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# The Microbial Metabolism of $C_1$ Compounds

## THE CYTOCHROMES OF *PSEUDOMONAS* AM1

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*Pseudomonas* AM1 contains cytochromes *a*, *b* and *c* and more than one CO-binding pigment (cytochrome $a_3$, cytochrome *c* and possibly a cytochrome *o*). The soluble cytochrome *c* has been purified; its isoelectric point is low and its molecular weight is 20000. This cytochrome is reduced in whole bacteria by all oxidizable substrates at rates determined by the primary dehydrogenases. A mutant lacking cytochrome *c* oxidizes all substrates except methanol, ethanol and methylamine; these no longer support growth. The role of cytochrome *c* in electron transport in *Pseudomonas* AM1 is discussed.

*Pseudomonas* AM1 is a typical facultative methylotroph (Colby & Zatman, 1972; Anthony, 1975), able to grow on a wide range of carbon and energy sources (Peel & Quayle, 1961) including $C_1$ compounds. The pathway for assimilation of $C_1$ compounds has been investigated in some detail in these bacteria (Quayle, 1972; Dunstan *et al.*, 1972*a*,*b*; Dunstan & Anthony, 1973; Salem *et al.*, 1973), but little work has been reported on their electron-transport systems (see Davey & Mitton, 1973). The primary dehydrogenases involved in oxidation of methanol, methylamine, formaldehyde and formate have been described. Formate dehydrogenase is the only one of these soluble dehydrogenases that is NAD-linked (Johnson & Quayle, 1964). The prosthetic group of methanol dehydrogenase is probably a pteridine (Anthony & Zatman, 1967*b*; Anthony, 1971), whereas that of the primary amine dehydrogenase is probably a pyridoxal derivative (Eady & Large, 1968, 1971). Formaldehyde oxidation is mediated by a soluble dehydrogenase which is assayed with the artificial electron acceptors phenazine methosulphate or 2,6-dichlorophenol-indophenol (Johnson & Quayle, 1964); this enzyme is possibly a flavoprotein.

The present paper describes work aimed at an understanding of the relationship between these dehydrogenases and the cytochrome chain of *Pseudomonas* AM1. Preliminary reports of some of this work have been published (Anthony, 1970; Anthony & Dunstan, 1971).

## Methods

### *Maintenance of cultures and growth media*

Media were prepared as described by Anthony & Zatman (1964). Stock cultures were maintained on methylamine–agar slopes (or succinate–agar slopes for mutants PCT76 and PCT761) and subcultured every 2 months. Carbon sources were used at 0.2%, except for methanol (0.4%) and methylamine (0.4%).

### *Growth and harvesting of cultures*

Cultures were grown in shaken flasks at 30°C; the inoculum was 5% (v/v) of a culture of bacteria grown on the same carbon source. For large-scale work a laboratory fermenter was used (L. H. Engineering Co. Ltd.) or the bacteria were grown at the Microbiological Research Establishment, Porton Down, Wiltshire, U.K. (financed by a grant from the S.R.C.). Bacteria were harvested at 2°C at the end of the exponential phase, washed twice in 20mM-sodium–potassium phosphate buffer, pH 7.0, then resuspended in buffer and stored when necessary, either at 2°C for whole cell experiments or at −15°C for preparation of cell-free extracts.

### *Isolation of mutants and of revertants*

Mutants were isolated (with the help of Dr. P. M. Dunstan) by a method based on that of Heptinstall & Quayle (1970) designed to select for mutants able to grow on succinate but not on methanol. Bacteria treated with *N*-methyl-*N*-nitro-*N*-nitrosoguanidine were expressed in succinate medium and then treated three times with penicillin (1000i.u./ml of medium) in the presence of methanol. Attempts were made to isolate revertant organisms by treating with *N*-methyl-*N*-nitro-*N*-nitrosoguanidine and plating approx. $10^8$ bacteria on to methanol–agar or methylamine–agar plates.

### *Measurement of $O_2$ uptake by bacterial suspensions*

$O_2$ uptake was measured by using a Rank $O_2$ electrode. The incubation vessel contained 50$\mu$mol

of 2-(*N*-2-hydroxyethylpiperazin-*N'*-yl)ethanesulphonic acid buffer (Hepes buffer, pH 7.0, and 0.05–2.0 mg dry wt. equivalent of washed bacteria in a total volume of 2.0 ml. After measurement of the endogenous rate of $O_2$ uptake, substrate was injected and $O_2$ uptake measured for 5–10 min. Sufficient substrate was used to give the maximum rate with that substrate (this was usually 15 $\mu$mol but for succinate it was 40 $\mu$mol, and for formate 80 $\mu$mol was necessary). Rates of $O_2$ uptake were calculated by assuming that 0.445 $\mu$l of $O_2$ is dissolved in 1 ml of buffer at 30°C.

In experiments on phosphate inhibition bacteria were grown on the appropriate substrate, e.g. methylamine oxidation was measured by using methylamine-grown organisms. Sodium–potassium phosphate was prepared by neutralization of $KH_2PO_4$ with NaOH. Bacteria were preincubated with potential inhibitors for 10 min at 30°C before addition of substrate.

*Induction of enzymes involved in the metabolism of $C_1$ compounds*

Some of these enzymes are inducible (Large & Quayle, 1963; Eady & Large, 1968). For their measurement in mutants PCT76, PCT761 and PCT29, which are unable to grow on methanol or methylamine, succinate-grown bacteria were incubated overnight in growth medium containing methanol (0.4%) or methylamine (0.4%). Induction required bacteria harvested from the mid-exponential phase of growth.

*Preparation of sonic extracts*

Washed bacterial suspensions (100 mg wet wt./ml) were sonicated for 10 min in 2–3 min periods in a 100 W MSE ultrasonic disintegrator at 20 kHz, with a probe tip of 9 mm. Throughout treatment the suspension was cooled in an ice bath. The extract was centrifuged at 40000*g* for 1 h at 2°C and enzymes were assayed immediately in the supernatant.

*Protein determination*

Protein was assayed by the method of Lowry *et al.* (1951); crystalline bovine serum albumin (fraction V) was used as standard.

*Enzyme assays*

Enzymes were measured as previously described: methanol dehydrogenase and hydroxypyruvate reductase (Dunstan *et al.*, 1972*a*); primary amine dehydrogenase (Eady & Large, 1968); formate dehydrogenase (Johnson & Quayle, 1964); glycerate kinase (Heptinstall & Quayle, 1970); serine–glyoxylate aminotransferase (Blackmore & Quayle, 1970); malate dehydrogenase and $\beta$-hydroxybutyrate dehydrogenase (Dunstan & Anthony, 1973). Formaldehyde dehydrogenase was assayed in the $O_2$ electrode by using the reaction mixture described by Johnson & Quayle (1964) except that indophenol dye was omitted and phenazine methosulphate (1 mM) was included. Succinate dehydrogenase was assayed in the $O_2$ electrode by the method of Veeger *et al.* (1969).

*Estimation of cytochromes*

The molar extinction coefficients were assumed to be similar to those for mammalian cytochromes *a*, $a_3$, *b* and *c* (Chance, 1957) or bacterial cytochrome *o* (Chance, 1961).

*Purification of the cytochrome c*

The final stage of purification of the methanol dehydrogenase from *Pseudomonas* sp. M27 and *Pseudomonas* AM1 is the removal of cytochrome *c* by gel filtration (Anthony & Zatman, 1967*a*; Anthony, 1971). The two methods for cytochrome purification described here are based on the procedures for purification of the methanol dehydrogenase (summarized in Table 2).

Washed bacteria (30 g dry wt.) were suspended in 120 ml of 50 mM-sodium–potassium phosphate buffer (pH 7.0), sonicated at 2°C for 10 min then centrifuged for 15 min at 3000*g*. The soluble fraction from the supernatant was obtained by centrifugation (at 2°C) for 3 h at 100000*g*. The pH was lowered carefully to 4.0 with 1 M-HCl at room temperature, the heavy precipitate was removed by centrifugation at 40000*g* for 20 min and the pH of the supernatant raised by addition of 1 M-NaOH to either pH 8.0 (method 1) or pH 6.0 (method 2).

*Method* 1. The acid-treated extract was passed through a column of DEAE-cellulose (2 cm × 26 cm) equilibrated with 20 mM-Tris–HCl buffer, pH 8.0, and 3 ml fractions were collected. The cytochrome was not absorbed but was eluted just behind the solvent front together with the methanol dehydrogenase. The fractions containing cytochrome *c* were pooled and concentrated by ultrafiltration through an Amicon UM 20E membrane and the concentrated material was passed down a column (2 cm × 40 cm) of Sephadex G-75. The pooled fractions yielded about 40% of the cytochrome *c* initially present in the soluble fraction of the suspension of broken bacteria.

*Method* 2. After acidification the pH was raised to 6.0 and protein precipitated by addition of solid $(NH_4)_2SO_4$ (at 20°C) to 85% saturation. The precipitate was dissolved at 20 mM-Tris–HCl buffer, pH 8.0, and used for DEAE-cellulose chromatography, ultrafiltration and gel filtration as described

above. A second gel filtration was sometimes necessary; pooled fractions from the first Sephadex column were concentrated by ultrafiltration through an Amicon UM 20E membrane and the concentrated material passed down a column (2cm×40cm) of Sephadex G-75.

*Measurement of absorption spectra*

Spectra were measured in a Unicam SP.8000 recording double-beam spectrophotometer calibrated with a holmium filter at ambient temperature (usually approx. 25°C) unless otherwise stated. The second sample position was used for bacterial or membrane suspensions. Bacterial suspensions were prepared in 25mM-Hepes buffer, pH7.0. Spectra at 77°K were obtained by placing Perspex spectrophotometer cuvettes containing the suspension in a pre-cooled cell holder and then totally immersing the holder in liquid $N_2$ for 2min. The Perspex cuvettes (1.5mm light-path) were made by separating the two optical faces with a 'former' of Perspex 1.5mm thick. The cell was 'glued' with chloroform.

*Measurement of difference spectra and estimation of cytochrome c in bacterial suspensions*

Reduced-minus-oxidized difference spectra were obtained by measuring spectra of bacterial suspensions (in 25mM-Hepes buffer, pH7.0) plus sodium dithionite (a small amount of solid) with the reference cuvette containing (instead of dithionite) 0.05ml of 0.03% $H_2O_2$ (when 1mM-potassium ferricyanide was used instead this is stated on the legend for the Figure). Care was taken to disperse bubbles in the reference cuvette before measurements. When anaerobic conditions were required for cytochrome reduction by substrate, $N_2$ was bubbled through the bacterial suspension for 5–15min before recording of the spectrum. For measurement of (reduced plus CO) minus reduced difference spectra, suspensions in both cuvettes were reduced with dithionite, or by endogenous or added substrate (followed by the bubbling of $N_2$ through the suspensions for 5min); after reduction of cytochromes, CO was bubbled through the suspension in one cuvette and $N_2$ bubbled through that in the reference cuvette. When $N_2$ or CO were used SubaSeal caps were fitted to the spectrophotometer cuvettes.

Baselines were always checked before recording difference spectra, by recording the spectrum obtained with untreated bacterial suspensions in both cuvettes. All experiments were repeated with at least two different amounts of bacterial suspension to check that the peak heights in spectra were not artifacts related to the light-scattering obtained with dense suspensions.

*Measurement of rates of cytochrome c reduction in whole bacteria*

The relative rates of cytochrome *c* reduction by various substrates were obtained from difference spectra of whole bacteria measured aerobically. Substrate was added to the test cuvette and an equivalent amount of water to the reference cuvette. Air was slowly bubbled through both cuvettes for a constant time (usually 2min). The amount of cytochrome in the reduced state is governed by the rate of its reduction by substrate and by the rate of its reoxidation by $O_2$. In the reference cuvette the amount of cytochrome *c* reduced by endogenous substrate was usually small. This endogenous rate of reduction varied with growth substrate and was proportional to the rate of endogenous respiration. A measure of the rate of reduction of cytochrome *c* by added substrate is given by $\Delta E_{550}$ measured from the substrate-reduced minus endogenous substrate-reduced difference spectrum. Depending on the substrate and the bacterial preparation being used this value varied between zero and that measured from the dithionite-reduced minus $H_2O_2$-oxidized difference spectrum.

## Results

*Prosthetic group of the methanol dehydrogenase of Pseudomonas AM1*

The absorption and fluorescence spectra of this enzyme and its prosthetic group were shown to be identical with those from *Pseudomonas* sp. M27 (Anthony & Zatman, 1967*a*,*b*).

*Effect of phosphate and other ions on respiration in Pseudomonas AM1*

Phosphate ion was unsuitable for use as a pH buffer because it inhibited respiration with some substrates; methanol oxidation was particularly susceptible to this inhibition. Other ions were not as active as phosphate (Table 1). Batches of cells sometimes differed in the amount of phosphate required for a given degree of inhibition. Inhibition by phosphate of methanol oxidation also occurs in *Pseudomonas methanica* and was used by Higgins & Quayle (1970) to accumulate methanol during methane oxidation by this bacterium.

*A mutant of Pseudomonas AM1 unable to form carotenoid pigments (mutant PCT7)*

*Pseudomonas* AM1 forms a red carotenoid pigment which complicates spectrophotometric measurement. A number of colourless mutants was therefore isolated. They never arose spontaneously but were observed during the selection of auxotrophic mutants of *Pseudomonas* AM1. One such mutant was isolated

Table 1. *Effect of phosphate and other ions on the oxidation of methanol by Pseudomonas AM1*

Inhibition is expressed as a percentage of the rate of oxidation of methanol in the absence of phosphate (after correction for the endogenous rate of $O_2$ uptake).

| Potential inhibitor | Concentration (mM) | % inhibition |
|---|---|---|
| Sodium–potassium phosphate | 100, 200 | 100 |
| Sodium–potassium phosphate | 50 | 80 |
| Sodium–potassium phosphate | 25 | 50 |
| $K_2SO_4$ | 25 | 0 |
| $NaNO_3$ | 25 | 0 |
| $NaHSO_4$ | 25 | 0 |
| $K_2SO_4$ | 100 | 60 |
| $NaNO_3$ | 100 | 50 |
| Sodium arsenate | 100 | 72 |

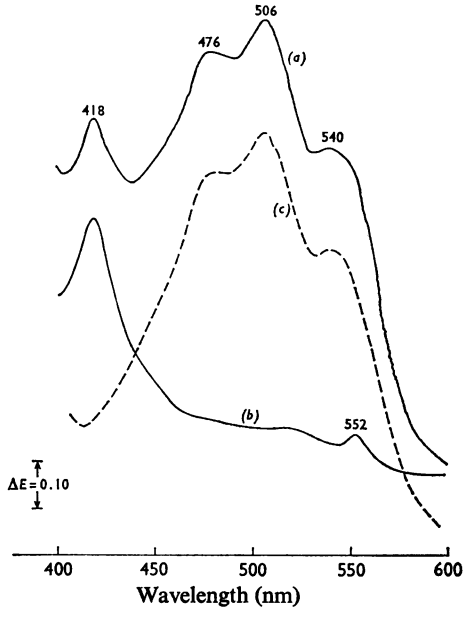


Fig. 1. *Absorption spectra of Pseudomonas AM1 and the carotenoid mutant PCT7*

(*a*) Wild-type *Pseudomonas* AM1 (19.5mg/ml); (*b*) mutant PCT7 (19.5mg/ml); (*c*) spectrum of wild-type organisms with mutant PCT7 in the reference cuvette.

and used in the present work (PCT7). Its growth and oxidative properties were identical with those of the wild-type bacterium. Fig. 1 shows the spectrum of whole cells of mutant PCT7 compared with that of the wild-type bacterium. The spectrum of the carotenoid pigment is obtained by drawing the spectrum of a suspension of wild-type *Pseudomonas* AM1 with a suspension of mutant PCT7 in the reference cuvette (the broken line in Fig. 1). In the mutant and $\alpha$ and $\gamma$ bands of cytochrome *c* are clearly observable; in the wild-type strain, by contrast, the $\alpha$ region is completely obscured by the carotenoid pigment. No pigments were detected in chloroform–methanol extracts of mutant PCT7 (prepared by the method of Peel & Quayle, 1961) and t.l.c. of concentrated extracts [with silica gel G and light petroleum b.p. 60–80°C or chloroform–methanol as solvent] did not show the seven coloured bands seen in similar extracts of wild-type *Pseudomonas* AM1.

*Mutants deficient in cytochrome c (mutants PCT76 and PCT761)*

Mutant PCT76 was isolated (with the help of Dr. P. M. Dunstant) from *Pseudomonas* AM1 as described in the Methods section. This mutant did not grow on methanol, ethanol or methylamine (even in the presence of glyoxylate or glycollate). With formate and oxalate growth was slower than with the wild-type bacterium. The mutant was identical with *Pseudomonas* AM1 in its response to all other growth substrates and also in its pink pigmentation.

During attempts to obtain revertants of mutant PCT76 on methylamine–agar a non-pigmented mutant (PCT761) was obtained from it. Although lacking carotenoid pigments it had the growth and oxidative properties of its parent (PCT76) and was therefore used for subsequent studies of cytochrome spectra.

Suspensions of mutant PCT76 did not oxidize methanol, ethanol or methylamine, which accounts for its failure to grow on these substrates. Formaldehyde was oxidized at 30–60% of the rate measured with wild-type bacteria, but other oxidative properties were unchanged.

The specific activity of the methanol dehydrogenase (31 $\mu$mol of substrate oxidized/h per mg of protein) was the same as that in wild-type bacteria, but primary amine dehydrogenase was not detectable, which explains the failure of mutant PCT76 to oxidize this substrate.

The failure of mutant PCT76 to oxidize methanol or ethanol is probably because it lacks cytochrome *c* normally found in wild-type *Pseudomonas* AM1. Although cytochrome *c* is absent from this mutant cytochromes *a* and *b* are present; these are most readily seen in the non-pigmented derivative (mutant PCT761, Fig. 2*d*).

When grown on succinate and then incubated overnight with methanol or methylamine the mutant formed low activities of the enzymes involved in assimilation of $C_1$ compounds and oxalate (hydroxypyruvate reductase, serine–glyoxylate aminotransferase and glycerate kinase). Activities of these enzymes in the mutant grown on formate were also

lower than in the wild-type grown on the same substrate. The specific activities of these three enzymes were similar to, or slightly less than, those measured in succinate-grown *Pseudomonas* AM1 by Dunstan *et al.* (1972*b*).

Mutant PCT76 is clearly either a pleiotropic or a multiple mutant. The nature of such mutants is usually elucidated by a study of revertant strains. A few revertants indistinguishable from the wild-type were obtained on methylamine–agar, but not methanol–agar. More information is required before the nature of this mutant can be fully understood. It is unlikely that it would have completely lost both primary amine dehydrogenase and cytochrome *c* and also suffered a mutation leading to low activities of the serine-pathway enzymes. One explanation is that mutant PCT76 has lost cytochrome *c* and also some regulatory function; this regulatory function may involve cytochrome *c* itself.

*Cytochromes of the a, b, c and o types in Pseudomonas AM1*

A major problem in the study of cytochromes in *Pseudomonas* AM1 is the presence of carotenoid pigments which absorb in the same spectral region as the $\alpha$, $\beta$ and $\gamma$ bands of reduced cytochromes. The use of carotenoid-deficient mutants PCT7 and PCT761 considerably diminished this problem. All results obtained were confirmed in parent strains; to do this it was sometimes necessary to dilute cell suspensions and measure difference spectra at 77°K. This increases the absorption and resolution of complex spectra (the absorption peaks may also be moved to a lower wavelength) (Chance, 1957). The intense absorption of cytochrome *c* in the 400–580nm range tended to obscure the absorption due to other cytochromes. A clearer demonstration of cytochromes of the *a* and *b* types was therefore obtained with membrane fragments of the wild-type containing only traces of cytochrome *c*. The best material for demonstration of cytochrome *b* was mutant PCT761.

*Cytochrome a.* Cytochromes of the *a* type have characteristic spectra in the reduced state with $\alpha$ and $\gamma$ bands at 600–605nm and 440–445nm respectively (Bartsch, 1968; Kamen & Horio, 1970; Horio & Kamen, 1970); the $\alpha$ and $\gamma$ bands in the reduced-minus-oxidized difference spectra are in the same spectral range.

Absorption bands of the *a* type ($\alpha$) are observed in spectra of all wild-type and mutant organisms (Fig. 2). All the cytochrome *a* was found in the pellet after centrifugation of sonic extracts at 180000*g* for 1h (Figs. 3*b* and 3*c*). Quantitative evaluation of the data is difficult, but if the molecular extinction coefficients of Lemberg (1969) are assumed then these results indicate a mixture of cytochromes of type *a* and type $a_3$ in *Pseudomonas AM1*.

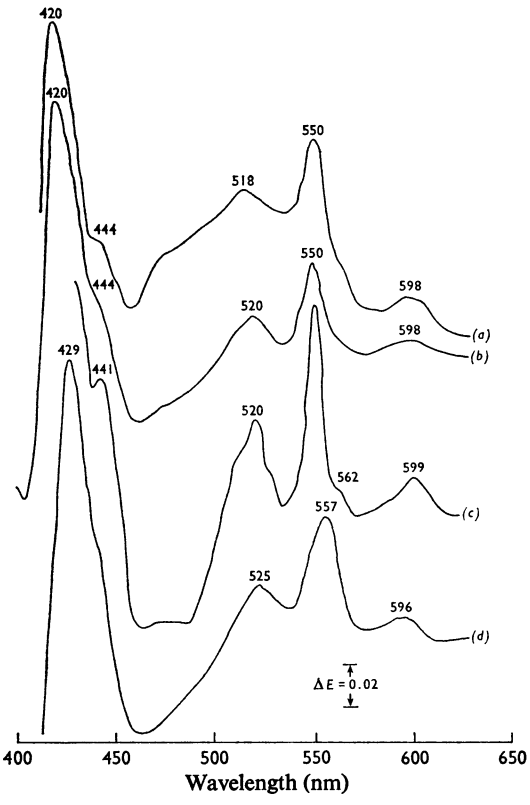


Fig. 2. *Reduced-minus-oxidized difference spectra of whole organisms*

These were prepared as described in the Methods section. (*a*) Wild-type organisms (60.4mg dry wt./ml; 25°C); (*b*) mutant PCT7 (15.9mg dry wt./ml; 25°C); (*c*) mutant PCT7 (24mg dry wt./ml; 77°K); (*d*) mutant PCT761 (38.4mg dry wt./ml; 77°K).

*Cytochrome b.* Cytochromes of the *b* type have characteristic absorption spectra in the reduced state, with $\alpha$, $\beta$ and $\gamma$ peaks at 556–565nm, 525–535nm and 430nm, the peaks in the reduced-minus-oxidized difference spectrum being in the same spectral regions.

Some absorption due to a cytochrome of the *b* type is seen (as a shoulder in the $\alpha$ region at 562nm) in difference spectra of whole wild-type bacteria (Fig. 2*a*), but is clearer in difference spectra of membrane fragments of the wild-type bacteria and of mutant PCT7 obtained as the pellet after centrifugation of bacterial extracts for 1h at 180000*g* (Figs. 3*b* and 3*c*). Cytochrome *b* is more obvious in mutants deficient in cytochrome *c*, e.g. in mutant PCT761 (Fig. 2*d*).

*Cytochrome c.* Cytochromes of the *c* type have characteristic absorption spectra in the reduced state,

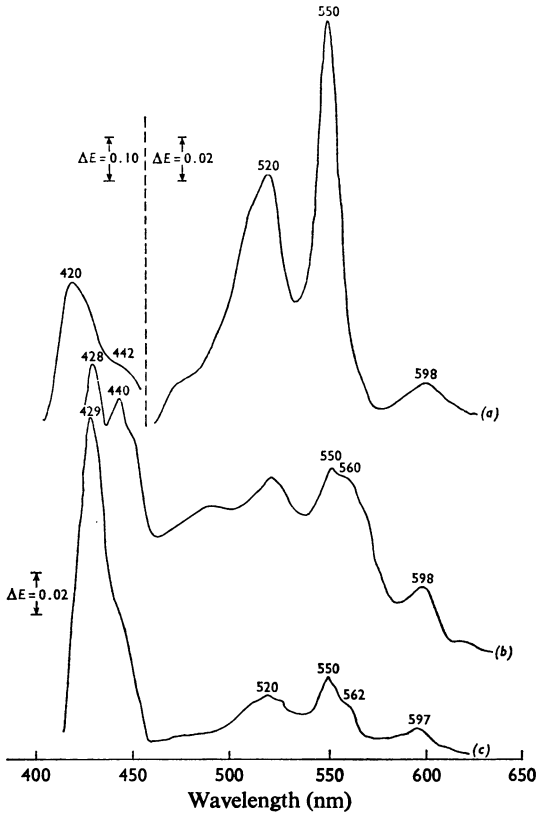


Fig. 3. *Reduced-minus-oxidized difference spectra of cell-free extracts*

(*a*) Wild-type organism; supernatant after centrifugation of sonic extract for 20min at 15000*g* (21.7mg of protein/ml; 25°C); (*b*) wild-type organism; pellet obtained after centrifugation of sonic extract for 1h at 180000*g*, suspended in 20mM-phosphate buffer, pH7.0 (16mg of protein/ml; 77°K); (*c*) mutant PCT7; pellet obtained after centrifugation of sonic extract for 1h at 180000*g*, suspended in 20mM-phosphate buffer, pH7.0 (22.4mg of protein/ml; 88°K).

with $\alpha$, $\beta$ and $\gamma$ peaks at 550–552nm, 520–523nm and 416–418nm respectively; the peaks in the reduced-minus-oxidized difference spectra are in the same spectral regions. This cytochrome is the most obvious one in whole organisms and in sonic extracts of *Pseudomonas* AM1 (Figs. 1, 2*a*, 2*b*, 2*c* and 3*a*). It was found mostly in the supernatant fraction of sonic extracts centrifuged at 180000*g* for 4h. Washing the pellet with 20mM-phosphate buffer, pH7.0, removed most of the cytochrome *c*. Difference spectra of whole organisms (at 77°K) showed only one *c*-type cytochrome. Its purification and some of its properties are described below.

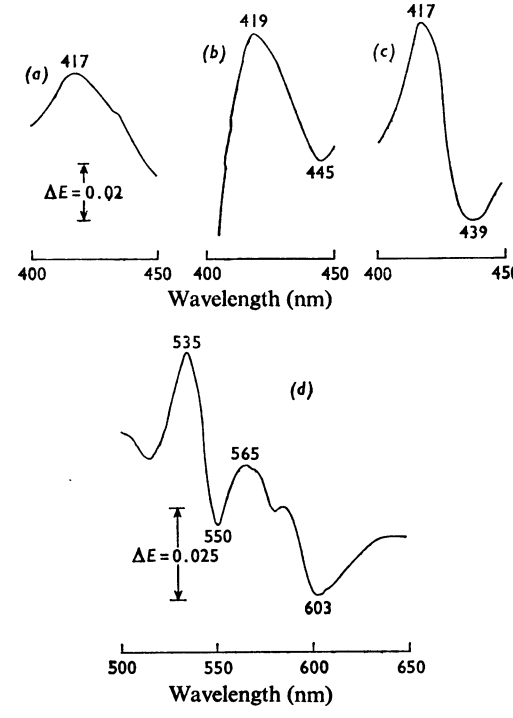


Fig. 4. (*Reduced plus CO*) *minus reduced difference spectra*

(*a*) Wild-type organisms (12.4mg dry wt./ml; 25°C); (*b*) wild-type organism; pellet obtained after centrifugation of sonic extract for 1h at 180000*g* suspended in phosphate buffer, pH7.0 (22.1mg of protein/ml; 25°C); (*c*) whole organisms of mutant PCT761 (19.2mg dry wt./ml; 25°C); (*d*) wild-type organisms (53mg dry wt./ml; 25°C).

*Carbon monoxide-binding cytochromes of Pseudomonas AM1*

The presence of more than one CO-binding cytochrome in *Pseudomonas* AM1 is indicated by the (reduced plus CO) minus reduced difference spectra shown in Fig. 4. The presence of a *c*-type cytochrome able to bind CO is clearly shown by the absorption peak at 535nm and the associated trough at 550nm (Fig. 4*d*) (see Weston & Knowles, 1973). The presence of cytochrome $a_3$ is indicated by the troughs at about 440nm and at 603nm. Cytochrome *o* usually exhibits a peak at 420nm and a trough at 430nm (Castor & Chance, 1959) and the peaks at about 418nm in Figs. 4(*a*) and 4(*b*) could perhaps be the result of a mixture of a cytochrome *o*, cytochrome *c* and cytochrome $a_3$. That these peaks are not due solely to a mixture of the cytochrome $a_3$ plus cytochrome *c* is indicated by the similar spectrum (Fig. 4*c*) of mutant PCT761, which has no cytochrome *c*.

Table 2. *Purification of the cytochrome c*

Details of the methods are given in the Methods section. One unit of cytochrome *c* is defined as the amount giving an extinction at 550nm (10mm light-path) of 0.01; the preparations were reduced with a grain of sodium dithoinite before measurement. Specific activity is given in units per mg of protein.

| Purification step | Volume (ml) | Specific activity | Purification | Yield (%) |
|---|---|---|---|---|
| Method 1 | | | | |
| Supernatant after high-speed centrifugation | 100 | 3.6 | — | 100 |
| Supernatant after acid treatment | 106 | 10.2 | 2.8 | 92 |
| Pooled fractions from DEAE-cellulose | 77 | 10.5 | 2.9 | 45 |
| Pooled fractions from gel filtration | 48 | 62.8 | 17.5 | 41 |
| Method 2 | | | | |
| Supernatant after high-speed centrifugation | 110 | 2.6 | — | 100 |
| Supernatant after acid treatment | 110 | 7.8 | 3.0 | 96 |
| 0–85%-satd.-$(NH_4)_2SO_4$ precipitate | 29 | 3.1 | 1.2 | 33 |
| Pooled fractions from DEAE-cellulose | 16 | 9.0 | 3.5 | 26 |
| Pooled fractions from first gel filtration | 28 | 45.0 | 17.8 | 18 |
| Pooled fractions from second gel filtration | 22 | 65.0 | 25.2 | 8 |

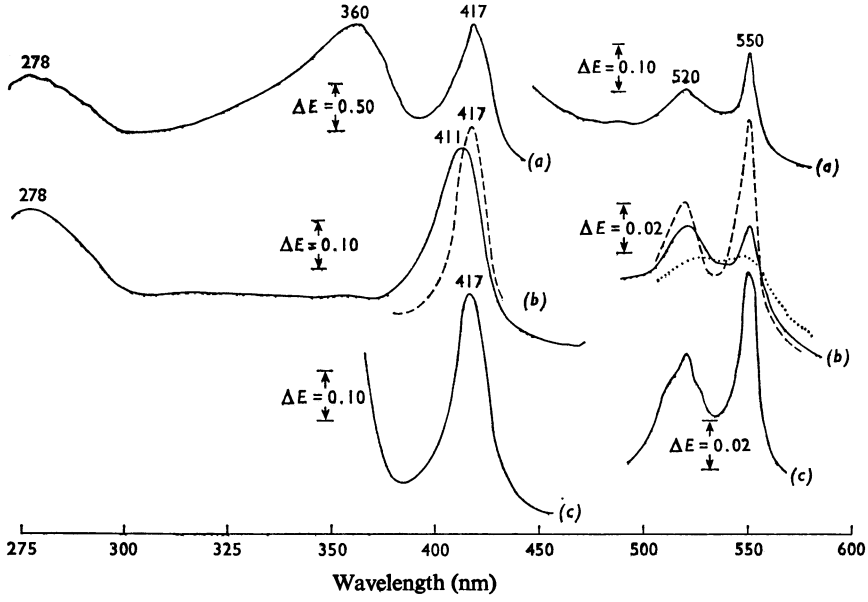


Fig. 5. *Spectra of purified cytochrome c*

(*a*) Absorption spectrum of final preparation obtained after gel filtration in method 1 (0.5mg of protein/ml); (*b*) absorption spectrum of final preparation obtained after the second gel-filtration step in method 2 (0.14mg of protein/ml), ——, untreated cytochrome, – – – –, after reduction with dithionite, · · · ·, after oxidation with 1mM-potassium ferricyanide; (*c*) reduced-minus-oxidized difference spectrum of purified cytochrome *c* (same material as used for Fig. 5*b*). No absorption was observed at wavelengths greater than 600nm.

Preliminary results (D. Widdowson & C. Anthony, unpublished work) have shown that the soluble cytochrome *c* of *Pseudomonas* AM1 (described above) is able to slowly bind CO.

*Purification and properties of cytochrome c*

Table 2 summarizes the two purification methods. The first gives a higher recovery, but the product has a high absorption peak at 360nm. (Absorbance at 360nm was 3.6 in extracts containing 10mg of protein/ml). The nature of the responsible material is not known; it is the major spectral component at wavelengths higher than 300nm in all crude extracts. In both methods DEAE-cellulose was used in the early

stages; the cytochrome *c* did not absorb to the cellulose. By contrast, after separation from the methanol dehydrogenase (by gel filtration) the cytochrome absorbed to the DEAE-cellulose at pH 8.0 (in 20mM-Tris–HCl). In 5mM buffer cytochrome *c* was absorbed even in the presence of methanol dehydrogenase. In method 2 the yield obtained after $(NH_4)_2SO_4$ precipitation was low, because even at 85% saturation with $(NH_4)_2SO_4$ precipitation of cytochrome *c* was not complete.

An estimate of the purity of the cytochrome *c* is obtained from a consideration of the absorption spectrum of the purest preparation given by method 2 (Fig. 5*b*). Assuming an extinction coefficient of $19 cm^{-1} \cdot mM^{-1}$ at 550nm the concentration of the final preparation was calculated to be 5$\mu$M and the concentration of protein 7$\mu$M by using the Folin assay for protein (with bovine serum albumin as standard) and the measured molecular weight of 20000 (see below).

The preparations of purified cytochrome were in the partially reduced state. The reduced cytochrome *c* has absorption peaks at 417, 520 and 550nm (Figs. 5*a*, 5*b* and 5*c*). Spectra obtained at −77°K indicated the presence of only one cytochrome of the *c* type (confirming similar observations of whole cells and of sonic extracts).

The low isoelectric point of the cytochrome *c* was demonstrated by its behaviour as an acidic protein during electrophoresis on cellulose acetate strips at pH 6.0. The failure of acidic cytochrome *c* (with a net negative charge) to bind to DEAE-cellulose at pH 8.0 in the presence of methanol dehydrogenase is possibly because the two proteins bind preferentially to one another; the isoelectric point of methanol dehydrogenase is between pH 7.0 and 8.0 and this protein does not bind to DEAE-cellulose at pH 8.0 (Anthony & Zatman, 1967*a*).

The molecular weight of the purified cytochrome was 20000 when measured by using Sephadex G-75 (Andrews, 1964); standard proteins were bovine cytochrome *c*, serum albumin, pepsin, ovalbumin, chymotrypsinogen, chymotrypsin A, trypsin and trypsin inhibitor. No specific attempts were made to dissociate the cytochrome into subunits.

The redox potential of the purified cytochrome *c* was similar to those from other sources, being reduced by sodium ascorbate and in being oxidized by 2,6-dichlorophenol-indophenol and by potassium ferricyanide. No significant oxidation by molecular $O_2$ was measured.

Reaction of the cytochrome *c* with methanol dehydrogenase could not be unequivocally demonstrated. Addition of pure methanol dehydrogenase immediately reduced purified cytochrome *c* (oxidized). Added methanol was not required for this process, possibly because methanol dehydrogenase always carries bound methanol.

*Reduction of cytochrome c by oxidizable substrates in whole bacteria*

Bacterial suspensions of *Pseudomonas* AM1 and of mutants PCT7 and PCT29 were used to elucidate the role of the soluble cytochrome *c* in electron transport. Mutant PCT29 is deficient in methanol dehydrogenase and was isolated and described by Dunstan *et al.* (1972*a*). The general conclusions to be drawn from these experiments are placed in the Discussion section. The substrates used were methanol, ethanol, formaldehyde, formate, L-malate, succinate and DL-$\beta$-hydroxybutyrate. Usually mutant PCT7 was used first and essential conclusions were confirmed with wild-type organisms. Bacteria deficient in one primary dehydrogenase were achieved by growth on a variety of substrates and by using mutant bacteria.

The total amount of cytochrome *c* in whole bacteria was the same in *Pseudomonas* AM1 and mutant PCT7 and was unaffected by growth substrate or phase of growth. All of the cytochrome *c* was available for reduction by endogenous or added substrate, the difference spectrum with substrate as reductant (measured anaerobically or in the presence of 1mM-KCN) being identical with that with dithionite.

The rates of reduction of cytochrome *c* by various substrates were obtained from difference spectra of whole bacteria measured aerobically (see the Methods section). For a given batch of bacteria the rates of substrate oxidation were proportional to the extent of reduction of cytochrome *c* with that substrate ($\Delta E_{550}$, substrate-reduced minus endogenous substrate-reduced difference spectrum). The extent of cytochrome *c* reduction by the same substrate in different batches of bacteria was proportional to the rate of substrate oxidation.

Some batches (particularly of mutant PCT7) when stored at 2°C for a few weeks lost all oxidative activity, perhaps because cytochrome oxidase was destroyed; cytochrome *c* remained reduced even after extensive aeration of the suspension.

*Oxidation of malate, succinate and* DL-*β-hydroxybutyrate.* The rate of oxygen uptake in the presence of malate, succinate and hydroxybutryate was three to six times higher with bacteria grown on the respective substrate compared with the rates obtained with bacteria grown on methanol. The extent of reduction of cytochrome *c* by the growth substrate was always much higher than that measured with the same substrate in methanol-grown bacteria and was unaffected by 100mM-phosphate. Variations in rates of $O_2$ uptake were due to variations in specific activities of the primary dehydrogenases.

*Oxidation of formaldehyde and formate.* The rates of oxidation of formaldehyde and formate were independent of the nature of the growth substrate

(e.g. methanol, methylamine, formate, succinate, malate or β-hydroxybutyrate), but occasional batches of bacteria gave high or low rates of oxidation. A high oxidation rate was correlated with a greater proportion of the cytochrome *c* in the reduced state after addition of substrate. Phosphate (100mM) inhibited formaldehyde oxidation by about 50% and diminished formaldehyde reduction of cytochrome *c*. Phosphate did not inhibit reduction of cytochrome *c* by formate and it had no effect on $O_2$ uptake in the presence of formate.

*Oxidation of methanol and ethanol.* Growth on methanol induced the activity of methanol dehydrogenase to five times that produced by growth on succinate; these dehydrogenase activities were reflected in the rates of $O_2$ uptake in the presence of alcohols by whole bacteria. When methanol or ethanol was added to methanol-grown bacteria a high proportion of the cytochrome *c* was reduced, whereas only a small proportion was reduced in bacteria grown on succinate. Phosphate (100mM) inhibited methanol oxidation by more than 90% and almost abolished reduction of cytochrome *c* by methanol and ethanol. That cytochrome *c* reduction by methanol is mediated by methanol dehydrogenase was confirmed by the failure of methanol or ethanol to reduce the cytochrome in mutant PCT29, which lacks methanol dehydrogenase and is thus unable to oxidize alcohols (Anthony & Zatman, 1965; Dunstan *et al.*, 1972*a*). Reduction of cytochrome *c* by all other substrates in this mutant was the same as in wild-type bacteria.

*Oxidation of methylamine.* Succinate-grown bacteria do not oxidize methylamine because they have negligible amounts of primary amine dehydrogenase. Methylamine failed to reduce the cytochrome *c* in these bacteria, but completely reduced it in methylamine-grown bacteria, in which the methylamine dehydrogenase is induced. Phosphate (100mM) stimulated both cytochrome *c* reduction by methylamine and also methylamine oxidation.

*Inhibition of respiration and of cytochrome c oxidation by cyanide.* $O_2$ uptake in the presence of the following substrates was completely inhibited by 1mM-KCN: methanol, ethanol, formaldehyde, formate, methylamine, β-hydroxybutyrate, succinate and malate. Endogenous respiration was inhibited 50% by 1mM-KCN. In aerobic conditions in the presence of cyanide the cytochrome *c* in whole cells became completely reduced, the rate of reduction being greater in the presence of oxidizable substrates.

## Discussion

The results show an interesting complexity in the electron-transport system of *Pseudomonas* AM1. A multiplicity of CO-binding pigments is not unusual in bacteria (White & Sinclair, 1971) and their function in *Pseudomonas* AM1 is being studied. It will be of interest to determine whether there is more than one terminal oxidase and whether there are alternative terminal oxidation pathways for oxidation of different substrates.

The results with cytochrome *c* are of particular interest with reference to growth on the $C_1$ compounds, methanol and methylamine. The experiments with whole cells suggest that all oxidizable substrates reduce the cytochrome *c* in *Pseudomonas* AM1 and are oxidized by a cyanide-sensitive pathway. The activity (usually the amount) of primary dehydrogenase determines both the rate of $O_2$ uptake with a given substrate and also the rate of reduction of cytochrome *c*. All of the cytochrome *c* is available for reduction by all (oxidizable) substrates, and all of this cytochrome is oxidized by a cyanide-sensitive pathway. The unusual effects on methanol and methylamine oxidation by phosphate are mediated at a site(s) before cytochrome *c* in the electron-transport chain.

These results suggest a role for cytochrome *c* in the oxidation of all substrates by *Pseudomonas* AM1, but the results with mutant PCT76 indicate that this is not the case. This mutant, which contains all the cytochromes except cytochrome *c*, oxidizes all substrates except methanol, ethanol and methylamine. The results with methylamine are difficult to interpret, because primary amine dehydrogenase is also absent from this mutant. The only substrates shown to require cytochrome *c* for their oxidation are methanol and ethanol, which are both oxidized by the same primary dehydrogenase. Even here it is possible that cytochrome *c* is only involved in binding methanol dehydrogenase to the electron-transport chain and is not involved as an obligatory electron-transport component. Little is known about reactions of the primary dehydrogenases with the electron-transport chain. The dehydrogenases for oxidation of methanol, methylamine and formaldehyde are all soluble; this is probably the reason for the extreme difficulty in preparing cell-free systems capable of oxidizing these substrates with molecular $O_2$ as electron acceptor. Whether soluble cytochrome *c* is ever an essential part of the electron-transport system to $O_2$ is unknown, but it is evident that all substrates reduce this cytochrome, and its function thus remains of interest in the study of electron transport from $C_1$ compounds in *Pseudomonas* AM1.

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