Sources of Conductance Changes during Bacterial Reduction of Trimethylamine Oxide to Trimethylammonium in Phosphate Buffer

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The sources of conductance changes during reduction of trimethylamine oxide to trimethylamine by *Escherichia coli* with formate as electron donor and in the presence of phosphate buffer were investigated. Theoretical considerations and experimental results suggest that the major source of conductance change is the conversion of dihydrogen phosphate to hydrogen phosphate. This transformation contributes almost twice as much to the total conductance change as does the conversion of uncharged trimethylamine oxide to charged trimethylammonium.

INTRODUCTION

The monitoring of conductance changes in microbial cultures is finding increasing application as a relatively rapid, automated method for the enumeration of micro-organisms in routine food and medical samples (McMurdo & Whyard, 1984). But wider use of the methodology is hindered by a lack of understanding of the mechanisms by which microbial growth brings about changes in the conductance of culture media (Firstenberg-Eden & Eden, 1984).

Easter *et al.* (1982) described a conductance method for studying the reduction of trimethylamine oxide (TMAO) by bacterial cultures or enzyme preparations. They demonstrated a linear relationship between reduction of TMAO to trimethylammonium (TMAH⁺) and change in the conductance of the medium. They ascribed this change in conductance 'mainly to the molecular charge of TMA compared with TMAO' and stated that 'It is not simply due to a pH change or the apparatus being a sensitive pH meter'. However, since their assay systems contained phosphate buffer, it is possible that much of the change in conductance was, in fact, due to changes in the proportions of dihydrogen phosphate and hydrogen phosphate ions. Since the amount of phosphate transformed will be proportional to the amount of TMAO reduced, this does not affect the validity of the assay method. But, if the rational formulation of culture media for conductimetric assays is to be attempted, it is important that the sources of conductance changes in cultures are correctly distinguished.

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Abbreviations: TMAH⁺, trimethylammonium; TMAO, trimethylamine oxide.

THEORY

Taking formate as the electron donor, the reduction of TMAO in the presence of phosphate buffer (in the range pH 7.0–8.2) in a sealed system (to retain carbon dioxide) can be represented as:

At pH 7.0 approximately 80% of the carbon dioxide will be present as the bicarbonate ion and at higher pH values this percentage increases. Most of the remainder will be present as carbon dioxide or undissociated carbonic acid (pK_1 , 6.38; pK_2 , 10.32). Hence, if the carbon dioxide and carbonic acid are neglected the sum of the above equations may be written:

Current is carried in solution only by molecules bearing a net charge and thus the conductance of the system will change due to: (i) reduction of uncharged TMAO to charged TMAH⁺, (ii) oxidation of formate to bicarbonate, and (iii) conversion of dihydrogen phosphate to hydrogen phosphate. Thus the change in conductance of the system per mole of TMAO reduced is given by:

$$\Delta\Lambda = \Lambda_{\text{TMAH}^+} + (\Lambda_{\text{HCO}_3^-} - \Lambda_{\text{HCOO}^-}) + (\Lambda_{\text{HPO}_4^{2-}} - \Lambda_{\text{H}_2\text{PO}_4^-}) \quad (2)$$

where Λ is the molar conductivity of the respective ions.

It is not possible to calculate accurately the molar conductivities of ions in complex mixtures. However, as a first approximation, it may be assumed that the molar conductivities of the different ions are proportionately decreased by similar amounts from their values at infinite dilution. Thus, for the purposes of comparing the relative contributions of each of the above transformations to the total change in conductance, molar conductivities at infinite dilution may be used. Substituting the values given by Erdey-Gruz (1974), Parsons (1959) or Stark & Wallace (1982) in equation (2):

$$\begin{aligned}\Delta\Lambda_0 &= 4.2 + (4.45 - 5.46) + (11.4 - 3.6) \\ &= 4.2 + (-1.01) + 7.8 \\ &= 10.99 \text{ mS m}^2 (\text{mol TMAO reduced})^{-1}\end{aligned} \quad (3)$$

It is evident that, in this system, the contribution of the phosphate transformations to the total conductance change is almost double that due to the production of TMAH⁺.

Transformation of the phosphates must necessarily be accompanied by change in the pH of the mixture. Calculation of the extent of this change must take account of buffering by both phosphate and carbonic acid/bicarbonate. From equation (1), the pH following the reduction of a particular amount of TMAO is given by:

$$\text{pH} = \text{pK}_2^{\text{P}'} + \log \left(\frac{[\text{HPO}_4^{2-}]_0 + [\text{TMAH}^+]_1}{[\text{H}_2\text{PO}_4^-]_0 - [\text{TMAH}^+]_1} \right) \quad (4)$$

where $\text{pK}_2^{\text{P}'}$ is the second dissociation constant for phosphoric acid at the prevailing ionic strength, I [i.e. $\text{pK}_2^{\text{P}'} = \text{pK}_2^{\text{P}} - 1.53\{I^{1/2}(1 + I^{1/2})^{-1} - 0.2I\}$ in water at 25 °C; Butler, 1982], $[\text{HPO}_4^{2-}]_0$ is the initial concentration of hydrogen phosphate, $[\text{H}_2\text{PO}_4^-]_0$ is the initial concentration of dihydrogen phosphate, and $[\text{TMAH}^+]_1$ is the concentration of TMAH⁺ formed. But since, at pH values below about 8, the bicarbonate is in equilibrium with carbonic acid (actually mainly present as carbon dioxide) the change in pH is greater than given by (4):

Thus, allowing for this, the pH is given by:

$$\text{pH} = \text{pK}_2' + \log \left(\frac{[\text{HPO}_4^{2-}]_0 + [\text{TMAH}^+]_1 + [\text{H}_2\text{CO}_3]_1}{[\text{H}_2\text{PO}_4^-]_0 - [\text{TMAH}^+]_1 - [\text{H}_2\text{CO}_3]_1} \right) \quad (5)$$

and since

$$[\text{H}_2\text{CO}_3]_1 + [\text{HCO}_3^-]_1 = [\text{TMAH}^+]_1$$

$$\text{pH} = \text{pK}_2' + \log \left(\frac{[\text{HPO}_4^{2-}]_0 + 2[\text{TMAH}^+]_1 - [\text{HCO}_3^-]_1}{[\text{H}_2\text{PO}_4^-]_0 - 2[\text{TMAH}^+]_1 + [\text{HCO}_3^-]_1} \right) \quad (6)$$

where $[\text{H}_2\text{CO}_3]_1$ is the concentration of carbonic acid after reduction of the TMAO and $[\text{HCO}_3^-]_1$ is the concentration of bicarbonate after reduction of the TMAO. The only unknown in this equation, apart from pH, is $[\text{HCO}_3^-]_1$ which may be determined as follows.

Since
$$[\text{H}^+]_1 = \frac{K_1^C(C_1^C - [\text{HCO}_3^-]_1)}{[\text{HCO}_3^-]_1}$$

and
$$[\text{H}^+]_1 = \frac{K_2^P[\text{H}_2\text{PO}_4^-]_1}{[\text{HPO}_4^{2-}]_1}$$

then
$$\frac{K_1^C(C_1^C - [\text{HCO}_3^-]_1)}{[\text{HCO}_3^-]_1} = \frac{K_2^P([\text{H}_2\text{PO}_4^-]_1 + [\text{HCO}_3^-]_1)}{[\text{HPO}_4^{2-}]_1 - [\text{HCO}_3^-]_1} \quad (7)$$

where K_1^C is the first dissociation constant for carbonic acid at the prevailing ionic strength [$\text{pK}_1^C = \text{pK}_1^C + 0.51\{I^{1/2}(1 + I^{1/2})^{-1} - 0.2I\}$ in water at 25 °C], C_1^C is the total amount of CO_2 produced (i.e. $[\text{H}_2\text{CO}_3]_1 + [\text{HCO}_3^-]_1 = [\text{TMAH}^+]_1$),

$$[\text{H}_2\text{PO}_4^-]_1 = [\text{H}_2\text{PO}_4^-]_0 - 2[\text{TMAH}^+]_1, \text{ and}$$

$$[\text{HPO}_4^{2-}]_1 = [\text{HPO}_4^{2-}]_0 + 2[\text{TMAH}^+]_1.$$

Rearranging:

$$(K_1^C - K_2^P)([\text{HCO}_3^-]_1)^2 - (K_1^C C_1^C + K_1^C[\text{HPO}_4^{2-}]_1 + K_2^P[\text{H}_2\text{PO}_4^-]_1)[\text{HCO}_3^-]_1 + K_1^C C_1^C[\text{HPO}_4^{2-}]_1 = 0 \quad (8)$$

$[\text{HCO}_3^-]_1$ can thus be calculated by solving this quadratic equation and the new pH value of the solution determined with equation (6). Iteration of this procedure yields values for the final concentrations of all the components of the system.

The work reported in this paper attempts to confirm the above interpretation by monitoring the conductivity and pH changes during the reduction of TMAO by *Escherichia coli* with formate as electron donor and in the presence of phosphate buffer.

METHODS

Measurement of conductivity. Conductivity of solutions was measured with a Schott Gerate conductivity meter (model CG857; Boro Laboratories, Aldermaston, Berks, UK) using alternating current at 1000 Hz and a dip type electrode having a cell constant of approximately 1 cm^{-1} (Schott Gerate LF3100). An accurate cell constant was determined using 0.01 and 0.1 M-KCl and recorded conductivity values were appropriately corrected. The KCl solutions were made with freshly collected distilled water and were stored under soda lime tubes but further precautions to exclude CO_2 were not taken. Measurements were made in a constant temperature room at 25 °C.

Chemicals. TMAO was obtained from Sigma and other chemicals were from BDH or Fisons and were of Analar grade or the purest grade otherwise available. Before use they were dried either at 105 °C or over P_2O_5 at ambient temperature.

Distilled water was made by distilling water from a reverse osmosis water purifier (Millipore RO60) in a glass still (Corning Aquatron; Bibby Science Products, Stone, Staffs., UK). Freshly collected distilled water had a conductivity of $0.12\text{--}0.2 \text{ mS m}^{-1}$ at 25 °C.

Bacterial suspension. *Escherichia coli* K12 was grown in a medium containing (g l^{-1}): tryptone (Oxoid L42), 10; yeast extract (Oxoid L21), 5; K_2HPO_4 , 12; KH_2PO_4 , 3; NaCl, 5; glycerol, 5; TMAO, 10; pH, 7.3. The medium, without TMAO, was sterilized by autoclaving at 121 °C for 10 min and filter sterilized TMAO solution, 100 g l^{-1} , was then aseptically added to it. The medium was aseptically dispensed into 250 ml screw-capped bottles which were completely filled. Cultures were incubated at 37 °C for 1 d and the cells from 1 litre of culture were then

Fig. 1. Relationship between final conductivity value and initial concentration of TMAO following reduction of TMAO to TMAH⁺ by *E. coli* in phosphate buffer.

harvested by centrifugation. Cells were washed twice in 10 mM-potassium phosphate buffer, pH 6.9, containing 2–3 drops of β -mercaptoethanol l⁻¹, resuspended in 40 ml of the same buffer, and stored at 4 °C until required. The suspension contained 5.6 g dry matter l⁻¹.

Bacterial reduction of TMAO. Reaction mixtures comprised (ml): 10 mM-potassium phosphate buffer pH 6.9, 9; 50 mM-sodium formate, 4; bacterial suspension, 1; 40 mM-TMAO, as required; distilled water, to 20. Mixtures were contained in 150 × 16 mm test-tubes and partially sealed from air by floating a small test-tube on the liquid surface and closing the mouth of the 16 mm test-tube with a rubber bung. Tubes were incubated at 25 °C and the pH and conductivity measured after 2 and 3 h. Each test was done in duplicate. The presence of TMAH⁺ in tubes after incubation was obvious due to its very distinctive smell.

RESULTS

The final conductivities of reaction mixtures containing different initial amounts of TMAO are shown in Fig. 1. The results show the expected linear relationship between conductivity change and initial concentration of TMAO, with a slope of 6.9 mS m² (mol TMAO reduced)⁻¹. The final pH values of the mixtures were very close to those predicted by calculation using equation (6) (Table 1). Except in the tubes containing the highest initial concentration of TMAO, the pH and conductivity values after 3 h incubation were similar to those recorded after 2 h, showing that the reactions had gone to completion.

DISCUSSION

The closeness of the final pH values of the experimental reaction mixtures to the predicted values provides good evidence for the reaction pathways proposed.

Although the observed change in conductivity of 6.9 mS m² (mol TMAO reduced)⁻¹ is substantially less than the theoretical maximum of 10.99 mS m² mol⁻¹ at infinite dilution, it is almost double the theoretical maximum obtainable from production of TMAH⁺ alone without any contribution from phosphate transformations. It is clear, therefore, that phosphate transformations are a major source of conductance change in the system studied and that this will generally be the case for the reduction of TMAO with most electron donors in the presence of phosphate buffer. It may be presumed, from the theoretical predictions, that the contribution from the phosphate transformations is nearly double that due to the formation of TMAH⁺. It also follows that the sensitivity of conductance change as an assay for the reduction of TMAO is higher (10.99 mS m² mol⁻¹ at infinite dilution) in the presence of pH changes in phosphate buffer than it would be in the absence of such changes (3–4 mS m² mol⁻¹). However, the exploitation of such increased response is dependent on a clear understanding of the reactions involved, since with different electron donors and/or oxidized products quite different changes in conductivity per mole of TMAO reduced may be obtained (Table 2).

Table 1. *Calculated and experimentally determined final pH values of reaction mixtures following the bacterial reduction of TMAO to TMAH⁺ in phosphate buffer*

The initial reaction mixture contained 9.5 mM-phosphate buffer, 50 mM-sodium formate, *E. coli* suspension, and TMAO as required. Calculated values were determined as described in the text, taking $pK_2^{\text{f}} = 7.21$ and $pK_1^{\text{f}} = 6.38$.

Initial TMAO concn (mM)	pH after reduction of TMAO to TMAH ⁺		
	Calculated	Experimental (duplicates)	
0	6.86*	6.87	6.90
1	7.06	7.07	7.08
2	7.26	7.23	7.25
3	7.48	7.43	7.46
4	7.74	7.67	7.67
5	8.33	7.98	7.98

* Initial experimental value.

Table 2. *Calculated change in conductivity at infinite dilution due to the reduction of TMAO with different electron donors in the presence of excess phosphate buffer*

Oxidation-reduction reaction	H ⁺ consumed [mol (mol TMAO) ⁻¹]	$\Delta\Lambda_0$ [mS m ² (mol TMAO) ⁻¹]
CH ₃ CHO + TMAO → CH ₃ COO ⁻ + TMAH ⁺	0	8.3
C ₆ H ₁₂ O ₆ + 12TMAO + 6H ⁺ → 6HCO ₃ ⁻ + 12TMAH ⁺	0.5*	10.3*
HCOO ⁻ + TMAO + H ⁺ → HCO ₃ ⁻ + TMAH ⁺	1*	11.0*
NADH + TMAO + 2H ⁺ → NAD ⁺ + TMAH ⁺ + H ₂ O	2	19.8†

* Calculated on the assumption that all CO₂ is present as HCO₃⁻.

† Not including contribution from NAD⁺.

Very many metabolic activities of micro-organisms lead to pH changes in culture media and it is possible, therefore, that a substantial proportion of the conductance change that occurs in cultures during growth might also be due to changes in the conductance of phosphate ions or other buffer compounds. If this is the case, it suggests that conductance changes in microbial cultures could be enhanced by the use of appropriate pH buffer compounds, with a consequent decrease in the time required for the enumeration of a given microbial population. The techniques used in this paper to estimate the relative contributions of different reactions to the overall conductance change provide a means for the selection of suitable buffers and for the rational formulation of culture media for conductimetric assays.

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