

## The electron-transport chains of the obligate methylotroph *Methylophilus methylotrophus*

Andrew R. CROSS\* and Christopher ANTHONY  
Department of Biochemistry, University of Southampton, Southampton SO9 3TU, U.K.

(Received 18 February 1980/Accepted 18 July 1980)

The cytochrome complement of *Methylophilus methylotrophus* and its respiratory properties were determined during batch culture and in continuous culture under conditions of methanol-, nitrogen- and O<sub>2</sub>-limitation. About 35% of the cytochrome *c* produced by the bacteria was released into the growth medium, and of the remaining cytochrome *c* about half was membrane-bound and half was soluble. Two cytochromes *c* were always present on membranes (redox potentials 375 mV and 310 mV), and these probably correspond to the soluble cytochromes *c* described previously [Cross & Anthony (1980) *Biochem. J.* 192, 421–427]. A third minor component of cytochrome *c* (midpoint potential 356 mV) was only detected on membranes of methanol-limited bacteria. *M. methylotrophus* always contained two membrane-bound cytochromes *b* with  $\alpha$ -band absorption maxima of about 556 and 563 nm (measured at room temperature) and midpoint potentials of 110 and 60 mV respectively. There appeared to be relatively more of the cytochrome *b*<sub>563</sub> in methanol-limited bacteria. A third *b*-type cytochrome with an  $\alpha$ -band absorption maximum at 558 (at 77 K) reacted with CO and had a high midpoint redox potential (260 mV); it is thus a potential oxidase and hence is called cytochrome *o*. The roles of these cytochromes in electron transport were confirmed by investigating the patterns of respiratory inhibition. It is proposed that two cytochromes are physiological oxidases: cytochrome *a* + *a*<sub>3</sub>, which is present only in methanol-limited conditions, and the cytochrome *o*, which is induced 10-fold in conditions of methanol excess. Schemes for electron transport from methanol and NAD(P)H to O<sub>2</sub> in *M. methylotrophus* under various limitations are proposed. Spectra and potentiometric titrations of cytochromes in whole cells and membranes of *M. methylotrophus* grown under various nutrient limitations have been deposited as Supplementary Publication SUP 50111 (10 pages) at the British Library Lending Division, Boston Spa, Wetherby, West Yorkshire LS23 7BQ, U.K., from whom copies can be obtained on the terms indicated in *Biochem. J.* (1978) 169, 5.

*Methylophilus methylotrophus* is an obligate methylotroph assimilating methanol by way of the ribulose monophosphate pathway, and it is thus the type of methylotroph giving greatest cell yields on methanol (van Dijken & Harder, 1975; Goldberg *et al.*, 1976; Anthony, 1978, 1980); in this respect it is similar to *Pseudomonas C* (Goldberg *et al.*, 1976), bacterium C2A1 (Colby & Zatman, 1973) and organism W1 (Dahl *et al.*, 1972).

A full description of the three soluble cytochromes *c* of this bacterium is given in the preceding paper (Cross & Anthony, 1980), and the present

paper describes the spectral characteristics of the cytochromes of *M. methylotrophus* grown in batch culture and in continuous culture under conditions of methanol-, nitrogen- and O<sub>2</sub>-limitation; these cytochromes have also been fully characterized with respect to their midpoint redox potentials. The results given in the present paper indicate that membranes of *M. methylotrophus* contain at least two cytochromes *c*, two cytochromes *b* and two potential oxidases, cytochromes *o* and *a* + *a*<sub>3</sub>; both oxidases may be involved in the oxidation of methanol and NAD(P)H, but the relative contributions of these oxidases to respiration are determined by the growth conditions. A scheme for electron transport from methanol and NAD(P)H to

\* Present address: Department of Biochemistry, University of Bristol Medical School, Bristol BS8 1TD, U.K.

O<sub>2</sub> in *M. methylotrophus* under various growth conditions is proposed.

A preliminary report of some of this work has been published (Cross & Anthony, 1978).

### Materials and methods

Unless otherwise stated these were as previously described (Cross & Anthony, 1980).

#### *Growth and harvesting of bacteria*

The growth medium was as previously described except that for batch culture (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> was used at 1.8 g/litre; 21 mM-potassium/sodium phosphate buffer, pH 6.8, was used; MgSO<sub>4</sub>·7H<sub>2</sub>O was used at a final concentration of 0.2 g/litre, and only 2 ml of trace-element solution was used.

During growth in continuous culture the concentration of methanol in the flow medium was always 1% (v/v). The (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> concentration in the flow medium was normally 3.6 g/litre; nitrogen-limitation was achieved by lowering the concentration to 1.0 g/litre. O<sub>2</sub> was normally supplied as air at a flow rate of 6 litres/min; O<sub>2</sub>-limitation was achieved by lowering the flow rate to 1.5 litres/min. The culture volume in the 5-litre fermenter was 3 litres.

The 1-litre batch cultures were grown in 2-litre shake flasks at 37°C in minimal medium supplemented with 1% (v/v) methanol. Cells were harvested in the late-exponential phase of growth, except where stated, by centrifugation at 10000g for 10 min at 4°C. Unless otherwise stated cells were washed twice with 25 mM-Mops (4-morpholinepropanesulphonic acid) buffer, pH 6.8, resuspended in buffer and stored at 0–4°C. Cells were always used within 6 h of harvesting.

#### *Protein determinations*

Protein concentrations of samples were determined by the method of Lowry *et al.* (1951); procedures were those of DeMoss & Bard (1957). Crystalline bovine serum albumin (fraction V) was used as standard.

#### *Cytochrome determinations*

Cytochrome spectra were recorded on a Cary 118C dual-beam spectrophotometer (Varian Associates, Walton-on-Thames, Surrey, U.K.) at ambient temperature or at 77K with a 2 mm-light-path low-temperature attachment. Reduced-minus-oxidized difference spectra were obtained by recording the spectra of samples (whole-cell suspensions, membrane and soluble cell fractions and washings), reduced with Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub> (a few crystals of solid) with the reference sample oxidized with approx. 0.05 ml of a 0.03% solution of H<sub>2</sub>O<sub>2</sub> or a small amount of solid K<sub>3</sub>Fe(CN)<sub>6</sub>. In some cases a few microlitres of a saturated solution of quinol were used to reduce

preparations instead of Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub>. Aeration of bacterial suspensions was achieved by passing air through cuvettes or by vigorous shaking immediately before recording the spectrum against an untreated or reduced sample. Spectra were recorded at the temperature of liquid N<sub>2</sub> (77K) by plunging samples into a Dewar flask containing liquid N<sub>2</sub> and running the spectrum while maintaining the base of the aluminium cell holder in a reservoir of the liquid gas. For the measurement of CO-binding pigments, suspensions were reduced with Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub> and, after reduction, CO was passed through the test cuvette and the (reduced-plus-CO)-minus-reduced spectrum was read. Base-lines were always checked before recording spectra by recording the spectrum with untreated samples in both cuvettes. The proportion of cytochrome *c* released into the medium during growth was calculated by measuring the quantity of cytochrome *c* (by means of the reduced-minus-oxidized difference spectra) in whole culture, in whole bacteria obtained by centrifugation and in the supernatant after removal of whole bacteria. Cytochrome contents were calculated by using the molar absorption coefficients given by Chance (1957): cytochrome *a* + *a*<sub>3</sub>, 601–635 nm, 16 mm<sup>-1</sup>·cm<sup>-1</sup>; cytochrome *b*, 556–575 nm, 22 mm<sup>-1</sup>·cm<sup>-1</sup>; cytochrome *c*, 549–535 nm, 19 mm<sup>-1</sup>·cm<sup>-1</sup>. Binding of CO to cytochrome *c* was calculated by using a molar absorption coefficient of 55 mm<sup>-1</sup>·cm<sup>-1</sup> (peak to trough). All spectra have been deposited in Supplement SUP 50111 with the British Library Lending Division.

#### *Measurement of midpoint redox potentials*

The procedures used for the measurement of midpoint redox potentials of the cytochromes *c* in soluble or membrane preparations were essentially those of Dutton (1971) with the purpose-built dual-wavelength spectrophotometer (Applied Photophysics, London W.1, U.K.) as described in the preceding paper (Cross & Anthony, 1980). Membrane preparations were reduced with ascorbate or NADH and oxidized with ferricyanide.

The following mediators were used: quinol, 25 μM (*E*<sub>m7</sub>, 280 mV); 2,3,5,6-tetramethyl-*p*-phenylenediamine, 50 μM (*E*<sub>m7</sub> 200 mV); phenazine methosulphonate, 25 μM (*E*<sub>m7</sub> 80 mV); phenazine ethosulphate, 25 μM (*E*<sub>m7</sub> 55 mV); pyocyanine, 6 μM (*E*<sub>m7</sub> –34 mV); 2-hydroxy-1,4-naphthoquinone, 20 μM (*E*<sub>m7</sub> –145 mV). Soluble preparations were titrated in the presence of 2,3,5,6-tetramethyl-*p*-phenylenediamine, quinol and 50 μM-K<sub>3</sub>Fe(CN)<sub>6</sub>. All redox titrations have been deposited as Supplement SUP 50111 with the British Library Lending Division.

#### *Preparation of membrane fractions*

Membrane preparations were made from approx. 250 ml samples of bacteria taken directly from the

chemostat pot. The bacteria were harvested by centrifugation and washed with ice-cold 25 mM-Mops buffer, pH 7.0, containing 5 mM-MgCl<sub>2</sub> and 5 mM-methanol and resuspended in the same buffer to a density of about 30 mg dry wt./ml. The bacterial suspension was passed twice through a French pressure cell press (Aminco, Silver Spring, MD, U.S.A.) at 135 MPa (20 000 lb/in<sup>2</sup>) and the resulting extract was centrifuged at 6000g for 10 min to remove whole bacteria and cell debris. The cell-free extract was centrifuged at 35 000g for 1 h to separate the particulate and soluble fractions. The particulate fractions were washed twice in buffer and stored in 0.7 ml lots in liquid N<sub>2</sub>.

#### Effect of inhibitors on respiration

Respiratory activities of whole bacteria and cell fractions were measured in a Clark-type oxygen electrode (Rank Bros., Bottisham, Cambs., U.K.) in a 2 ml reaction volume in the presence of 25 mM-Mops buffer, pH 7.0, with substrates at the following concentrations: methanol, 7.5 mM; NADH, NADPH, 1 mM; ascorbate + tetramethylphenylenediamine, 2 mM and 0.2 mM respectively. Ascorbate and tetramethylphenylenediamine were mixed at least 10 min before use; this was found to give the lowest non-enzymic oxidation rate. The following inhibitors were used: KCN (in 0.5 mM-Tris/HCl buffer, pH 8.0); NaN<sub>3</sub> (in 5 mM-Tris/HCl buffer, pH 8.0); antimycin A (in dimethyl sulphoxide); rotenone (in dimethyl sulphoxide); *n*-heptyl-4-hydroxyquinoline *N*-oxide (in dimethyl sulphoxide). Inhibitors were incubated with samples until a new linear rate was obtained or for 10 min if no inhibition was observed. Initial oxidation rates (before the addition of inhibitors) were always greater than 30 μl of O<sub>2</sub>/h (22.3 nmol of O<sub>2</sub>/min) to ensure that rates in the presence of inhibitors were large enough to measure accurately.

## Results

### Cytochromes of *Methylophilus methylotrophus*

All spectra and redox titrations have been deposited as Supplement SUP 50111 with the British Library Lending Division (see the Materials and methods section).

#### Cytochrome *a* + *a*<sub>3</sub>

A membrane-bound cytochrome of the *a*-type was present in bacteria grown in methanol-limited continuous culture and in batch cultures (Table 1). This cytochrome was probably a mixture of cytochromes *a* and *a*<sub>3</sub>, since CO binding was observed (see below). No cytochrome *a* + *a*<sub>3</sub> was detected in bacteria (or bacterial membranes) grown under conditions of O<sub>2</sub>- or nitrogen-limitation (when methanol was in excess).

Table 1. Effect of growth conditions on the cytochromes of *Methylophilus methylotrophus*

Cytochrome	Absorption maxima in difference spectra (nm)						Quantity			
	22°C		77K		(nmol/mg dry wt. of bacteria)	% of cytochrome binding CO	(nmol/mg of membrane protein)	Variable, N.D.-160	N.K.	N.K.
	<i>α</i>	<i>γ</i>	<i>α</i>	<i>γ</i>						
Cytochrome <i>a</i> + <i>a</i> <sub>3</sub>	Batch culture	602	440	600.5	440	Variable, N.D.-160	0.2	—	—	—
	Methanol-limitation	602	440	600.5	440	160	0.2	—	—	—
	Methanol-excess	N.D.	—	—	—	—	—	—	—	—
Cytochrome <i>b</i>	All conditions	563	428	563	428	220 (total)	0.55 (total)	—	—	N.K.
		555	428	558*	428					
Cytochrome <i>c</i>	Whole bacteria	550	420	548.5/545.5	420	835	—	—	—	30
	Membranes	550	420	548.5/545.5	420	400	1.22	—	—	31
	Soluble preparations	550	420	548.5/545.5	420	432	—	—	—	33
	Growth medium	550	420	548.5/545.5	420	480	—	—	—	35

\* This cytochrome *b*<sub>558</sub> peak was not present in methanol-limited preparations.

† Cytochrome *c* did not show splitting on membranes from methanol-limited bacteria.

### Cytochrome *b*

Two species of cytochrome *b* were identified in reduced-minus-oxidized difference spectra of whole bacteria. Cytochrome *c* usually obscured the absorption bands of the cytochromes *b*, and their demonstration depended on the fact that, after aeration, cytochrome *c* became reduced before the cytochromes *b* by endogenous reductants. The total amount of *b*-type cytochromes was not markedly different in bacteria grown under different conditions.

Reduced-minus-oxidized difference spectra of bacterial membranes at 77K revealed the presence of three *b*-type cytochromes with  $\alpha$ -band absorption maxima at 563, 558 and 554.5 nm. There appeared to be more of the cytochrome  $b_{558}$  in conditions where methanol was in excess. It is possible that this cytochrome was cytochrome *o*, because the quantity of the CO-binding cytochrome *b* appeared to be higher in these growth conditions (see below).

Spectra (at 77K) of quinol-reduced minus spectra of ferricyanide-oxidized membrane preparations showed reduction of all the cytochrome components with the exception of cytochrome  $b_{563}$ , indicating that this cytochrome had the lowest redox potential, and this was consistent with conclusions drawn from direct measurements of redox potentials (see below).

### Cytochrome *c*

Cytochrome(s) *c* having absorption maxima in reduced-minus-oxidized difference spectra at 550, 520 and 420 nm was always produced by *M. methylotrophus* in high quantities, and an unusual feature was that 35% of the total was released into the growth medium in both batch and continuous culture (Table 1). This amount was far too high to be attributed to cell lysis. Washing of the cells did not remove significantly more cytochrome, nor did it affect the rate of methanol oxidation. The cytochrome released into the growth medium was fully reduced when kept in the dark, but on exposure to light the cytochrome was reversibly photo-oxidized. This effect was lost on concentration (7-fold) by using a 10000-mol.wt.-cut-off filter and restored on addition of the ultrafiltrate, indicating that the molecular weight of at least one of the components involved in the photo-oxidation was less than 10000 (the cytochrome was retained by the filter). The 'spent' growth medium and ultrafiltrate showed a green fluorescence in u.v. light, and it is possible that the photo-oxidation was catalysed by a molecule such as flavin, which may have been released into the growth medium. The nature of this cytochrome *c* has not been further investigated.

Disruption of the bacteria by passage through a French pressure cell released further quantities of cytochrome(s) *c*, but, even after washing of parti-

culate fractions with concentrated salt solutions (500 mM-KCl) and further sonication to disrupt vesicles, 48% of the cytochrome *c* remained bound to the membranes (Table 1). The cytochromes remaining bound to the membrane did not appear to be different types on the basis of redox potentials and CO binding (see below).

It has been previously shown that the soluble cytochrome *c* released by passage through the French pressure cell consists of three distinct cytochromes *c* (cytochromes  $c_H$ ,  $c_{LM}$  and  $c_L$ ), and these have been completely purified and characterized (Cross & Anthony, 1980). The two major components both show  $\alpha$ -band splitting at 77K in the pure state, and the soluble cell fraction of both methanol-limited and methanol-excess bacteria ( $O_2$ - or nitrogen-limited) also exhibited a split cytochrome *c*  $\alpha$ -peak at low temperature. The  $\alpha$ -peak of cytochrome *c* was also split on membranes of methanol-excess ( $O_2$ - or nitrogen-limited) bacteria, giving a peak at 548.5 nm and a shoulder at 545.5 nm; these are similar to the split peaks of the pure cytochromes *c* (Cross & Anthony, 1980), indicating that the  $\alpha$ -peaks of some of the cytochromes *c* also split at 77K when the cytochromes are bound to the membrane. On membranes of methanol-limited bacteria no splitting of the  $\alpha$ -peak was observed. However, there is insufficient evidence to distinguish between the possibility that the cytochromes are arranged on the membrane in such a way that splitting no longer occurs and the possibility that the small amount of a third different cytochrome *c* (that does not show  $\alpha$ -splitting) present on these membranes might obscure the splitting of the  $\alpha$ -band of the other cytochromes.

Membranes of methanol-limited bacteria always had an unidentified component detected at 77K in the (dithionite-reduced)-minus-( $H_2O_2$ -oxidized) difference spectrum at 450 nm that was absent from membranes of methanol-excess bacteria.

Because spectra (at 77K) were identical with NADH or dithionite as reductant, it can be concluded that there is an electron-transport pathway between NADH and all the cytochromes demonstrated above.

### Reaction of the cytochromes of *Methylophilus methylotrophus* with CO

Cytochrome  $a_3$  was indicated by a trough at 440 nm in spectra of methanol-limited bacteria. Under growth conditions where cytochrome  $a + a_3$  was not present, no CO-binding *a*-type cytochrome was detected.

In bacteria grown under conditions of methanol-excess, a *b*-type cytochrome capable of binding CO was indicated by a trough at 429 nm and a shoulder at 556 nm; a small amount of this CO-binding cytochrome *b* was also indicated in the spectra of

methanol-limited bacteria. Results presented below support the conclusion that this *b*-type cytochrome has an oxidase function, and it is therefore referred to throughout the rest of this work as cytochrome *o*.

The presence of a *c*-type cytochrome able to bind CO was indicated by a trough at 550 nm and peaks at 535 and 410 nm. This binding was exhibited to the same extent (about 30%; Table 1) in whole bacteria, in growth medium and in soluble and particulate cell fractions.

Exposure to CO for 15 s was sufficient to cause maximal binding to cytochrome  $a_3$ . Approx. 30 s exposure was necessary for the maximal formation of the cytochrome *o*-CO complex. Cytochrome *c* required at least 10 min incubation with CO for full combination.

#### *Redox potentiometry of the membrane-bound cytochromes b and c*

From the results of the redox titrations summarized in Table 2 it can be seen that under conditions of methanol-excess (nitrogen-limitation) there were three cytochromes *b* with midpoint redox potentials of 260, 109 and 60 mV, which contributed to the absorption changes at 563–570 nm in the proportion 25, 37 and 39% respectively. By contrast, under conditions of methanol-limitation only two components were found, with midpoint potentials of 110 mV and 61 mV, contributing 16 and 84% respectively. Thus under conditions of methanol-excess an extra *b* cytochrome was present with a midpoint potential of 260 mV. This *b* cytochrome is almost certainly the cytochrome *o* (CO-binding cytochrome *b*) shown to be present under these conditions (methanol-excess) to a much greater extent than in conditions of methanol-

limitation, where cytochrome  $a + a_3$  was the terminal oxidase (see above). Cytochromes *o* typically have high redox potentials (greater than 100 mV), which are consistent with their oxidase function.

The percentage contributions to the absorbance changes during the redox titrations only reflect the relative amounts of each component if the absorption maxima are very similar and if the molar absorption coefficients are the same. With the cytochromes *b* considered here these conditions do not apply. The change in percentage contributions in membranes from bacteria grown in different growth conditions do, however, reflect changes in the relative proportions of the cytochrome *b* with changing growth conditions. Thus the proportions of the two cytochromes *b* with midpoint redox potentials of 60 and 110 mV are clearly different in membranes of bacteria grown in different growth conditions; in bacteria grown in methanol-excess conditions (nitrogen-limited) the proportion of the lower-potential (60 mV) to higher-potential (110 mV) cytochrome *b* is relatively lower than in bacteria grown under methanol-limitation. Although it is not possible to measure accurately the total amount of cytochrome *b*, the total was not markedly different in bacteria grown in different conditions. Spectra of samples reduced with quinol suggested that cytochrome  $b_{563}$  had the lowest midpoint redox potential, and thus the most likely assignment of wavelengths to the *b*-type cytochrome is as listed in Table 2.

Resolution of the data for the cytochrome *c* titrations was difficult because of the proximity of the midpoint potentials, but the best fit was obtained by assuming two components in the membranes from methanol-excess (nitrogen-limited) bacteria and three components in membranes from methanol-

Table 2. Summary of the properties of membrane-bound cytochromes of *Methylophilus methylotrophus*

The midpoint redox potentials were taken from titrations published in Supplement SUP50111 (see the text). The wavelength pairs were 550 and 540 nm and 563 and 570 nm. Because the cytochromes *c* have virtually the same  $\alpha$ -band absorption maxima at room temperature, the percentage contributions are a measure of the amount of each cytochrome *c* present (assuming similar molar absorption coefficients). This does not apply to the *b*-type cytochromes (see the text).

Growth conditions	Cytochrome	$E_{m7}$ (mV)	$\alpha$ -Band absorption maximum (at 77 K) (nm)	% contribution to absorbance change
Methanol excess	Cytochrome $c_H$	375	548/545.5 (sh)	69
	Cytochrome $c_L$	313	548/545.5 (sh)	31
	Cytochrome <i>o</i>	260	558	25
	Cytochrome <i>b</i>	109	554	36
	Cytochrome <i>b</i>	60	563	39
Methanol limitation	Cytochrome $c_H$	374	548	69
	Cytochrome $c_{LM}$	356	548	15
	Cytochrome $c_L$	310	548	16
	Cytochrome <i>b</i>	110	554	16
	Cytochrome <i>b</i>	61	563	84
	Cytochrome $a + a_3$	>400	601	

limited bacteria. Two components appeared to be the same in both samples, with midpoint redox potentials of 375 and 310 mV; it is probable that these are cytochromes  $c_H$  and  $c_L$  respectively, since the midpoint redox potentials were the same as those found for the purified cytochromes (Cross & Anthony, 1980). The third component (midpoint redox potential 356 mV), which was present only in membranes from methanol-limited bacteria, was possibly cytochrome  $c_{LM}$ , which, in the soluble form, has a midpoint redox potential of 336 mV (Cross & Anthony, 1980). The total amount of cytochrome  $c$  was not markedly different in bacteria grown in different conditions, and the results in Table 2 indicate that about 70% of the membrane-bound cytochrome  $c$  was cytochrome  $c_H$  and that the proportion of cytochrome  $c_L$  might be lower in methanol-limited conditions.

It was not possible to determine the midpoint redox potential of the cytochrome  $a + a_3$ , as the potential required to oxidize the cytochrome was greater than could be satisfactorily reached with ferricyanide, suggesting that the midpoint redox potential was greater than 400 mV.

#### *Respiratory properties of Methylophilus methylophilus*

The results with bacteria grown under methanol-excess conditions were exactly the same when either

$O_2$  or the nitrogen source ( $NH_4^+$ ) was the limiting nutrient. For convenience only one Figure is presented to illustrate the effects of inhibitors on respiration (Fig. 1); all conclusions in the present paper were based on similar Figures. The inhibitor concentrations giving 50% inhibition of respiration are summarized in Table 4.

#### *Respiratory activities of whole bacteria and membranes*

Whole *M. methylophilus* cells were found to oxidize methanol, formaldehyde, formate, NADH, NADPH and ascorbate + *NNN'N'*-tetramethylphenylenediamine (Table 3). Oxidation of NADH and NADPH by whole bacteria is unusual, but has been observed previously in whole cells of *Haemophilus parainfluenzae* (White & Sinclair, 1970). The rates of oxidation of all substrates were similar in all growth conditions with the exception that the rate with ascorbate + tetramethylphenylenediamine was greater in conditions of methanol-excess ( $O_2$ - or nitrogen-limitation) than in methanol-limitation.

Membrane preparations oxidized NADH, NADPH and ascorbate + tetramethylphenylenediamine (Table 3), but methanol was oxidized at very low rates or not at all, although methanol dehydrogenase was present in these preparations (specific activity 225 nmol of methanol oxidized/min per mg of protein). Ascorbate + tetramethylphenyl-

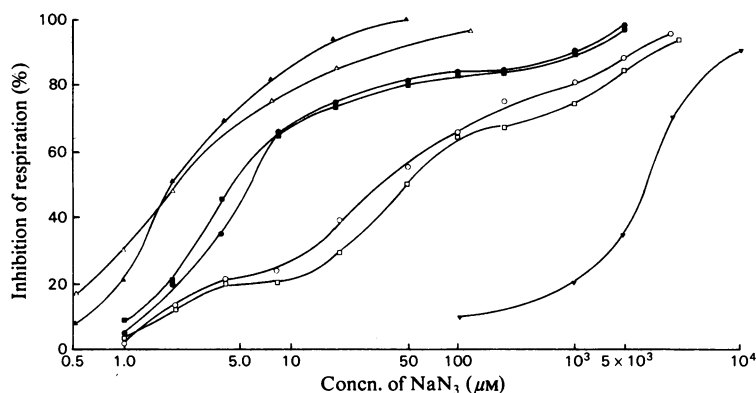


Fig. 1. Inhibition by azide of respiration in whole bacteria, membranes and soluble bacterial extracts

The inhibition was determined as described in the Materials and methods section. ▲, Ascorbate + tetramethylphenylenediamine oxidation by membranes from methanol-excess ( $O_2$ -limited) bacteria (no cytochrome  $a + a_3$ ); the uninhibited oxidation rate was 2604 nmol of  $O_2$ /min per mg dry wt. of bacteria. Δ, Ascorbate + tetramethylphenylenediamine oxidation by membranes from methanol-limited bacteria (cytochrome  $a + a_3$  present); the uninhibited oxidation rate was 170 nmol of  $O_2$ /min per mg dry wt. of bacteria. ■, Methanol oxidation by methanol-excess ( $O_2$ -limited) bacteria (no cytochrome  $a + a_3$ ); the uninhibited oxidation rate was 251 nmol of  $O_2$ /min per mg dry wt. of bacteria. □, Methanol oxidation by methanol-limited bacteria (cytochrome  $a + a_3$  present); the uninhibited oxidation rate was 225 nmol of  $O_2$ /min per mg dry wt. of bacteria. ●, NADH oxidation by methanol-excess ( $O_2$ -limited) bacteria (no cytochrome  $a + a_3$ ); the uninhibited oxidation rate was 225 nmol of  $O_2$ /min per mg dry wt. of bacteria. ○, NADH oxidation by methanol-limited bacteria (cytochrome  $a + a_3$  present); the uninhibited oxidation rate was 222 nmol of  $O_2$ /min per mg dry wt. of bacteria. ▼, Methanol oxidation by the soluble cell fraction from methanol-excess ( $O_2$ -limited) bacteria; the uninhibited oxidation rate was 3 nmol of  $O_2$ /min per mg of protein.

Table 3. Respiratory activities of membrane preparations and whole cells of *Methylophilus methylotrophus* grown in continuous culture under various growth limitations

Respiration rates are expressed as nmol of O<sub>2</sub>/min per mg dry wt. of bacteria or per mg of membrane protein. There was no endogenous rate in the absence of substrate. Respiration rates have been corrected for the non-enzymic rate of ascorbate + tetramethylphenylenediamine (TMPD) oxidation, which was always less than 5% of the respiratory rate. N.D., Not determined.

Respiratory substrate	Limiting growth substrate	...	Respiration rate		
			Methanol	Nitrogen	O <sub>2</sub>
<b>Whole bacteria</b>					
Methanol			251	254	288
Ascorbate + TMPD			47	222	238
NADH			171	202	201
NADPH			62	67	65
Formaldehyde			130	N.D.	119
Formate			25	N.D.	28
<b>Membrane preparations</b>					
Methanol			3.7	—	—
Ascorbate + TMPD			245	2615	2361
NADH			66	59	73
NADPH			31	26	36
Formaldehyde			0	0	0
Formate			0	0	0

enediamine was oxidized at an extremely high rate in membranes prepared from bacteria grown under methanol-excess conditions. Under these conditions there was no cytochrome *a* + *a*<sub>3</sub> and the concentration of cytochrome *o* was markedly increased (to 200 pmol of cytochrome *o*/mg of membrane protein). The rate of oxidation of ascorbate + tetramethylphenylenediamine was only 10% of this rate in membranes prepared from bacteria grown under methanol-limitation. The lower rate of oxidation of ascorbate + tetramethylphenylenediamine measured in whole bacteria compared with these membrane preparations was probably due to a relatively low permeability of whole bacteria to this substrate.

#### Methanol oxidation in soluble preparations

Methanol was slowly oxidized by the soluble cell fraction after the removal of the membrane fraction. The only detectable cytochrome was cytochrome *c*. The pattern of inhibition was monophasic (Fig. 1) with 50% inhibition at 90 μM-cyanide or 1.1 mM-azide, consistent with the suggestion that the cyanide- and azide-resistant oxidation of methanol and NAD(P)H in whole cells and membranes could be due to this autoxidizable cytochrome *c*. This oxidation was probably by way of cytochrome *c*<sub>L</sub>, which is the most autoxidizable cytochrome *c* and the cytochrome that is reduced most rapidly by methanol dehydrogenase (Cross & Anthony, 1980).

#### Reduction of cytochromes in whole bacteria and membranes by methanol and NADH

In methanol-limited bacteria the cytochromes did

not become reduced in the absence of added substrate. Addition of NADH to an aerobic suspension of *M. methylotrophus* caused a rapid reduction of all the cytochromes; the addition of methanol to an aerobic suspension of bacteria caused a rapid reduction of cytochrome(s) *c*, cytochrome *a* + *a*<sub>3</sub> and some *b*-type cytochrome (presumably the cytochrome *o*). The remainder of the cytochrome *b* became reduced only after a period of several minutes. Similar results were obtained with bacteria grown in batch cultures, or under conditions of methanol-excess, except that there was sufficient endogenous substrate present to reduce fully all the cytochrome *c*; the rate of endogenous reduction was always lower than in the presence of added substrate. NADH and NADPH, but not methanol, rapidly reduced all cytochromes on membrane preparations.

#### Inhibition by cyanide and azide of respiration in bacteria grown in continuous culture methanol-excess conditions

The oxidation of ascorbate + tetramethylphenylenediamine was powerfully inhibited by cyanide and azide. The inhibition pattern with whole cells and membranes was monophasic (suggesting one predominant oxidase), with 50% inhibition occurring at 0.9 μM-KCN and 2 μM-azide (Fig. 1 and Table 4). As cytochrome *a* + *a*<sub>3</sub> was absent from these bacteria, this cyanide- and azide-sensitive respiration was presumably by way of the cytochrome *o* induced to high concentrations in methanol-excess conditions (see above).

The oxidation of NAD(P)H differed from that of

Table 4. *Inhibitor concentrations required to cause 50% inhibition of oxidase activity in whole bacteria and membrane preparations*

The methods used to measure inhibition of respiratory activity are described in the Materials and methods section. The substrates used were NADH, NADPH, ascorbate + tetramethylphenylenediamine (for whole bacteria and membranes) and methanol (whole bacteria only).

	Concn. of KCN ( $\mu\text{M}$ )	Concn. of $\text{NaN}_3$ ( $\mu\text{M}$ )
Potential oxidase		
Cytochrome $a + a_3$	0.4–2.0	50
Cytochrome $o$	0.4–2.0	2–4
Cytochrome $c$	120	1000

ascorbate + tetramethylphenylenediamine in that inhibition by cyanide and azide was biphasic (Fig. 1); the pattern for NADH and NADPH was always very similar. In whole bacteria and in membrane preparations about 75% of respiration was by way of an oxidase that was very sensitive to cyanide and azide (50% inhibition at  $1.9\mu\text{M}$ -KCN and  $5\mu\text{M}$ -azide), indicating that cytochrome  $o$ , responsible for the oxidation of ascorbate + tetramethylphenylenediamine, was also involved in the oxidation of NAD(P)H.

The inhibition of oxidation of methanol by whole bacteria was also biphasic, 70% of respiration being very sensitive to cyanide and azide (50% inhibition at  $0.8\mu\text{M}$ -KCN and  $4\mu\text{M}$ -azide) (Fig. 1); thus methanol oxidation also appears to be by way of cytochrome  $o$ .

As stated above, inhibition by cyanide and azide of oxidation of methanol in whole bacteria, and NADH in whole bacteria and membranes, was biphasic after growth in methanol-excess conditions. In the presence of sufficient inhibitor to prevent respiration by the cyanide-sensitive oxidase (cytochrome  $o$ ) respiration could continue at up to about 30% of the uninhibited rate. This respiration, which was probably non-physiological, was sensitive only to high concentrations of inhibitors, 50% inhibition being obtained with  $100\mu\text{M}$ -KCN and about  $1\text{mM}$ -azide; this inhibitor-insensitive respiration was probably by way of a slowly autoxidizable cytochrome  $c$  (Cross & Anthony, 1980). It may be concluded from the results above that ascorbate + tetramethylphenylenediamine was oxidized exclusively by way of cytochrome  $o$ , which is very sensitive to cyanide and azide (50% inhibition at less than  $5\mu\text{M}$ ), and that this oxidase was also involved in the oxidation of methanol and NAD(P)H. The reason that the inhibition of oxidation of ascorbate + tetramethylphenylenediamine was not biphasic may be that the maximum rate of cytochrome  $c$  autoxidation is very low compared with the high rate of ascorbate + tetra-

methylphenylenediamine oxidation by way of cytochrome  $o$  and hence is not readily observed in curves of inhibition concentration plotted against percentage inhibition of respiration.

*Inhibition by cyanide and azide of respiration in bacteria grown in methanol-limited continuous culture*

Bacteria grown under methanol limitation differed from those grown under conditions of methanol-excess ( $\text{O}_2$ - or nitrogen-limited) by having an extra potential oxidase, cytochrome  $a + a_3$ , and by having much less cytochrome  $o$  (Tables 1 and 2).

The inhibition by azide of ascorbate + tetramethylphenylenediamine oxidation by whole cells and membranes was monophasic (with 50% inhibition at  $2.2\mu\text{M}$ -azide) (Fig. 1) and hence similar to that found in methanol-excess cultures. By contrast, during oxidation of methanol and NAD(P)H by membranes a second oxidase was involved, as shown by the complex inhibition pattern seen with azide (Fig. 1). That there was usually some inhibition of methanol and NAD(P)H oxidation by this low concentration of azide suggests that the same oxidase (cytochrome  $o$ ) was involved in the oxidation of all these substrates. The second phase of inhibition (50% inhibition at about  $50\mu\text{M}$ -azide) was presumably due to inhibition of the oxidase only present in methanol-limited bacteria, i.e. cytochrome  $a + a_3$ . The third phase of inhibition occurring in the presence of high concentrations of azide corresponded to that due to the same non-physiological autoxidation of cytochrome  $c$  as was found in methanol-excess bacteria (50% inhibition at  $1.5\text{mM}$ -azide), which have no cytochrome  $a + a_3$  to confuse interpretation of results.

Whereas the pattern of inhibition by azide of methanol and NAD(P)H oxidation was triphasic, the pattern with cyanide was biphasic, with respiration being almost completely inhibited by low concentrations of cyanide (50% inhibition at  $0.4$ – $2\mu\text{M}$ -cyanide). In the presence of high concentrations of cyanide some respiration occurred by way of a cytochrome  $c$  with similar characteristics to those found in methanol-excess preparations (50% inhibition at  $80$ – $150\mu\text{M}$ -KCN). The oxidation of ascorbate + tetramethylphenylenediamine was completely inhibited by  $2\mu\text{M}$ -cyanide, and the lack of cyanide-resistant respiration suggests either that there is no cytochrome  $c$ -dependent respiration from this substrate or that the rate is too low to be detected.

These results with cyanide, taken together with those for azide, were consistent with the cytochrome  $a + a_3$  having the same sensitivity to cyanide as the cytochrome  $o$ , but different sensitivities to azide (see Table 4 for summary).

*Inhibition of respiration by n-heptyl-4-hydroxyquinoline N-oxide, antimycin A and rotenone*

Similar results were obtained for these inhibitors with bacteria grown in all conditions.

About 80% of NADH oxidation by membranes was inhibited by low concentrations of heptylhydroxyquinoline *N*-oxide and antimycin A, with 50% of this inhibition occurring at 4.5  $\mu\text{M}$ -heptylhydroxyquinoline *N*-oxide and 5  $\mu\text{M}$ -antimycin A. About 20% of the oxidation was insensitive to these inhibitors and may be by way of an inhibitor-resistant site. Unexpectedly, ascorbate + tetramethylphenylenediamine oxidation was also inhibited by heptylhydroxyquinoline *N*-oxide and antimycin A but at higher concentrations than those required to inhibit NADH oxidation, with 50% inhibition occurring at 22  $\mu\text{M}$ -heptylhydroxyquinoline *N*-oxide and 45  $\mu\text{M}$ -antimycin A. High concentrations of heptylhydroxyquinoline *N*-oxide inhibited virtually all ascorbate + tetramethylphenylenediamine oxidation, but antimycin A inhibited only 70% at the highest concentration used (400  $\mu\text{M}$ ). Neither inhibitor was effective with whole bacteria. In view of the different concentrations required to inhibit NADH and ascorbate + tetramethylphenylenediamine oxidation, it is likely that they are reacting at two sites, at low concentration between cytochromes *b* and *c* (inhibiting NADH oxidation) and at higher concentrations at cytochrome *o* (inhibiting ascorbate + tetramethylphenylenediamine oxidation).

Rotenone was effective in inhibiting NADH oxidation by membranes, 50% inhibition occurring at 0.5 mM; the effect of rotenone on NADPH oxidation was not tested. Rotenone did not inhibit oxidation of any substrate by whole cells nor the oxidation of ascorbate + tetramethylphenylenediamine by membranes.

**Discussion**

The results with inhibitors show that *M. methylotrophus* contains up to three potential oxidases, cytochromes *c*, *o* and  $a + a_3$ , and Table 5 indicates the maximum proportions of electron transport able to go by way of each of these. It should be noted that

the percentage of respiration able to go through a particular route does not mean that the route is usually active, because this will be determined by the  $K_m$  for  $\text{O}_2$  of each potential oxidase, by the redox state of the oxidase and by the concentration of  $\text{O}_2$  present.

It should be noted that respiration by way of cytochrome *c* could only be demonstrated unequivocally in the presence of sufficient cyanide or azide to inhibit oxidation by way of cytochromes  $a + a_3$  and/or *o*. In these conditions the cytochrome *c* could be more reduced than in the absence of inhibitors and the rate of its autoxidation could have been higher than ever might occur in the absence of inhibitors. Because membranes always contained the oxidases cytochrome *o* and/or cytochrome  $a + a_3$ , the rate of autoxidation of the membrane-bound cytochrome *c* could not be measured. It is not known if the slightly autoxidizable soluble cytochrome  $c_L$  (Cross & Anthony, 1980) is the cytochrome *c* responsible for oxidation by membranes, but the similarity in sensitivity to inhibition by KCN and azide of methanol oxidation by the soluble fraction suggests that it is. It is unlikely that respiration by way of cytochrome *c* is physiologically significant.

During growth in excess-methanol conditions there was no cytochrome  $a + a_3$ , and so an alternative oxidase must have been operating. It is appreciated that the absence of a spectrally detectable cytochrome does not necessarily mean that it is completely absent; a component present in low concentrations with a high turnover might still be functional. However, in these excess-methanol conditions an alternative oxidase was induced 10-fold as determined by the increase in the rate of oxidation of ascorbate + tetramethylphenylenediamine. This increase was concomitant with the appearance in membranes of an extra, high-potential (260 mV), *b*-type cytochrome seen as a peak at 558 nm in spectra measured at 77 K. It may thus be concluded that this inducible *b*-type cytochrome is an alternative oxidase, cytochrome *o*. The high turnover number for ascorbate + tetramethylphenylenediamine oxidation of 218  $\text{s}^{-1}$  lies within the range of other oxidases (Chance & Williams, 1956; Smith,

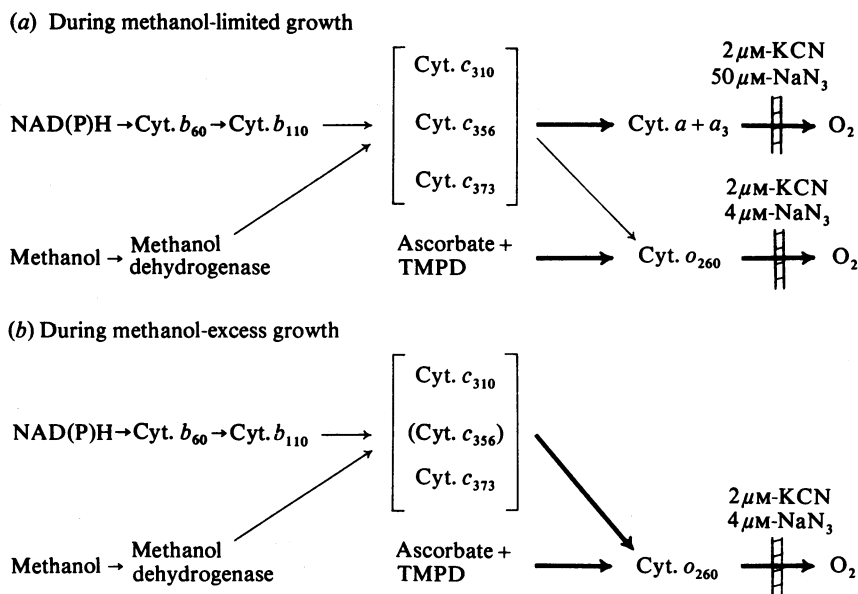
Table 5. *Estimated maximum proportion of electron transport to oxygen via each potential oxidase*  
The methods used to measure respiratory inhibition are described in the Materials and methods section, and the proportions below were calculated from curves of the type illustrated in Fig. 1.

Growth conditions	Proportion of electron transport (%)		
	Cytochrome $a + a_3$	Cytochrome <i>o</i>	Cytochrome <i>c</i>
Methanol-limitation	60	15	25
Methanol-excess	0	75	25

1961), and indicates either that the cytochrome *o* (midpoint redox potential 260mV) reacts directly with ascorbate + tetramethylphenylenediamine (midpoint redox potential of tetramethylphenylenediamine 255mV) or that a cytochrome *c* is present in the membrane that is capable of very rapid reaction with both ascorbate + tetramethylphenylenediamine and with cytochrome *o*. This is probably unlikely; if such a cytochrome *c* is present it cannot be the autoxidizable cytochrome *c* observed in inhibition studies with methanol with NADH because of the monophasic inhibition pattern by cyanide and azide of ascorbate + tetramethylphenylenediamine oxidation. The cytochrome *o* is unusual in being inhibited by heptylhydroxyquinoline *N*-oxide and antimycin A; in this respect it resembles the cytochrome *o* of *Acetobacter suboxydans* (Daniel, 1970). The simplest scheme that is consistent with all the evidence presented above and published previously (Cross & Anthony, 1980) is shown in Scheme 1. The key proposals of this scheme are that the branch

point for the entry of electrons from methanol dehydrogenase is at cytochrome *c* (after cytochromes *b*<sub>60</sub> and *b*<sub>110</sub>) and that the branch to cytochrome *o* and *a* + *a*<sub>3</sub> is from cytochrome *c* and not from cytochrome *b*. The proposal that electrons from methanol dehydrogenase enter the electron-transport chain at the level of cytochrome *c* is consistent with the systems that have been proposed for other methylotrophs, and there is nothing to indicate that *M. methylotrophus* differs from other methylotrophs in this respect.

The direct evidence that cytochrome *c* is involved in the oxidation of methanol by *M. methylotrophus* is that methanol rapidly reduces all the cytochrome *c* in whole bacteria, that the soluble fraction, in which the only cytochrome is the slightly autoxidizable cytochrome *c*, oxidizes methanol, and that pure methanol dehydrogenase reacts directly with soluble cytochrome *c*<sub>L</sub> (this occurs in the absence of added methanol; Cross, 1980). Further, extensive evidence showing that cytochrome *c*, but not cytochrome *b*, is



Scheme 1. Proposed pathways for electron transport in *Methylophilus methylotrophus*

The arrows indicate flow of electrons; they do not necessarily imply a direct reaction between components. The hatched bars indicate the site of action of inhibitors (inhibition concentrations are for 50% inhibition; see Table 4). The subscripts on the cytochromes refer to the midpoint redox potential measured at pH 7.0. The thickness of the lines indicates the probably relative importance of the alternative oxidases. Although ascorbate + tetramethylphenylenediamine may be oxidized to some extent by way of cytochrome *c*, the monophasic inhibition of this oxidation suggests that the cytochrome *c* with which it reacts does not react with cytochrome *a* + *a*<sub>3</sub> but that it must react very rapidly with cytochrome *o*. For convenience the cyanide- and azide-insensitive autoxidation of cytochrome *c* is not included (50% inhibition occurs at 120 μM-KCN or 1 mM-NaN<sub>3</sub>). Whether the three high-potential cytochromes *c* (Cross & Anthony, 1980) have separate roles is not known. It should be noted that, in addition to the membrane-bound cytochrome *c*, there is an equal amount of cytochrome *c* in solution. Abbreviations: TMPD, *NNN'*-tetramethylphenylenediamine; Cyt., cytochrome.

involved in the oxidation of methanol in other methylotrophs has been published elsewhere (Tonge *et al.*, 1974; Anthony, 1975; Widdowson & Anthony, 1975; O'Connor *et al.*, 1977; Bamforth & Quayle, 1978; O'Keefe & Anthony, 1978; van Verseveld & Stouthamer, 1978; Higgins, 1979; Keevil & Anthony, 1979; Netrusov & Anthony, 1979; O'Keefe & Anthony, 1980*a,b*).

Scheme 1 proposes that cytochrome *c* is also involved in the oxidation of NAD(P)H; this is suggested from the experiments (above) showing that NADH rapidly reduced all the cytochrome *c* in whole bacteria and that, in the presence of concentrations of cyanide and azide sufficient to inhibit cytochromes *a+a<sub>3</sub>* and *o*, a small amount of oxidation continued via cytochrome *c*. The involvement of cytochrome *c* in both methanol and NAD(P)H oxidation places the branch point to cytochrome *o* at the level of cytochrome *c* and not at the level of cytochrome *b*. This is consistent with most typical cytochrome *c*-containing heterotrophic bacteria, where branching to alternative inhibitor-sensitive oxidases occurs at the level of cytochrome *c* (Jones, 1977).

The proposed scheme for *M. methylotrophus* differs from those proposed for other methylotrophs in that cytochrome *o* is the main oxidase when bacteria are grown under methanol-excess conditions; cytochrome *o* has not been shown to be involved in methanol oxidation in other methylotrophs.

In *M. methylotrophus*, the regulation of the terminal oxidases cytochromes *a+a<sub>3</sub>* and *o* appears to be in response to the methanol concentration experienced by the bacteria and not by the O<sub>2</sub> concentration. When methanol was the growth-limiting substrate, cytochrome *a+a<sub>3</sub>* was maximally induced (and cytochrome *o* repressed). When nitrogen or O<sub>2</sub> supply became growth-limiting, cytochrome *o* was induced (approx. 10-fold) and cytochrome *a+a<sub>3</sub>* was repressed. The regulatory molecule is not known. The response of the bacteria to an excess of methanol may be to oxidize this substrate rapidly to reduce its toxic effects. Unfortunately, preliminary results of respiration-driven proton translocation experiments (Cross, 1980) were not sufficiently consistent to determine whether the substitution of one oxidase (cytochrome *a+a<sub>3</sub>*) by another (cytochrome *o*) alters the phosphorylation efficiency of the *M. methylotrophus* with a resulting change in yield, but it is known that this organism gives higher yields when grown under methanol-limited conditions than when grown under conditions of methanol-excess (Brooks & Meers, 1973).

We thank The Royal Society for financial assistance, the Science Research Council for a C.A.S.E. studentship

(with ICI Ltd.) to A. R. C., and Dr. G. M. Tonge (industrial supervisor at ICI Ltd.).

## References

- Anthony, C. (1975) *Biochem. J.* **146**, 289–298  
 Anthony, C. (1978) *J. Gen. Microbiol.* **104**, 91–104  
 Anthony, C. (1980) in *Hydrocarbons in Biotechnology* (Higgins, I. J., ed.), pp. 35–57, Hayden, London  
 Bamforth, C. & Quayle, J. R. (1978) *Arch. Microbiol.* **119**, 91–97  
 Brooks, J. D. & Meers, J. L. (1973) *J. Gen. Microbiol.* **77**, 513–519  
 Chance, B. (1957) *Methods Enzymol.* **4**, 273–329  
 Chance, B. & Williams, G. R. (1956) *Adv. Enzymol. Relat. Subj. Biochem.* **17**, 65–134  
 Colby, J. & Zatman, L. J. (1973) *Biochem. J.* **132**, 101–112  
 Cross, A. R. (1980) Ph.D. Thesis, University of Southampton  
 Cross, A. R. & Anthony, C. (1978) *Proc. Soc. Gen. Microbiol.* **5**, 42  
 Cross, A. R. & Anthony, C. (1980) *Biochem. J.* **192**, 421–427  
 Dahl, J. S., Mehta, R. J. & Hoare, D. S. (1972) *J. Bacteriol.* **109**, 916–921  
 Daniel, R. M. (1970) *Biochim. Biophys. Acta* **216**, 328–341  
 DeMoss, R. D. & Bard, R. C. (1975) in *Manual of Microbial Methods*, p. 179, Society of American Bacteriologists, Committee on Bacteriological Techniques, McGraw-Hill, London  
 Dutton, P. L. (1971) *Biochim. Biophys. Acta* **226**, 63–80  
 Goldberg, I., Rock, J. S., Ben-Bassat, A. & Mateles, R. I. (1976) *Biotechnol. Bioeng.* **18**, 1657–1668  
 Higgins, I. J. (1979) *Int. Rev. Biochem.* **21**, 300–353  
 Jones, C. W. (1977) *Symp. Soc. Gen. Microbiol.* **27**, 23–59  
 Keevil, C. W. & Anthony, C. (1979) *Biochem. J.* **182**, 71–79  
 Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275  
 Netrusov, A. I. & Anthony, C. (1979) *Biochem. J.* **178**, 353–360  
 O'Connor, M. L., Wopat, A. & Hanson, R. S. (1977) *J. Gen. Microbiol.* **98**, 265–272  
 O'Keefe, D. T. & Anthony, C. (1978) *Biochem. J.* **170**, 561–567  
 O'Keefe, D. T. & Anthony, C. (1980*a*) *Biochem. J.* **190**, 481–484  
 O'Keefe, D. T. & Anthony, C. (1980*b*) *Biochem. J.* **192**, 411–419  
 Smith, L. (1961) in *The Bacteria* (Gunsalus, I. C. & Stanier, R. Y., eds.), vol. 2, p. 364, Academic Press, New York and London  
 Tonge, G. M., Knowles, C. J., Harrison, D. E. F. & Higgins, I. J. (1974) *FEBS Lett.* **44**, 106–110  
 van Dijken, J. P. & Harder, W. (1975) *Biotechnol. Bioeng.* **17**, 15–30  
 van Verseveld, H. W. & Stouthamer, A. H. (1978) *Arch. Microbiol.* **118**, 13–20  
 White, D. C. & Sinclair, P. R. (1970) *Adv. Microb. Physiol.* **5**, 173–211  
 Widdowson, D. & Anthony, C. (1975) *Biochem. J.* **152**, 349–356