Trimethylamine Oxide: A Terminal Electron Acceptor in Anaerobic Respiration of Bacteria

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Trimethylamine oxide (TMAO) stimulated both the anaerobic growth rate and the growth yield of *Proteus* NTHC 153. The molar growth yield from glucose and pyruvate in tryptone/yeast extract medium doubled in the presence of TMAO, and the organism grew anaerobically on the non-fermentable substrates L-lactate and formate when TMAO was added to the medium. We conclude that TMAO stimulated growth by serving as a terminal electron acceptor in an oxidative phosphorylation process.

INTRODUCTION

Trimethylamine oxide (TMAO) is a constituent of marine animals, including both fish and invertebrates (Groninger, 1959; Yamada, 1967). *Post mortem*, TMAO is reduced by bacteria, notably Gram-negative rods, to trimethylamine (TMA) which gives rise to the characteristic off-flavour of marine fish in the early stage of decay (Beatty & Gibbons, 1937; Tarr, 1954). The bacteria can use a variety of hydrogen donors for TMAO reduction according to the general equation (Watson, 1939*a*, *b*; Unemoto *et al.*, 1964):

$$(CH_3)_3NO + AH_2 \longrightarrow (CH_3)_3N + A + H_2O$$
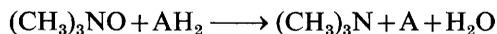
We have previously reported that TMAO stimulates the anaerobic growth of bacteria in fish muscle extracts (Strøm, 1971). Promotion of anaerobic growth of bacteria by TMAO has also been reported for *Salmonella typhimurium* (Kim & Chang, 1974), *Escherichia coli* (Yamamoto & Ishimoto, 1977) and *Rhodopseudomonas capsulata* (Madigan & Gest, 1978). We have now determined the growth yields of *Proteus* NTHC 153 under various conditions to determine whether TMAO can serve as electron acceptor for redox reactions coupled to energy conservation: if so, this would be a new example of the general phenomenon of anaerobic respiration.

METHODS

Organism and growth. Proteus NTHC 153 was isolated from an enrichment culture made by inoculating a small piece of herring gill into a sterile extract of herring muscle and incubating at 15 °C under anaerobic conditions until maximum bacterial growth was reached (Strøm, 1971; Strøm & Larsen, 1979). It was grown anaerobically at 25 °C on glass-stoppered culture tubes which were completely filled. The media contained (g l^{-1} in tap water): KH_2PO_4, 1·2; NH_4Cl, 0·8; $MgSO_4.7H_2O$, 0·08; NaCl, 2·4; tryptone (Difco), 1·6; yeast extract (Oxoid), 0·4; Tris, 5·6. When used, TMAO was added to give 50 mM. Solutions of D-glucose, sodium lactate, sodium pyruvate and sodium formate were sterilized by filtration and added to give the concentrations specified in the text. The pH was adjusted to 7·1 and 7·6 for media with and without TMAO, respectively. The media were inoculated with 2 % (v/v) of a culture actively growing in the same medium.

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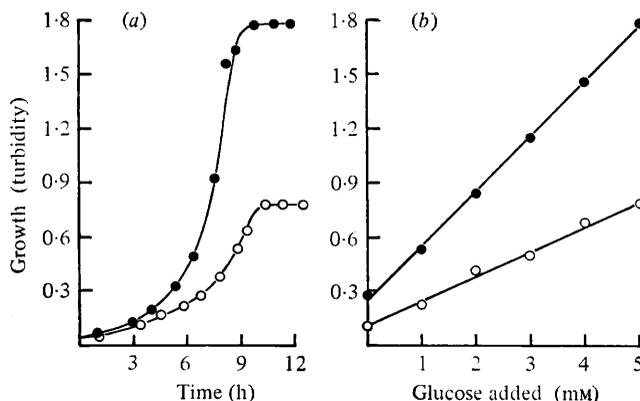
Fig. 1. Effect of TMAO on anaerobic growth of *Proteus* NTHC 153. The organism was grown at 25 °C in glucose/tryptone/yeast extract medium in the presence (●) or absence (○) of 50 mM-TMAO. (*a*) Growth with glucose at an initial concentration of 5 mM. (*b*) Maximum density with different initial concentrations of glucose.

Determination of glucose assimilation. To the above medium containing 5 mM-glucose, with or without TMAO, D-[U-^{14}C]glucose (194 mCi mmol^{-1}; The Radiochemical Centre, Amersham) was added to give 0·13 μM. The media, completely filling the glass-stoppered culture tubes, were inoculated with the organism and incubated at 25 °C. Soon after growth had ceased, bacteria were harvested by centrifuging (10000 *g* for 10 min, 4 °C), washed once with water and treated with formamide (Neujahr & Ewaldson, 1964). Scintillation liquid (Bray, 1960) was added, and the solution was counted in a Packard model 3375 Tri-Carb liquid scintillation spectrometer. Counts per minute were converted to disintegrations per minute by the channels ratio method.

Determination of growth yield. Growth was measured in an EEL portable colorimeter with filter no. 626. Dry weight was determined by transferring washed bacteria, resuspended in water, to a membrane filter (Sartorius SM11305) and drying at 105 °C. Cell nitrogen was determined by the Kjeldahl method.

Chemical analyses. Glucose, pyruvate and formate were assayed by colorimetric methods according to Bartlett (1959), Friedemann (1957) and Wood & Gest (1957), respectively. L-Lactate was determined by an enzymic method (Hohorst, 1962). Acetate was measured with a Varian Aerograph model 204 gas chromatograph equipped with a flame ionization detector and a 1·5 m × 3·2 mm (o.d.) stainless steel column filled with 10 % (w/w) neopentyl glycol succinate plus 2 % (w/w) H_3PO_4 on Firebrick, 60 to 80 mesh (Varian Aerograph). TMAO and TMA were determined by the microdiffusion technique (Conway & Byrne, 1933) and CO_2 by van Slyke's manometric method (N. N., 1956).

RESULTS

Growth on glucose

Proteus NTHC 153 grew more rapidly and to twice the final cell density in the presence of TMAO than in its absence (Fig. 1*a*). During exponential growth the generation time was 1·5 h in the medium containing TMAO, compared with 2·2 h in its absence. Estimations of dry weight and cell nitrogen indicated that growth was increased in the presence of TMAO by 84 and 87 %, respectively. These results support the contention that TMAO stimulated normal growth rather than the accumulation of metabolites such as reserve material.

In unsupplemented cultures, the pH decreased during growth, but in the presence of TMAO the pH increased due to the formation of TMA. In order to counteract the opposite pH changes the media were well buffered, and a lower initial pH was chosen for the cultures supplemented with TMAO (7·1 instead of 7·6). The pH values in fully grown cultures were 7·5 and 6·8, respectively. Since *Proteus* species grow profusely over a considerably wider pH range than this, it is unlikely that the stimulation of growth by TMAO was due to a pH effect.

Table 1. *Quantitative characteristics of the anaerobic growth of Proteus* NTHC 153
on various substrates in complex medium with or without trimethylamine oxide

Substrate and hydrogen acceptor	Molar growth yield [μg dry wt (μmol substrate)$^{-1}$]	Products [μmol (μmol substrate)$^{-1}$]			ATP formation at substrate level [μmol (μmol substrate)$^{-1}$]	Y_{ATP} [μg dry wt (μmol ATP)$^{-1}$]
		TMA	CO_2	Acetate		
Glucose	38·5	0	·	0·97	2+0·97 = 2·97	13·0
Glucose+TMAO	77·0	3·50	·	1·76	2+1·76 = 3·76	—
Pyruvate	5·9	0	0·38	0·76	0·76	7·8
Pyruvate+TMAO	14·7	0·94	0·89	0·75	0·75	—
L-Lactate+TMAO	13·0	1·82	0·83	0·77	0·77	—
Formate+TMAO	10·3	1·06	0·97	0	0	—

Complex media can provide many of the biosynthetic precursors required for bacterial growth and glucose is therefore used predominantly as an energy source. This was demonstrated in experiments with [U-14C]glucose in which it was established that 17 and 7%, respectively, of added glucose was assimilated by the bacteria in media with and without TMAO; the rest (83 and 93%, respectively) was presumably utilized in the dissimilation process.

Growth was glucose-limited up to at least 5 mM (Fig. 1b) and with this initial concentration only 40% of the TMAO had been reduced by the fully grown cultures. A small amount of growth occurred in the absence of glucose, presumably because nutrients in the complex medium served as energy sources (Fig. 1b).

For molar growth yield determinations (Table 1), glucose was used at an initial concentration of 5 mM. Corrections have been made for the 'background' growth in the absence of glucose and for the glucose assimilated. The former correction was based on the determination of turbidity in cultures with and without glucose (Fig. 1b). The molar growth yield from glucose doubled in the presence of TMAO. The quantities of acetate and TMA produced (Table 1) have been corrected for the amounts of products formed in the absence of added glucose. More acetate accumulated when TMAO was added to the glucose medium.

Enterobacteria ferment glucose by the glycolytic pathway in which two molecules of ATP are formed per molecule of glucose dissimilated to pyruvate (Wood, 1961). A further molecule of ATP is formed when acetate is generated from acetyl phosphate. For *Proteus* NTHC 153 the Y_{ATP} value, defined by Bauchop & Elsden (1960), was 13·0 during anaerobic growth with glucose (Table 1). Similar values have been found by other investigators for other strains of enterobacteria grown in similar media (Stouthamer, 1969, 1977; Forrest & Walker, 1971).

Growth on formate, lactate and pyruvate

To obtain more information about the bacterial reduction of TMAO, *Proteus* NTHC 153 was grown in anaerobic tryptone/yeast extract medium with excess of TMAO (50 mM) and various amounts of the non-fermentable substrates L-lactate and formate. For comparison, the anaerobic growth on pyruvate, with or without TMAO, was also measured. Pyruvate and L-lactate were growth-limiting at 20 mM (Fig. 2). When formate was the substrate, there was an approximately linear relationship between the final density of the culture and the initial concentration of formate up to about 10 mM. The organism could not utilize formate or L-lactate as a substrate for anaerobic growth in media without TMAO; D-lactate did not support anaerobic growth even in media containing TMAO.

In the determinations of the molar growth yields (Table 1), lactate and pyruvate were used at an initial concentration of 20 mM and formate at 10 mM. The actual amounts of substrate converted by the bacteria were determined but not the amounts of substrate assimilated. All results have been corrected for 'background growth' and dissimilation
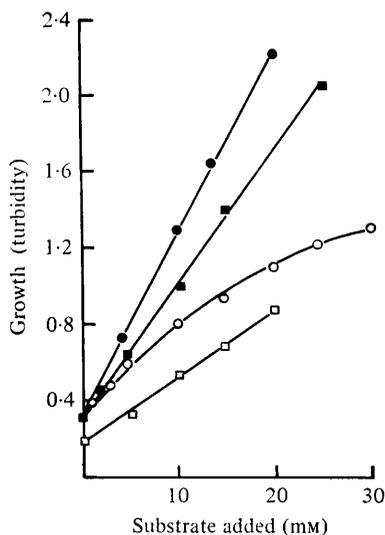
Fig. 2. Maximum density of *Proteus* NTHC 153 grown at 25 °C in tryptone/yeast extract basal medium containing: □, pyruvate; ■, pyruvate plus TMAO; ○, formate plus TMAO; ●, L-lactate plus TMAO. The initial concentration of TMAO was 50 mM.

products (acetate, CO_2 and TMA) formed in the absence of added substrate. The molar growth yield from pyruvate more than doubled in the presence of TMAO and was about the same as the molar growth yield from L-lactate in the presence of TMAO. From the results for growth on pyruvate in the absence of TMAO, a Y_{ATP} value of 7·8 was calculated.

The data for the conversion of formate fit the stoicheiometry of the equation:

$$(CH_3)_3NO + HCOOH \longrightarrow (CH_3)_3N + CO_2 + H_2O$$
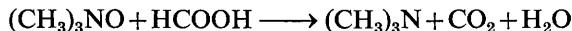
The molar growth yield from formate was 10·3. A corresponding value of 7·0 has been determined for *E. coli* (Yamamoto & Ishimoto, 1977).

Distribution of TMAO respiration among bacterial species

Seven strains of bacteria were isolated from an anaerobic enrichment culture on fish extract as described for *Proteus* NTHC 153 (see Methods). The organisms were all Gram-negative rods but differed with respect to the appearance of colonies, size of cells and motility. They were not identified. None grew when inoculated into the glucose/tryptone/yeast extract medium and incubated anaerobically. However, when TMAO or nitrate was added to the medium, all seven organisms grew well. These observations indicated that the ability to utilize TMAO as terminal electron acceptor during anaerobic growth might be fairly common among bacteria, as is the ability to utilize nitrate.

DISCUSSION

When enterobacteria metabolize carbohydrate anaerobically, a number of externally added hydrogen acceptors such as nitrate, nitrite, thiosulphate and tetrathionate can stimulate the production of acetate (Forget & Pichinoty, 1964; Hadjipetrou & Stouthamer, 1965; Stouthamer & Bettenhausen, 1972). An increased production of acetate from acetyl phosphate leads to an increased production of ATP, and in this way an externally added hydrogen acceptor may stimulate anaerobic growth (Stouthamer, 1969). However, this 'acetate effect' can account for only a small part of the substantial increase in growth yield

of *Proteus* NTHC 153 obtained on adding TMAO to the glucose medium: the increase in estimated production of ATP by substrate-level phosphorylation was 27%, whereas the increase in molar growth yield was 100% (Table 1). Furthermore, bacterial conversion of formate to CO_2 cannot support ATP synthesis by any known mechanism of substrate-level phosphorylation. The only reasonable explanation seems to be that an extra mechanism for the production of ATP has come into operation in which TMAO acts as a terminal electron acceptor in a redox system coupled to ATP production. Evidence for TMAO respiration has also been reported for other strains of enterobacteria (Kim & Chang, 1974; Yamamoto & Ishimoto, 1977).

An NADH-dependent TMAO reductase system has been detected in cell-free extracts of *Vibrio parahaemolyticus* (Unemoto *et al.*, 1965). Formate, NADH and NADPH were effective electron donors for the reduction of TMAO by extracts of *E. coli* (Sakaguchi & Kawai, 1977), and formate, NADH and D-lactate can be used to reduce nitrate (for review, see Haddock & Jones, 1977). The redox potential of the TMAO/TMA couple is about $+0.13$ V (Castell, 1950); E_0' for CO_2/formate is -0.43 V (Netter, 1969), that of NAD/NADH is -0.32 V and that of pyruvate/lactate is -0.19 V (Lehninger, 1970). Thus $\Delta G°$ for the reduction of TMAO by formate, NADH and lactate will be, respectively, about -26, -21 and -15 kcal mol^{-1} (-6.2, -5.0 and -3.6 kJ mol^{-1}). Thermodynamically, it is therefore feasible that the reduction of TMAO by these electron donors can be coupled to oxidative phosphorylation.

The Y_{ATP} values calculated from our data for growth with glucose and pyruvate were 13.0 and 7.8, respectively (Table 1). Stouthamer (1977) reported that Y_{ATP} values depend on the growth conditions; this implies that an accurate P/2e$^-$ ratio cannot be calculated from our data. However, the molar growth yield from formate (10.3) compared with the Y_{ATP} value determined from growth on the simple substrate 'pyruvate (7.8) indicates that the P/2e$^-$ ratio was at least 1 when formate was the electron donor for the reduction of TMAO. We cannot conclude whether electrons liberated from 3-phosphoglyceraldehyde during glucose dissimilation were coupled to oxidative phosphorylation, since the increased growth yield obtained on adding TMAO to glucose media may be due to oxidation of formate formed from glucose. Furthermore, our results indicate that no ATP is formed during the initial step in the oxidation of L-lactate by TMAO because the molar growth yields from L-lactate and pyruvate were similar in the presence of TMAO (Table 1). The initial oxidation of L-lactate is presumably catalysed by an NAD-independent flavoprotein (Kline & Mahler, 1965). It thus seems probable that *Proteus* NTHC 153 may also have a TMAO reduction system which is not coupled to translocation of protons and consequent ATP production.

The content of TMAO in various marine animals varies within wide limits, but is often 2 to 4% of the dry weight of a teleost and 3 to 7% of the dry weight of an elasmobranch (Groninger, 1959; Yamada, 1967). TMAO is thus one of the most common organic molecules in the marine environment. When a marine animal dies, aerobic bacteria will probably start the decay process but oxygen will soon become a limiting factor. Apparently a number of the bacteria can easily switch from aerobic respiration to anaerobic respiration using the readily available TMAO as an electron acceptor. Such bacteria would have a great advantage over those which can only switch from aerobic respiration to a simple fermentation.

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