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FACTORS AFFECTING PROTON TRANSLOCATION BY FACULTATIVE METHYLOTROPHS *

ROGER C. HAMMOND **, BRIAN REES and I. JOHN HIGGINS ***

Biological Laboratory, University of Kent, Canterbury CT2 7NJ (U.K.)

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The general properties of respiration-driven proton translocation by the two facultative methylotrophs, *Pseudomonas* AM1 and *Pseudomonas extorquens*, were similar to those of other bacteria. The stoichiometry of H^+ extruded/O atom consumed ($\overleftarrow{H^+}/O$) during respiration with a particular substrate depended, however, on the concentration of the permeant anion SCN^- used to abolish the membrane potential and on the physiological state of the organism. This variability makes the use of proton translocation data of dubious value in the elucidation of electron-transport pathways, at least in these species, unless the physiological condition of the organisms can be accurately described and reproduced. Methanol oxidation was inhibited by SCN^- but substitution of valinomycin for most of the SCN^- during pulse oxidant experiments allowed measurement of proton translocation when methanol was the substrate. Starved organisms were used to eliminate ambiquity as to whether added test substrates or endogenous reserve materials were being oxidised. Viability remained high during starvation and endogenous O_2 uptake remained detectable long after endogenously driven proton translocation was undetectable. In the absence of endogenously driven proton translocation, measured $\overleftarrow{H^+}/O$ stoichiometries differed substantially from those when it was present, suggesting that the physiological state of the organisms is an essential parameter in assessing proton translocation data.

Introduction

Microorganisms utilising reduced C_1 compounds are currently of considerable academic and industrial interest. For example, methane-utilising bacteria have surprisingly extensive metabolic capabilities towards complex organic compounds [1,2] which together with other properties makes them candidates for industrial exploitation as biocatalysts. Methanol-utilising bacteria are already used for production of single-cell protein [3]. This paper describes studies of respiration-driven proton translocation by two facultative methylotrophs, *Pseudomonas* AM1 and *Pseudomonas extorquens*.

Measurement of $\overleftarrow{H^+}/O$ and ATP/O ratios can yield information on the arrangement of respiratory carriers in the electron-transport chain, on the number of sites of ATP synthesis and hence on expected growth yields according to the chemiosmotic hypothesis of Mitchell [4,5]. However, NAD(P)H limitation in serine pathway organisms may mean that such ratios are less important in predicting molar growth

* A preliminary report of some of these results has been published [23].

** Present address: Unilever Research, Colworth House, Sharnbrook, Bedford MK 44 ILQ, U.K.

*** Present address: Biotechnology Unit, Industrial Science Centre, Cranfield Institute of Technology, Cranfield, Bedford MK43 OAL, U.K.

Abbreviations: $\overleftarrow{H^+}/O$, number of protons ejected from organisms per oxygen atom consumed; ATP/O, number of ATP molecules synthesised per oxygen atom consumed; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; ΔpH, transmembrane pH difference; $\Delta\psi$, transmembrane difference in electrical potential.

yields [6,7]. While some analyses of relationships between growth yields and the number of sites of ATP synthesis are available [6,8–10], relatively few attempts have been made to determine ATP/O ratios in methane- and methanol-utilising bacteria. ATP/O measurements have been made directly on cell-free membrane preparations [11,13] or inferred from the measured $\overleftarrow{H}^+/O$ ratio [18,19] although the various stoichiometries of electron transport, proton translocation and oxidative phosphorylation are currently subjects of very considerable controversy (see Refs. 17 and 20).

Pseudomonas AM1 and *P. extorquens* are two similar facultative methylotrophs able to grow on a range of C_1 compounds excluding methane as carbon and energy sources as well as multicarbon compounds such as succinate, malate and 3-hydroxybutyrate. Both organisms use the serine pathway [21] during growth on C_1 substrates for incorporation of carbon at the oxidation levels of formaldehyde and CO_2. In the few previous studies of $\overleftarrow{H}^+/O$ ratios during oxidation of various substrates by *Pseudomonas* AM1 [15, 22], difficulties were experienced in obtaining organisms suitable for unequivocal $\overleftarrow{H}^+/O$ measurements and in measuring proton translocation during methanol oxidation. Some of these difficulties are explored here and an alternative regime is described which allows measurement of proton translocation with methanol as test substrate.

Methods

Organisms and culture conditions. Pseudomonas AM1 (NCIB 9133) and *P. extorquens* (NCIB 9399) were grown in liquid culture on either the salts medium of Jayasuriya [24] or the minimal salts medium described by Downs and Harrison [25]. Carbon and energy sources were succinate (sodium salt; 0.5%, w/v) or methanol (0.5 or 1%, v/v). Batch cultures (750 ml) in 2 l flasks were grown at 30°C with shaking at 175 rev./min. *P. extorquens* used in some experiments was from carbon-limited continuous cultures ($D = 0.15\ h^{-1}$) using the above minimal salts medium.

Harvesting and starvation of microorganisms. Batch cultures were harvested by centrifugation at similar cell densities (A_{470} 0.5–1.0), thoroughly washed by resuspension in either growth medium without carbon source or in 1 mM Tris-HCl, pH 7.0, containing 140 mM KCl and finally resuspended in fresh washing buffer. Cell suspensions were then starved by shaking aerobically at 175 rev./min for varying times or until proton pulses (see below) due to endogenous substrate were no longer detectable. Harvesting, washing and starvation were done at 30°C. After starvation, the organism concentration was adjusted, if necessary, by centrifugation and resuspension, to 5–10 mg dry wt./ml in 1 mM Tris-HCl, pH 7.0, containing 140 mM KCl prior to measurement of the apparent $\overleftarrow{H}^+/O$ ratio.

Measurement of proton translocation. Apparent $\overleftarrow{H}^+/O$ ratios were determined using the technique of Mitchell and Moyle [26] as described by Drozd [27] except that a Pye Model 290 pH meter (Pye Unicam Ltd., Cambridge, U.K.) fitted with a backing-off control and a Russell type CMW 72 pH probe (Russell pH Ltd., Fife, U.K.) were used. KSCN (150 mM) was used routinely to abolish the membrane potential except when methanol was test substrate. Methanol-driven proton translocation was measured using a mixture of KSCN and valinomycin as described in the text. Residual O_2 in the bacterial suspensions was removed by the endogenous respiration which remains after starvation (see below). Suspensions were incubated after additions until the pH was constant before additions of air-saturated 140 mM KCl were made. The pH scale was standardised by additions of N_2-sparged 0.01 M KOH. Values of $\overleftarrow{H}^+/O$ ratios are shown as the mean ± S.D. with, in brackets, the number of determinations with different batches of cells.

Measurement of bacterial respiration. Whole organism respiration rates were measured using an oxygen electrode (Rank Bros., Bottisham, Cambridge, U.K.) at 30°C. Respiration rates with added substrates were corrected for endogenous respiration and for the basal uptake of the electrode.

Determination of viability of organisms. Viability of *P. extorquens* after various starvation times was determined by standard plating techniques on nutrient agar (Oxoid).

Chemicals. Valinomycin was obtained from Calbiochem, C.P. Laboratories Ltd., Bishop's Stortford, Hertfordshire, U.K.; sodium L-ascorbate, carbonic anhydrase (carbonate hydro-lyase, EC 4.2.1.1), TMPD and CCCP were from Sigma (London) Chemical Co. Ltd., Poole, Dorset, U.K. Formaldehyde was freshly prepared before use from paraformaldehyde

[28] and all other chemicals were of the purest grades available.

Results and Discussion

Proton pulse measurements were made in the presence of a variety of substrates, most of which are both growth substrates and intermediates in the pathway of methanol oxidation, and also with the artificial electron-donating system, sodium L-ascorbate plus TMPD.

Most test substrates gave rapid, transient acidifications of the bulk medium (proton pulses) of the form typically reported on addition of small volumes (100 μl or less) of air-saturated 140 mM KCl to anaerobic suspensions of both *Pseudomonas* AM1 and *P. extorquens*. These typical pulses decayed exponentially with half-lives of about 45 s. Addition of the uncoupler CCCP (10 μM, final concentration) at the peak of a pulse caused a rapid decay of that pulse and prevented further pulses on subsequent additions of air-saturated saline. The presence of carbonic anhydrase in incubations of either bacteria had no effect on the observed $\overleftarrow{H}^+/O$ ratios and was not routinely used. The presence of Na^+ or the addition of small volumes of N_2-sparged 2 M NaCl caused no significant pH changes, suggesting the absence of an Na^+/H^+ antiporter in *Pseudomonas* AM1 and *P. extorquens*. The general properties of proton translocation by these two methylotrophs are similar to those reported for *Pseudomonas* AM1 by O'Keeffe and Anthony [15].

The proton-motive force generated by electron transport is composed of a ΔpH component and a $\Delta\psi$ component which must be dissipated in order to measure maximum proton translocation [29]. In proton pulse studies this is usually achieved by the inclusion of a permeant ion such as SCN^- although a K^+ uniporter such as valinomycin may be suitable. O'Keeffe and Anthony [15] studied the effect of varying SCN^- concentration on apparent $\overleftarrow{H}^+/O$ ratios in unstarved *Pseudomonas* AM1. Maximum proton translocation was observed between 50 and 150 mM KSCN; 66 mM KSCN was routinely used. We have also studied the variation of the apparent $\overleftarrow{H}^+/O$ ratio with SCN^- concentration during formate oxidation by starved suspensions of succinate-grown *P. extorquens* (Fig. 1). Even low (15 mM) concentrations of KSCN allowed pulses as great as those with 150 mM KSCN but between these extremes the apparent $\overleftarrow{H}^+/O$ ratio was much lower. The reason for this variation and the apparent discrepancy between this result and that of O'Keeffe and Anthony is not clear, although it could reflect the different physiological state of the cells. KSCN at 150 mM was routinely used in this study; this concentration has previously been used in this and other laboratories [14,27].

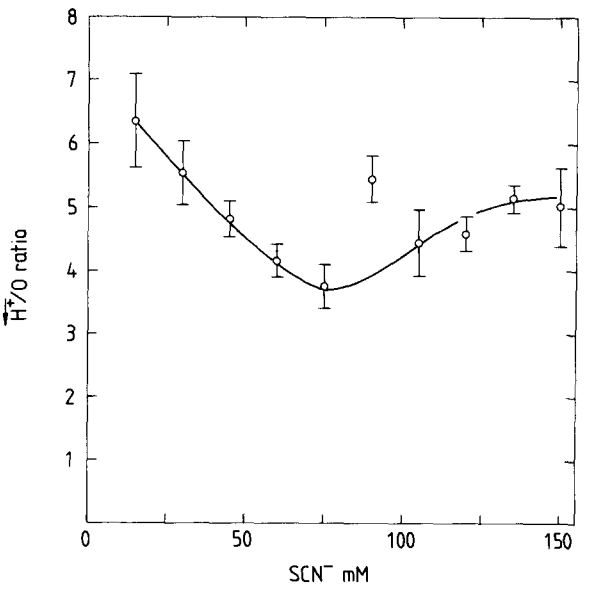

Fig. 1. Variation of apparent $\overleftarrow{H}^+/O$ ratio with SCN^- concentration. $\overleftarrow{H}^+/O$ measurements of starved, succinate grown *P. extorquens* with 5 mM formate as test substrate were made as described in Methods but using various KSCN concentrations. Points shown are the mean ± S.D. of different determinations using the same batch of organisms.

It has been reported [15] that KSCN has the undesirable effect of inhibiting methanol oxidation by *Pseudomonas* AM1 and thus prevented measurement of apparent $\overleftarrow{H}^+/O$ ratios which this substrate. This inhibition was confirmed in both *Pseudomonas* AM1 and *P. extorquens* but proton translocation could still be measured if valinomycin replaced most of the SCN^- (Fig. 2). The presence or absence of 4.3 μM valinomycin had little effect when using the usual concentration of SCN^- (150 mM): a slow pH change which did not decay (Fig. 2a) was observed. Valinomycin alone (Fig. 2b) allowed a pH change which was more rapid but again did not decay. Such pH changes

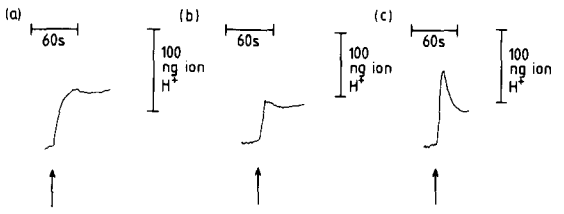

Fig. 2. Effect of valinomycin and thiocyanate on proton pulses of methanol-grown *P. extorquens* with 2 mM methanol as test substrate. (a) 4.3 μM valinomycin plus 150 mM KSCN, (b) 4.3 μM valinomycin, (c) 4.3 μM valinomycin plus 15 mM KSCN. Arrows indicate addition of 100-μl volumes of air-saturated 140 mM KCl. Conditions other than uniport concentrations were as described in Methods. Cell suspensions were incubated with valinomycin for at least 10 min before addition of KCl. Organisms used were grown on 0.5% (v/v) methanol.

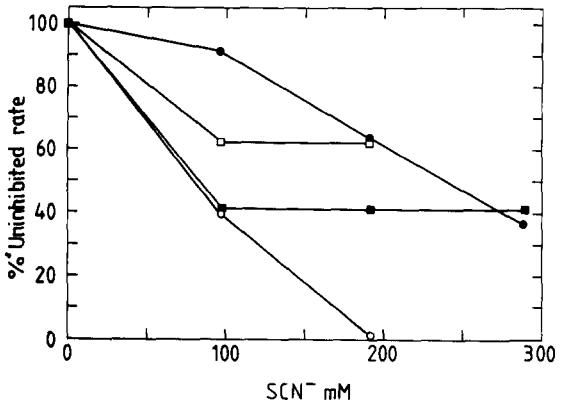

Fig. 3. Effect of SCN^- and valinomycin on whole cell methanol oxidation by *Pseudomonas* AM1 and *P. extorquens* grown on methanol (0.5%, v/v). Respiration rates were determined as described in Methods. The methanol concentration was 2.5 mM and suspensions were incubated with valinomycin (final concentration, 4.1 μM) for at least 10 min before measurements were made. (o——o) *P. extorquens*, (•——•) *P. extorquens* plus valinomycin, (□——□) *Pseudomonas* AM1, (■——■) *Pseudomonas* AM1 plus valinomycin. Respiration rates are expressed as % uninhibited rate in the absence of KSCN.

could not be used to measure apparent $\overleftarrow{H}^+/O$ ratios. In the presence of both 4.3 μM valinomycin and 15 mM KSCN, however, relatively normal pulses with exponential decay were recorded although the decay was sometimes rapid (apparent half-times of about 30 s) and incomplete (Fig. 2c). Only pulses showing essentially complete decay were used for calculation of apparent $\overleftarrow{H}^+/O$ ratios with methanol as test substrate. This apparent protective effect of valinomycin against SCN^- inhibition of methanol oxidation was reflected in whole organism methanol oxidation (Fig. 3). Methanol oxidation by *P. extorquens* was strongly inhibited by SCN^- but the presence of valinomycin lessened the inhibition. In contrast, valinomycin increased slightly the inhibition by SCN^- of methanol oxidation by *Pseudomonas* AM1, although this inhibition was always less than 60% at those concentrations tested. Although the protective effect of valinomycin was probably small at the SCN^- concentration used for proton pulse determinations, the conditions described in the legend to Fig. 2c were used routinely with both bacteria to measure apparent $\overleftarrow{H}^+/O$ ratios when methanol was test substrate. While apparent $\overleftarrow{H}^+/O$ ratios may be calculated under these conditions, the concentrations of KSCN and valinomycin required to maximise the pH change were not determined. Kell [31] calculated that at low concentrations of SCN^-, only about 10% of the observed stimulation of H^+ ejection into the bulk phase during an oxygen pulse experiment could be accounted for by the charge-collapsing transmembrane migration of SCN^-, the remainder being attributed to a surface effect of the chaotropic SCN^- within the unstirred boundary layers of the cell membrane. The possibility that SCN^- might act in several ways may help to clarify the dependence of the apparent $\overleftarrow{H}^+/O$ value on $[SCN^-]$ (Fig. 1). Different surface effects of SCN^- and K^+/valinomycin in the interphase between membrane and bulk phase [31] might be an alternative explanation of why a combination of these two treatments allows measurement of pH changes in the bulk phase while individually they are ineffective (data of Fig. 2).

In the previous study [15], it was not possible to remove endogenous respiration in *Pseudomonas* AM1 by incubation in the absence of carbon source for up to 24 h. The precaution of using starved organisms is important, since the presence of endogenous substrate during incubations with an added test substrate leads to uncertainty as to whether the endogenous or the added substrate or, perhaps, some of each is being oxidised. This is illustrated in Table I. Lower, reproducible apparent $\overleftarrow{H}^+/O$ values were obtained with sus-

TABLE I

EFFECT OF STARVATION ON APPARENT $\overleftarrow{H}^+/O$ RATIOS

Proton translocation by succinate-grown *P. extorguens* was measured as described in Methods with 150 mM KSCN after harvesting and washing ('unstarved') and after subsequent starvation until proton translocation due to endogenous substrate was no longer detectable ('starved'). n.d., not detectable.

Substrate	$\overleftarrow{H}^+/O$ starved	$\overleftarrow{H}^+/O$ unstarved
Endogenous	n.d.	6.44 ± 0.36 (9)
Succinate (5 mM)	2.23 ± 0.48 (7)	variable, 4.8–6.5
Formate (5 mM)	3.19 ± 0.45 (7)	variable, 4.9–6.3

pensions of *P. extorquens* lacking endogenous proton translocation compared with the variable values for unstarved suspensions. Apparent $\overleftarrow{H}^+/O$ values obtained with unstarved suspensions were often as great in the presence of exogenous substrates as those for endogenous substrates, suggesting that the cells continued to oxidise their endogenous substrates, at least in part. Variations of apparent $\overleftarrow{H}^+/O$ ratios when endogenous proton translocation was present might reflect different holding times of the cells between harvesting and measurement of proton translocation. Dawson and Jones [30] studied formaldehyde oxidation by *Methylophilus methylotrophus* and calculated that the observed $\overleftarrow{H}^+/O$ value was a result of contributions from both NADH oxidase and methanol oxidase.

In contrast to a previous report [23], it has since proved possible to starve succinate-grown *P. extorquens* of endogenous proton translocation by prolonged incubation in the absence of carbon source. Methanol-grown *Pseudomonas* AM1 and *P. extorquens* and succinate-grown *Pseudomonas* AM1 could be starved more rapidly. Fig. 4 shows the effect of starvation period on apparent $\overleftarrow{H}^+/O$ ratios with endogenous and added substrates. In this experiment, pulses due to endogenous respiration were absent after 4 days of starvation although different batches varied in the time required to remove endogenous proton translocation. The apparent $\overleftarrow{H}^+/O$ values with the tricarboxylic acid cycle intermediates, succinate and malate, were lower in the absence of endogenous pulses than when they were present while formate

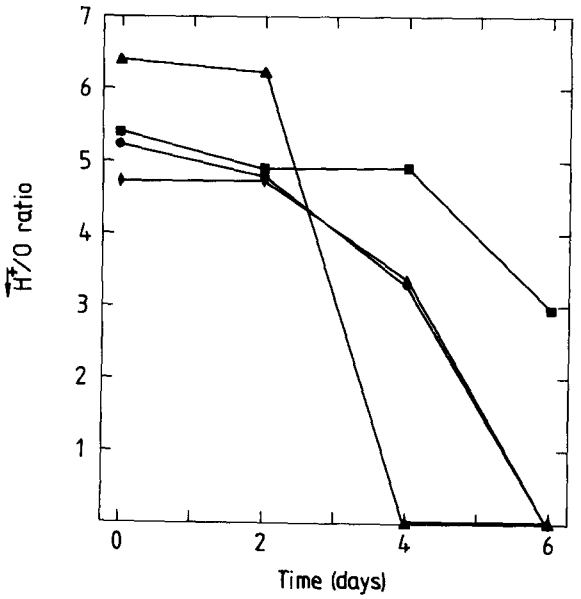

Fig. 4. Effect of starvation on apparent $\overleftarrow{H}^+/O$ ratios of succinate-grown *P. extorquens*. Apparent $\overleftarrow{H}^+/O$ ratios during oxidation of endogenous or added substrates were measured as described in Methods after different times of starvation. Points shown are the mean values obtained with several different batches of organisms. (▲——▲) endogenous substrate, (■——■), 5 mM formate, (●——●) 5 mM succinate, (◆——◆) 5 mM malate.

pulses appeared relatively unaffected. Further starvation, however, resulted in the abolition of succinate and malate pulses and a lower apparent $\overleftarrow{H}^+/O$ ratio with formate. The latter finding might suggest that the cells are losing viability during prolonged starvation. Fig. 5 however, shows that the viability remains constant during prolonged starvation and also that endogenous oxygen uptake remains high for a considerable period after endogenous proton translocation has ceased (usually after 4 days of starvation). While the nature of this apparently uncoupled endogenous respiration has not been investigated, it is clear that the use of endogenous respiration as a criterion of starvation is unreliable. Indeed, the actual respiration rate during a pH pulse is low and it would be extremely difficult to prove from polarographic data that a culture had a substantially lower endogenous respiration rate than this and could hence be termed 'fully starved'. These points may serve to explain why other workers [15] reported their inabil-

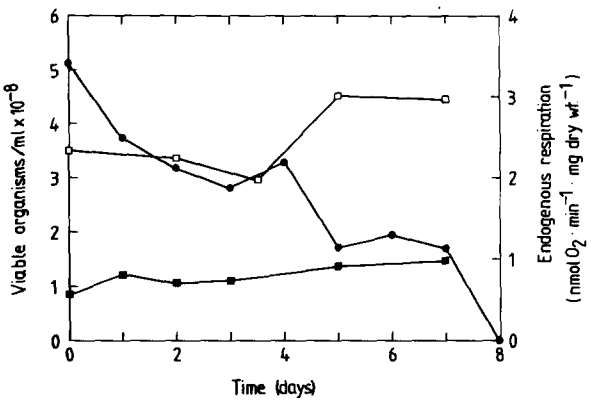

Fig. 5. Effect of starvation on viability and endogenous respiration of succinate-grown *P. extorquens*. Viability and endogenous respiration rate measurements were made as described in Methods after different times of starvation. Separate samples of continuously grown organisms were used for viability and respiration rate determinations. (●———●) Endogenous respiration of continuously grown organisms, (□———□) viability of continuously grown organisms, (■———■) viability of batch-grown organisms.

ity to starve *Pseudomonas* AM1 of endogenous respiration. Use of endogenously driven proton translocation as a criterion of adequate starvation is obviously more relevant if proton translocation is to be monitored.

Porte and Vignais [33] also experienced difficulty in assessing the contribution of endogenous substrates to the observed $\overleftarrow{H}^+/O$ ratio of spheroplasts of *Paracoccus denitrificans* in the presence of added substrates. They claimed that lowering the amount of spheroplasts used allowed the identification of 'experimental conditions where the effect of endogenous substrates can be completely eliminated'. The data presented show that at low spheroplast concentrations (about 1 mg protein per 5 ml reaction volume) proton translocation due to endogenous substrate was not detectable whereas an apparent $\overleftarrow{H}^+/O$ ratio could be measured with added substrates. At greater spheroplast concentrations, apparent $\overleftarrow{H}^+/O$ ratios with exogenous substrates reached a plateau: presumably the organism concentration was then so high that the oxygen injected was rapidly reduced, as is required for rapid, measurable proton pulses. In none of the data shown, however, did the plateau of apparent $\overleftarrow{H}^+/O$ ratio with added substrate coincide with a zero or basal apparent $\overleftarrow{H}^+/O$ ratio due to endogenous substrate only. Hence, the effect of endogenous respiration could be minimised but not completely eliminated. In the present study, a high organism concentration (5–10 mg dry wt./ml) was used as even large additions (50 μl) of air-saturated saline gave rapid pulses and small variations in organism concentration had no effect on apparent $\overleftarrow{H}^+/O$ ratios.

Two points are worthy of mention in regard to the use of starved organisms. Firstly, at the point during starvation at which endogenous pulses disappear, oxygen uptake is still relatively high and pulses can be measured with substrates such as succinate, which is probably actively transported into the organism [32]. Secondly, the loss of pulses with succinate usually occurs when residual endogenous respiration becomes undetectable. It is important then to make $\overleftarrow{H}^+/O$ determinations as soon as possible after removal of endogenous pulses so that exogenous substrates may enter the cells. The prolonged holding of organisms at low temperature before $\overleftarrow{H}^+/O$ measurements as reported by O'Keeffe and Anthony [15] is probably to be avoided. The variations in the time taken to starve endogenous proton translocation may reflect differences in concentrations of storage materials such as poly(3-hydroxybutyrate) after different growth conditions or, perhaps, differing metabolite leakage from lysing cells during starvation. In our hands, methanol-grown cells could be starved more rapidly than succinate-grown ones. Cells grown in carbon-limited continuous culture at near μ_{max} require starvation periods similar to those of batch-grown organisms and gave the same apparent $\overleftarrow{H}^+/O$ ratios to within experimental scatter.

Information on the effects of starvation period on observed $\overleftarrow{H}^+/O$ ratios has served to rationalise highly variable proton pulse data obtained with facultative methylotrophs in this laboratory. In Table II are shown apparent $\overleftarrow{H}^+/O$ ratios measured during oxidation of various test substrates by *P. extorquens*. Starving suspensions were tested frequently for endogenously driven proton extrusion and measurements made as soon as this was undetectable. A starvation period of 2–3 days was required for methanol-grown *P. extorquens* while succinate-grown cultures required 4–5 days starvation. In general, apparent $\overleftarrow{H}^+/O$ ratios with a particular test substrate were the same in succinate- and methanol-grown organisms. The main

TABLE II

APPARENT $\overleftarrow{H}^+/O$ RATIOS OF *P. EXTORQUENS* DURING OXIDATION OF VARIOUS TEST SUBSTRATES

Suspensions of starved *P. extorquens* after growth on succinate or methanol were used for $\overleftarrow{H}^+/O$ determinations as described in Methods. KSCN was present at 150 mM except for methanol-driven pulses when the regime described in Fig. 2c was used. Acidification, when the sodium L-ascorbate/TMPD couple served as electron donor, was monitored at pH 5.6. For further details see text.

Test substrate	Growth substrate	
	Succinate	Methanol
Succinate (5 mM)	2.25 ± 0.27 (6)	2.15 ± 0.23 (3)
Formate (5 mM)	2.76 ± 0.30 (5)	2.99 ± 0.13 (3)
Formaldehyde (1 mM)	3.66 ± 0.28 (3)	3.63 ± 0.27 (7)
Malate (5 mM)	3.74 ± 0.40 (3)	4.11 ± 0.10 (4)
3-Hydroxybutyrate (5 mM)	5.60 ± 0.30 (3)	4.40 ± 0.28 (3)
Methanol (2 mM)	1.17 ± 0.12 (3)	2.33 ± 0.10 (4)
Sodium L-ascorbate (1.5 mM) + TMPD (0.4 mM)	2.40 ± 0.18 (3)	2.44 ± 0.30 (3)

exception was when oxidising methanol; after growth on methanol, the apparent $\overleftarrow{H}^+/O$ ratio was double that after growth on succinate. If this difference in the apparent $\overleftarrow{H}^+/O$ ratio reflects a doubling in the number of coupling sites during methanol oxidation by methanol-grown *P. extorquens* as compared with succinate-grown organisms, then it would seem that the extra site(s) must be situated before cytochrome *c* as acidifications in the presence of ascorbate/TMPD, an artificial system donating electrons at the level of cytochrome *c*, were similar in organisms grown on methanol or succinate. It may be that the observed dependence of methanol-driven proton translocation on growth substrate is a reflection of a different arrangement of cytochromes in succinate- and methanol-grown *P. extorquens*. Anthony [34] has recently summarised some of the changes in electron transport which are engendered in methylotrophs by growth conditions.

The data in Table II also suggest a small difference in energy conservation between the two cultures with different growth substrates when exogenous 3-hydroxybutyrate was energy source. More strikingly, however, other substrates such as formate and malate expected to donate electrons to the respiratory chain via NADH gave apparent $\overleftarrow{H}^+/O$ values which were essentially the same in organisms grown on either succinate or methanol but which differed considerably between themselves. A considerable difference in apparent $\overleftarrow{H}^+/O$ ratio with formaldehyde and succinate was also observed. Reducing equivalents from the latter two substrates were supposed by O'Keeffe and Anthony [15] to follow similar routes. Further studies will be required to elucidate the physiological basis of these differences. Extrapolation from these observed $\overleftarrow{H}^+/O$ values to ATP/O ratios is subject to a number of possible inaccuracies as discussed above. We consider, however, that this detailed study on the effects of preparation of cultures for these measurements demonstrates the importance of such considerations for future proton translocation studies.

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