The Respiratory System of *Chromobacterium violaceum* Grown under Conditions of High and Low Cyanide Evolution

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SUMMARY

The particulate fraction of disrupted *Chromobacterium violaceum* grown under cyanide-evolving conditions was unable to oxidize ascorbate plus *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD), but oxidized NADH and succinate by a linear respiratory pathway which was very resistant to inhibition by cyanide. When the bacteria were grown under conditions where little cyanide evolution occurred, particulate fractions developed the ability to oxidize ascorbate-TMPD by a pathway highly sensitive to cyanide inhibition; respiratory activity with NADH and succinate proceeded via both the cyanide-sensitive and -resistant pathways. Studies with respiratory inhibitors, and the cytochrome compositions of the fractions derived from cultures grown under both conditions, are presented. A soluble, carbon monoxide-binding cytochrome *c* was found, and this appears similar to those found recently in *Beneckea natriegens*, methylotrophic bacteria and the marine pseudomonad B16.

INTRODUCTION

The respiratory systems of eukaryotic and prokaryotic organisms are usually highly sensitive to inhibition by cyanide (Gel'man, Lukyanova & Ostrovskii, 1967; Slater, 1967). In contrast, respiration in some plant and eukaryotic micro-organisms has been found to be cyanide insensitive (von Jagow & Klingenberg, 1970; Bendall & Bonner, 1971; Lambowitz & Slayman, 1971; Ray & Cross, 1972; Downie & Garland, 1973). This is due to the presence of an 'alternate', cyanide-insensitive oxidase in addition to a normal cyanide-sensitive cytochrome oxidase. The alternate oxidases may be inhibited specifically by hydroxamic acids (Schonbaum, Bonner, Storey & Bahr, 1971). Oxidation of ascorbate plus *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) occurs exclusively via the cyanide-sensitive cytochrome oxidase.

Cyanide-resistant, aerobic respiration has been observed in several bacteria (Arima & Oka, 1965; Jones & Redfearn, 1967; McFeters, Wilson & Strobel, 1970; Weston, Collins & Knowles, 1974). Respiratory branching occurs in *Azotobacter vinelandii* (Jones & Redfearn, 1967; Ackrell & Jones, 1971) and in *Beneckea natriegens* (Weston *et al.* 1974); in both cases one branch of the respiratory system is resistant to inhibition by cyanide.

We have investigated the respiratory system of *Chromobacterium violaceum* which evolves cyanide under certain growth conditions; in fact, cyanide production is a diagnostic test for this organism (Sneath, 1966). This paper describes some of the properties of the respiratory system of *C. violaceum* grown under conditions where it evolves cyanide, and under conditions where little cyanide is produced.

METHODS

Chromobacterium violaceum (strain D252, NCIB9131) was kindly supplied by Dr Dorothy Jones of the University of Leicester. The complex medium used consisted of 100 mM-glycerol in nutrient broth (25 g Oxoid dehydrated medium/l). The minimal medium consisted of 100 mM-glycerol, M-9 salts (Miller, 1972) and 1 ml trace metal salts/l (Bauchop & Elsdon, 1960). With both media 18 ml of a stationary-phase culture were used to inoculate 900 ml medium in a 2 l conical flask; incubation was at 30 °C, in a gyrotary shaker (200 rev./min). Growth was allowed to proceed to the stationary phase (16 and 40 h, on complex and minimal media respectively).

The bacteria were harvested by centrifugation at 2000 g for 20 min. They were resuspended in 50 mM- Na_2HPO_4 - KH_2PO_4 buffer, pH 7.5, recentrifuged (23 000 g, 10 min) and resuspended in fresh buffer to about 250 mg wet wt/ml. The bacteria were disrupted by sonication (MSE sonicator, 150 W, for 4×1 min at 0 °C) and, after centrifugation (12 000 g, 10 min) to remove the debris, were fractionated into particulate and supernatant fractions as described previously for *B. natriegens* (Weston & Knowles 1973, 1974). The particulate fraction was resuspended in 50 mM-phosphate buffer pH 7.5, at 20 to 60 mg protein/ml.

Difference spectra of the cytochromes ($\text{Na}_2\text{S}_2\text{O}_4$ -reduced minus H_2O_2 - or ferricyanide-oxidized, and $\text{Na}_2\text{S}_2\text{O}_4$ -reduced-plus-CO minus $\text{Na}_2\text{S}_2\text{O}_4$ -reduced) at both room and liquid nitrogen temperatures and pyridine haemochrome spectra of extracts and residues from acid-acetone treatment were determined as described by Weston & Knowles (1973, 1974).

Respiration was measured at 30 °C with an oxygen electrode (Rank Bros, Bottisham, Cambridge). The incubation mixture contained up to 6 mg particulate-fraction protein with 50 mM- Na_2HPO_4 - NaH_2PO_4 buffer, pH 7.8, in a total volume of 2.9 ml. The reaction was initiated by the addition of 0.1 ml substrate, to give final concentrations of 2.5 mM-NADH, 30 mM-succinate and 1.5 mM-ascorbate, plus either 1.0 mM-TMPD, 1.0 mM-2,6-dichlorophenolindophenol (DCIP) or 0.5 mM-horse heart cytochrome *c*. Water-insoluble inhibitors were added in 100 μl dimethylformamide. An aqueous solution of KCN (pH 7.8) was freshly prepared for each experiment. The maximum concentrations of cyanide and azide used were 10 and 100 mM, respectively, as higher concentrations were found to alter the electrode response time.

Protein was assayed by the modified Biuret method of Gornall, Bardawill & David (1949), and cyanide by the method of Epstein (1947). Bacterial growth was measured by the absorbance at 680 nm using 10 mm cuvettes in a SP500 spectrophotometer (Pye-Unicam Ltd, Cambridge).

High-purity CO was obtained from the British Oxygen Co., London. 2-Heptyl-4-hydroxy-quinoline-*N*-oxide (HOQNO), antimycin A, NADH (grade III), horse heart cytochrome *c* (grade III) and DCIP were from Sigma. TMPD was obtained from Eastman Kodak Co., Liverpool, and salicylhydroxamic acid from Aldrich Chemical Co., Wembley, Middlesex. Whenever possible, all other reagents were of analytical grade; glass-distilled water was used throughout.

RESULTS

Growth and cyanide evolution

Chromobacterium violaceum grew well on both glycerol-nutrient broth complex medium and glycerol-minimal salts medium (Fig. 1). Growth on complex, but not minimal, medium was accompanied by the production of the pigment violacein (Sneath, 1966), which has a series of broad absorbance peaks throughout the visible region of the spectrum. This

Fig. 1. Growth curves and cyanide evolution for *C. violaceum*. Growth on complex (●) and minimal (○) media was monitored at 680 nm; cyanide evolution during growth on complex (▲) and minimal (△) media was measured by the method of Epstein (1947).

Table 1. Oxidase activities of the particulate fractions derived from cultures grown on complex and minimal media

Substrate	Oxidase activity (n-atoms oxygen uptake/min/mg protein)	
	Complex medium	Minimal medium
NADH	130	40
Succinate	120	20
Ascorbate-TMPD	6	300

resulted in a growth yield apparently greater than that on minimal medium, though in fact the cell yields using either medium were 3.5 to 4.0 wet wt/l.

On complex medium, vigorous cyanide evolution occurred during the late-exponential phase of growth (see Michaels & Corpe, 1965), the concentration reaching a maximum of about 1.8 mM at the start of the stationary phase (Fig. 1). Growth on minimal medium, however, was accompanied by little cyanide evolution (about 30 μ M-cyanide by the onset of the stationary phase).

Oxidase activities

The particulate fractions derived from cultures grown on complex or minimal medium actively oxidized NADH and succinate (Table 1), with higher activities in the particulate fraction of cultures from the complex medium. The particulate fraction of cultures from minimal medium oxidized ascorbate-TMPD at a high rate, but the particulate fraction of cultures from complex medium oxidized ascorbate-TMPD at only about 5% the rate of NADH and succinate oxidation. Ascorbate-DCIP and ascorbate-horse heart cytochrome *c* were not oxidized by the particulate fraction of cultures from complex medium. Ascorbate-horse heart cytochrome *c* was also not oxidized by the particulate fraction of cultures from minimal medium, but ascorbate-DCIP was oxidized at a similar rate to ascorbate-TMPD.

The supernatant fractions from cultures grown on both media possessed negligible NADH, succinate or ascorbate-TMPD oxidase activities. Addition of the supernatant fraction to the particulate fraction caused no stimulation of any of the oxidase activities.

Fig. 2. Effect of cyanide, CO and azide on NADH and succinate oxidase activities of the particulate fraction from *C. violaceum* grown on complex medium. The reaction vessel contained, in 2.9 ml, particulate fraction and various concentrations of the inhibitors in 50 mM-sodium phosphate buffer pH 7.8. The reactions were started by adding 0.1 ml of either 75 mM-NADH (○) or 900 mM-succinate (●). The incubation temperature was 30 °C.

We found it difficult to measure the oxidase activities of washed, intact bacteria utilizing exogenous substrates. In contrast to the highly reproducible oxidase activities found in extracts, extremely high and variable endogenous activities were found in intact bacteria which had been extensively washed and/or starved. Variability occurred between oxygen electrode experiments, even when using samples from the same batch of bacteria. We were therefore unable to measure the effectiveness of inhibitors with intact bacteria.

Inhibitor studies

Figure 2 shows the effects of cyanide, CO and azide on NADH and succinate oxidation by the particulate fraction of cultures from complex medium. Plots of the reciprocal of fractional inhibition against the reciprocal of inhibitor concentration for cyanide (Fig. 2*a*), carbon monoxide (Fig. 2*b*) and azide (Fig. 2*c*) all intercept the ordinate at 1.0, indicating that total inhibition may be effected at infinite concentrations of each of these inhibitors. However, respiration was very resistant to inhibition by all these compounds; for 50% inhibition, concentrations of the inhibitors were required 1 to 4 orders of magnitude greater than are typically needed to inhibit bacterial respiratory systems (but see Pudek & Bragg, 1974). The rate of oxidation of ascorbate-TMPD was too low for effective inhibitor studies. Table 2 gives the concentrations of the inhibitors required for 50% ($I_{0.5}$) and 90% ($I_{0.9}$) inhibition of respiration, derived from the reciprocal plots.

Oxidation of ascorbate-TMPD by the particulate fraction of cultures grown on minimal medium was highly sensitive to inhibition by both cyanide (Fig. 3) and azide (Fig. 4). With both inhibitors, reciprocal plots intercepted the ordinate at 1.0, indicating 100% inhibition at infinite inhibitor concentrations (Figs. 3*c*, 4); $I_{0.5}$ and $I_{0.9}$ values are given in Table 2. When the rate of respiration of ascorbate-TMPD was lowered to less than 10% of maximal activity by reducing the TMPD concentration, similar concentrations of cyanide or azide were still required for 90% inhibition (see Pudek & Bragg, 1974).

Oxidation of NADH and succinate by the particulate fraction of cultures grown on minimal medium was inhibited in a biphasic manner by both cyanide and azide. Low concentrations of these inhibitors caused partial inhibition of activity; much higher concentrations were required for substantial inhibition to be effected, the two phases being separated by a 'plateau' region (e.g. Fig. 3*a*, *b*). These inhibition curves are similar to those reported recently for *B. natriegens* (Weston *et al.* 1974).

Table 2. Concentrations of cyanide, azide and CO required for 50% ($I_{0.5}$) and 90% ($I_{0.9}$) inhibition of oxidase activities of the particulate fraction of *C. violaceum* grown on complex medium, and concentrations of cyanide and azide required for 50% inhibition (and also 90% with ascorbate-TMPD) of the two inhibition phases of the particulate fraction of cultures grown on minimal medium

Results are taken from the reciprocal plots in Figs. 2 to 4.									
Growth medium	Inhibitor	Substrate	First phase			Second phase			
			Percentage of total activity	Concn for an inhibition of		Percentage of total activity	Concn for an inhibition of		
				$I_{0.5}$	$I_{0.9}$			$I_{0.5}$	
Complex	Cyanide	NADH	100	3.90 mM	34.5 mM	—	—		
		Succinate	100	2.32 mM	21.1 mM	—	—		
	Azide	NADH	100	87 mM	790 mM	—	—		
		Succinate	100	87 mM	790 mM	—	—		
	CO	NADH	100	65 μ M	0.59 mM	—	—		
		Succinate	100	149 μ M	1.35 mM	—	—		
Minimal	Cyanide	NADH	43	4.0 μ M	—	57	1.65 mM		
		Succinate	50	4.0 μ M	—	50	2.25 mM		
		Ascorbate-TMPD	100	4.0 μ M	36 μ M	—	—		
	Azide	NADH	56	40.5 μ M	—	44	51.3 mM		
		Succinate	56	40.5 μ M	—	44	51.3 mM		
		Ascorbate-TMPD	100	35.0 μ M	315 μ M	—	—

Fig. 3. Effect of cyanide on NADH, succinate and ascorbate-TMPD oxidase activities of the particulate fraction from *C. violaceum* grown on minimal medium. The reaction vessel contained, in 2.9 ml, particulate fraction and various concentrations of cyanide in 50 mM-sodium phosphate buffer pH 7.8. The reactions were started by adding 0.1 ml of either 75 mM-NADH (○) or 900 mM-succinate (●), or by adding 0.05 ml 90 mM-ascorbate followed by 0.05 ml 60 mM-TMPD (△). The incubation temperature was 30 °C.

Fig. 4. Effect of azide on NADH, succinate and ascorbate-TMPD oxidase activities of the particulate fraction from *C. violaceum* grown on minimal medium. The reaction vessel contained, in 2.9 ml, particulate fraction and various concentrations of azide in 50 mM-sodium phosphate buffer pH 7.8. The reactions were started by adding 0.1 ml of either 75 mM-NADH (○) or 900 mM-succinate (●), or by adding 0.05 ml 90 mM-ascorbate followed by 0.05 ml 60 mM-TMPD (△). The incubation temperature was 30 °C.

Double reciprocal plots of the inhibition of NADH and succinate oxidases by cyanide and azide (Figs. 3c and 4) were also biphasic. Analysis of the reciprocal plots shows that the phase more sensitive to inhibition is 50% inhibited by similar concentrations of cyanide and azide to those required for 50% inhibition ($I_{0.5}$) of ascorbate-TMPD oxidation (Table 2). The less sensitive phase is 50% inhibited by concentrations of cyanide and azide similar to those required for 50% inhibition ($I_{0.5}$) of oxidation of NADH and succinate by the particulate fraction of cultures grown on complex medium (Table 2).

Oxidation of NADH, succinate or ascorbate-TMPD by the particulate fraction of cultures grown on minimal medium was resistant to inhibition by CO; 0.5 mM-CO caused less than 30% inhibition of all three oxidases, ascorbate-TMPD oxidation being slightly less inhibited than NADH or succinate.

Fig. 5. Effect of antimycin A and HOQNO on NADH and succinate oxidase activities of the particulate fractions from *C. violaceum* grown on complex and minimal media. The reaction vessel contained, in 2.9 ml, particulate fraction from either bacteria grown on complex (*a, b*) or minimal (*c, d*) media, various concentrations of the inhibitors in 0.1 ml dimethylformamide, and 50 mM-sodium phosphate buffer pH 7.8. The reactions were started by adding 0.1 ml of either 75 mM-NADH (○) or 900 mM-succinate (●). The incubation temperature was 30 °C.

Oxidation of NADH or succinate by the particulate fractions of cultures of bacteria grown on both media was inhibited by both antimycin A and HOQNO (Fig. 5). These two substrates had a similar sensitivity to antimycin A, irrespective of growth conditions. However, for HOQNO inhibition, NADH oxidase was more sensitive than succinate oxidase, and the difference between the two was greater for the particulate fraction of cultures grown on complex medium than for the particulate fraction of cultures grown on minimal medium. These results suggest that antimycin A and HOQNO act either at different sites, or at the same site with HOQNO having a further site of action on the pathway of NADH oxidation, possibly in the dehydrogenase region. As expected, antimycin A and HOQNO had no effect on ascorbate-TMPD oxidation by the particulate fraction of cultures grown on minimal medium.

Cytochrome composition

Room-temperature reduced minus oxidized difference spectra of the particulate fraction of *C. violaceum* grown on complex medium had peaks at 552 and 523 nm corresponding to a *c*-type cytochrome, with shoulders at 559 and 528 nm due to a *b*-type cytochrome. In addition, there were peaks at 623 to 628 nm (cytochrome *d*) and at 595 to 600 nm (cytochrome *a*₁). The Soret peak at 429 nm, due to the fused γ -peaks of the *b*- and *c*-type cytochromes, had a shoulder at 438 nm due to *a*-type cytochromes.

The spectra were considerably sharpened when measured at 77 °K in 0.8 M-sucrose (Fig. 6); in particular, there were separate peaks at 556 and 528 nm due to the *b*-type cytochromes. All peaks were shifted 2 to 3 nm towards the ultraviolet region. The Soret peak was at 423 nm and had a shoulder at 440 nm due to cytochrome *a*₁ (not shown).

Fig. 6

Fig. 7

Fig. 6. $\text{Na}_2\text{S}_2\text{O}_4$ -reduced minus $\text{K}_3\text{Fe}(\text{CN})_6$ -oxidized difference spectra of the particulate and supernatant fractions from *C. violaceum* grown on complex medium. Particulate fraction (5 mg protein/ml) or supernatant fraction (8 mg protein/ml) were suspended in 50 mM-sodium/potassium phosphate buffer pH 7.5, containing 0.8 M-sucrose; addition of a few grains of either $\text{Na}_2\text{S}_2\text{O}_4$ or $\text{K}_3\text{Fe}(\text{CN})_6$ was used to reduce or oxidize the fractions. The light path was 2 mm and the temperature 77 °K. Upper line, particulate fraction; lower line, supernatant fraction.

Fig. 7. $\text{Na}_2\text{S}_2\text{O}_4$ -reduced minus $\text{K}_3\text{Fe}(\text{CN})_6$ -oxidized difference spectra of the particulate and supernatant fractions from *C. violaceum* grown on minimal medium. Particulate fraction (2 mg protein/ml) or supernatant fraction (4.8 mg protein/ml) was suspended in 50 mM-sodium/potassium phosphate buffer, pH 7.5, containing 0.8 M-sucrose; addition of a few grains of either $\text{Na}_2\text{S}_2\text{O}_4$ or $\text{K}_3\text{Fe}(\text{CN})_6$ was used to reduce or oxidize the fractions. The light path was 2 mm and the temperature 77 °K. Upper line, particulate fraction; lower line, supernatant fraction.

Room-temperature reduced minus oxidized difference spectra of the supernatant fractions of cultures grown on complex medium had peaks at 551 and 523 nm due to a *c*-type cytochrome, with rather indistinct shoulders at 559 and 528 nm due to a small quantity of *b*-type cytochrome. The Soret peak was at 427 nm, with a small shoulder at 436 to 437 nm. When measured in 0.8 M-sucrose at 77 °K (Fig. 6), the α - and β -peaks of the cytochrome *c* shifted to 549 and 521 nm, whilst there were distinct, but smaller, peaks at 557 and 528 nm due to lower concentrations of cytochrome *b*. Also present was a small shoulder at 567 nm due to an unidentified component. In the Soret region (not shown), there was a peak at 424 nm and a shoulder at 436 nm.

Pyridine-haemochrome spectra of acid-acetone extracts and residues from the particulate fraction of cultures grown on complex medium indicate that similar concentrations of *b*- and *c*-type cytochromes were present (not shown). However, pyridine-haemochrome spectra of the supernatant fraction indicate that the *b*-type cytochrome was present at only about 15 % of the *c*-type cytochrome in this fraction. This value is less than the relative

Fig. 8. $\text{Na}_2\text{S}_2\text{O}_4$ -reduced-plus-CO minus $\text{Na}_2\text{S}_2\text{O}_4$ -reduced difference spectra of the particulate and supernatant fractions from *C. violaceum* grown on complex medium. Particulate fraction at 5 mg protein/ml (visible region) or 2.5 mg protein per ml (Soret region), or supernatant fraction (8 mg protein/ml) was suspended in 50 mM-sodium/potassium phosphate buffer pH 7.5 containing 0.8 M-sucrose, and reduced by the addition of a few grains of $\text{Na}_2\text{S}_2\text{O}_4$. CO treatment was by bubbling for 1 min followed by 10 min incubation before freezing to 77 °K and recording. The cuvette light path was 2 mm. Upper lines, particulate fraction; lower lines, supernatant fraction.

concentration of cytochrome *b* to cytochrome *c* estimated from the cytochrome α -peaks in reduced minus oxidized difference spectra (Fig. 6 and Table 3). This was probably due to overlap of the cytochrome *b* peak in the difference spectra by the dominant cytochrome *c* peak.

Room-temperature reduced-plus-CO minus reduced difference spectra of the particulate fraction of bacteria grown on complex medium had a peak at 420 nm, a shoulder at 429 nm and a trough at 441 nm in the Soret region; there were also a series of indistinct peaks and troughs in the visible region. When the spectra were measured at 77 °K in 0.8 M-sucrose, the Soret peak was at 419 nm, with a large shoulder at 428 to 429 nm and a trough at 438 nm (Fig. 8). In the visible region, there was a series of distinct peaks and troughs. The peak at 636 nm corresponded to cytochrome *d* and the peak at 595 nm (plus the shoulder at 428 to 429 nm on the Soret peak) was due to cytochrome a_1 . The W-shaped curve in the 570 to 540 nm region was presumably due to two components with overlapping peaks, possibly two *o*-type (protohaem) cytochromes or, alternatively, an *o*-type (trough at 556 nm) plus a CO-binding *c*-type (c_{co}) cytochrome (trough at 548 nm) (see Daniel, 1970; Weston & Knowles, 1973). The Soret peak at 419 nm was due to the fused γ -peaks of these components. The nature of the 648 nm absorbing component is not known.

Room-temperature reduced-plus-CO minus reduced difference spectra of the supernatant fraction of cultures grown on complex medium had a peak at 419 nm and a trough at 436 nm, with an indistinct trough at about 549 nm. When measured at 77 °K in 0.8 M-sucrose, the Soret peak was at 419 nm, with a very small shoulder at 428 nm (Fig. 8). In the

Table 3. *Distribution of total and CO-binding cytochromes of the particulate and supernatant fractions of C. violaceum grown on complex and minimal media*

Growth medium	Cell fraction	Total cytochrome (pmol/mg protein)				CO-binding cytochrome (pmol/mg protein)			
						Component with 419 nm Soret peak estimated as:		Other CO-binding cytochromes	
		cyt. <i>b</i>	cyt. <i>c</i>	cyt. <i>a</i> ₁	cyt. <i>d</i>	cyt. <i>o</i>	cyt. <i>c</i> _{CO}	cyt. <i>a</i> ₁	cyt. <i>d</i>
Complex	Particulate	616	647	+	+	52	80	+	+
	Supernatant	53	202	—	—	194	300	—	—
Minimal	Particulate	504	784	—	—	116	180	—	—
	Supernatant	67	319	—	—	164	254	—	—

The total concentrations of cytochromes *b* and *c* were determined from room temperature $\text{Na}_2\text{S}_2\text{O}_4$ -reduced minus $\text{K}_3\text{Fe}(\text{CN})_6$ -oxidized difference spectra using the extinction coefficients given by Jones & Redfearn (1966). The concentrations of cytochromes *a*₁ and *d* were too low for accurate determination; + indicates their presence and — their absence. The CO-binding components with Soret peaks at about 419 nm were determined from their 'peak to plateau' absorbance and estimated as cytochrome *o* or cytochrome *c*_{CO} from room temperature $\text{Na}_2\text{S}_2\text{O}_4$ -reduced-plus-CO minus $\text{Na}_2\text{S}_2\text{O}_4$ -reduced difference spectra using the extinction coefficients given by Chance (1961) and Bartsch (1967) ($E_{\text{max}} = 85$ or 55/haem group, respectively). Note that the concentrations of total cytochromes *b* in the supernatant fractions may be overestimated due to peak overlap (see text).

visible region, there were peaks at 566, 552 and, especially, at 534 nm, and troughs at 556 and 548 nm. Like the particulate fraction, the complex curve in the 540 to 570 nm region and the Soret peak at 419 nm were probably due to cytochrome(s) *o* and/or *c*_{CO}. The much greater absorbance at 534 nm than at 566 nm would suggest that the component with the trough at 548 nm was present at a higher concentration than the other component. There are no peaks in the 570 to 650 nm region due to *a*- or *d*-type cytochromes.

The total concentrations of the cytochromes in the particulate and supernatant fractions derived from bacteria grown on complex medium were estimated from reduced minus oxidized difference spectra using published extinction coefficients (Table 3). Similarly, the concentration of the CO-binding cytochrome(s) with a Soret peak at about 420 nm was estimated by assuming that it is either a cytochrome *o* ('peak-to-plateau' $E_{\text{max}} = 85$ /haem group; Chance, 1961) or a CO-binding cytochrome *c* ($E_{\text{max}} = 55$ /haem group; Bartsch, 1967). Even taking into account possible peak overlap and partial enhancement or cancellation of the peak, as well as errors in the assumed extinction coefficients, it is clear that the CO binding of the supernatant fraction was due principally to CO binding by the cytochrome *c*, as there was insufficient total cytochrome *b* present to account for the observed extent of CO binding. The trough at 548 nm in the low-temperature CO spectra (Fig. 8) was therefore most probably due to CO-binding cytochrome *c*, whilst the trough at 556 nm could have been due to lower concentrations of cytochrome *o*.

Room-temperature reduced minus oxidized difference spectra of the particulate fraction of cultures grown on minimal medium had peaks at 552 and 523 nm due to cytochrome *c*, and shoulders at 559 and 529 nm due to cytochrome *b*; the Soret peak at 427 nm had an exceedingly small shoulder at 439 nm. In low temperature spectra, the shoulders due to cytochrome *b* were resolved as separate peaks at 556 and 528 nm (Fig. 7). The Soret peak at 426 nm (not shown) had a very small shoulder at 438 nm; there were no discernible peaks in the 580 to 650 nm region, indicating the almost complete absence (cf. shoulder at

Fig. 9. $\text{Na}_2\text{S}_2\text{O}_4$ -reduced-plus-CO minus $\text{Na}_2\text{S}_2\text{O}_4$ -reduced difference spectra of the particulate and supernatant fractions from *C. violaceum* grown on minimal medium. Particulate fraction (7.7 mg protein/ml) or supernatant fraction (4.5 mg protein/ml) was suspended in 50 mM-sodium/potassium phosphate buffer pH 7.5, containing 0.8 M-sucrose, and reduced by the addition of a few grains of $\text{Na}_2\text{S}_2\text{O}_4$. CO treatment was by bubbling for 1 min followed by 10 min incubation before freezing to 77 °K and recording. The cuvette light path was 2 mm. Upper lines, particulate fraction; lower lines, supernatant fraction.

438 nm) of *a*- or *d*-type cytochromes. The relative concentration of cytochrome *c* to cytochrome *b* (Table 3) was greater than that found for the particulate fraction of cultures grown on complex medium.

Room-temperature reduced minus oxidized difference spectra of the supernatant fraction of cultures grown on minimal medium had peaks at 551 and 523 nm due to cytochrome *c*, and rather indistinct shoulders at 559 and 529 nm due to a small quantity of cytochrome *b*; the Soret peak was at 426 nm with a small shoulder at 436 nm. In low-temperature spectra the visible-region peaks were shifted 2 to 3 nm towards the ultraviolet region (Fig. 7), and in addition a small but distinct shoulder was seen at 556 nm, which was due to the presence of some cytochrome *b*; the Soret peak at 422 nm (not shown) had a shoulder at 435 nm.

Room-temperature reduced-plus-CO minus reduced difference spectra of the particulate fraction of cultures grown on minimal medium had an indistinct trough in the visible region at about 556 nm, with a shoulder at 550 nm. In the Soret region, there was a trough at 441 nm and a peak at 420 nm. In spectra at the temperature of liquid nitrogen, the Soret peak was at 419 nm and the visible region trough was resolved into two components with minima at 556 and 548 nm (Fig. 9). Unlike the particulate fraction of cultures grown on complex medium, there were no peaks in the 580 to 650 nm region, and only a very small shoulder at 429 nm on the Soret peak, which confirms the almost complete absence of *a*- and *d*-type cytochromes, as noted in the reduced minus oxidized difference spectra (Fig. 7).

Room-temperature reduced-plus-CO minus reduced difference spectra of the supernatant fraction of cultures grown on minimal medium had troughs at 551 and 437 nm and a Soret peak at 419 nm. At low temperatures, the visible-region trough was at 547 nm and the Soret peak, which possessed an exceedingly small shoulder at 429 nm, was at 419 nm (Fig. 9). No extra bands were observed, unlike the low-temperature CO spectra of the supernatant

fraction of cultures grown on complex medium, where a small trough at 556 nm was resolved (Fig. 8). This could have been due to an absence of cytochrome *o*, or a much lower relative concentration of it such that it was not spectrally resolved, in the supernatant fraction of cultures grown on minimal medium. Analysis of the total concentrations of cytochromes *b* and *c* relative to the CO-binding cytochrome (Table 3) indicated that there was too little cytochrome *b* to account for the CO binding which must, as expected from the position of the visible-region troughs, have been due principally to CO binding by the *c*-type cytochrome.

DISCUSSION

Respiratory resistance to cyanide inhibition may be induced in *Achromobacter* (Arima & Oka, 1965) and *Bacillus cereus* (McFeters *et al.* 1970) by growth in the presence of cyanide. In both organisms, induction of cyanide resistance is accompanied by an increase in the concentration of cytochrome *d* (Arima & Oka, 1965; Niven and Knowles, unpublished observations); cytochrome *d* may be the cyanide-resistant oxidase. Unfortunately these papers give no evidence for the exact pathways of electron flow in *Achromobacter* or *Bac. cereus*.

On the other hand, cyanide-resistant respiration occurs in *A. vinelandii* (Jones & Redfearn, 1966; Ackrell & Jones, 1971) and *B. natriegens* (Weston *et al.* 1974), despite growth in the absence of cyanide. In both organisms, respiration occurs via a terminally branched respiratory system, with one branch sensitive to cyanide and the other resistant. Ascorbate-TMPD or ascorbate-DCIP oxidation occurs exclusively via the cyanide-sensitive pathway; a similar situation prevails in branched mitochondrial respiratory systems (e.g. Lambowitz & Slayman, 1971).

Growth of *C. violaceum* under cyanide-synthesizing and -evolving conditions might therefore be expected to result in the development of a respiratory system that is resistant to cyanide and perhaps other inhibitors of cytochrome oxidase (azide and CO). Furthermore, since ascorbate-TMPD oxidation usually occurs via a cyanide-sensitive oxidase, it would also seem likely that such a pathway would not be induced under these conditions. This is found to be the case, since ascorbate-TMPD oxidase activity was negligible (Table 1) and respiration of NADH and succinate was highly resistant to inhibition by cyanide, azide and CO (Fig. 2). Analysis of the inhibition curves show that, at infinite inhibitor concentrations, total inhibition of oxidation occurs (Fig. 2) and the monophasic, double-reciprocal plots suggest that a conventional, linear respiratory pathway is operative. Despite the resistance to inhibition by cyanide, azide and CO, the oxidation of NADH and succinate is respiratory-linked, as their oxidation is sensitive to inhibition by antimycin A and HOQNO.

The particulate fraction of cultures grown on minimal medium actively oxidizes ascorbate-TMPD. This activity is highly sensitive to inhibition by cyanide and azide (Figs. 3 and 4) but uninhibited by CO. In addition, cyanide and azide inhibition curves for NADH and succinate are no longer monophasic, but have inhibitor-sensitive and -insensitive components (Figs. 3, 4). The inhibitor-resistant component displays a degree of resistance similar to that found for the inhibition of NADH and succinate oxidation by the particulate fraction of bacteria grown on complex medium (Table 2), suggesting that this component is common to organisms grown under both conditions.

The simplest interpretation of these results is that terminal branching of the respiratory system occurs, with one pathway resistant to cyanide and azide inhibition and the other sensitive. Oxidation of ascorbate-TMPD would then occur exclusively via the latter pathway. Branching also occurs after the site(s) of antimycin A and HOQNO inhibition (Fig. 5).

This interpretation is in agreement with the previously observed respiratory branching in extracts of *A. vinelandii* (Jones & Redfearn, 1967) and *B. natriegens* (Weston *et al.* 1974).

An alternative explanation could be that there are two parallel pathways of oxidation of NADH and succinate, one sensitive to cyanide and azide inhibition (with associated ascorbate-TMPD oxidase activity) and the other more resistant to their inhibitory action. However, such an interpretation would require excessive synthesis of dehydrogenases, etc., and being unnecessarily wasteful of the cell's resources would seem less likely. Furthermore, the respiratory systems of *A. vinelandii* and *B. natriegens*, which have similar inhibitor characteristics, have been shown to be branched.

The oxidation of NADH and succinate by the particulate fraction of *C. violaceum* grown on complex medium, is relatively resistant to CO inhibition, although there is considerable inhibition at high CO concentrations (Fig. 2 *b*). However, the oxidation of NADH and succinate by the particulate fraction of cultures grown on minimal medium is essentially insensitive to CO. These data suggest that CO acts, but with relatively low efficiency, on the cyanide/azide-resistant pathway, and the lack of a CO inhibitory effect on the particulate fraction of cultures grown on minimal medium is due to electron flow occurring preferentially via the cyanide-sensitive pathway. Some increase in inhibition of NADH and succinate oxidation occurred when CO plus low (100 μ M) cyanide were added together, but the results were found to be somewhat variable, as was previously found for *B. natriegens* (Weston *et al.* 1974).

Hydroxamic acids act as specific inhibitors of the cyanide-resistant alternate oxidase of branched, mitochondrial respiratory systems (Schonbaum *et al.* 1971). Salicylhydroxamic acid (up to 10 mM) had negligible effect on NADH or succinate oxidation by *C. violaceum*, grown on minimal or complex medium, when present either alone or in combination with cyanide, azide or CO.

In view of the two respiratory pathways displayed by the particulate fraction derived from *C. violaceum* grown under conditions of low cyanide evolution, it might be expected that under these conditions the bacterium would synthesize a larger number of different cytochromes, especially the oxidases, than during growth under high-cyanide-evolving conditions, when only the cyanide-resistant pathway is present. Surprisingly, the reverse seems to be the case (Table 3); growth on complex medium yields bacteria with a particulate fraction containing cytochromes a_1 , d , c (CO-binding) and o as the CO-binding cytochromes and hence possible oxidases, whereas the particulate fraction of cultures grown on minimal medium contains essentially only cytochromes o and c (CO-binding). The concentration of cytochrome c (CO-binding) (plus cytochrome o) of the latter particulate fraction is double that of the former. In addition, while CO-binding is generally taken as an indication that a cytochrome is an oxidase, it could, perhaps, be functioning as an oxygenase or oxygen carrier, or the cytochrome may be denatured. It is therefore difficult to ascertain the exact roles of the different cytochromes without further data from kinetic and CO-action spectra experiments, as well as a knowledge of the redox potentials of the cytochromes.

It is of interest that the larger portion of the CO-binding cytochrome c occurs in the supernatant fractions of *C. violaceum*. Addition of supernatant fraction to particulate fraction did not result in any stimulation of oxidase activity. This could be due to sufficient cytochrome c remaining on the membrane for maximal activity, the relative concentration of this cytochrome in the supernatant fraction being too low, it having no direct role in respiration, or to the cytochrome c being inactive, perhaps through dislocation from the membrane. Studies on the supernatant-fraction, CO-binding cytochrome c are complicated

by the presence of lower concentrations of *b*-type cytochromes: the latter may be due to low concentrations of contaminating, non-sedimented, material.

Beneckea natriegens contains a high potential CO-binding cytochrome *c* (Weston & Knowles, 1973, 1974). This cytochrome is also located principally in the supernatant fraction from disrupted cells, and again respiration occurs almost exclusively in the particulate fraction. The bacterium also possesses a cyanide-resistant respiratory pathway (Weston *et al.* 1974). Methane- and methanol-oxidizing bacteria grown on methane or methanol synthesize a CO-binding cytochrome *c* (also found in the supernatant fraction) as their major cytochrome component (Tongue *et al.* 1974). Marine pseudomonad B16 contains large quantities of a CO-binding, *c*-type cytochrome but no other CO-binding cytochromes (Knowles, Calcott & MacLeod, 1974). Again, this cytochrome is located in the supernatant fraction of bacteria disrupted by sonication or in a French press. However, selective wall-stripping procedures have shown the CO-binding cytochrome *c* to be located in the periplasmic space of B16 (Knowles *et al.* 1974), which suggests that the similar cytochromes observed in *B. natriegens*, the methylotrophs and *C. violaceum* may also occur in the periplasm rather than being soluble cytoplasmic enzymes. We have therefore done some preliminary experiments to determine the cellular location of the CO-binding cytochrome *c* of *C. violaceum*. Sphaeroplasts of *C. violaceum* (grown on complex medium) were prepared by lysozyme-EDTA treatment of bacteria suspended in 0.9 M-sucrose. Assay of release of the cytoplasmic enzymes, isocitrate dehydrogenase and glutamate dehydrogenase, showed that there was little lysis (0 to 12 %). However, 60 to 70 % of the CO-binding cytochrome *c*, usually found in the supernatant fraction, was released, with essentially no release of cytochrome *b* or other cytochromes. Spectral assay of the supernatant fraction of sonicates of sphaeroplasts showed a corresponding decrease in concentration of the CO-binding cytochrome *c*, and the ratio of cytochrome *c* to cytochrome *b* was about 1:1, whereas the ratio found in the supernatant fraction derived from untreated bacteria was about 4:1 (Table 3). Thus, we tentatively conclude that the CO-binding cytochrome *c* of *C. violaceum* is periplasmic in origin. The cytochromes remaining in the supernatant fraction are presumably contaminating, non-sedimented, particulate fraction cytochromes, which would explain the presence of the cytochrome *b* shoulder on the cytochrome *c* peak (Figs. 6, 7) of the supernatant fraction derived from intact bacteria.

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