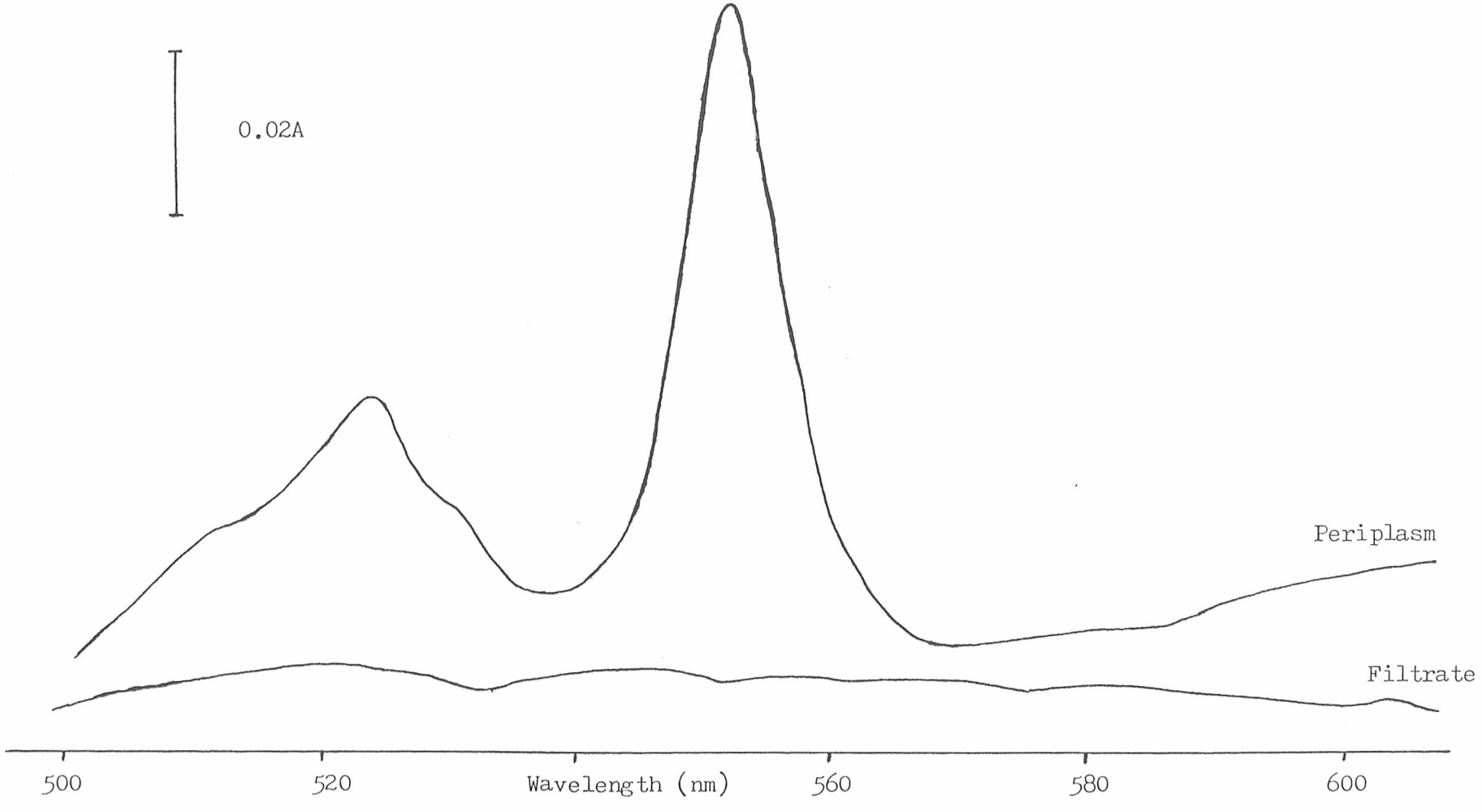


Figure 23 : **Reduced minus oxidised** difference spectra of the periplasm and the filtrate.

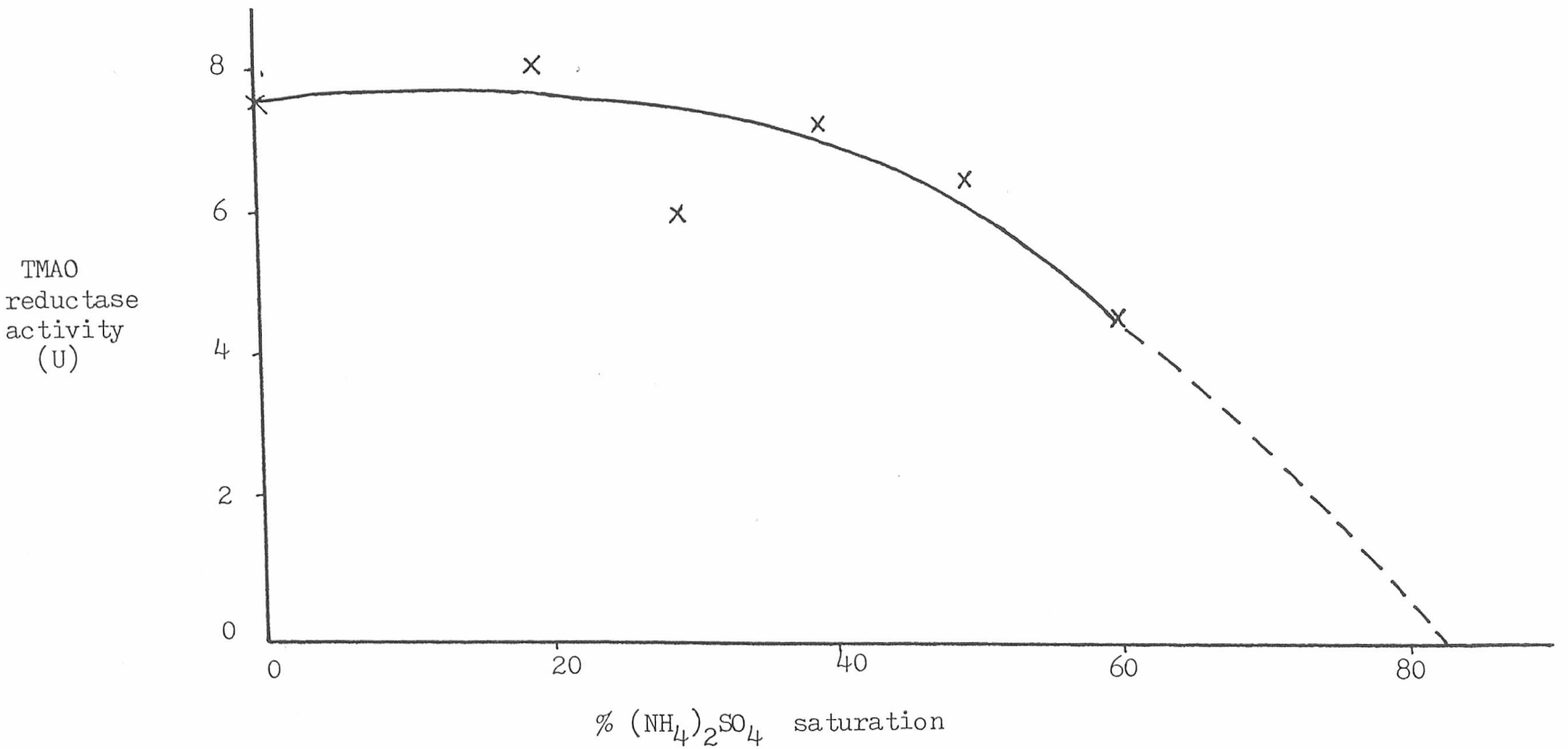


Figure 24 : Ammonium sulphate precipitation of periplasmic TMAO reductase.

eluted from the column quickly. The eluant was assayed for protein and TMAO reductase activity and the results are shown in Fig. 25. The first two protein peaks to be eluted contained TMAO reductase, the second peak contained most activity. Cytochrome c_{552} was also present in these two fractions. The retention time of TMAO reductase in the column was short. This suggests that the enzyme was a large protein, possibly in the range 10^5 - 10^6 daltons. Alternatively, the fast elution of TMAO reductase may have been a consequence of the column being overloaded. However, this was unlikely as gel filtration columns are generally loaded at 2-4% (v/v) of the bed volume, and in some instances, up to 33% (v/v). In the present experiment, the column was loaded to ca 2% (v/v), well within its working capacity. The presence of two peaks of TMAO reductase activity suggested that either there were two proteins with enzyme activity, or that TMAO reductase was part of a large dissociable protein complex. The resolution of the technique was poor, but it could be improved by using a longer column and/or slower flow rates. However, this technique was not pursued further, since a more specific method of purification was devised.

An affinity matrix was produced using AH-sepharose 4B as the support and betaine as the ligand. Carbodiimide was used as the catalyst for coupling the C-terminal of betaine to the 6 carbon spacer arm of the sepharose support. Thus the N-terminal group of betaine, the trimethyl-ammonium group, was the effective ligand of the affinity matrix. The concentrated periplasm was applied to the column in phosphate buffer and TMAO reductase was bound to the matrix presumably through its recognition of a possible substrate, the $(CH_3)_3N$ group. However, some TMAO reductase was detected in the initial elutant and so was not bound to the matrix, this suggests that the purification may be only partly effective or that the column was overloaded. Cytochrome c_{552} was eluted from the column

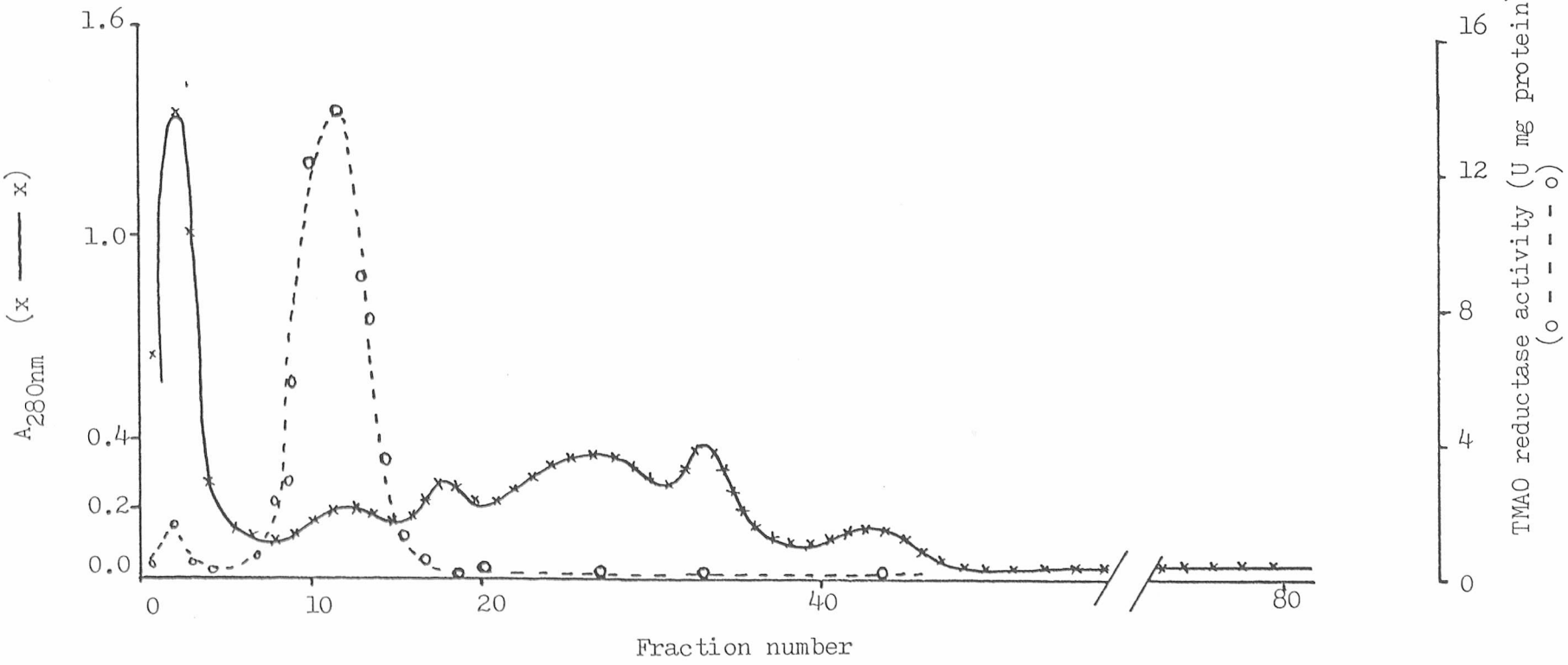


Figure 25 : Fractionation of the periplasm by gel filtration.

during sample application, but the top 1cm of the affinity matrix itself was coloured orange-pink which might indicate the presence of a cytochrome.

TMAO reductase was inhibited in whole cells by Tris-HCl which possibly acted as a competitive inhibitor. Therefore, it was used to elute TMAO reductase from the affinity matrix but the enzyme was not released. In the presence of Tris-HCl and a NaCl gradient 0.2-0.8M, protein was eluted from the column in 3 peaks and the orange-pink colour was dispersed. The elution profile is shown in Fig. 26. The first protein peak contained TMAO reductase, the second contained cytochrome c_{552} but the identity of the third peak could not be established by activity assays. TMAO reductase was purified 18 fold (Table 21).

At room temperature, ca 50% of the TMAO reductase activity loaded on to the column was recovered in the eluted fractions, but at 1°C in the presence of 5mM glutathione and 5mM ascorbic acid, the recovery was increased to 66%. The loss of recovered activity may have been due to the separation of the 3 eluted proteins, which were subsequently recombined to determine whether they affected the activity of the reductase. Aliquot samples (50-200 µl) of the eluted fractions were combined with 50 µl of the fraction containing TMAO reductase, and the activity compared with the TMAO reductase fraction alone. Neither the cytochrome c_{552} nor the unidentified protein fractions, or a combination of the two, had any effect on the activity of TMAO reductase, and could not restore full enzyme activity. Similarly the unbound TMAO reductase was recombined with the immobilized enzyme fraction, and on one occasion, the activity detected was greater than the sum of their individual activities. However, this result was not obtained when the enzyme was purified in the presence of ascorbic acid and glutathione, and further studies are required.

The spectral properties of cytochrome c_{552} eluted with TMAO reductase from the affinity column are shown in Fig. 27a; the γ soret peak was observed at 409 nm and when reduced with dithionite, it shifted to 418 nm

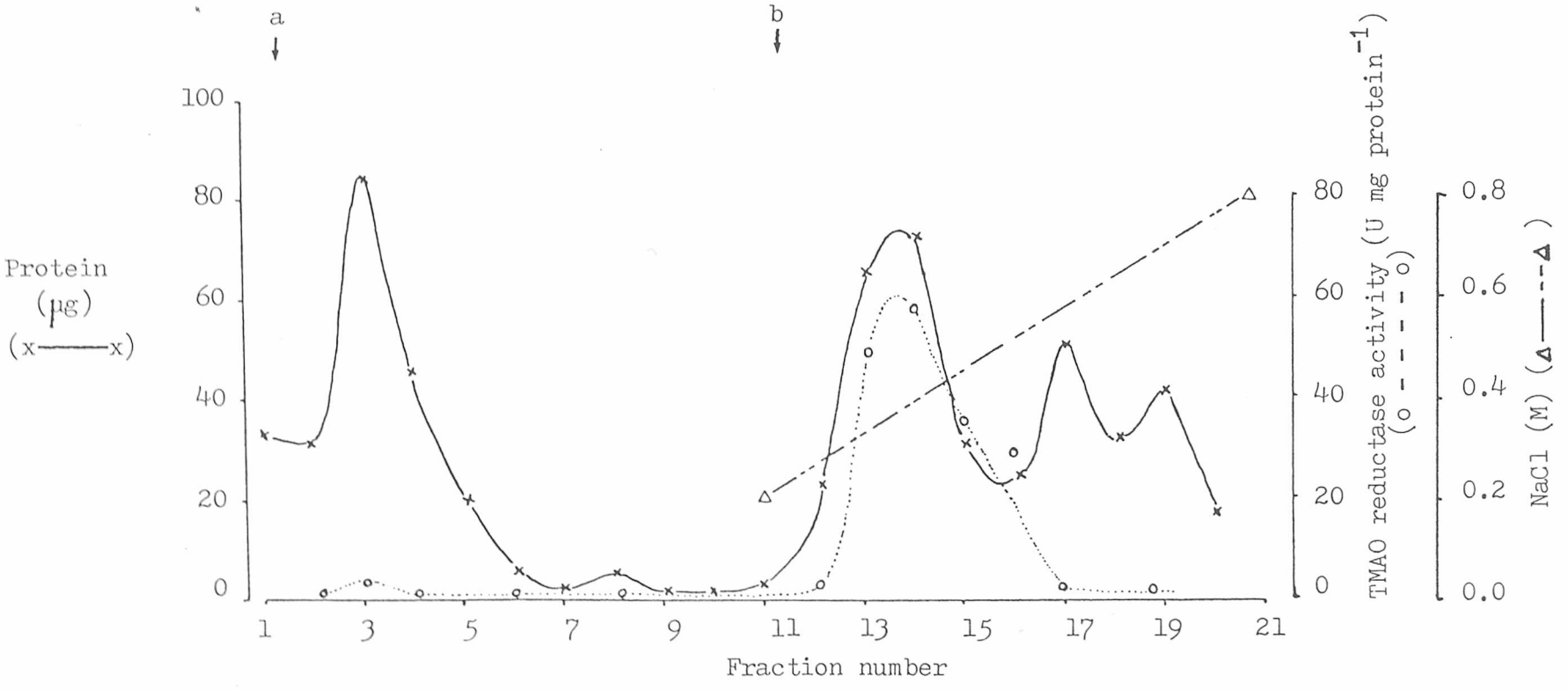


Figure 26 : Affinity chromatography of periplasmic TMAO reductase.

(a) periplasm applied in 50mM phosphate buffer, pH 6.5

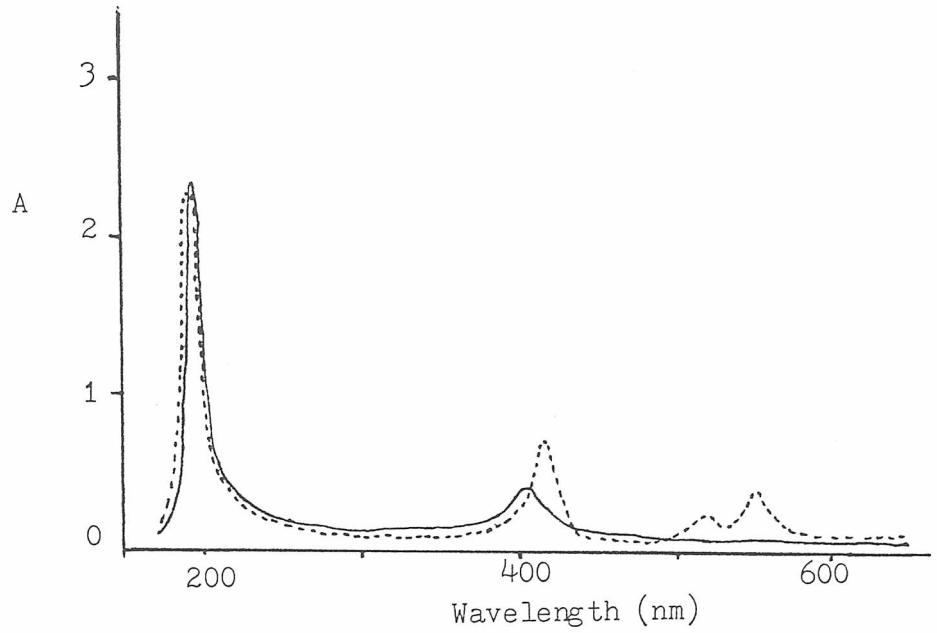
(b) elution with 50mM Tris-HCl, pH 7.5 and 0.2 - 0.8M NaCl gradient.

Both buffers contained 5mM ascorbic acid and 5mM glutathione and the purification was performed at 1°C.

TABLE 21 : The purification of TMAO reductase by affinity chromatography.

Source of TMAO Reductase	Total Activity (U)	Yield (%)	Specific Activity ($U\ mg\ protein^{-1}$)	Purification
Whole Cells	411	100	3.18	1.0
Periplasm	295	72	5.15	1.6
Affinity Chromatography	195	47	57.00	18.0

(a)



(b)

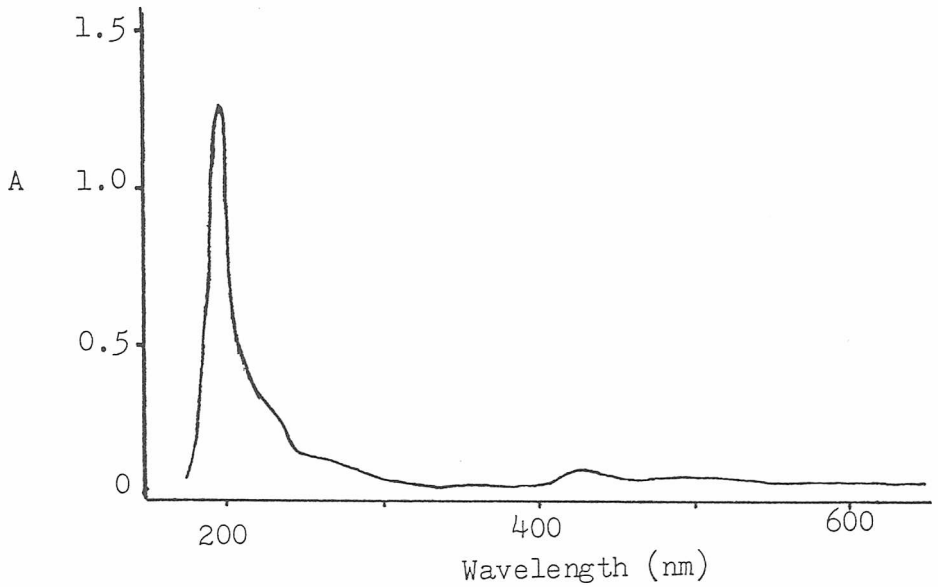


Figure 27 : Spectra of fractions from affinity chromatography:

(a) cytochrome c_{552}, the second protein peak;

(b) TMAO reductase, the first protein peak.

——— air oxidised – H_2O blank

- - - - dithionite reduced – H_2O blank

and two additional peaks, the β and α soret peaks, appeared at 523 and 551-552 nm, respectively. On one occasion, a small peak at 409 nm was observed in the fraction containing TMAO reductase (Fig. 27b) which would suggest that cytochrome c_{552} might be co-purified with TMAO reductase.

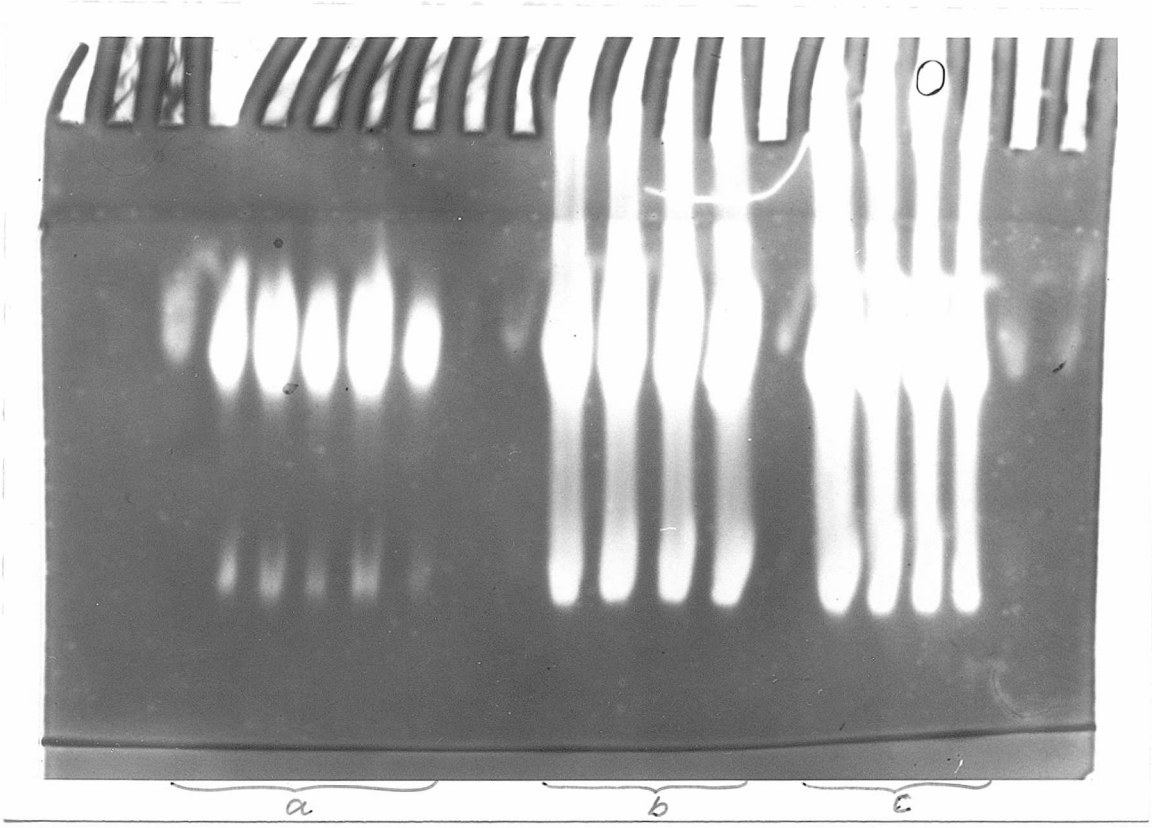
3.7.2 Analysis of the purified preparation

The fractions containing TMAO reductase obtained from affinity chromatography were pooled and concentrated using the Amicon Centriflo Cones (exclusion limit = 25k daltons) before the determination of the enzyme's biophysical properties.

The proteins of a sonicated extract of whole cells, the periplasm and the TMAO reductase preparation from affinity chromatography, were separated by electrophoresis on polyacrylamide gel. The gel was stained for both enzyme activity and protein content after electrophoresis. The enzyme activity or zymogram stain showed that all 3 samples contained 2 zones with TMAO reductase activity (Plate 2) and their relative mobilities (R_f) were 0.25 and 0.69; they are referred to as protein A and B respectively. The intensity of the zymogram stain of protein A was greater than that of protein B, but in the purified preparation, the activity of protein B was markedly reduced. The loss of activity of protein B might also explain the poor recovery of TMAO reductase activity during affinity chromatography (see section 3.6.1). The two zones of enzyme activity indicate that the periplasm contains 2 TMAO reductases, or the enzyme was multimeric and the chaotropic effect of NaCl and/or electrophoresis separated this complex into a large and a small unit both of which contained active protein.

The gel was stained for protein after the stain for enzyme activity, but the results were inconclusive due to the diffusion of the protein bands, and their low concentration. Therefore a larger volume of sample

PLATE 2 : Zymogram stain for TMAO reductase activity after PAGE.



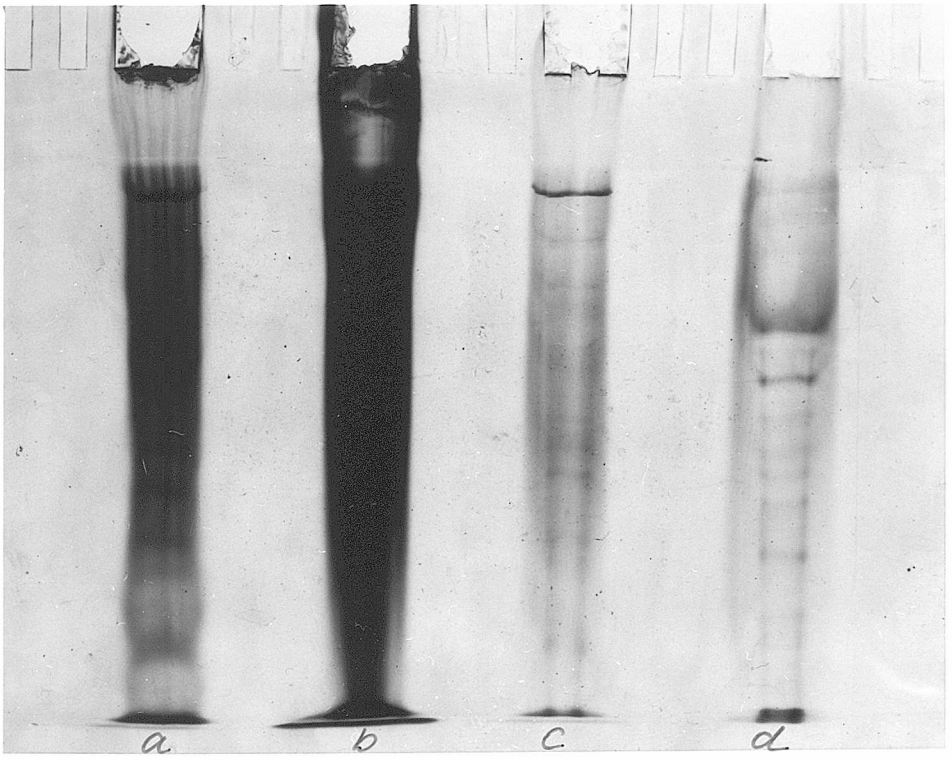
Four replicates of 3 samples were applied to the gel, (a) purified preparation of TMAO reductase from affinity chromatography, (b) periplasm and (c) sonicate. Areas of decolourization were due to TMAO reductase activity : the larger zone was called protein A and the smaller zone with greater mobility was called protein B.

was applied to the gel, and stained for protein immediately after electrophoresis. Plate 3 shows that the purified preparation of TMAO reductase contained 9-10 protein bands, and 3 major proteins were observed with R_f values of 0.29, 0.38 and 0.70, two of which corresponded to the zones of TMAO reductase activity observed in the zymogram stain i.e. proteins A and B. The presence of many protein bands shows that the purified preparation containing TMAO reductase was a multi-component complex of which only two components on their own were capable of TMAO reduction. Alternatively, the TMAO reductase preparation may be impure.

Cell fractions and the purified preparation were boiled with 2.5% (w/v) SDS and 5mM dithiothreitol for 2-3 min to separate the proteins into their subunits. Electrophoresis in the presence of SDS, separated the proteins on the basis of their size, and molecular weight determinations were made by calibrating the gels with proteins of known size as shown in Fig. 28. TMAO reductase activity was lost by boiling with SDS and zymogram stains were not carried out. The purified preparation was found to contain four subunits, one of which was distinct, but the other three appeared as diffuse protein bands (Plates 4 and 5). Similar results were obtained on 2 occasions and the poor definition was probably due to the low concentration of protein in the sample applied to the gel. The major subunit of the preparation had a molecular weight of 95-102k daltons, and this protein was 2-3 times more concentrated than the other protein bands. The molecular weights of the other 3 subunits were 86-89k, 72-77k and 53k daltons. A very faint band was also present at ca 40k daltons. The total molecular weight was estimated to be 300-350k daltons, assuming a 1:1:1:1:1 ratio of the subunits, or 400-450k daltons assuming that the major subunit was present at twice the concentration of the other subunits.

The subunit structure of TMAO reductase was also determined by electrophoresis of the periplasm, and excising the two active bands

PLATE 3 : Separation of the protein components of TMAO reductase preparations by PAGE.



Sample (a) sonicate, (b) concentrated periplasm, (c) periplasm and (d) TMAO reductase preparation purified by affinity chromatography. Samples (a) and (b) were over stained due to their high protein concentration.

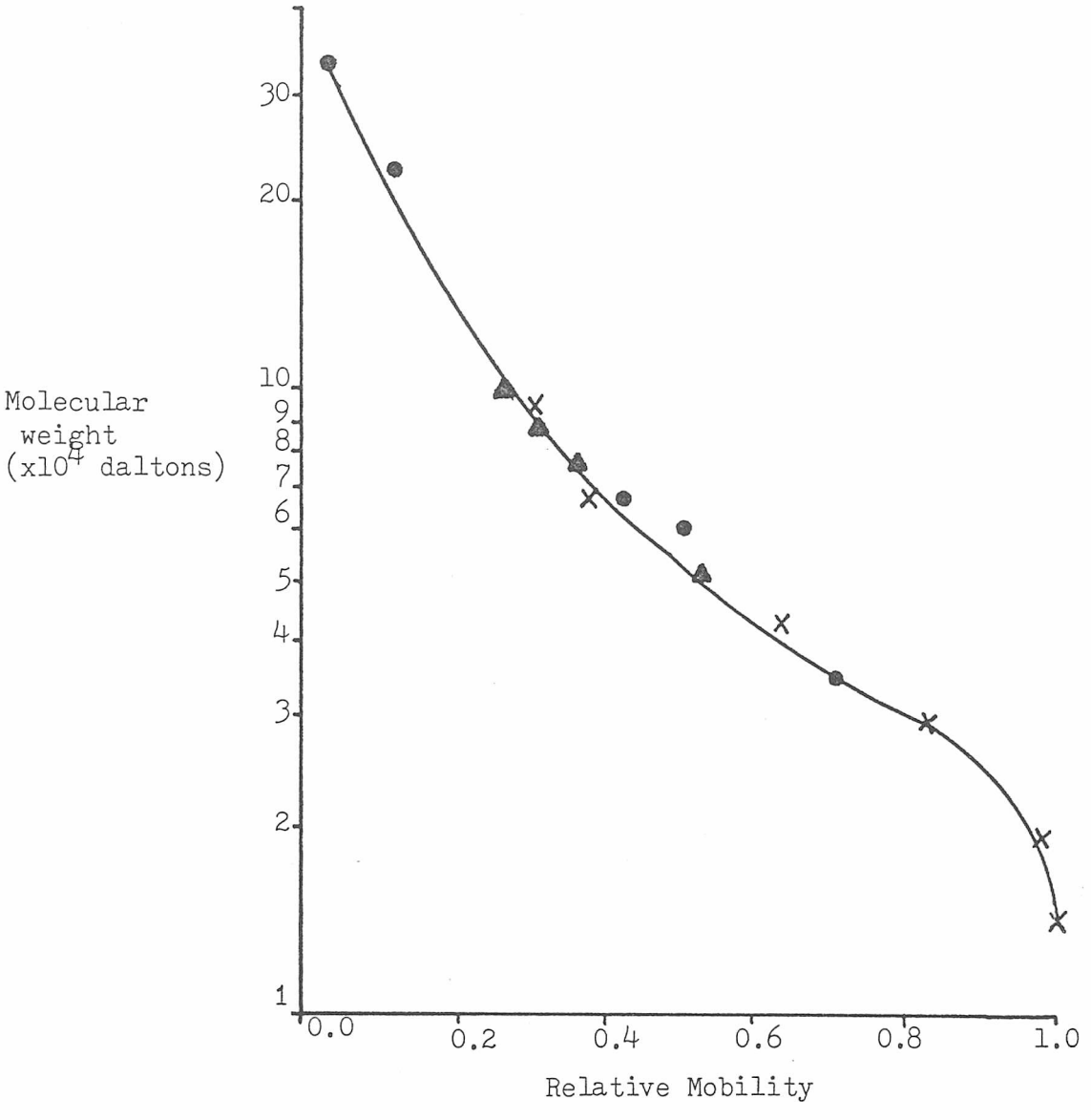


Figure 28 : Molecular weight determinations by SDS PAGE.

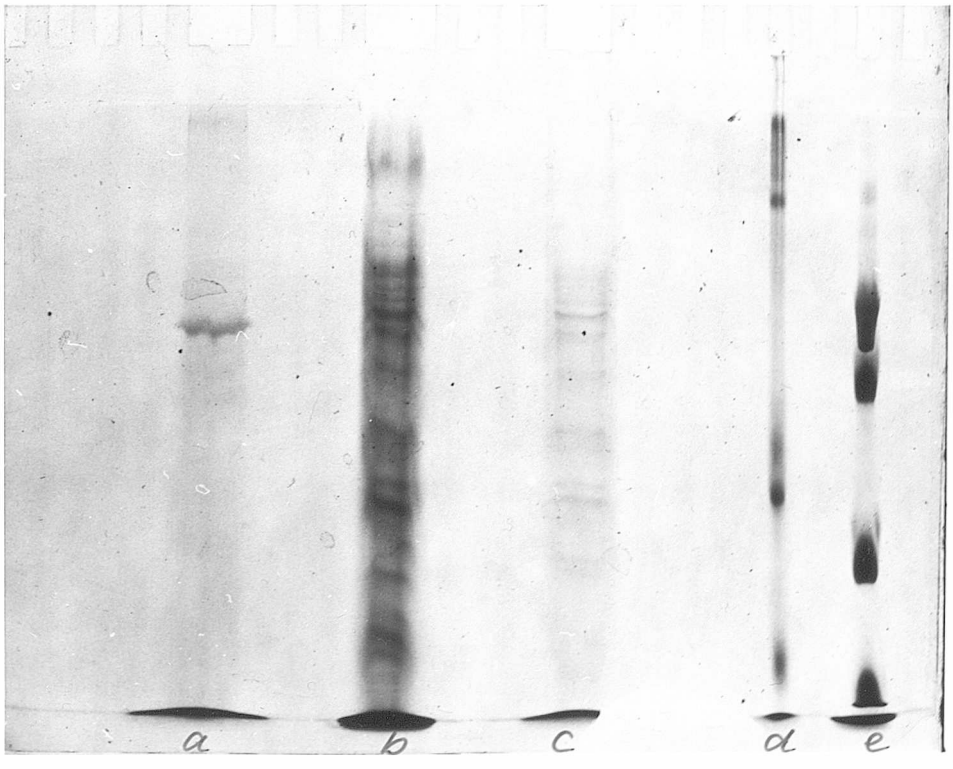
▲ , subunits of the purified TMAO reductase preparation

● , high molecular weight calibration kit containing (k daltons) thyroglobin (333), ferritin (220), albumin (67), lactate dehydrogenase (36) and catalase (60).

× , low molecular weight calibration kit containing (k daltons) phosphorylase b (94), albumin (67), ovalbumin (43), carbonic anhydrase (30), trypsin inhibitor protein (20.1), ∝-lactalbumin (14.4).

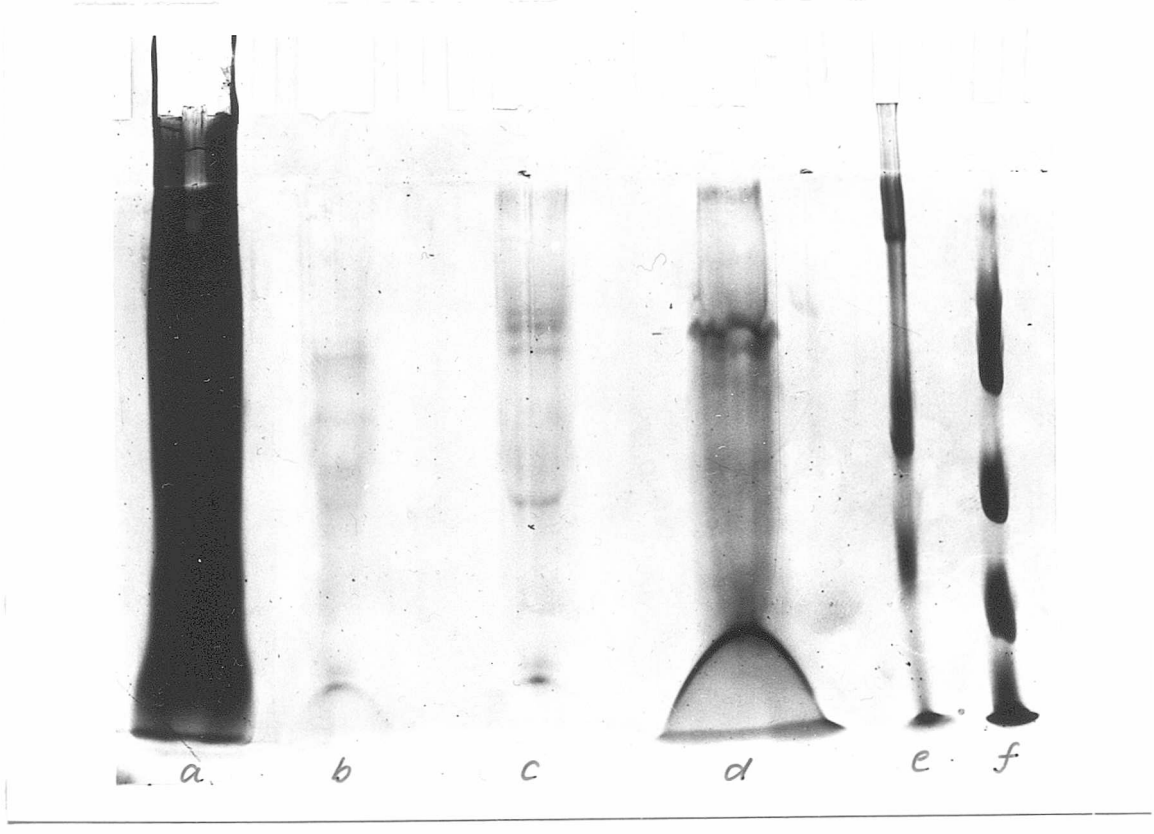
$$\text{Relative Mobility} = \frac{\text{Distance moved by protein band.}}{\text{Distance moved by solvent front.}}$$

PLATE 4 : Determination of the subunit composition of TMAO reductase using SDS PAGE.



Sample (a) purified preparation from affinity chromatography, (b) concentrated periplasm, (c) periplasm. The method was calibrated using the high and low molecular weight marker proteins i.e. samples (d) and (e) respectively.

PLATE 5 : Subunit composition of the TMAO reductase proteins A and B.



Sample (a) concentrated periplasm (overstained due to high protein concentration), (b) protein B, (c) protein A, (d) purified preparation from affinity chromatography. Samples (e) and (f) were the high and low molecular weight calibration proteins respectively.

(proteins A and B) from the gel. These bands were treated with SDS (as described previously) and their subunits were separated by electrophoresis in the presence of SDS. Proteins A and B each contained 4 major subunits and in all six different subunits were detected (Plate 5). Two subunits, 86-89k daltons and 48k daltons, were common to both protein A and B (Table 22); the larger protein was also one of the subunits of the purified preparation, and might be the subunit responsible for TMAO reduction. Two other subunits from proteins A and B were also present in the purified preparation i.e. 98-102k and 53k dalton subunits respectively. Conversely, some subunits of proteins A and B appeared to be absent from the purified preparation e.g. 110k dalton subunit of protein A, and the common subunit at 48k daltons. However, the high density of the 95-102k dalton subunit in the purified preparation may represent the incomplete separation of the 102 and 110k dalton subunits of protein A. The TMAO reductase preparation from affinity chromatography also contained a subunit of 72k daltons, which was not present in either protein A or B; the only similarly sized subunit was one of 64k daltons from protein B. Although excising proteins A and B from the gel was subject to error and contamination by unrelated proteins, the results suggest that the purified preparation contained subunits from both these proteins.

Proteins of the purified preparation were also separated on the basis of charge using isoelectric focusing, from which their isoelectric points (pI) could be determined using the calibration recommended by LKB Ltd., as shown in Fig. 29. The gel was stained for both enzyme activity and protein after running for 1.5 - 2.0 h at 1200V., and the results are shown in Plates 6 and 7. The purified preparation, the periplasm and the sonicated extract were all found to contain 1 clear band of TMAO reductase activity (Plate 6), and its pI was estimated to be pH 5.45. Some enzyme activity was also detected at the sample application site; this was

TABLE 22 : Subunit composition of TMAO reductase preparations.

TMAO reductase preparation	Relative mobility (R_f)*	Molecular weight (x 10^3 daltons)
Protein A	main bands = 0.23, 0.25, 0.29, 0.56 others = 0.76, 0.86, 0.89	110, 102 89, 48 32, 26.5, 25.5
Protein B	main bands = 0.30, 0.43, 0.51, 0.56 others = 0.63, 0.86, 0.89	87, 64, 53.5 48 42, 26.5, 25.5
Purified by affinity chromatography	main band = 0.26 others= 0.31, 0.38, 0.53 (0.63?)	98 86, 72, 53 (42.5?)

* R_f = $\frac{\text{Distance travelled by band}}{\text{" " by the solvent front.}}$

For calibration of R_f and molecular weight, see fig. 28.

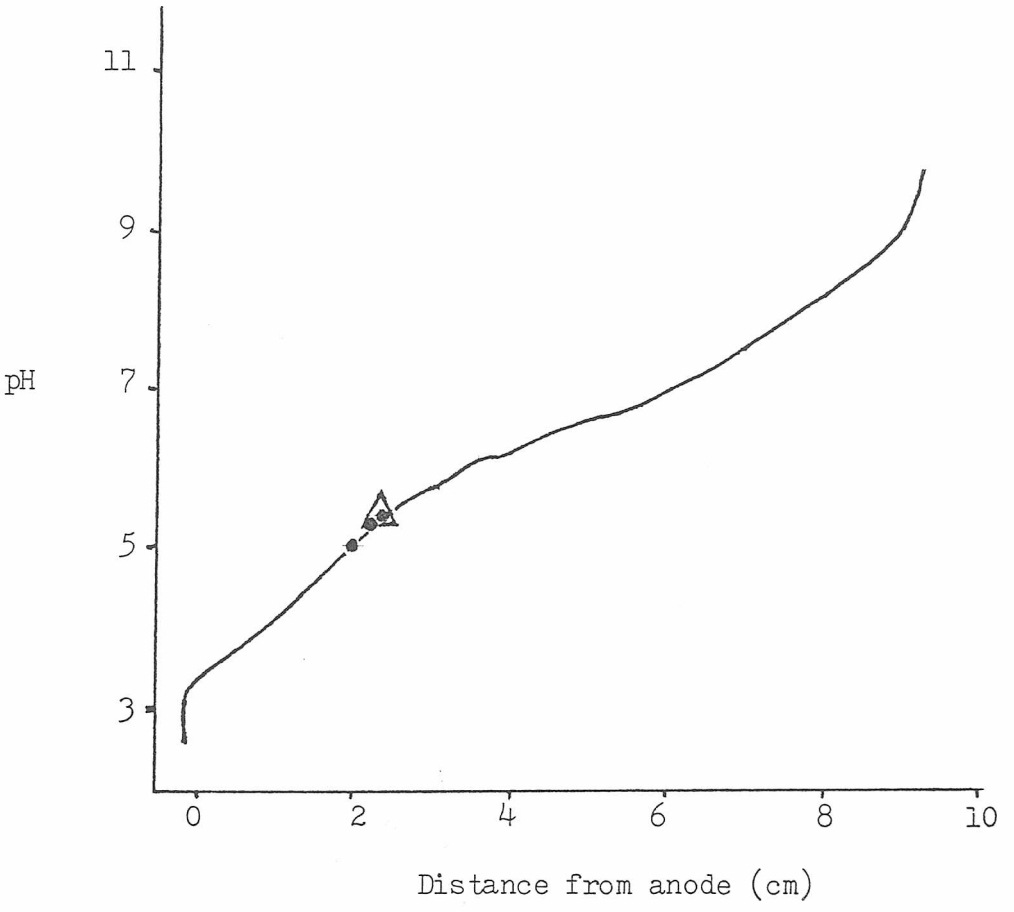


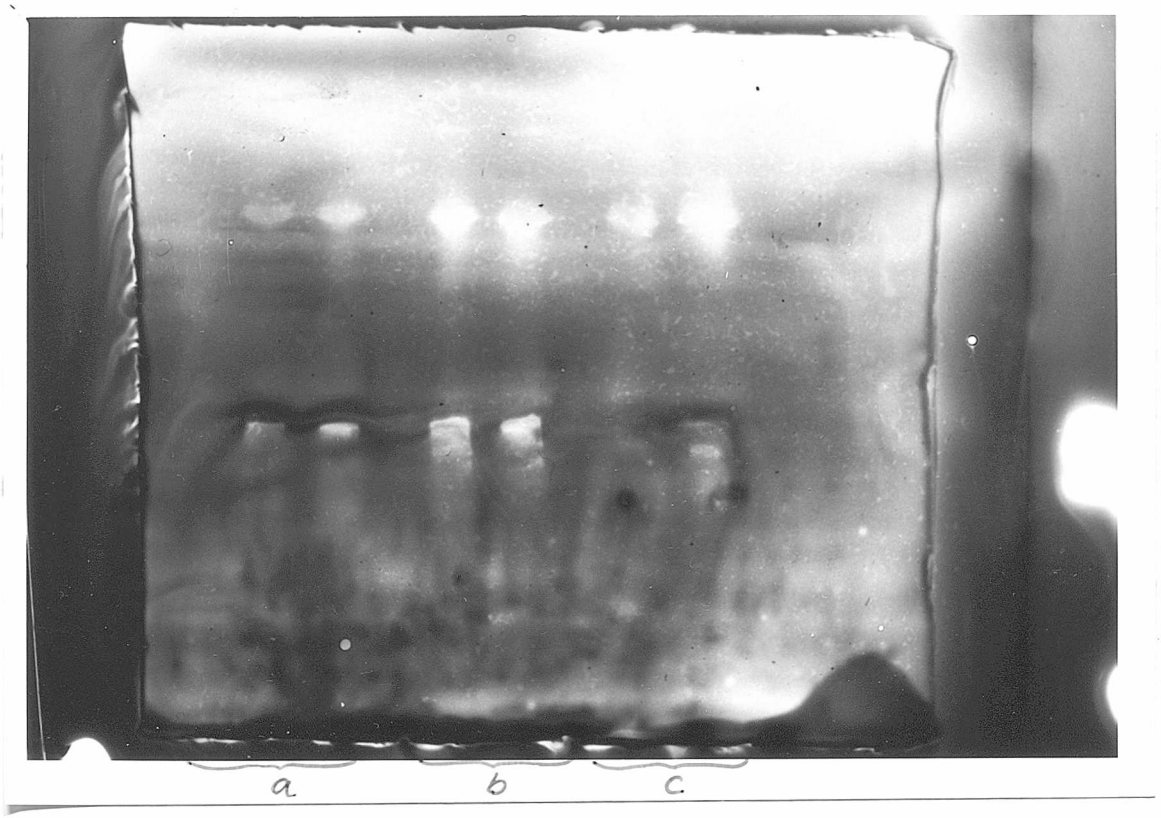
Figure 29 : Isoelectric focusing of TMAO reductase.

△ , zone of decolourization due to TMAO reductase activity.

● , 3 main protein bands of the purified TMAO reductase preparation.

PLATE 6 : Zymogram stain for TMAO reductase activity after isoelectric focusing.

ANODE (+)

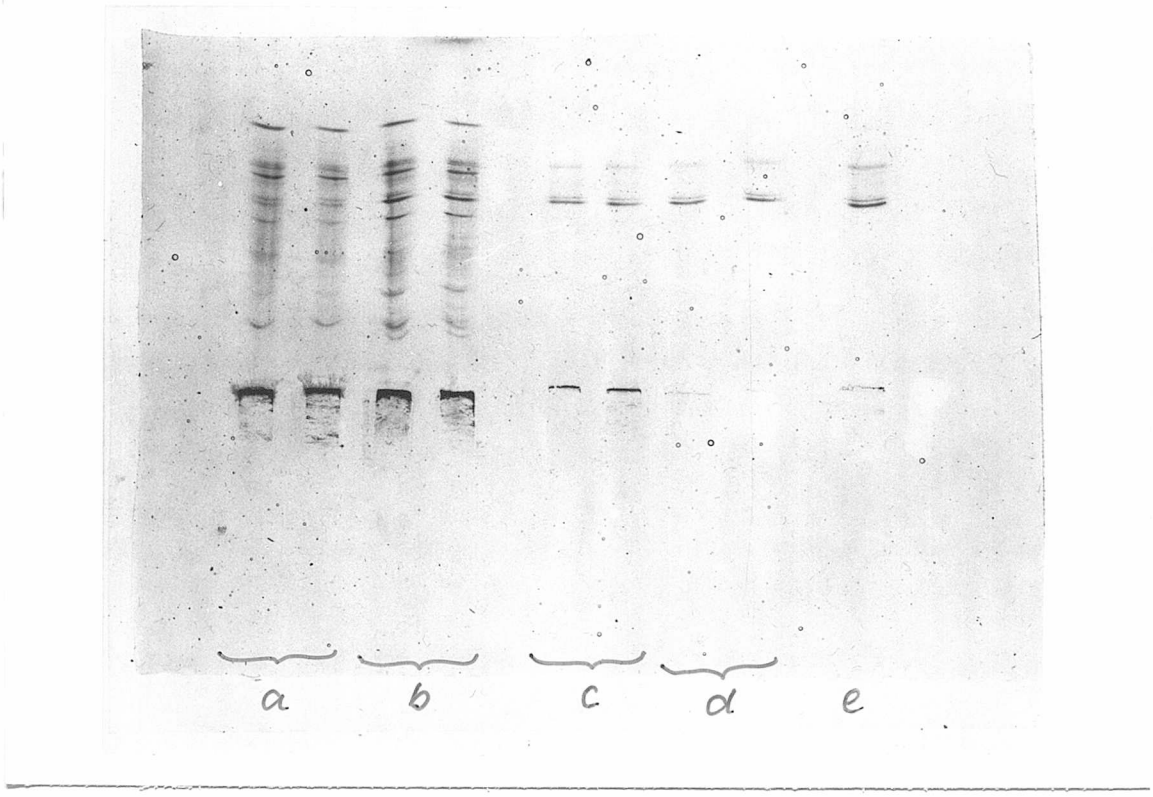


CATHODE (-)

Samples (in duplicate): (a) purified preparation from affinity chromatography, (b) periplasm and (c) sonicate. Zones of decolourization were due to TMAO reductase activity and correspond to a pI = 5.45.

PLATE 7 : Separation of the protein components of TMAO reductase preparations by isoelectric focusing.

ANODE (+)



CATHODE (-)

Samples (in duplicate) : (a) sonicate and (b) periplasm were applied in ca 50µl amounts. The preparation purified by affinity chromatography was applied in amounts of 25, 50 and 75µl (1 replicate) i.e. samples (c), (d) and (e) respectively.

probably due to precipitation of the enzymes, since similar results were obtained at different sample loading positions. The zymogram stain also showed very slight activity near the cathode at a point corresponding to a pI of 9.0, but this was only evident after 15-20 min incubation and might be an artefact. These results contrast with those from Page113 where 2 zones of enzyme activity were detected, however, the protein stain after isoelectric focusing showed 2 bands in the region of the enzyme activity, and their pI values were estimated at 5.45 and 5.50. Therefore it is possible that both proteins contained enzyme activity, and due to their close proximity only 1 zone of decolourization was observed in the zymogram stain. Three main protein bands were detected with pI values of 5.10, 5.45 and 5.50, and four minor bands were also perceptible with pI values of 4.50, 4.85, 5.12 and 5.30. No protein bands were observed near the cathode; thus the existence of another band of TMAO reductase with a high pI = 9.0 seems unlikely.

Attempts were made to isolate TMAO reductase from the periplasm using ion exchange chromatography and high voltage paper electrophoresis, to determine whether the periplasm contains 1 or 2 enzymically active proteins of markedly different isoelectric points. A cation exchanger was used, so that at pH 7.0, TMAO reductase (pI = 5.45) should be anionic and bind to the resin. Other proteins with isoelectric points above 7.0 should be cationic and be eluted from the column. Figure 30 shows that no TMAO reductase was bound to the column and all the activity was recovered in the initial fractions. Cytochrome c_{552} was also present in the same fraction. These results suggest that the pI of TMAO reductase was > 7.0 which contradicts that obtained from isoelectric focusing.

The results from high voltage paper electrophoresis were inconclusive because poor background staining prevented the detection of TMAO reductase activity using the zymogram stain. Thus the existence of two proteins with TMAO reductase activity and different isoelectric points could not be ascertained using this technique.

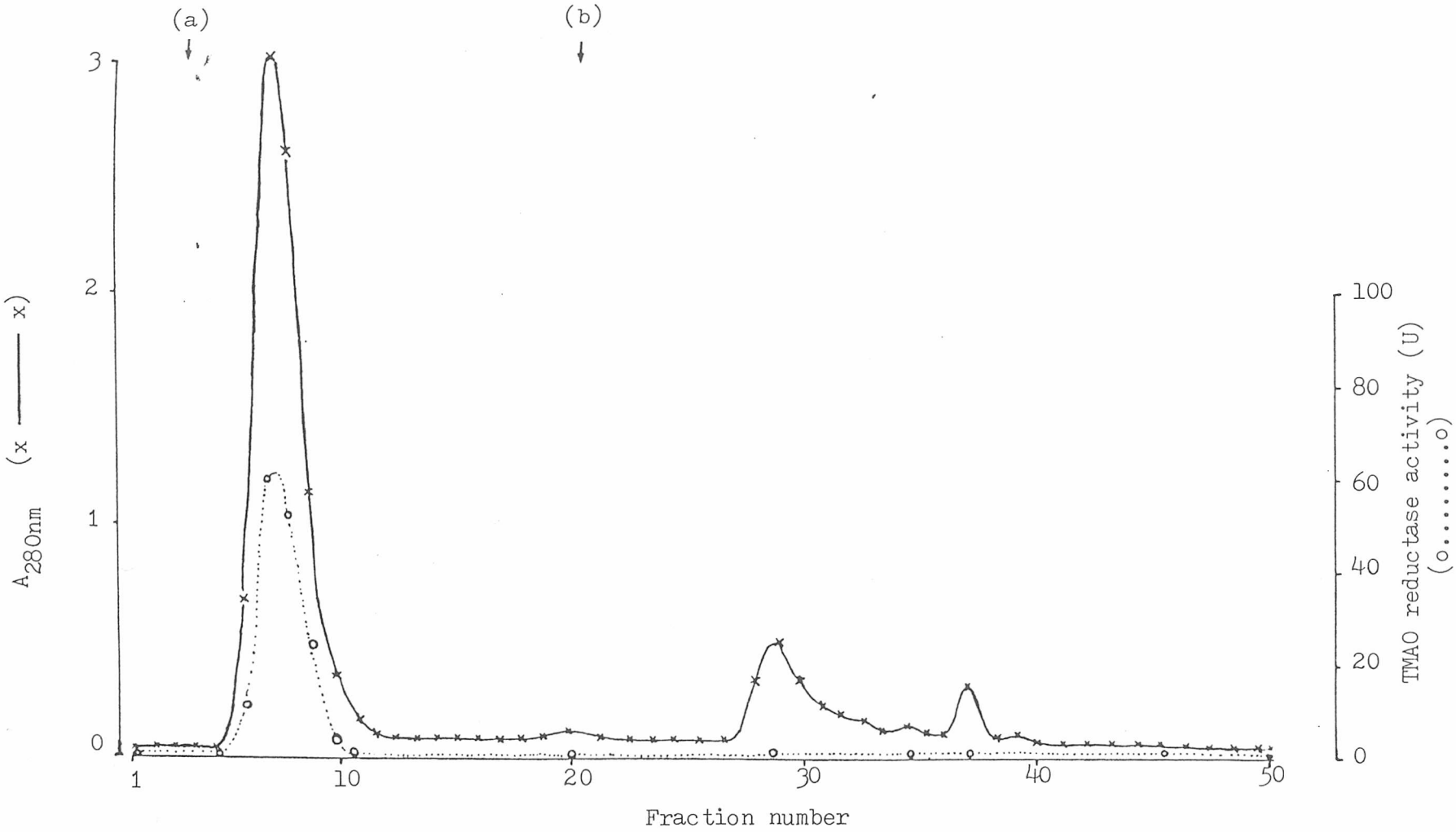


Figure 30 : Separation of periplasmic proteins by ion exchange chromatography.
(a) periplasm applied in 50mM citrate/phosphate buffer, pH 7.0
(b) elution with 50mM glycine/NaOH buffer, pH 10.0

3.7.3 Properties of the purified preparation

The ability of the purified preparation to use various substrates and the effect of several compounds on its ability to reduce TMAO was determined.

The specificity of the purified preparation was tested by replacing TMAO with other N-oxides e.g. $NaNO_2$, adenosine-N-oxide, nicotinamide-N-oxide and pyridine-N-oxide, and measuring the decolourization of $MV^{\cdot +}$. TMAO was the principle electron acceptor for this enzyme but some activity (ca 70%) was also detected with adenosine-N-oxide. No activity was detected with the other N-oxides. In the presence of both TMAO and adenosine-N-oxide the rate of dye oxidation was additive, suggesting that the purified preparation had 2 activities; one specific for TMAO and the other specific for adenosine-N-oxide.

The rate of dye oxidation was measured in the presence of various concentrations of TMAO ranging from 0.075-1.0mM, to determine the Km_{TMAO}. No activity could be detected in the presence of $\leqslant$ 0.05mM TMAO and the results are shown as a double reciprocal plot in Fig. 31. The Km_{TMAO} was estimated to be 150 μM, a similar value to that of 93 μM obtained in whole cells (section 3.6.1).

The effect of several compounds on the TMAO reductase activity was tested and the results are shown in Table 23.

TMAO reduction was inhibited by some N-oxides e.g. nicotinamide-N-oxide and pyridine-N-oxide, but the enzyme was relatively resistant to cyanide e.g. 5mM KCN caused only 40% inhibition. The activity was sensitive to inhibition by p-chloromercuribenzoate at 20 μM, confirming that a thiol group was involved in TMAO reduction.

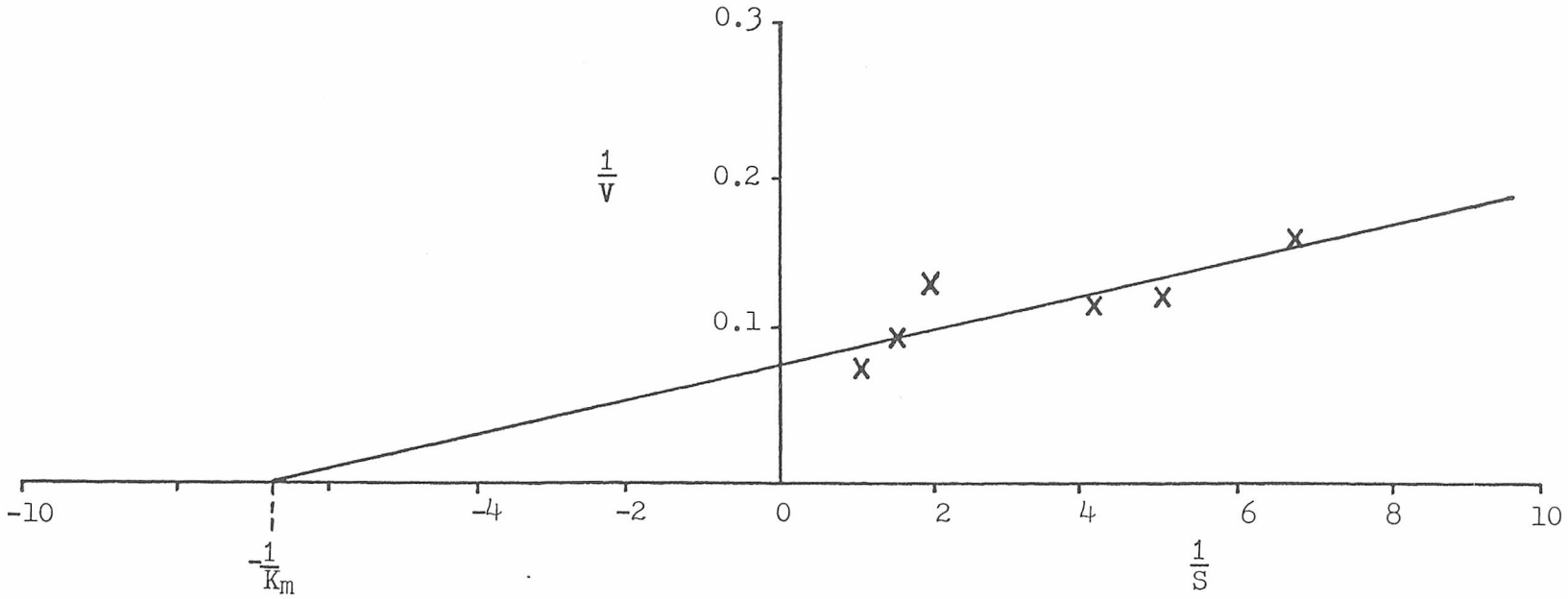


Figure 31 : Michaelis-Menten double reciprocal plot for the purified TMAO reductase preparation.

TABLE 23 : Inhibitors of the purified TMAO reductase preparation.

Compound	Concentration (mM)	TMAO reductase activity (U)	Inhibition (%)
Control	-	10.60	-
Pyridine-N-oxide	1	7.37	30
Nicotinamide-N-oxide	1	6.46	39
KCN	1	10.58	0
	5	6.36	40
p-chloromercuri-benzoate	0.02	3.18	70

3.8 Micro-aerobic growth supported by adenosine-N-oxide

The purified preparation was shown to reduce both TMAO and adenosine-N-oxide, and since TMAO had been shown to support anaerobic bacterial growth, the ability of adenosine-N-oxide to do likewise was examined. The medium of Wood & Baird (1943) was supplemented with adenosine-N-oxide (as described in section 2.3) to give a concentration range of 1-10mM. The micro-aerobic growth of *Alteromonas* sp., NCMB 400, was determined spectrophotometrically after 48 h at 20°C.

Adenosine-N-oxide was shown to support the growth of the alteromonad (Fig. 32), and the extent of growth was dependent upon the initial adenosine-N-oxide concentration (Fig. 32b). These results were similar to those obtained for the micro-aerobic growth of the alteromonad on TMAO (section 3.2.3). At equimolar concentrations, adenosine-N-oxide supported more growth than TMAO.

In the presence of adenosine-N-oxide and TMAO, the growth yield was greater than that obtained with either of these 2 compounds. A conductance experiment produced a similar result and also showed that in the presence of both electron acceptors the growth rate was increased by *ca* 30%; the mean generation time being 0.65 h instead of 0.95 h (Fig. 32c). The additive effect of TMAO and adenosine-N-oxide was also demonstrated in enzyme assays involving the purified preparation from affinity chromatography (section 3.7.3) and whole cells (section 3.6.1, Table 17).

The activity of TMAO reductase in whole cells grown with and without TMAO and/or adenosine-N-oxide was determined using the viologen dye assay. TMAO induced the enzyme but no activity was detected in cells grown on adenosine-N-oxide alone. This suggests that adenosine-N-oxide was not a gratuitous inducer of TMAO reductase. In the presence of both N-oxides, there was no change in the induced level of enzyme activity.

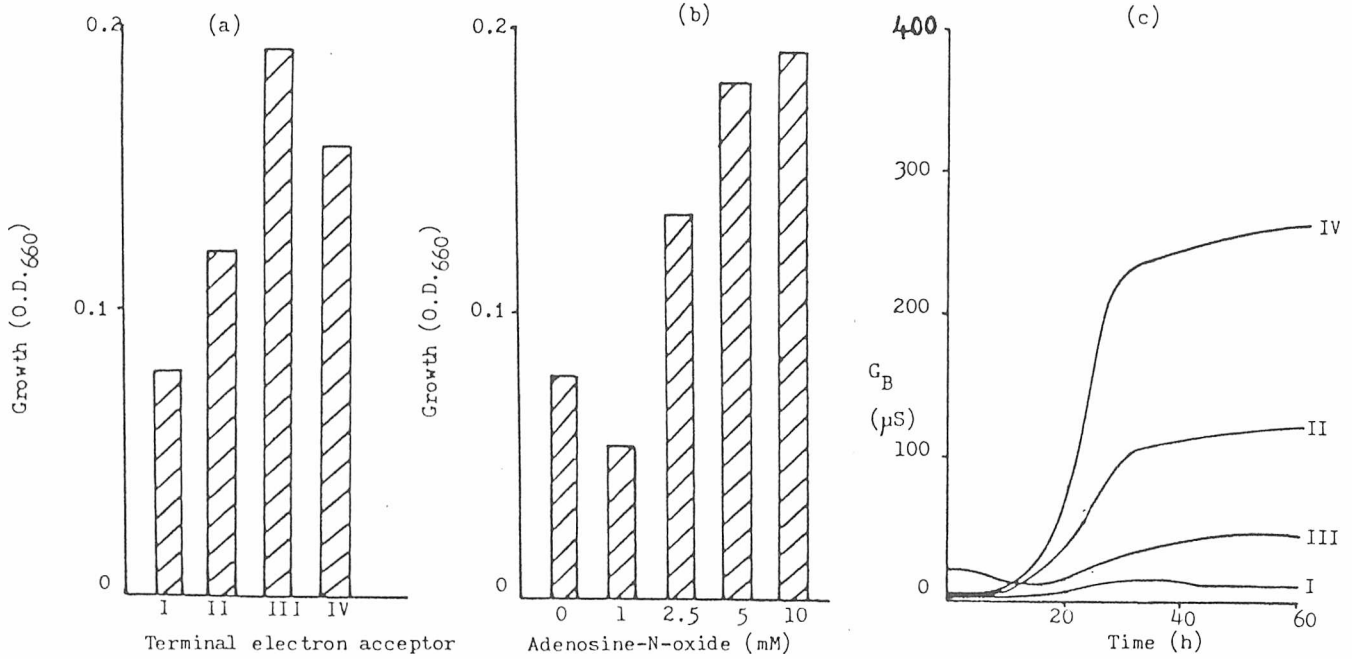


Figure 32 : Micro-aerobic growth of Alteromonas sp., NCMB 400, on TMAO and/or adenosine-N-oxide.

(a) TMAO and/or adenosine-N-oxide as terminal electron acceptor.

(b) effect of adenosine-N-oxide concentration.

(c) conductance change (G_B) with TMAO and/or adenosine-N-oxide.

I, no terminal electron acceptor; II, TMAO (10mM); III, adenosine-N-oxide(10mM); IV, TMAO and adenosine-N-oxide.

CHAPTER 4

DISCUSSION

Many observations have been made over the past 70 years regarding the reduction of TMAO by bacteria and its relationship to fish spoilage. A respiratory function was first ascribed to TMAO by Beatty (1938), but the mechanisms involved were not understood. A better understanding of bacterial metabolism in the 1970's rekindled interest in TMAO reduction, and it is now thought that TMAO acts as a terminal electron acceptor and supports anaerobic bacterial growth in a manner analogous to nitrate and fumarate. Most recent studies on TMAO reduction have involved organisms whose physiology and biochemistry are well understood such as E. coli, but these organisms are rarely associated with the spoilage of iced marine fish. Thus the objective of this study was to determine the properties of TMAO reduction in typical spoilage bacteria, Alteromonas spp. A prerequisite to the study of TMAO reduction is the development of methods for the quantitative analysis of TMA and TMAO.

4.1 Assay methods for bacterial TMAO reduction.

The absorption maximum of TMAO and TMA is at 193 and 191 nm respectively: spectroscopy could not be used directly to assay for TMA and TMAO since the presence of protein (and other ultra-violet absorbing material) would cause interference. TMAO reduction was therefore determined by measuring the amount of TMA formed per unit time. The methods for TMA detection and quantitation were compared (see Table 4). Glc was the most sensitive and specific assay of TMA, but the assay of TMAO was difficult due to the inactivation of the column by $TiCl_3$. TMA was the only amine detected in spent culture media, and it was equimolar to the TMAO added. Sakaguchi & Kawai (1975a) reported similar results for E. coli. Accordingly, the metabolism of TMAO by Alteromonas spp. appears to be a reduction rather than a demethylation.

The picrate assay was the most frequently used method of TMA analysis since it was relatively sensitive, reproducible, moderately fast and easy to set up. However, a recently developed electrical method, the gas sensor, was found to be more sensitive and once calibrated, it was a relatively fast method giving results within 5-15 min. The gas sensor, like the picrate assay, was non-specific, but modifications are currently being made so as to separate TMA, DMA and NH_3 (Storey, personal communication). Thus, the gas sensor may facilitate a faster, more sensitive method for the assay of TMAO reduction in the future.

A more sophisticated electronic method was used to monitor bacterial TMAO reduction. Conductance measurements were shown by Richards et al. (1978) to be a valid means of monitoring bacterial growth, and similar results were obtained for Alteromonas sp., NCMB 400. The relationships between G_B and TMAO reduction/TMA production were also established (equations 4-10), and conductance was shown to be a valid measure of bacterial TMAO reduction. The magnitude of G_B was due mainly to the molecular charge difference between TMAO and TMA, and the amount of TMAO available which limited bacterial growth. In comparison with the other methods used for the study of TMA production (section 2.8), the conductance assay has several advantages. It is automatic, sensitive, non-perturbative, non-destructive, provides a continuous indication of TMAO reduction and requires relatively few operator manipulations. It was capable of measuring TMA production in the presence of $\geqslant 1.5\mu$mol TMAO.

The conductance method was analogous to that described by Alison, Anderson & Cole (1938) who used conductivity measurements to follow ammonia production by P. fluorescens growing on skimmed milk, the end product (NH_3) being a much better charge carrier than its precursors (presumably amino acids). Conductance assays have been used for following other enzyme mediated activities e.g. Baernstein (1928) who studied

the digestion of egg white by pepsin and papain. Thus the conductance method may be applicable to any assay in which there is a net change in the charge of the reactants. It was therefore particularly useful in determining the properties of TMAO reduction by whole cells of NCMB 400, and in combination with the spot test it was used successfully to detect the ability of microorganisms to reduce TMAO.

4.2 Bacterial growth

All the alteromonads tested were Gram negative rods of varying length and their optimum growth temperature was 20°C. However, the temperature profiles of the 5 NCMB strains showed an additional peak at 5°C, which may be a function of a psychrophilic growth mechanism. The mechanisms involved in the adaption of bacteria to growth at low temperatures are poorly understood. Enzyme activities decrease with lowering the temperature and this may be compensated for by increased enzyme synthesis or modified enzyme reaction rates (Ingraham & Stokes, 1959; Elliot & Michener, 1965). Morita & Burton (1963) suggested that at low temperatures, whole cells of a marine psychrophilic vibrio have some mechanism for permitting malate dehydrogenase to function at rates which are higher than those of cell free extracts. The structure and composition of certain macromolecules may also be altered to facilitate growth at low temperatures. Herbert & Bkahoo (1979) reviewed some of the properties of psychrophilic bacteria and showed that the fatty acid composition of the cell changed in response to temperature. This may have been a regulatory response for the maintenance of membrane fluidity and substrate transport, but the full significance of these results has not yet been elucidated. Therefore, if the growth of Alteromonas spp. at 5°C represents an adaption to psychrophilic growth, then these strains should provide a useful tool for the study of psychrophily.

A morphology change i.e. an elongation of the cell, was observed in several alteromonads, when they were grown to stationary phase or at high temperatures e.g. 25° and 30°C. The reasons for this were not known but it is possible that stationary phase cells were in a poor physiological condition and the staining procedure produced the elongated curved rods as an artefact, or that cell wall synthesis and cell division were out of synchrony. Colonies of most bacteria were white to grey-white in colour, except NCMB 1520 which produced two types of colonies after prolonged storage at 4°C, when they were white or yellow in colour. Variation in morphology and physiology are frequently observed phenomena that occur in pure culture of similar bacteria. The reasons for such variations are not well understood, but it has been proposed that they are caused by spontaneous mutation (Greenfield, Hines & Boral, 1962; Gow, DeVoe & MacLeod, 1973).

Gram negative bacteria from several genera including fermentative and non-fermentative types could reduce TMAO (Table 7). They were isolated from several environments, thus TMAO reduction is not a property restricted to marine bacteria as might be expected in view of the natural occurrence of TMAO. Conversely the reduction of TMAO is not a property common to all marine bacteria. Many alteromonads were found to reduce TMAO, confirming the findings of Lee et al. (1977). All the marine isolates of A. putrefaciens reduced TMAO but some of the non-marine strains could not. Lee et al. (1977) used TMAO reduction as a characteristic for classifying Alteromonas spp. into phenons D and E, the latter being A. putrefaciens. The results presented here show that TMAO reduction was a variable property in Alteromonas spp., and so it may be of less use in taxonomy and identification than Lee et al. (1977) proposed.

In non-fermentative bacteria such as Alteromonas spp., the ability to reduce TMAO provides a method of anaerobic growth. Thus under conditions of low oxygen tension, and in the presence of TMAO, TMAO reducers would have a competitive advantage over non-reducers. Such a situation may exist during fish spoilage.

Nitrate, fumarate and TMAO are known to support anaerobic bacterial growth in E. coli (Yamamoto & Ishimoto, 1977) and their ability to support anaerobic growth of Alteromonas spp. was examined. Most strains could grow anaerobically on fumarate but the ability to reduce nitrate and TMAO was only present in certain strains (Table 5). TMAO reduction was limited to strains in phenons D and E, and nitrate reduction was not always a property of TMAO reducing strains. The ability to grow on both TMAO and nitrate was present in 67% of the TMAO reducers tested, and the remaining 33% could grow anaerobically only with TMAO (Table 6).

Kim & Chang (1974) have shown that both TMAO and nitrate reduction were properties of a wild type strain of S. typhimurium, but both activities were absent in chlorate resistant mutants. However, the addition of molybdate to the medium resulted in the recovery of both activities. They concluded that the nitrate and TMAO reductase systems shared some common component(s) e.g. a molybdoprotein, and that each system also has some unique protein components. Shimokawa & Ishimoto (1979) showed that tungstate inhibited and molybdate recovered the activity of a tertiary amine-N-oxide reductase which can be regarded as TMAO reductase. Therefore TMAO reductase is probably a molybdoprotein and may have similar genetic loci to nitrate reductase.

In the presence of both TMAO and nitrate, the growth yield was not additive, and in several alteromonads growth was less than that observed with TMAO alone. Both TMA and nitrite were detected in the medium, and the results might be explained by the inhibitory effect of nitrite on growth and TMAO and nitrate reduction (as shown in Figs. 7 and 8). Such

an explanation also infers that both TEA were reduced simultaneously which is an unlikely event. Dyer (1949) also showed that nitrite inhibited TMA production and demonstrated that TMAO was reduced before nitrate by fish spoilage bacteria. If the same is true of Alteromonas spp., and since nitrate inhibited TMAO reduction (Table 17), then these properties might account for the poor growth yield of Alteromonas spp. in the presence of TMAO and nitrate.

The ability of adenosine-N-oxide to support bacterial growth was examined since the purified TMAO reductase preparation from Alteromonas sp., NCMB 400, was also active in the presence of adenosine-N-oxide. The N-oxide was shown to support the micro-aerobic growth of Alteromonas sp. but this has not been demonstrated in other bacteria. Sagai & Ishimoto (1973) have suggested that the reduction of adenosine-N-oxide to hypoxanthine via adenosine may be a form of anaerobic respiration, but the exact physiological function of this N-oxide is unknown. It is possible that it is used as a source of hypoxanthine, an intermediate in the biosynthesis of nucleotide bases. Certain N-oxides of purines and pyrimidines are thought to be either oncogenic agents or to have an antitumour action (Bickel, 1969; Sagai & Ishimoto, 1973). Thus the reduction of adenosine-N-oxide may represent a detoxification.

4.3 Cellular location of TMAO reductase

The intracellular location of TMAO reductase in Alteromonas sp., NCMB 400, was of importance because of its proposed respiratory function. Since energy conservation is thought to be coupled to TMAO reduction, the enzyme would be expected to be found in the cytoplasmic membrane, as shown for V. parahaemolyticus (Unemoto et al., 1965) and E. coli (Sakaguchi & Kawai, 1975b). It was necessary to fractionate the bacteria

into their major subcellular components i.e. the cytoplasm, the inner membrane and the periplasm, in order to determine the location of TMAO reductase. Cell fractionation involved the formation of sphaeroplasts which was the critical part of the fractionation procedure and several methods were tried with varying degrees of success. This was probably due to the precise structure and composition of the outer membrane, and the toxicity of EDTA.

The conventional method of sphaeroplast formation from Gram negative bacteria such as E. coli (method A, section 2.6.1), did yield some sphaeroplasts of the alteromonad, but it was not reproducible as lysis was difficult to control. Asbell & Eagon (1966) and Costerton (1970), suggested that EDTA chelates divalent cations of the outer membrane resulting in the dissociation of lipopolysaccharides and polypeptides. Thus EDTA effectively breaks down the major permeability barrier of the cell and allows lysozyme access to the rigid peptidoglycan layer. The action of lysozyme then converts the rod-like bacterium into a sphere i.e. a sphaeroplast. Gilleland (1977) and Matsushita, Adachi, Shinagawa & Ameyama (1978), reported that EDTA was not fatal to E. coli , but with P. aeruginosa it caused gradual damage to the inner membrane, resulting in lysis and death. A similar explanation may account for the lysis of Alteromonas sp., NCMB 400, and the observation that Mg^{++} in the prewash decreased the lytic effect.

Cheng et al. (1971) described a technique for forming sphaeroplasts from P. aeruginosa not involving EDTA. Their method did not produce sphaeroplasts of Alteromonas sp., NCMB 400, and it was modified to incorporate a sucrose wash (method B, section 2.6.1), since Costerton et al. (1967) showed that sucrose weakened the outer membrane. However, sphaeroplasts were not formed by this modified method and it was concluded that EDTA was necessary for sphaeroplast formation. Costerton et al. (1967) also showed that a prewash with 0.5M NaCl enhanced the effect of sucrose on the outer membrane, probably due to the ability of Na^{+} to

displace Mg^{++} from the outer membrane, leading to a weakened structure which breaks up into fragments more readily. Thus method B may be improved if cells were washed with NaCl, and then treated with sucrose before the lysozyme and osmotic shock treatments.

Sphaeroplast formation from A. haloplanktis, NCMB 19, was described by Costerton et al. (1967). The outer membrane was removed by washing with NaCl and sucrose (as described above) and sphaeroplasts were formed by further treatment with either lysozyme or lysozyme and EDTA. Very few sphaeroplasts of Alteromonas sp., NCMB 400, were formed using lysozyme alone, but lysis occurred in the presence of lysozyme and EDTA. Lysis was probably due to the effect of EDTA on the cytoplasmic membrane as discussed above. Since EDTA was necessary for sphaeroplast formation, the method was modified (method C, section 2.6.1) such that the chelating agent was present during sucrose treatment, an earlier and less critical stage. The extent of sphaeroplast formation was dependent upon pH. Maximum sphaeroplast formation (ca 100%) was obtained using the sucrose/EDTA treatment at pH9.0-10.0; this was probably attributable to EDTA itself which is known to be particularly effective at alkaline pH's. Although this method was modified and used successfully to produce sphaeroplasts, it was not used in the cell fractionation procedure because the periplasm was very dilute, and the high pH of the sucrose/EDTA treatment could possibly result in the loss of enzyme activity due to protein denaturation.

EDTA was necessary for sphaeroplast formation using the method of Birdsell & Cota-Robles (1967), and the importance of inorganic cations in this method was also demonstrated. Magnesium and high (0.5M) concentrations of Na^{+} prevented lysis, and Mg^{++} also inhibited sphaeroplast formation. Rayman & MacLeod (1975) suggested that the rigidity of the peptidoglycan layer of A. haloplanktis was mainly due to Mg^{++} cross-linkages. Matsushita et al. (1978) inferred that Mg^{++} stabilised the cytoplasmic membrane of P. aeruginosa and aided the retention of

extrinsic membrane proteins. Thus Mg^{++} plays a major role in the structure and stability of the cell wall of certain Gram negative bacteria. A similar explanation might account for its effect on sphaeroplast formation and lysis in Alteromonas sp., NCMB 400. The high concentration of Na^{+} may have prevented lysis by stabilizing the cytoplasmic membrane perhaps due to its ability to replace Mg^{++}. Alternatively, Na^{+} may have counteracted the effect of excess EDTA. In conclusion, a correct balance between the presence of Mg^{++} and treatment with EDTA was required for maximal sphaeroplast formation and the prevention of lysis in Alteromonas sp., NCMB 400.

A reproducible method of sphaeroplast formation from the alteromonad was obtained using method D (modified from Birdsell & Cota-Robles, 1967). In comparison with method C, it had fewer manipulations (at pH's nearer neutrality) and took less time to perform, so that losses in enzyme activity were minimized.

The marker enzymes were found in their expected location, although some isocitrate dehydrogenase activity was detected in the periplasm probably due to some cell lysis. TMAO reductase was located along with alkaline phosphatase and cytochrome c_{552} in the periplasmic fraction of Alteromonas sp., NCMB 400. Some TMAO reductase activity remained with the sphaeroplasts (ca 35%), and it was solubilised by sonication, suggesting that the enzyme might be loosely associated with the cytoplasmic membrane. The cytochrome c content of the sphaeroplasts was less easily solubilised by sonication, and the alteromonad was shown to contain both periplasmic and membrane bound cytochrome c_{552}. Therefore, from this evidence, TMAO reductase should be regarded as a periplasmic enzyme, or possible a peripheral enzyme having a tenuous association with the cytoplasmic membrane.

Osmotic shock was sufficient to release most of the alkaline phosphatase activity from the cells, but only 25% of the TMAO reductase

activity was released. The retention of TMAO reductase might have been due to the molecular seiving of the outer membrane, which could suggest that TMAO reductase was a larger molecule than alkaline phosphatase, i.e. > 80-89000 daltons (Simpson, Vallee & Tait, 1968). EDTA was required for the further release of TMAO reductase and for complete sphaeroplast formation, which suggests that TMAO reductase was more firmly bound in the periplasm than alkaline phosphatase. In the periplasm of the marine alteromonad NCMB 19, alkaline phosphatase is bound to lipopolysaccharide by a Mg^{++}-linkage (Thomson & MacLeod, 1974b), and washing with NaCl and sucrose was sufficient to liberate the enzyme. A similar explanation may account for its release during the formation of sphaeroplasts from Alteromonas sp., NCMB 400. TMAO reductase is probably held in the periplasm by stronger forces than Mg^{++} cross-linkages, but its precise location is unknown.

It has been mentioned that EDTA might have adverse effects on the cytoplasmic membrane resulting in lysis; thus the release of TMAO reductase by EDTA during sphaeroplasting could be the result of a membrane mediated affect. Beacham (1979) also stated that certain transport enzymes were released by osmotic shock and that these proteins were probably associated with the cytoplasmic membrane. It is therefore possible that TMAO reductase was an extrinsic enzyme linked to the outer aspect of the cytoplasmic membrane, an attractive possibility in view of its proposed respiratory function.

Japanese workers have provided further evidence for the membrane location of TMAO reductase. Unemoto et al. (1965) showed that after sphaeroplast formation of V. parahaemolyticus, TMAO reductase was predominantly located in the cytoplasmic membrane. Sakaguchi & Kawai (1975b) claimed that in E. coli (strain IFO 3301 ≡ K12) TMAO reductase was membrane bound, but it was not particularly evident from the data presented in their short paper. Shimokawa & Ishimoto (1979) purified an amine N-oxide reductase from E. coli K10 Hfr which reduced TMAO,

and they considered TMAO to be the natural substrate. After sonication 33% of this enzymes activity was found in the soluble fraction and 67% of its activity was membrane bound. The different results obtained from E. coli TMAO reductase, may reflect the different properties of the 2 strains used and/or differences in the conditions for growth and membrane preparation. Accordingly, it appears from the work reported here and in the literature that TMAO reductase is located near or on the cytoplasmic membrane of Gram negative bacteria. However, the degree of association with the membrane varies between genera and possibly between strains of a species.

TMAO reduction is thought to provide a method of anaerobic respiration and since respiratory enzymes are usually associated with the cytoplasmic membrane it was surprising to find that TMAO reductase was located in the periplasm. However, this does not mean that TMAO reduction cannot be coupled to energy production via oxidative phosphorylation, since a respiratory function has been ascribed to other periplasmic enzymes e.g. nitrite reductase (Wood, 1978; Stouthamer, 1980) and possibly methanol dehydrogenase (Alefounder & Ferguson, 1981).

4.4 Induction of TMAO reductase

Enzyme activities are generally controlled in 2 ways (i) before synthesis e.g. transcriptional control of induction and repression, and (ii) after synthesis i.e. post-translational control e.g. feedback inhibition and allosteric effectors that are usually a feature of constitutive enzymes. The control of TMAO reductase in Alteromonas sp. was investigated and compared with that of E. coli.

TMAO reductase was induced both by TMAO and by conditions of low oxygen tension, the latter can also be described as derepression by

anaerobiosis. Oxygen (100% saturation) inhibited TMAO reduction by Alteromonas sp., NCMB 400, and both the activity and synthesis of TMAO reductase were inhibited by oxygen in E. coli (Sakaguchi & Kawai, 1976). Oxygen limitation was therefore required for the induction and activity of the reductase, so accounting for TMAO reductase being a property of anaerobic bacterial growth.

Oxygen is the preferred terminal electron acceptor in facultative anaerobes (Hamilton, 1979), and its presence represses the formation of other enzymes capable of utilizing alternative electron acceptors e.g. nitrate reductase. The formation of a particular terminal reductase is prevented when a hydrogen/electron acceptor with a higher energy-yielding potential is also present. This regulatory phenomenon therefore ensures that the largest amount of energy will be released during catabolism (Stouthamer, 1976). However, TMAO reduction was observed under aerobic growth conditions when the oxygen tension was still 40-60% saturation (Fig. 15). The most probable reason for this was that the biochemical oxygen demand by the culture (10^6 - 10^7 bacteria ml^{-1}) exceeded the oxygen supply, so that the energy demand was satisfied using another terminal electron acceptor i.e. TMAO. Such an explanation infers that (i) oxygen does not repress TMAO reductase below 60% oxygen saturation, and/or (ii) the repressive effect of oxygen was overridden by another controlling factor such as the redox potential as suggested by Sakaguchi & Kawai (1977b). Wimpenny & Cole (1967) suggested that the redox potential of the medium rather than the presence of a specific TEA, regulates enzymes involved in energy metabolism and electron transport pathways. However, the mechanisms involved are not fully understood. De Groot & Stouthamer (1970a,b) have proposed that the factor regulating the synthesis of reductases is the oxidation/reduction state of the components of the respiratory chain. They suggested that when electron flow to a redox enzyme was impossible, repression of its

formation occurred, possibly due to the oxidised enzyme acting as its own repressor; this regulatory mechanism was called "autogenous regulation of gene expression". Evidence in support is provided from work with mutants of E. coli with an unidentified defect in the electron transport chain, where it was shown that nitrate reductase can be formed and can function in the presence of oxygen, if electron transport to oxygen is restricted. Accordingly, when the oxygen supply is limited, electron transport to oxygen and to nitrate can occur simultaneously (Stouthamer, 1976).

Nitrate did not repress the synthesis of TMAO reductase, but inhibited the activity of the enzyme in Alteromonas sp., NCMB 400. In the presence of both TMAO andnitrate, the final growth yield was equal to that on TMAO alone, and both TMA and nitrite were produced. Thus it appears that both electron acceptors were utilised, but it was not determined which compound was preferentially used or whether they were simultaneously reduced. In E. coli however, nitrate did not inhibit TMAO reduction but repressed the synthesis of the enzyme (Sakaguchi & Kawai, 1975c; Nishida & Kobayashi, 1953). Therefore, TMAO reduction was controlled by two different mechanisms depending on species: Alteromonas sp. showed post-translational control while E. coli used transcriptional control.

Castell & Snow (1951) also showed that nitrate inhibited TMAO reduction, but the extent of inhibition varied with different species. They noted that nitrate strongly inhibited TMAO reduction in bacteria which were very active nitrate reducers e.g. E. coli and A. putrefaciens, but it caused relatively little inhibition in bacteria that could not reduce or slowly reduced nitrate e.g. Achromobacter sp. This may be interpreted as the preferential use of a particular TEA, e.g. in fish spoilage bacteria, TMAO was used in preference to nitrate (Dyer, 1949) whereas the converse is probably true of E. coli. Such characteristics may be explained by the different mechanisms controlling induction and

repression in these organism and may also account for the so-called "sparing-effects" of oxygen and nitrate (Watson, 1939a,b).

Fumarate was found to repress TMAO reductase in whole cells of Alteromonas sp., NCMB 400, (Fig. 16) and it was also shown to support the anaerobic growth of several other alteromonads (Table 5). Thus fumarate may be of greater biological importance to these organisms than TMAO. However, during fish spoilage, it is unlikely that fumarate would be present to cause repression of TMAO reductase.

In the presence of nitrate and TMAO, or fumarate and TMAO, the specific activity of TMAO reductase was higher than that induced by TMAO alone (Fig. 16). These results were obtained using formate as the electron donor in the whole cell assay for TMAO reductase. It is known that formate dehydrogenase is a major component of both the nitrate and fumarate reduction systems (Haddock & Jones, 1977), and its involvement in TMAO reduction has been proposed by Sakaguchi & Kawai (1977a). Thus the presence of nitrate or fumarate might have led to the further induction of formate dehydrogenase, which could have resulted in an apparent increase in the rate of TMAO reduction due to the nature of the enzyme assay.

Although TMAO reductase and cytochrome c_{552} were both located in the periplasm of Alteromonas sp., NCMB 400, their induction patterns were different. TMAO induced the cytochrome under micro-aerobic conditions and repressed it under aerobic conditions. Nitrite reductase was found to have a similar induction pattern (Table 14). Both nitrite reductase and cytochrome c are located in the periplasm of other bacteria e.g. P. aeruginosa (Wood, 1978), and c-type cytochromes are thought to be involved with the electron transport chain to nitrite reductase (Wood, 1978; Stouthamer, 1980). Therefore, it seems unlikely that periplasmic cytochrome c_{552} is involved in the electron transport to TMAO reductase in Alteromonas sp., but unequivocal data has yet to be obtained.

The membrane bound cytochrome c_{552} of the sphaeroplasts was induced by TMAO under aerobic and micro-aerobic conditions, and repressed by oxygen. This induction pattern was very similar to that of TMAO reductase, which might suggest that this cytochrome was involved in the reduction of TMAO by Alteromonas sp., NCMB 400. In E. coli it was shown that membrane bound haem c and TMAO reductase were simultaneously induced (Sakaguchi et al., 1979) and that cytochrome c_{552} was oxidised by TMAO (Ishimoto & Shimokawa, 1978). Sakaguchi et al. (1979) then proposed that cytochrome c constituted part of the electron transport chain to TMAO reductase (see Fig. 1), such that under anaerobic conditions energy production could be coupled to TMAO reduction by oxidative phosphorylation. A similar explanation could be envisaged for Alteromonas sp., NCMB 400. The redox potential of the membrane bound cytochrome c_{552} would then be expected to be $< 0.13V$, the standard oxidation/reduction potential of the TMAO/TMA couple (Castell, 1950), but no such measurements have been made.

4.5 Properties of TMAO reductase

The properties of TMAO reductase from Alteromonas sp., NCMB 400, were investigated using resting cell suspensions, the periplasmic fraction and a partially purified preparation from affinity chromatography. The results obtained were in close agreement.

The viologen dye assay was used to assay TMAO reductase from various cell fractions, and the results were comparable to those determined by the chemical assay of TMA. The reaction rate was shown to be dependent on both the enzyme and substrate concentrations i.e. typical enzyme kinetic results, but the overall rate of decolourization of $MV^{\cdot +}$ was not linear. Similar observations were made by Thorneley (1974) who

suggested that the oxidation of $MV^{\cdot +}$ by air exhibited second order kinetics that might be composed of a slow-fast sequence of two successive, one equivalent electron transfer reactions. This proposal may also account for the rate of $MV^{\cdot +}$ oxidation observed during TMAO reduction.

The pH optimum of TMAO reductase was estimated to be pH 6.8-6.9 which is slightly lower than that reported for other bacteria. Unemoto et al. (1965), Tomizawa (1951) and Castell & Snow (1951) reported values between pH 7.0 - 7.5. The variation can probably be explained as the differences in test organism, and the culture and assay conditions used, but all values were near neutrality.

The TMAO reductase of Alteromonas sp., NCMB 400, was found to have a high affinity for its substrate TMAO since the Km_{TMAO} was very low, 93-150μM. In E. coli and V. parahaemolyticus, the Km_{TMAO} was 1.5-1.7mM (Shimokawa & Ishimoto, 1979; Unemoto et al., 1965), i.e. ca. 10 fold higher than that of the alteromonad. The higher affinity for TMAO by Alteromonas sp., NCMB 400, may reflect a greater dependence on this mode of anaerobic growth, since it is non-fermentative.

The ability of TMAO reduction to support anaerobic bacterial growth, the lack of end product inhibition, the low Km_{TMAO} and the inhibition of enzyme activity by cyanide and azide suggest that TMAO reductase is a respiratory enzyme. HOQNO inhibited TMAO reduction in Alteromonas sp., NCMB 400,and E. coli (Sakaguchi & Kawai, 1978a). It is known to inhibit electron transport at or near b-type cytochromes in respiratory chains (Heinen, 1972), and Alteromonas sp., NCMB 400,was shown to contain cytochrome b_{560}(Fig.17). Sakaguchi & Kawai (1977a) and Sakaguchi et al. (1979) obtained similar results with E. coli, and suggested that the b-type cytochrome may function as an electron carrier in the respiratory chain to TMAO reductase (see Fig. 1). A similar mechanism could be envisaged for Alteromonas sp. As several other N-oxides were capable of inhibiting TMAO reduction, it is possible that HOQNO is a competitive inhibitor of TMAO reductase. Alternatively the enzyme may

have a broad substrate specificity as proposed by Tarr (1940). The TMAO reductase preparation purified by affinity chromatography was found to contain two activities i.e. both TMAO and adenosine-N-oxide supported the oxidation of the viologen dye. Sagai & Ishimoto (1973) and Shimokawa & Ishimoto (1979) obtained similar results with E. coli and they purified a tertiary amine-N-oxide reductase which reduced several N-oxides including TMAO and adenosine-N-oxide. Therefore, the enzyme responsible for TMAO reduction in Alteromonas sp., NCMB 400, may also be described as a non-specific tertiary amine-N-oxide reductase.

Nitrite has been shown to be an effective preservative of herring, however, it was also reported that $NaNO_2$ exerted little or no bacteriostatic action but suppressed the formation of TMA (Tarr, 1954). Nitrite was shown to inhibit TMAO reduction in Alteromonas sp., NCMB 400, but its mode of action was unknown. Dyer (1949) suggested that inhibition by nitrite was due to enzyme inactivation by free nitrous acid. Rake & Eagon (1980) have shown that nitrite affected the permeability of protons through the cytoplasmic membrane of Pseudomonas spp. It has been suggested that TMAO reduction is coupled to oxidative phosphorylation, which can be explained in terms of proton translocation by the chemiosmotic hypothesis. Therefore by reducing proton permeability and translocation in Alteromonas sp., nitrite may have inhibited TMA production. Similarly, the effect of nitrite could be regarded as indirect evidence for the coupling of oxidative phosphorylation to TMAO reduction.

Tris-HCl, tetramethylammonium chloride and other compounds containing the tris-(hydroxymethyl) group inhibited TMAO reduction, probably due to their structural similarities with TMAO causing steric hindrance at the active site of the enzyme.

Cyanide has been shown to inhibit TMAO reduction in several bacteria but the extent of inhibition varies between species. For example at 1mM

KCN, little inhibition (0-15%) was observed in Alteromonas sp., NCMB 400 (Table 17, 18 and 23) and V. parahaemolyticus (Unemoto et al., 1965), but in E. coli and Pr. vulgaris there was 25-100% inhibition (Tomizawa, 1951; Sakaguchi & Kawai, 1977a). Cyanide acts by chelating metal ions such that enzymes and cytochromes containing haem are inhibited by the complexing of Fe^{2+} (Lehninger, 1975). This would suggest that some strains are cyanide resistant or, metal ions and haem do not play an important role in TMAO reduction. It is interesting to note that both TMAO reductase and NADH oxidase of Alteromonas sp., NCMB 400, were relatively resistant to cyanide in comparison with other bacterial respiratory enzymes e.g. cytochrome oxidases of Paracoccus denitrificans (Stouthamer, 1980).

The effect of storage on the activity of periplasmic TMAO reductase was examined so that losses during purification and storage could be minimized. The enzyme was most stable at low temperatures and neutral pH, and under these conditions activity loss could be further prevented by the addition of TMA, Tris-HCl and TMAO to the periplasm which may stabilize the active site of the enzyme by their structural similarities. Glycerol and ethylene glycol markedly reduced the activity loss suggesting that TMAO reductase might be a hydrophobic protein, stabilized by non-polar solvents. Membrane bound proteins are also hydrophobic, and this may be further evidence for the membrane association of TMAO reductase. Similarly, the enzyme may be expected to contain a high proportion of hydrophobic amino acid residues e.g. alanine, valine, leucine, isoleucine, methionine, phenylalanine, tryptophan and proline (Lehninger, 1975). The enzyme also contains thiol groups since glutathione and dithiothreitol stabilized it, and p-chloromercuribenzoate inhibited activity. Unemoto et al. (1965) reported similar results for V. parahaemolyticus.

The ability of various compounds to support TMAO reduction in resting cell suspensions was investigated in Alteromonas sp., NCMB 400. Pyruvate, lactate and formate were the best electron donors. Watson (1939b) obtained similar results with fish spoilage bacteria. Glucose and glycerol are both known to support anaerobic growth and nitrate reduction in E. coli, but they were poor donors of TMAO reduction in the alteromonad. Formate is considered to be the principal physiological electron donor for other anaerobic respiratory systems e.g. nitrate and fumarate reduction (Wimpenny & Cole, 1967; Kröger, 1978; Haddock & Jones, 1977), and its involvement in TMAO reduction has been demonstrated using Alteromonas sp., Pr. morganii (Unemoto et al., 1964) and E. coli (Sakaguchi & Kawai, 1977a). Therefore, formate may also be the major electron donor for the in vivo reduction of TMAO. Pyruvate was a better donor than formate in whole cells. It may act indirectly as an electron donor possibly by yielding endogenous formate via the phosphoroclastic reaction; this system might also produce more energy via substrate level phosphorylation from acetyl phosphate, as originally suggested for nitrate reduction by Wimpenny & Cole (1967). The other good donors in Table 15, may also be metabolised to pyruvate and produce endogenous formate for the reduction of TMAO.

NADH was a poor electron donor and no activity could be detected with NADPH in Alteromonas sp. Unemoto et al. (1964) reported similar results for Pr. morganii, but Sakaguchi & Kawai (1976) showed that both NADH and NADPH were effective donors in E. coli. The poor activity of NADH:TMAO oxidoreductase in Alteromonas sp., NCMB 400, might be a consequence of its loose association with the cytoplasmic membrane. NADH is assumed to donate electrons to the electron transport chain via NADH dehydrogenase which is located in the membrane; therefore separation of the reductase from the membrane would decrease the NADH: TMAO oxidoreductase activity.

The substrates classified as poor and moderate electron donors for TMAO reduction in Table 15 were also oxidised by soluble NAD(P)-linked dehydrogenases (Lee, 1973). It is possible that NAD(P)H was the effective electron donor for such substrates, and that the oxidation of NAD(P)H itself was either the rate limiting step or it was a poor electron donor (as discussed above). Therefore formate and NADH are probably the principle physiological electron donors for TMAO reduction in Alteromonas sp., NCMB 400.

It has already been mentioned that TMAO reductase may resemble nitrite reductase due to its periplasmic location and the involvement of c-type cytochromes. Both enzymes use the same electron donors, formate and NADH, and it is possible that they operate by a similar mechanism. Pope & Cole (1981) showed that a membrane potential was formed during the reduction of nitrite using formate as the donor, and suggested that this could be coupled to energy conservation via oxidative phosphorylation. Cole (1981) suggested that since NADH:nitrite reductase was not membrane bound, then it could not be coupled to oxidative phosphorylation, but probably acts as an electron sink for the regeneration of reducing equivalents. It is interesting to note that NADH:nitrite reductase requires a flavoprotein (FAD) for full activity (Jackson, Cornish-Bowden & Cole, 1981). A similar explanation may account for the poor activity of NADH:TMAO oxidoreductase.

4.6 Purification of TMAO reductase.

Proteins of the periplasmic fraction from Alteromonas sp., NCMB 400, were separated to purify TMAO reductase and determine its properties. Historically, the precipitation of proteins by $(NH_4)_2SO_4$ has been widely used for the purification of single, globular proteins. The gradual

precipitation of TMAO reductase over a fairly wide range of $(NH_4)_2SO_4$ concentrations (40-80% saturation) suggests that the enzyme was composed of several proteins that were salted out at different ionic strengths. Alternatively, TMAO reductase may not be a pure protein but may be a lipoprotein, an attractive possibility in view of its association with the cell membrane, since lipid moieties would increase the solubility of certain proteins in the hydrophobic membrane. Formate dehydrogenase is a membrane bound enzyme which was purified by Linnane & Wrigley (1963) as a soluble complex with cytochrome b_1 using deoxycholate and $(NH_4)_2SO_4$. A similar method may also be useful for the purification of TMAO reductase.

Gel filtration separates proteins on the basis of their size and the rapid elution of TMAO reductase from the gel used suggested that it was very large, perhaps 10^5 - 10^6 daltons. Two peaks containing TMAO reductase activity were eluted, which suggests that either there were two enzymes present or that the enzyme was easily dissociated. The latter may be the more plausible in view of the implications raised from the poor precipitation by $(NH_4)_2SO_4$ but multiple enzyme forms are known e.g. the tertiary amine-N-oxide reductase (Shimokawa & Ishimoto, 1979).

Affinity chromatography is a powerful method of protein purification. It utilizes the biospecificity of macromolecules, and can give purification yields ranging from 10 to 10^3, often in a single step (see Cuatrecasas & Anfinsen, 1971). An affinity matrix was designed so that the immobilised ligand was similar to TMAO i.e. the $(CH_3)_3N$ group was available for enzyme attachment. TMAO reductase was strongly bound to the affinity matrix (as suggested by its low Km_{TMAO}), and could only be eluted from the column by treatment with NaCl, a chaotropic agent that reduces hydrophobic interactions. Two other proteins were eluted with the reductase suggesting that they may be part of a TMAO reductase complex which was dissociated by NaCl. However, they did not contribute to the enzyme activity. One of these proteins was identified as cytochrome

c_{552} and it is interesting to speculate that TMAO reductase and the cytochrome may be co-purified, an attractive possibility in view of the proposal that cytochrome c comprises part of the electron transport chain to TMAO reductase (Sakaguchi et al., 1979; see Fig. 1). Alternatively, the presence of cytochrome c_{552} and the unidentified protein may be accounted for by non-specific protein-protein interaction.

Some TMAO reductase activity was not bound to the affinity matrix and was lost. A similar elution pattern during affinity chromatography was reported by Izzo & Gantt (1977). Cuatrecasas & Anfinsen (1971) suggested that if very crude enzyme solutions with total protein concentrations greater than 20mg ml^{-1} are passed through affinity columns some of the enzyme escapes, probably because of interaction with the major protein fraction. A similar explanation may account for the results obtained from both affinity chromatography and gel filtration.

TMAO reductase was purified 18 fold in a single step using affinity chromatography and the yield was 47%. This may be considered small when compared with other examples of affinity chromatography, but Turner (1981) points out that purification to homogeneity in a single step is rarely achieved in practice. The purification of TMAO reductase is similar to that of other terminal reductases e.g. Enoch & Lester (1975) obtained a 30 fold purification of nitrate reductase with only a 20% yield in enzyme activity using 6 purification steps. MacGregor, Schnaitmann & Normansell (1974) also purified nitrate reductase and obtained a 112 fold purification with a 5.2% yield in 6 steps. Coleman, Cornish-Bowden & Cole (1978) obtained a 30 fold purification of nitrite reductase but Jackson et al. (1981) modified the method and obtained a purification of 121 fold with a much higher yield (20% compared with 0.4%) and specific activity. Therefore the purification of TMAO reductase is not dissimilar to that of other anaerobic respiratory enzymes, although the method involved only 2 steps. Optimization and/or

modification of the purification procedure together with other chromatographic techniques should increase the purity and yield of TMAO reductase.

The biophysical properties of the TMAO reductase preparation obtained from affinity chromatography were investigated using electrophoretic techniques that separated proteins on the basis of charge and/or size. The preparation contained 3 major proteins and 4-6 minor proteins, all had low isoelectric points (pH 4.5-5.5), but 2 showed TMAO reductase activity (proteins A + B). The pI of the two active proteins were probably 5.45 and 5.50 and consequently they could not be distinguished in the zymogram stain after isoelectric focusing. However they were clearly separated by PAGE suggesting that their molecular weight were significantly different, as confirmed by SDSPAGE: protein A had a minimum molecular weight of ca 350 k daltons and protein B was ca 250 k daltons. It is interesting to note that the results from ion exchange chromatography suggested that periplasmic TMAO reductase **had pI$<$7.0 and it is unlikely that there was a TMAO reductase with pI = 9.0. Therefore the faint zone of decolorisation observed near the cathode (corresponding to pI = 9.0) after isolectric focussing was probably an artefact.**

MacGregor et al. (1974) showed that during the purification of nitrate reductase from E. coli, protease activity caused the appearance of an additional protein subunit. A similar explanation might account for the presence of 2 TMAO reductase proteins. However, this is unlikely since both proteins A and B are present in the sonicate, the periplasm and the purified preparation, and they have largely different molecular weights. In addition the protease activity of E. coli was activated by an alkaline-heat treatment which was not used for the purification of TMAO reductase from Alteromonas sp. TMAO reductase may therefore be a multimeric enzyme consisting of two active proteins and the other protein

components of the preparation probably represent impurities. Shimokawa & Ishimoto (1979) reported similar results for a tertiary amine-N-oxide reductase that also reduced TMAO. Their enzyme consisted of 4 forms, one of which was constitu tive and the others being induced by TMAO.

The subunit composition and molecular weight of the TMAO reductase preparation was determined by SDS PAGE. It contained 4, or possibly 5, subunits with molecular weights of ca 98, 86, 72, 53 (and possibly 42.5) k daltons; the largest subunit was stained most densely and the total molecular weight was estimated to be 400-450 k daltons (as predicted from the results from gel filtration). Subunits from both proteins A and B were present in the purified preparation. If TMAO reductase in vivo contains both proteins, then its molecular weight would be 600 k daltons and it might be composed of 6 different subunits in a ratio of 2:2:1:1:1:1. However, the results obtained were not ideal and need to be repeated with samples of higher protein concentration.

The loss of enzyme activity during purification, particularly that of protein B (see Plate 2), was probably due to non-specific protein denaturation. The chaotropic effect of NaCl may also have caused some loss by dissociating the TMAO reductase complex, and may also account for the molecular weight of the TMAO reductase preparation being 400-450 k daltons instead of ca 600 k daltons.

If TMAO reductase has a molecular weight of 400-450 k daltons, it would be considered a large molecule in comparison with other periplasmic enzymes. Ames (1974) showed that periplasmic proteins generally have molecular weights of 10 - 150 k daltons. Alkaline phosphatase is the periplasmic enzyme studied most and its molecular weight is 80-89 k daltons (Simpson et al. 1968). Nitrite reductase is also periplasmic but its molecular weight has been estimated to be ca 190 k daltons (Coleman et al., 1978).

Therefore, on the basis of its molecular weight, TMAO reductase would not be considered a typical periplasmic enzyme.

The literature does not contain any reports on the purification of TMAO reductase. Sagai & Ishimoto (1973) and Shimokawa & Ishimoto (1979) have purified a tertiary amine-N-oxide reductase from E. coli, which reduces TMAO,and its properties are very similar to those of the TMAO reductase from Alteromonas sp. The properties are compared in Table 24, the major differences being the wider substrate range of the enzyme from E. coli. The enzymes of both organisms were induced by TMAO and repressed by oxygen. Adenosine-N-oxide was reduced by both enzymes but it did not act as an inducer. In E. coli the tertiary amine-N-oxide reductase was repressed by nitrate, and Sakaguchi & Kawai (1975c) obtained similar results for TMAO reductase. Since TMAO is considered to be the natural substrate for these 2 enzymes, and because of their striking similarities, they can be regarded as the same and should be called TMAO reductase. The significance of the ability to reduce several other N-oxides is not known but it may be a fortuitous property serving no physiological function.

Inorganic N-oxides are also reduced by bacteria and it is interesting to compare their reductases with that of TMAO reductase. The nitrate and nitrite reductase systems have been extensively studied since they support anaerobic bacterial growth, and provide a method of assimilating nitrogen (for reviews see Payne, 1973; Thauer, Jungerman & Decker 1977; Haddock & Jones, 1977). Nitrate reductase is a large membrane bound enzyme complex, consisting of 3 subunits (142, 60 and 19.5 k daltons) in a ratio of 1:1:1 and is thought to be a tetramer with a total molecular weight of 800-1000 k daltons (MacGregor, 1975). The smallest subunit is an apoprotein containing cytochrome b_{556} which forms part of the electron transport chain to nitrate reductase. The reductase is a molybdoprotein that also contains an Fe/S protein and is inhibited

TABLE 24 : Comparison of the N-oxide reductases from *Alteromonas* sp. and *E. coli*.

Characteristic	TMAO reductase of *Alteromonas* sp., NCMB 400	Tertiary amine-N-oxide* reductase of *E. coli*
Substrate specificity	Narrow	Wide
Inhibitors	Several N-oxides, Cyanide, Azide, HOQNO, p-chloro-mercuribenzoate	Few N-oxides, Cyanide, Azide, Tungstate, Cu^{++}; chelating agents
Inducers	TMAO	TMAO
Repressors	Oxygen	Oxygen and nitrate
Location	Periplasmic aspect of cytoplasmic membrane	Loose association with cell membrane
Properties of the purified enzyme		
No. of enzymes	2 (*ca.* 350 and 250 k daltons)	4 (220, 70, 70, 100 k daltons)
Molecular weight	400-450 (possibly 600) k daltons	440 k daltons
No. of subunits	possibly 4-6 ranging from 40 to 110 k daltons	2; 60 and 80 k daltons (for the largest enzyme form)
pI	5.45, 5.50	5.2
pH optimum	6.8 - 6.9	5.3
Km_{TMAO} (mM)	0.15	1.5

* Sagai & Ishimoto, 1973; Shimokawa & Ishimoto, 1979.

by tungstate, cyanide, azide, p-hydroxymercuribenzoate and oxygen (Enoch & Lester, 1975). TMAO reductase is affected by similar inhibitors, and although it is smaller than nitrate reductase, it possibly has a tenuous association with the membrane. Cytochrome c is probably involved in the electron transport chain to TMAO reductase, and in E. coli the reductase is thought to be a molybdoprotein (Shimokawa & Ishimoto, 1979).

Nitrite reductase is not membrane bound but is linked to the membrane via c-type cytochromes (Wood, 1978). The cytochrome c_{552} of nitrite reductase is not a firmly bound apoprotein, and the reductase is composed of two subunits (80 and 88 k daltons) and its molecular weight is ca. 190 k daltons (Coleman et al., 1978). Thus TMAO reductase has similar characteristics and properties to the inorganic N-oxide reductases. Its function also appears to be similar i.e. to catalyse the transfer of electrons to a TEA in such a way that energy is obtained to support anaerobic bacterial growth.

4.7 General discussion

It has been established that during anaerobic bacterial growth, TMAO is reduced to TMA suggesting that it is not used as a carbon and/or nitrogen source but acts as a terminal electron acceptor. Formate is the principal physiological electron donor for TMAO reduction. From calculations based on standard free energy changes (Table 25), it is thermodynamically possible to couple TMAO reduction to the production of ATP. TMAO reductase is located external to or on the outer aspect of the cytoplasmic membrane, and TMAO induces c-type cytochromes in the membrane which could be oxidised by the reductase. The TMAO reductase system has properties of a respiratory pathway in which electron transport could be coupled to oxidative phosphorylation. Therefore,

TABLE 25 : Standard oxidation/reduction potential of some electron donors and acceptors, and the potential for ATP production.

Redox Couple	Eo' (V)	Standard free energy change (ΔG°) using formate as the electron donor and various TEA*		
		TEA*	ΔG° ($kJmol^{-1}$)	Potential** for ATP formation (mol)
CO_2/Formate	- 0.42			
NAD^+/NADH	- 0.32	Nitrate	- 163	3
Pyruvate/lactate	- 0.19	Fumarate	- 86	1 - 2
Fumarate/Succinate	+ 0.03	TMAO	- 109	2
TMAO/TMA	+ 0.13			
NO_2/NO	+ 0.35			
NO_3/NO_2	+ 0.42			
O_2/H_2O	+ 0.82			

* TEA; Terminal electron acceptor

** Estimation base on ΔG ATP formation from ADP being +44 $kJmol^{-1}$ (Thauer et al., 1977)

(see Castell, 1950; Thauer et al., 1977; Yamamoto & Ishimoto, 1977; Strøm, Olafsen & Larsen, 1979).

TMAO reduction probably provides a method of anaerobic respiration. In the chemiosmotic hypothesis, the mechanism of energy coupling is explained in terms of a proton gradient but measurement of proton translocation during TMAO reduction has not yet been done.

The electron transport chain from formate to TMAO is probably composed of formate dehydrogenase, membrane bound cytochrome c_{552} and TMAO reductase. In addition, NADH and b-type cytochrome(s) may be involved. This electron transport system is similar to that proposed for E. coli by Sakaguchi et al. (1979) (see Fig. 1). In the alteromonad TMAO reductase was found in the periplasm, and has similar properties to nitrite reductase, in respect of the distribution and composition of electron transport components (Fig. 2), and energy conservation. Electron transfer from formate to TMAO may be coupled to oxidative phosphorylation but that from NADH may not be energy linked, in which case, TMAO may act as an electron sink.

The periplasmic location of TMAO reductase may be advantageous since the end product, TMA, is strongly basic and is free to diffuse away from the cytoplasm to which it may be toxic.

The reduction of TMAO to TMA is electrogenic, whereas the reduction of oxygen, nitrate, nitrite and fumarate is electroneutral. The chemiosmotic hypothesis states that the proton motive force ($^{\Delta}P$) can be a function of $^{\Delta}pH$ exclusively, of $^{\Delta}\psi$ exclusively or a combination of both (Haddock & Jones, 1977). If proton translocation occurs with TMA production, then both $^{\Delta}\psi$ and $^{\Delta}pH$ would be expected to contribute to the $^{\Delta}P$. Conversely, the electrogenic effect of TMA production may be sufficient to produce a $^{\Delta}P$ to which energy production could be linked. A possible mechanism for coupling energy conservation to formate:TMAO reduction in Alteromonas sp. is proposed in Fig. 33. It involves a membrane bound proton translocating formate dehydrogenase, linked to membrane bound c-type (and possibly b-type) cytochromes which carry

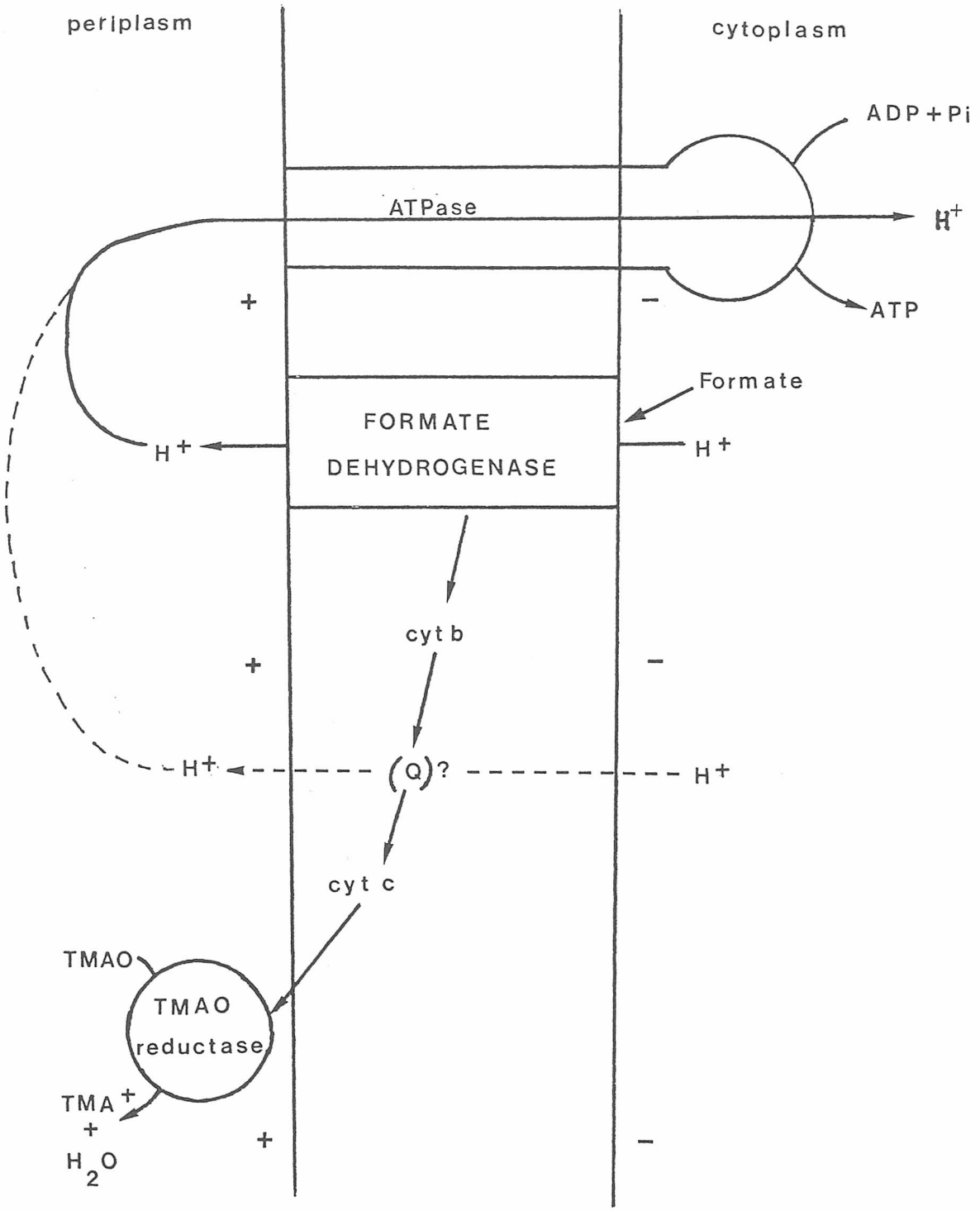


Figure 33 : Proposed mechanism for the coupling of energy conservation to TMAO reduction in Alteromonas sp. (For abbreviations see Fig.2)

electrons to TMAO reductase in the periplasm. The net effect in terms of the chemiosmotic hypothesis would be the generation of a ΔP consisting of a ΔpH and $\Delta\psi$ from which ATP could be produced *via* a membrane bound proton translocating ATPase. In other bacteria proton translocation is also thought to occur *via* quinones associated with b-type cytochromes in the electron transport chain to nitrate and fumarate (Haddock & Jones, 1977; Kröger, 1978), and to nitrite (Wood, 1978). It is not known whether the electron transport chain to TMAO contains quinones but their involvement might give rise to further proton translocation and an increase in ΔP. The model proposed (Fig. 33) does not take into account the electrogenic effect of TMA production which might increase $\Delta\psi$ and ΔP giving further energy conservation.

It is interesting to note that the anaerobic respiratory systems of some facultative anaerobes have similar electron transport components e.g. formate dehydrogenase and b-type cytochromes (see Thauer *et al.*, 1977; Haddock & Jones, 1977). This might suggest an evolutionary trend towards a branched electron transport chain (as proposed in Fig. 34), such that the organism could utilize a variety of TEA, so becoming more competitive in nature.

Castell (1946) and Ando & Inoue (1957) reported that some *Clostridium* spp. including *C. botulinum*, could reduce TMAO. However, they do not contain cytochromes (Jones, 1980), and so the mechanism of energy coupling in these strict anaerobes cannot be by electron transport and oxidative phosphorylation. Strøm, Olafsen & Larsen (1979) suggested that *Proteus* spp. contained 2 systems for TMAO reduction (i) an electron transport system from formate coupled to oxidative phosphorylation and (ii) a mechanism coupled to the oxidation of lactate to pyruvate which was not energy yielding. The latter method may account for TMAO

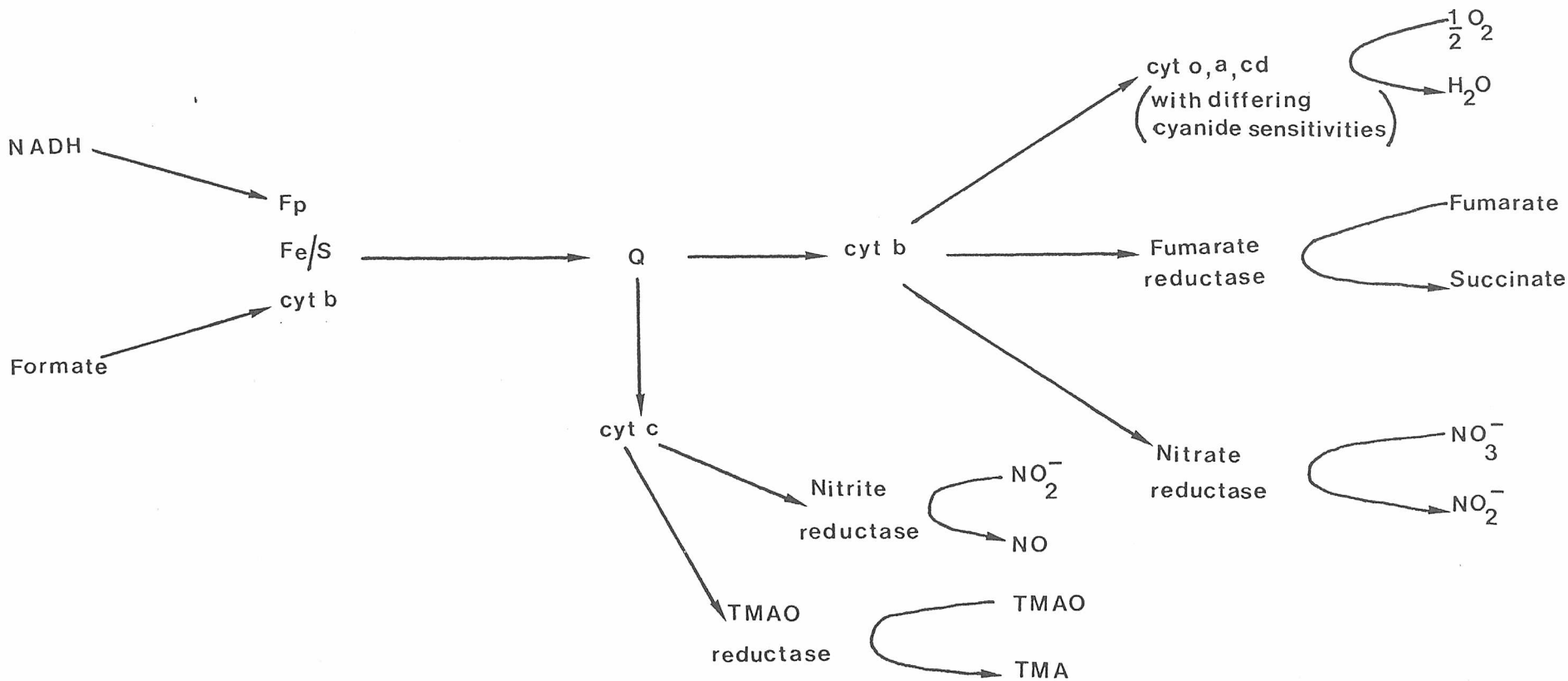


Figure 34 : A possible branched electron transport chain that might evolve in a highly competitive facultative anaerobe. (For abbreviations, see Fig.2).

reduction by Clostridium spp. and the energy for growth may have been obtained from substrate level phosphorylation by the fermentation of pyruvate. Alternatively, in clostridia, TMAO may be as an electron sink, and therefore it would be interesting to examine the reduction of TMAO in strict and facultative anaerobes.

The effect of nitrate on the control of TMAO reductase was different in E. coli and Alteromonas sp., NCMB 400. Nitrate repressed the reductase in E. coli and it was probably used in preference to TMAO. In Alteromonas sp. the reductase was not repressed by nitrate, and TMAO was probably reduced preferentially as shown for other fish spoilage bacteria (Dyer, 1949). Nitrate reduction has the potential to support more ATP production than the reduction of TMAO or fumarate (Table 25). and as it is energetically more favourable, it may contribute to the mechanisms of induction and repression. Yamamoto & Ishimoto (1977) showed that the molar growth yield of E. coli produced by the oxidation of formate was the same whether nitrate, fumarate or TMAO was used as TEA. They concluded that the energy yield was the same in all 3 cases and that there was probably a common site of ATP production e.g. between formate dehydrogenase and a cytochrome. In Alteromonas sp., nitrate did not repress TMAO reductase and supported less growth than TMAO or fumarate, suggesting that its reduction was not of great biological importance probably due to the toxicity of nitrite. Therefore, TMAO may have been used in preference to nitrate. Fumarate repressed TMAO reductase but the reasons for this were not known. It may be of greater biological importance than TMAO because the succinate produced might be a valuable source of carbon used for the biosynthesis of cytochromes and amino acids such as lysine, threonine, methionine and diaminopimelic acid. (Gest, 1981).

Shewan (1962) suggested that the accumulation of TMA in fish was fortuitous, but that it could be used as an indicator of fish freshness and quality. However, the work presented here shows that TMAO has an important physiological function in certain spoilage organisms. In the initial stages of spoilage bacterial growth would be aerobic, but as bacterial numbers increase, so the demand for oxygen would not be met resulting in localised anaerobiosis. Under such conditions, TMAO reductase would be induced and TMAO reduction would support anaerobic growth resulting in the accumulation of TMA. The TMAO reducers of the flora would therefore have a competitive advantage. Thus TMA production has significance in the spoilage of marine fish. The ability of TMAO to support anaerobic bacterial growth may explain the relative perishability of marine fish. Further studies may lead to a method for prolonging the shelf life of chilled marine fish.

4.8 Future Work

TMAO reduction was studied in bacteria typically associated with fish spoilage i.e. Alteromonas sp., and such investigations were performed at the optimum temperature for growth (20°C). The spoilage of iced marine fish occurs at ca. 0°C, and it was assumed that the properties of such spoilage organisms were the same at temperatures near freezing and at 20°C. Confirmatory experiments should be carried out at both temperatures and using fresh isolates that possess TMAO reductase activity from spoiling fish.

Yamamoto & Ishimoto (1977) and Kim & Chang (1974) have shown that TMAO reduction can support anaerobic bacterial growth and suggested that it may be analogous to the reduction of nitrate. Kim & Chang (1974) have shown that mutants lacking nitrate reductase also lack TMAO reductase suggesting that the two systems had similar components and/or genetic loci. However, no such studies have been performed with Alteromonas spp. and confirmation is required. The genetics and biochemistry of the nitrate reductase system have been studied extensively using chlorate resistant mutants of E. coli and are reviewed by Haddock & Jones (1977). Proposals have been made that the gene products of such mutants e.g. chl A, B and D code for the components of the molybdoprotein of formate dehydrogenase and nitrate reductase, and chl C codes for the nitrate reductase apoprotein (see Begg, Whyte & Haddock, 1977). A similar mechanism may be involved in TMAO reduction, and comparative studies should yield more information regarding the components and control of the system for TMAO reduction.

TMAO reduction supports anaerobic bacterial growth and it is currently believed that TMAO acts as a terminal electron acceptor. It is not known whether TMAO is an electron sink for the regeneration of reducing equivalents, or whether its reduction can be linked to

energy conservation (via oxidative phosphorylation) thus providing a method of anaerobic respiration. The evidence to date suggests that TMAO reduction probably provides a method of anaerobic respiration. The translocation of protons out of the cell and/or the generation of a membrane potential are considered to be evidence for the existence of a respiratory system, and the measurement of such parameters during TMAO reduction should be carried out. Proton translocation has been demonstrated during the reduction of nitrate and fumarate (see Haddock & Jones, 1977; Kröger, 1978), and the generation of a membrane potential has been observed during the reduction of nitrite (Pope & Cole, 1981). The TMAO reductase system has similar properties to nitrite reductase and similar mechanisms may be involved. If so, TMAO might act as an electron sink when NADH is the electron donor, and have a respiratory function when formate is the electron donor (see Cole, 1981; Pope & Cole, 1981), but confirmation is required. Information regarding the respiratory role of TMAO may also be obtained from studies using electron transport inhibitors and uncoupling agents. A respiratory function has been ascribed to TMAO since its presence increased the molar growth yields of *E. coli* (Yamamoto & Ishimoto 1977), *Proteus* sp. (Strøm, Olafsen & Larsen, 1979) and *S. typhimurium* (Kim & Chang, 1974) grown under anaerobic conditions. Similar studies should also be carried out with *Alteromonas* spp.

If TMAO reduction provides a method of anaerobic respiration, then the components of the electron transport chain to TMAO reductase should be determined. The work so far indicates that c-type (and possibly b-type) cytochromes are involved in TMAO reduction, and substantiation is necessary. Formate was one of the best donors supporting TMAO reduction in whole cells and it was assumed that electrons were donated to the electron transport system *via* formate dehydrogenase. The presence of this enzyme in *Alteromonas* sp. should be determined

and its involvement in TMAO reduction established. The latter may be achieved by the selection of TMAO reducing mutants lacking formate dehydrogenase as described for nitrate reducing strains of E. coli (Mandrand-Berthelot, Wee & Haddock, 1978; Begg et al., 1977).

Pyruvate was a better donor than formate in the whole cell assay for TMAO reduction, and it was suggested that this was due to the production of endogenous formate via the phosphoroclastic reaction i.e. pyruvate formate-lyase. This enzyme is present in E. coli, and like TMAO reductase, it operates under anaerobic conditions (Knappe, Blaschkowski, Gröbner & Schmitt, 1974), but its presence in Alteromonas spp.has yet to be demonstrated.

Strøm, Olafsen & Larsen (1979) suggested that there were two systems for TMAO reduction in Proteus spp. - one linked to the oxidation of lactate, and the other to formate oxidation, of which only the latter was linked to energy production. If this were true for Alteromonas sp. then the rate of TMAO reduction using lactate as the donor, might be expected to be twice that supported by pyruvate or formate. The rate of TMAO reduction in the presence of lactate, pyruvate or formate was very similar, therefore it seems unlikely that there are two methods for TMAO reduction in Alteromonas sp. However, further work is required to establish the existence of one or two systems for TMAO reduction, and such studies may involve the comparison of (i) TMAO reduction rates, (ii) molar growth yields and the amount of TMA produced and (iii) TMAO reduction in mutants lacking formate dehydrogenase.

During cell fractionation it was noted that exposure of the cells to magnesium ions and EDTA were important factors in controlling lysis and sphaeroplast formation. EDTA was also suspected of having a disruptive effect on the cell membrane which may have influenced the release of TMAO reductase. Therefore it would be interesting to examine the cellular location of TMAO reductase using milder conditions for sphaero-

plast formation to determine whether the enzyme has a weak association with the cell membrane. Useful techniques may include the modified method of Costerton et al. (1967) (method C, section 2.6.1.) or a modification of the method of Cheng et al. (1971). TMAO reductase was shown to be membrane-bound in E. coli and V. parahaemolyticus and its periplasmic location in Alteromonas sp., NCMB 400, was unexpected. It is possible that this alteromonad was atypical and the cellular location of TMAO reductase should be examined in other strains of Alteromonas spp.

TMAO reductase was partially purified by affinity chromatography and further work is required for the full characterization of the enzyme. The purity of the TMAO reductase preparation may be increased by using other techniques such as ion exchange chromatography and gel filtration, in addition to affinity chromatography. It was suggested that the enzyme may be a hydrophobic protein, and it is possible that hydrophobic interaction chromatography might be a useful technique for its purification. The identification and characterization of the protein components of TMAO reductase would also be useful for determining the proteins coded for by the TMAO reductase gene(s). This could be achieved by comparing the electrophoretic pattern of the enzyme proteins isolated from wild-type strains and mutants lacking TMAO reductase.

The ability of the preparation purified by affinity chromatography to reduce both TMAO and adenosine-N-oxide still raises the possibility that the enzyme is non-specific. Several N-oxides are reduced by the enzyme from E. coli (Shimokawa & Ishimoto, 1979) and the xanthine oxidase from liver and milk can reduce nicotinamide-N-oxide (Bickel, 1969). The significance of such N-oxide reduction(s) is not known, but it is interesting to note that the enzymes concerned have similar properties i.e. they are linked to flavoproteins, contain iron and molybdenum and have a molecular weight of ca. 300k daltons (Shimokawa &

Ishimoto, 1979; Lehninger, 1975). It is possible that there is a relationship between N-oxide reduction, the metabolism of purines and anaerobic respiration worthy of further investigation.

Further studies on TMAO reduction and its inhibition may result in the development and/or discovery of compounds that may be useful as preservatives of iced marine fish. Such compounds are likely to be competitive inhibitors and will probably resemble the structure of TMAO e.g. they may contain tri- or tetra-methyl moieties, or possibly tris-(hydroxymethyl) groups. Substances affecting membrane structure and/or permeability may also inhibit TMAO reduction, but they are also likely to be toxic to the consumer.

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