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ELECTRON TRANSPORT SYSTEMS OF *RHIZOBIUM JAPONICUM*

II. RHIZOBIUM HAEMOGLOBIN, CYTOCHROMES AND OXIDASES IN FREE-LIVING (CULTURED) CELLS

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SUMMARY

1. This work characterizes the cytochromes, other haemoproteins and oxidases of *Rhizobium japonicum* grown in non N_2-fixing pure culture, for comparison with the haemoproteins and oxidases found in Rhizobium bacteroids isolated from N_2-fixing legume root nodules.

2. In common with bacteroids, cultured cells possess cytochrome *c* and a cytochrome *b* (α-peak 559 nm at 20°, 556 nm at −196°) but they uniquely contain cytochromes a–a_3, an autoxidizable cytochrome *b* (possibly equivalent to cytochrome *o*) and a soluble 'Rhizobium haemoglobin'. They also contain traces of other CO-reactive haemoproteins.

3. Rhizobium haemoglobin is unrelated to leghaemoglobin, and is not a soluble, reactive form of cytochrome *o*.

4. Photochemical action spectra prove the oxidase functions of the CO and CN^--sensitive cytochromes a_3 and *o*; there may also be present a third oxidase pathway, with a component sensitive to CN^- and EDTA, but not to CO. The cytochrome a_3 co-exists with a CO and CN^--insensitive cytochrome *a*.

INTRODUCTION

The preceding paper[1] characterized some haemoproteins and other possible respiratory enzymes present in the bacteroids from N_2-fixing soybean root nodules. To aid the identification, in that paper, of bacteroid constituents which are directly or indirectly concerned with N_2 fixation, this work describes a corresponding study with cultured cells of the same Rhizobium strain, which are unable[2] to fix N_2.

In its own right this paper confirms the unusual[3] coexistence of the oxidase cytochromes *o* and a_3 in a single organism, and shows the essential similarity between the mitochondrial and Rhizobium cytochromes a–a_3 complexes. It also points out

Abbreviations and definitions: bacteroids, the form of Rhizobium isolated from N_2-fixing legume root nodules; cultured cells, the non N_2-fixing form of Rhizobium isolated from artificial culture; Hb^{3+}, Hb^{2+}, $Hb^{2+}O_2$, $Hb^{2+}CO$ and Lb^{3+}, Lb^{2+}, $Lb^{2+}O_2$, $Lb^{2+}CO$ represent the Fe^{3+}, Fe^{2+}, $Fe^{2+}O_2$ and $Fe^{2+}CO$ complexes of Rhizobium haemoglobin and leghaemoglobin respectively.

the difficulties in identifying the haemoglobin-like pigments found in Rhizobium cultured cells and elsewhere[4,5] as soluble cytochromes *o*.

MATERIALS AND METHODS

Preparation of cultured bacteria

Rhizobium japonicum strain 505 (Wisconsin) was grown on a shake table at 27° in 2-l flasks containing 400 ml culture medium of the following composition (*cf.* ref. 6): K_2HPO_4, 0.2 g; $MgSO_4 \cdot 7\,H_2O$, 0.08 g; crude NaCl, 0.04 g; $CaCO_3$, 0.04 g; Difco yeast extract, 0.3 g. The medium was adjusted to pH 7 before autoclaving and 10 ml of 30 % (w/v) sterile glucose added subsequently. After 12 days growth, cultures were filtered through Miracloth (Calbiochem), centrifuged at 500 × *g* to remove $CaCO_3$, then recentrifuged at 7000 × *g* for 20 min to sediment the bacteria. Polysaccharide slime was removed by repeatedly shaking the pellet in fresh 0.1 M phosphate (pH 6.8) and recentrifuging at 7000 × *g*. The washed bacteria were finally resuspended to 25 % (wet wt./v) in 0.1 M phosphate (pH 6.8) for immediate use or storage at —15°.

Other procedures

Cell rupture and fractionation, protein estimation, Warburg respirometry, spectrophotometry and quantitative calculation of pigment concentrations was carried out as described for bacteroids in the preceding paper[1]. In addition, from (reduced *minus* oxidized) difference spectra, the concentration of cytochromes a–a_3 was calculated by assuming that $\Delta A_{mM} = 13.1$, (604–630 nm), as for the mitochondrial cytochromes a–a_3 complex[7]. From (dithionite + CO *minus* dithionite) spectra the cytochrome a_3–CO complex concentration was calculated by assuming that $\Delta A_{mM} = 10.1$, (590–605 nm)[8], and from the (dithionite + CN^- *minus* dithionite) spectra, ferrous cytochrome a_3–CN^- concentration was calculated by assuming that $\Delta A_{mM} = 14.4$, (590–610 nm)[8]. The concentration of Rhizobium haemoglobin was calculated from CO difference spectra, assuming $\Delta A_{mM} = 94$, (416–480 nm) as found for pure leghaemoglobin[9] standardized by the pyridine haemochrome procedure.

Pyridine haemochromes were prepared by mixing haem or haemoprotein solutions with equal volumes of 4.4 M pyridine in 0.2 M NaOH. Concentrations were estimated from (dithionite-reduced *minus* $Fe(CN)_6^{3-}$-oxidized) difference spectra of such mixtures. For protohaemochrome, $\Delta A_{mM} = 23.4$, (556–539 nm) and for cytochrome-*c* haemochrome (if formed), $\Delta A_{mM} = 22.3$, (550–535 nm).

RESULTS

Cytochromes in fractions from cultured cells

In agreement with earlier work[6,10,11], Fig. 1 shows that cultured cells contain cytochrome *c* (α-peak at 551 nm), cytochrome *b* (α-peak at 559 nm), previously reported at 562 nm (ref. 10) and a cytochrome with α-peak at 603 nm which has been tentatively identified[11] as cytochromes a–a_3. This cytochromes a–a_3 complex is absent from bacteroids of the same Rhizobium strain isolated from N_2-fixing soybean root nodules[1]; its relatively low concentration even in cultured cells (Table I) suggests that cytochromes *b* and *c* might be linked with terminal oxidases other than cytochrome a_3.

At low temperature the (reduced *minus* oxidized) difference spectrum of whole cells (Fig. 2, solid trace) shows a cytochrome *b* component (α-peak, 556.0 nm at −196°) which is probably identical with bacteroid cytochrome *b* (ref. 1). A second cytochrome *b* (?) with α-peak at 559.5 nm (−196°) appearing in this spectrum, could not be detected in bacteroids[1] and the cytochromes *a*–a_3 peak appears at 597 nm (−196°).

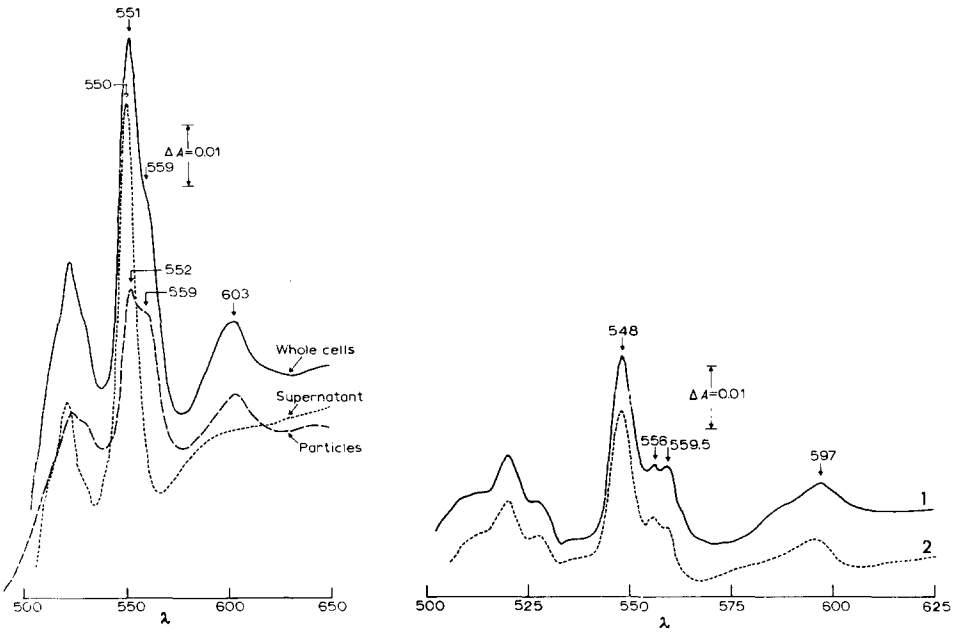

Fig. 1. Reduced *minus* oxidized difference spectra showing cytochrome distribution in Rhizobium cultured-cell fractions. Protein concns. (mg/ml) were: whole cells, 4.5; particles, 10; supernatant, 10.3.

Fig. 2. Low-temperature spectra of whole cells. Effect of CN^-. Trace (1) is the (succinate *minus* O_2) and trace (2) the (CN^- + succinate *minus* O_2) difference spectrum of whole cells (5 mg protein/ml, 2-mm light path) in 0.1 M phosphate (pH 6.8) at −196°, measured as described in ref. 1: MATERIALS AND METHODS.

As with bacteroids[1] the single α-peak of cytochrome *c* (548 nm at −196° and 551 nm at 20°) suggests the presence of only one cytochrome *c* component. However, as soluble cytochrome *c* has its α-peak at 550 nm (20°) and particle-bound cytochrome *c* at 552 nm (20°) as shown in Fig. 1, the possibility remains that more than one cytochrome *c* might be present. Fig. 1 and Table I show that most cytochrome *b* and all cytochromes *a*–a_3 remain particle-bound following cell disruption. The values of Table I express the relative concentration of pigments within each fraction, but because of the 'enhanced-absorption' phenomenon[1] they give only an approximate expression of relative purity between fractions.

Presence and distribution of CO-reactive pigments

Although the preceding paper[1] proved that the Rhizobium bacteroid–CO complex with a 414-nm absorption peak was unrelated to cytochrome *o* (refs. 12, 13) and that the bacteroid–CO complex with 428-nm absorption peak was unrelated to cyto-

TABLE I

HAEMOPROTEIN CONCENTRATIONS IN RHIZOBIUM CULTURED-CELL FRACTIONS

Concentrations of cytochromes and CO-reactive pigments, both expressed as μmoles haem/g protein, were estimated from the spectra of Figs. 1 and 2, as described in MATERIALS AND METHODS, and also in Table I of ref. 1. The symbols (++), (+) or (?) indicate respectively the estimated high or low concentration or doubtful presence of pigments with absorption bands partly obscured by those of other pigments.

Haemoprotein	*Whole cells*	*Particles*	*Supernatant*
Cytochrome *c*	0.51	0.48	0.27
Cytochrome *b*	0.45	0.60	0.02
Cytochromes a–a_3	0.15	0.22	0
CO-reactive pigments			
Cytochrome a_3	0.09	0.08	0
Cytochrome *o* (P-420)	+	+	0
P-450	?	?	<0.005*
P-428	?	?	+
Rhizobium haemoglobin	++	+	0.02*

* The concentrations of these two haemoproteins were estimated from CO difference spectra of semi-purified fractions isolated by chromatography on Sephadex G-75 (see text).

chrome a_3–CO complex, our previous work[11] suggested that both of these oxidases were present in the same Rhizobium strain isolated from pure culture. The CO difference spectrum of whole cultured cells, as now recorded by scattered-transmission spectrophotometry (Fig. 3, solid trace) is in fact consistent with the presence of both oxidases. The shoulders at 425–430 nm and at 590 nm, the trough at 445 nm and the inflection at 605 nm are characteristic of cytochrome a_3–CO complex[8] while the peaks at 415 nm, 539 nm and 574 nm, and the trough at 556 nm are characteristic of cytochrome *o* (ref. 13). In membrane fragments (particles) from disrupted cells (Fig. 3, dashed trace) the shoulder at 432 nm, and the trough at 446 nm are again consistent with the presence of cytochrome a_3–CO complex, but other absorption-band positions are slightly different from those ascribed to cytochrome *o* in whole cells. Thus the 416-nm peak is broadened, with a shoulder appearing at 420 nm while the 541- and 575-nm peaks and the 559-nm trough all show a shift to higher wavelengths compared with the spectrum of whole cells (Fig. 3). Conversely, a CO difference spectrum of the supernatant fraction (Fig. 3, dotted trace) shows a sharpened 415-nm peak with no 420-nm shoulder, and the 539- and 569-nm peaks and the 554-nm trough show a general shift to lower wavelengths as compared to the whole-cell or particle spectra. These results suggest the presence of at least two CO-reactive pigments other than cytochrome a_3 in whole cells. The first, particle-bound, pigment has a CO difference spectrum with ultraviolet peak at 420 nm and a trough at 559 nm which corresponds to an absorption peak of its reduced form. It is tentatively identified as cytochrome *o* (see below). The second pigment, with ultraviolet peak in its CO difference spectrum near 415 nm, and a trough at 554 nm, appears in the supernatant. It has many properties of a haemoglobin (see below) and is named 'Rhizobium haemoglobin'.

Chromatography of cultured-cell supernatants on Sephadex G-75 (Pharmacia, Sweden) proved the absence of a soluble, low molecular weight CO-reactive cyto-

chrome *c*-552 whose presence dominates the CO difference spectrum of Rhizobium bacteroids[1].

A surprising feature in the CO difference spectrum of cultured-cell supernatants (Fig. 3, dotted trace) is the appearance of an absorption shoulder at 427 nm, close to the expected peak position of the CO complex of the classically-insoluble cytochromes a_1 or a_3. However, the absence from this trace of a corresponding peak or shoulder near 590 nm shows that the 427-nm shoulder is not derived from cytochrome a_1 or a_3, so this pigment may resemble the soluble CO-reactive P-428 already described in Rhizobium bacteroids[1]. A preliminary examination of cultured-cell and bacteroid supernatants by gel filtration on Sephadex G-75 or G-200 suggests that these P-428 pigments both have a molecular weight of 200000 or greater.

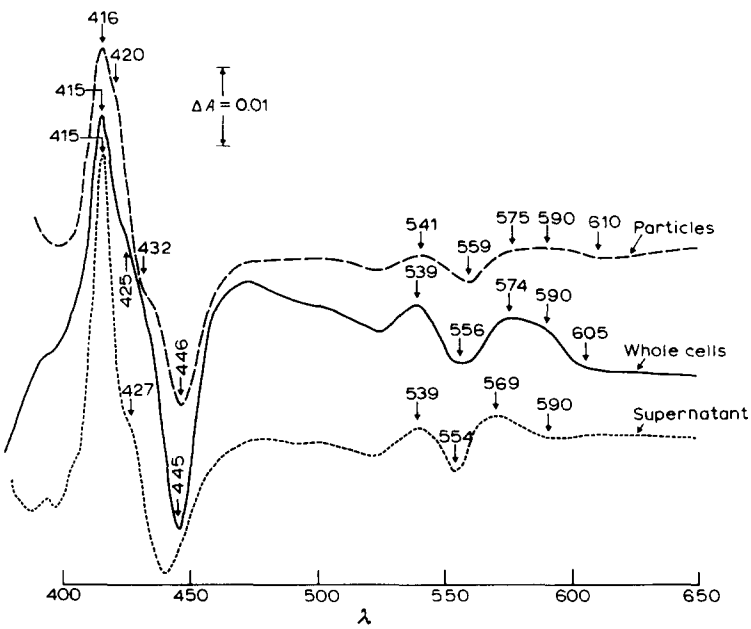

Fig. 3. CO difference spectra of dithionite-reduced Rhizobium cultured-cell fractions. All traces are of (dithionite + CO *minus* dithionite) spectra, and protein concns. (mg/ml) were: whole cells, 5.0; particles, 2.3; supernatant, 5.0.

The second part of Table I shows the relative content of CO-reactive pigments within each cell fraction; again the 'enhanced-absorption' phenomenon[1] prevents an exact comparison of purity between fractions. The concentration of CO-reactive cytochrome a_3 does not appear as half the total concentration of cytochrome a–a_3, as claimed for the mitochondrial enzyme[14]. This does not necessarily prove that non-equivalent amounts of cytochrome a and a_3 are present, since the Rhizobium pigments may have different spectral characteristics from those of the purified mitochondrial cytochromes a–a_3 complex on which the present calculations are based. Further evidence for the essential similarity between Rhizobium and animal-cell cytochromes a–a_3 is offered below.

Although none of the CO difference spectra of cultured-cell fractions (Fig. 3) show the presence of haemoprotein P-450, which is a major constituent of bacteroids[1],

the Sephadex G-75 chromatographic procedure used to purify Rhizobium haemoglobin (see below) showed the presence in cultured-cell supernatants of a very small amount (Table I) of CO-reactive pigment with CO complex absorbing at about 452 nm. As the absorption peak for the CO complex of bacteroid P-450 appears at 447–448 nm (refs. 15, 16) the pigments are not identical.

Biological reduction of pigments from cultured cells

As with the corresponding study on bacteroids[1], the biological reducibility of pigments in cultured cells is assumed to be evidence in favour of their undenatured state.

Thus, column 1 of Table II shows that endogenous respiration of whole cells under N_2 caused partial or complete reduction of all the haemoproteins previously oxidized by O_2. All particle-bound pigments were autoxidizable or were associated with oxidases, since they were completely oxidized in air, as evidence from (O_2 *minus* $Fe(CN)_6^{3-}$) difference spectra. The washed, O_2-equilibrated particles were deficient in substrate, since no pigment reduction occurred within 10 min under N_2 (Table II, column 2). Under CO, but in the absence of added substrate, some reduction and CO complex formation of cytochrome a_3, haemoglobin and possibly P-428 was detected. Particle-bound reductases were present for all the pigments since the addition of NADH to the washed, then N_2-equilibrated particles caused their immediate reduction (Table II, column 3).

In the supernatant from disrupted cells, the major pigment, cytochrome *c*, remained reduced (Table II, column 4) suggesting the absence of a soluble oxidase for cytochrome *c*. The Rhizobium haemoglobin, also present in this supernatant, apparently forms a stable ferrous–O_2 complex (see below), in contrast to its ready oxidation (indicated by inability to form a CO complex) when bound to O_2-equilibrated particles. In particular, the stable co-existence of reduced cytochrome *c* and

TABLE II

BIOLOGICAL REDUCTION OF CYTOCHROMES AND CO-REACTIVE PIGMENTS IN RHIZOBIUM CULTURED CELLS

The percent reduction of cytochromes or formation of CO complexes was measured as described in Table II of the preceding paper[1]. The symbol zero signifies no activity, whereas a blank space signifies the absence of pigment.

	Whole cells: endogenous minus O_2	*Particles*		*Supernatant: endogenous minus* $Fe(CN)_6^{3-}$
		Endogenous minus O_2	*NADH minus* O_2	
Reduction				
Cytochrome *c*	88	0	86	85
Cytochrome *b*	77	0	68	?
Cytochromes a–a_3	100	0	100	
Reduction and CO complex formation				
Cytochrome a_3	90	+	100	
Rhizobium haemoglobin	++	+	++	++
Cytochrome *o*	++	0	++	
P-428	?	?	?	0

of (oxy ?)-haemoglobin in the same supernatant establishes that this form of Rhizobium haemoglobin is not a cytochrome-*c* oxidase.

Effect of CO and other inhibitors on respiration; identification of cytochromes a_3 and o by photochemical action spectra

Our earlier work[11] showed CO inhibition of succinate respiration in both cultured and bacteroid forms of Rhizobium, with erratic photoreversal. Subsequently, TUZIMURA AND WATANABE[6] and APPLEBY[1] were unable to confirm the inhibition of bacteroid respiration by CO, so the corresponding experiments on cultured cells were also repeated. As with bacteroids[1], endogenous respiration was actually stimulated by CO (Table III, column 1) but the respiration of succinate in the dark (Table III, column 2) was 15–22 % inhibited by CO in the absence and 63–85 % inhibited by CO in the presence of 10 mM EDTA. Substantial relief of this inhibition occurred in the light, suggesting the operation of a CO-sensitive haemoprotein oxidase[13]. With the collaboration of Dr. WALTER D. BONNER Jr., the apparatus and technique of CASTOR AND CHANCE[13] was used to plot the photochemical action spectrum for relief of this CO-inhibited succinate respiration (Fig. 4). The peaks at 591, 551 and 431 nm are

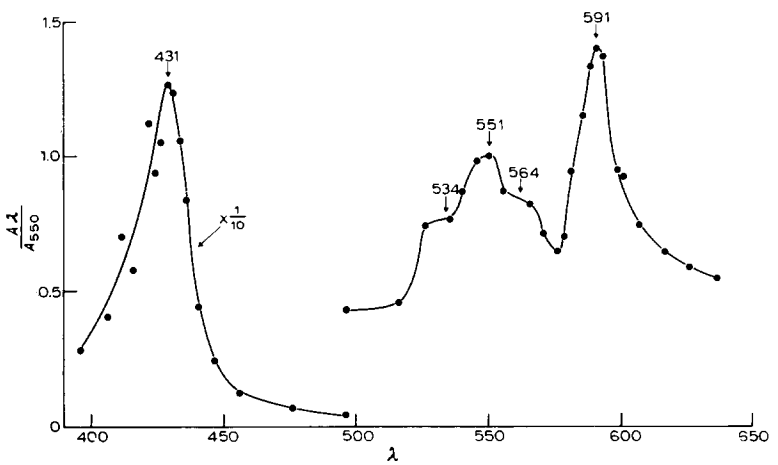

Fig. 4. Photochemical action spectrum of CO-inhibited respiration of Rhizobium cultured cells. This was determined as described in the text using a suspension of whole cells (6.5 mg protein/ml) in 0.1 M potassium phosphate (pH 6.8) at 25°, with sodium succinate (50 mM) as substrate. Gas phase was $CO-O_2$ (95:5, v/v). The ordinate scale, $A\lambda/A_{550nm}$, represents the relative efficiency of light at each wavelength (λ) compared with 550-nm light, in reversing the inhibition of O_2 uptake by CO. Experimental points are shown as closed circles.

characteristic of cytochrome a_3 (ref. 13), and the shoulders at 534 and 564 nm suggest the co-existence of cytochrome *o* (ref. 13). It is not possible to calculate the relative amounts of cytochromes *o* and a_3 from photochemical action spectra and the fact that cytochrome *o* and Rhizobium haemoglobin apparently show similar CO difference spectra also makes it impossible to calculate the relative concentrations of cytochrome a_3 and *o* from such CO difference spectra.

The ability of EDTA to accentuate the photosensitive CO inhibition of succinate respiration (Table III) suggests that a third oxidase pathway, not containing cytochrome a_3 or *o*, might be blocked by this reagent. Direct proof was not possible, as

TABLE III

INHIBITION OF RHIZOBIUM CULTURED-CELL RESPIRATION

The results are shown as percent inhibition (−) or activation (+) of O_2 respiration (Warburg manometry) compared with the appropriate control. In experiments including both CO and EDTA, the controls contained EDTA only. The $Q_{O_2}(N)$* value for uninhibited endogenous respiration was 70–120 and for gross respiration with succinate, 200–300. General conditions as for Table III of ref. 1.

Treatment	*Endogenous respiration*	*Net succinate respiration*
CO		
dark	+12	−15
light	0	−8
dark	+17	−22
CO + 100 μM EDTA		
dark	+14	−50
light	+24	−20
dark	+11	−22
CO + 10 mM EDTA		
dark	+13	−85
light	−3	−13
dark	+3	−63
EDTA		
100 μM	0	+6
1 mM	+21	−12
10 mM	+25	−15
40 mM	+21	−18
CN^-		
10 μM	−4	−100
100 μM	−58	−100
1 mM	−91	−100

* $Q_{O_2}(N)$ = μl O_2 uptake/h per mg protein N.

1–40 mM EDTA actually stimulated endogenous respiration (Table III) and 10 mM EDTA caused only 15% inhibition of 'net succinate' respiration. CN^- sensitivity (Table III) suggests that a metalloprotein may be involved in endogenous respiration, but the apparent insensitivity to CO makes it unlikely that this oxidase is a haemoprotein. The very efficient inhibition of succinate oxidation by CN^- (Table III) shows that the cytochrome a_3, cytochrome *o* and CO-insensitive oxidase pathways are all CN^- sensitive. Information about possible sites of CN^- action was gained from CN^- difference spectra (Figs. 2, 7 and 8) but interpretation of these spectra will be aided by prior consideration of Rhizobium haemoglobin and its properties.

Identification and properties of Rhizobium leghaemoglobin

The 144000 × *g* supernatant from disrupted cells was fractionated with $(NH_4)_2SO_4$ at pH 6.8 and most of the CO-reactive haemoprotein (Fig. 3; Table I) was precipitated between 0.25 and 0.5 saturation, with cytochrome *c* (Fig. 1) remaining in solution. The redissolved precipitate was passed through a calibrated column (*cf.* ref. 16) of Sephadex G-75 equilibrated with 0.1 M phosphate (pH 6.8).

Unlike the supernatant from Rhizobium bacteroids[1,16] this concentrated material, or the crude supernatant from cultured cells, contained no low molecular weight (approx. 13000) CO-reactive cytochrome *c*-552. Instead, there appeared in the fractions corresponding to a molecular weight of 20000–25000, a pigment whose (dithionite + CO *minus* dithionite) difference spectrum (Fig. 5A, trace (1)) has peaks at 417, 540 and 569 nm, and troughs at 434 and 556 nm, resembling those seen in the corresponding CO difference spectrum of leghaemoglobin (Fig. 5B, trace (1)). However, this pigment

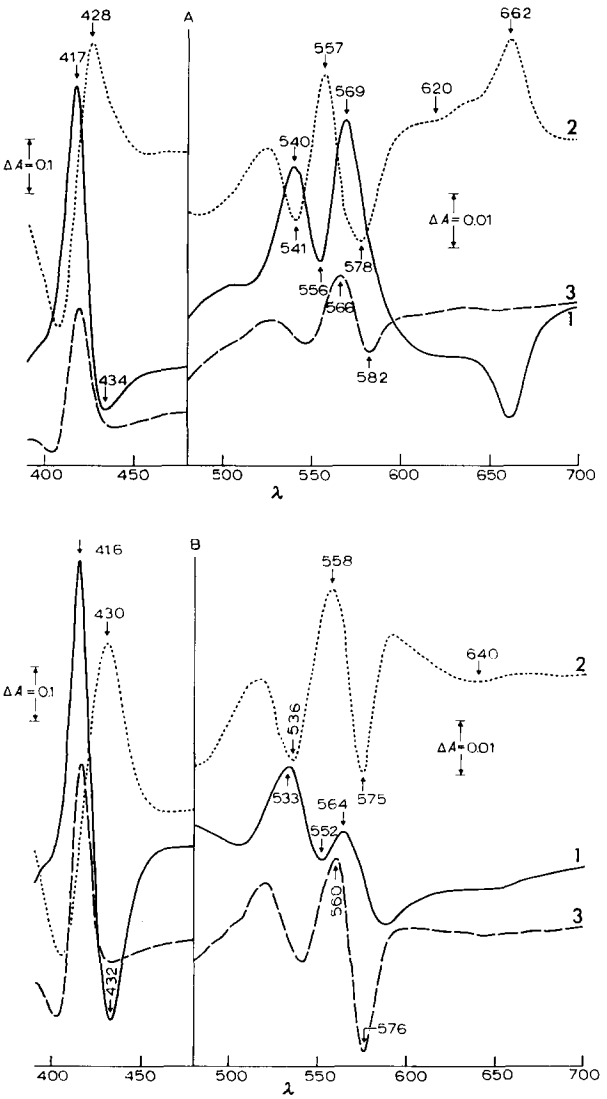

Fig. 5. Comparison of leghaemoglobin and Rhizobium haemoglobin difference spectra. A. Rhizobium haemoglobin (total haem approx. 4 μM) in 0.1 M phosphate (pH 6.8). (1), dithionite + CO *minus* dithionite; (2), dithionite-reduced *minus* air-equilibrated; (3), air then CO-equilibrated *minus* air-equilibrated. B. Leghaemoglobin (4 μM protohaem) in 0.1 M phosphate (pH 6.8). Key as for part A.

showed a complete absence of reaction when it was challenged with rabbit-serum antibodies prepared against authentic leghaemoglobin[17] obtained from the symbiotic tissue of soybean root nodules; the two pigments are, therefore, apparently unrelated. A pyridine haemochrome prepared from the most-concentrated Rhizobium haemoglobin fraction had its α-absorption peak at 553 nm, allowing the presence of a mixture of protohaem (α-peak at 556 nm) and haem *c* (α-peak at 550 nm) haemochromes. The absolute spectrum of this fraction, as eluted from the Sephadex G-75 column in air-equilibrated buffer is shown in Fig. 6, trace (2). It has sharp peaks at 550 and 523 nm, characteristic of reduced cytochrome *c*, but the shoulders at 539, 572 and 625 nm are not characteristic of reduced or oxidized cytochrome. From a comparison with the spectrum of oxy-leghaemoglobin ($Lb^{2+}O_2$) containing some ferric leghaemoglobin (Lb^{3+}), which is shown in Fig. 6, trace (1), it is concluded that the 539- and 572-nm shoulders could be due to the oxy form ($Hb^{2+}O_2$) and the 625-nm shoulder to the ferric form (Hb^{3+}) of a novel haemoglobin, named 'Rhizobium haemoglobin'.

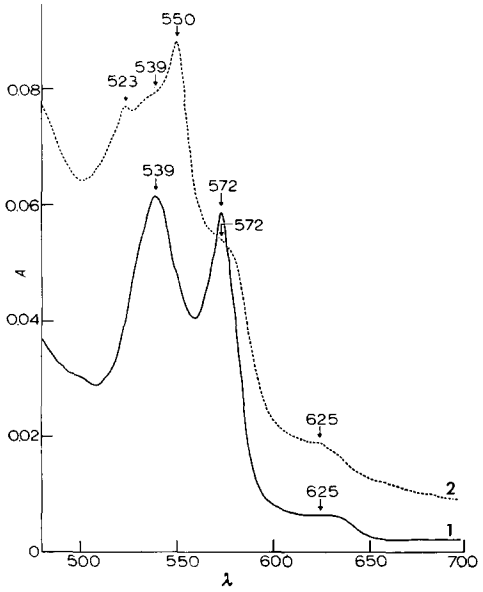

Fig. 6. Comparison of oxyleghaemoglobin and Rhizobium haemoglobin absolute spectra. Trace (1) is of $Lb^{2+}O_2$ containing about 10% Lb^{3+}, in 0.1 M phosphate (pH 6.8). Trace (2) is of crude Rhizobium haemoglobin prepared as described in the text.

To support this conclusion, difference spectra were recorded under conditions where cytochrome *c* would remain reduced in both cuvettes and hence cause no interference. Besides the CO difference spectrum of a dithionite-reduced preparation, already described, Fig. 5A shows, in trace (2), the (dithionite-reduced *minus* air-equilibrated) difference spectrum of this Rhizobium haemoglobin preparation. The peaks appearing at 428 and 557 nm are consistent with the formation of ferrous haemoglobin (Hb^{2+}) in the sample cuvette; the 662-nm peak will be discussed below. The absorption troughs in this spectrum at 541 and 578 nm are consistent with the presence of $Hb^{2+}O_2$ in the air-equilibrated reference cuvette, and the inflection at 620 nm to the presence of some Hb^{3+} in the reference cuvette. For comparison, Fig. 5B

trace (2) shows the equivalent difference spectrum of leghaemoglobin, namely: Lb^{2+} *minus* $Lb^{2+}O_2 + Lb^{3+}$.

In Fig. 5A, trace (3), the difference spectrum of a (CO-equilibrated *minus* air-equilibrated) preparation of Rhizobium haemoglobin, the absorption peak appearing at 566 nm and trough at 582 nm is consistent with the formation of $Hb^{2+}CO$ from $Hb^{2+}O_2$ in the sample cuvette, with $Hb^{2+}O_2$ remaining in the reference cuvette. The ($Lb^{2+}CO$ *minus* $Lb^{2+}O_2$) difference spectrum (Fig. 5B, trace (3)) has a similar appearance, although the displacement of all bands to lower wavelengths suggests that the O_2 and CO complexes of Lb^{2+} may have absorption peaks about 6 nm lower than those of the corresponding Rhizobium haemoglobin complexes. It was not possible from the spectrum of crude Rhizobium haemoglobin (Fig. 6, trace (2)) to measure the absolute positions of the supposed $Hb^{2+}O_2$ absorption bands. Any attempt at further purification of Rhizobium haemoglobin caused its rapid conversion to a pigment with an absorption band (when dithionite-reduced) at 662 nm; partial conversion had already occurred in the preparation used to record Fig. 5A. The modified haemoglobin is still CO reactive, since the 662-nm peak collapses in the presence of CO (Fig. 5A, trace (1)), but it is not analogous to choleglobin which has a major absorption band near 630 nm in the reduced form[19].

Distinction between the properties of cytochrome b, cytochrome o and Rhizobium haemoglobin by difference spectra using CN^- and other inhibitors

Following CN^- addition to an oxygenated suspension of whole cells, succinate respiration was able to reduce all cytochrome *c* (551-nm peak) but only about 30%

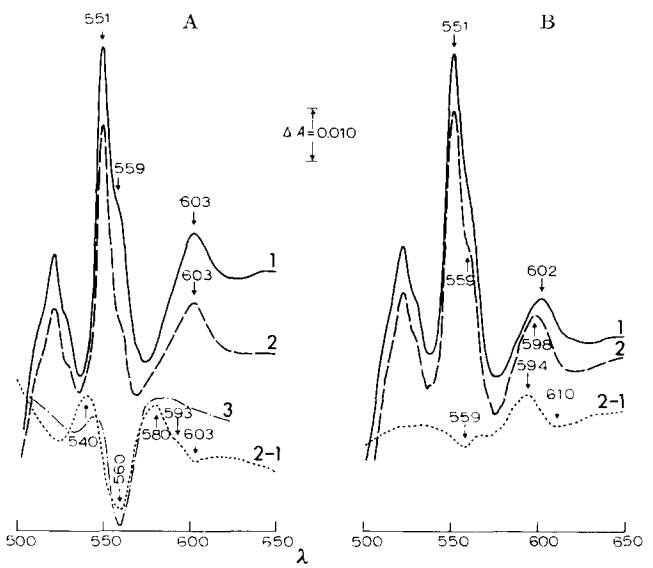

Fig. 7. Effect of CN^- on room-temperature spectra of Rhizobium cultured cells. A. Succinate-reduced *minus* oxidized. Trace (1) is the (succinate *minus* O_2) and trace (2) the (cyanide + succinate *minus* O_2) spectrum of whole cells (4.5 mg protein/ml). Trace (2) − (1) was obtained by subtraction and trace (3) shows for comparison the (oxidized *minus* reduced) spectrum of cytochrome b_1 (ref. 20). The spectra (1) and (2) were recorded 10 min after addition of succinate to sample cuvettes, which had previously been equilibrated with O_2 in the absence and presence, respectively, of CN^-. B. Dithionite-reduced *minus* oxidized. Traces (1), (2) and (2) − (1) correspond to those described in part A, but with dithionite replacing succinate as reductant.

of 'cytochrome *b*', assuming that in Fig. 7A, traces (1) and (2), absorption changes at 559 nm are due only to cytochrome *b*. By contrast, dithionite was able to reduce all cytochrome *b* even in the presence of 3 mM CN^- (Fig. 7B, traces (1) and (2)).

Already it is known that at −196°, the spectrum of succinate-reduced cultured cells (Fig. 2, trace (1)) allows the presence of two cytochrome *b* components, one with α-peak at 556 nm (−196°) and the other at 559.5 nm (−196°). When this difference spectrum was recorded in the presence of CN^- the 556-nm peak appeared at undiminished intensity but the 559.5-nm peak was partly collapsed (Fig. 2, trace (2)). It is proposed that cytochrome *b* (556 nm, −196°) represents a CN^--insensitive component also present[1] in bacteroids. The other, CN^--sensitive cytochrome *b* (559.5 nm, −196°), present only in cultured cells, was presumed to be responsible for the partial collapse of 559-nm absorption in Fig. 7A, trace (2), recorded at 20°.

The computed difference spectrum, shown as the dotted trace (2)−(1) in Fig. 7A, was expected to show the (oxidized *minus* reduced) difference spectrum of this CN^--sensitive cytochrome *b*. This spectrum did indeed show a trough at 560 nm but the peaks at 540 and 580 nm were not characteristic of the (oxidized *minus* reduced) spectrum of authentic cytochrome b_1 of *Escherichia coli*[20], drawn in for comparison (Fig. 7A, trace (3)). This trace (2)−(1) is very similar to that of (Lb^{2+} *minus* $Lb^{2+}O_2$), obtainable by reversing trace (2) of Fig. 5B. A simple explanation would be that in whole bacteria, Rhizobium haemoglobin stays as $Hb^{2+}O_2$ in the presence of CN^-, but can be deoxygenated to Hb^{2+} in the absence of this inhibitor. It is unlikely that this computed trace (Fig. 7A, trace (2)−(1)) contains any contribution from a CN^- complex of oxidized or reduced cytochrome *b* or Rhizobium haemoglobin; the CN^- difference spectrum of O_2-oxidized particle preparations is almost featureless in this region, as is the CN^- difference spectrum of dithionite-reduced whole bacteria (Fig. 7B, trace (2)−(1)). As discussed below the 594-nm peak in this latter spectrum is due to a cytochrome a_3–CN^- complex.

The CN^--sensitive cytochrome *b* of Fig. 7A cannot be positively identified as Rhizobium haemoglobin; autoxidizable cytochrome *o* would also contribute to the spectrum if it were CN^- sensitive.

Probable co-existence of cytochrome o and Rhizobium haemoglobin as CN^- and CO-sensitive haemoproteins. Although the cytochrome *o* components of *Rhodospirillum rubrum*[21] and of *Staphylococcus aureus*[22] are insensitive to CN^-, the spectra shown in Fig. 8 allow the separate existence of CN^--sensitive cytochrome *o* and haemoglobin in Rhizobium cultured cells. Trace (1) of this figure is the CO difference spectrum of succinate-reduced whole cells. It resembles the corresponding spectrum of dithionite-reduced cells (Fig. 3) in showing absorption bands due to cytochrome a_3–CO complex (see below), and also the peaks at 416, 540 and 573 nm and trough at 556 nm, which are thought to indicate the presence of at least two other CO-reactive pigments, as discussed with reference to Fig. 3. By contrast, trace (2) of Fig. 8, which is the CO difference spectrum of a succinate-reduced cell suspension previously treated with CN^-, lacks a trough near 556 nm. This shows that all CO-reactive pigments with absorption peaks (reduced form) in this region must have remained oxidized or otherwise unreactive with CO. The suppression by CN^- of CO complex formation is better shown by subtracting trace (2) of Fig. 8 from trace (1) and re-drawing the computed spectrum, shown as trace (1)−(2). By algebraic calculation (*cf.* the procedures of ref. 8) it was determined that this spectrum should show the (reduced + CO *minus*

reduced) difference spectrum of those pigments whose reduction was prevented by CN^-, or which were strongly bound by CN^- when reduced. There would be interference from the (reduced-CN^- complex *minus* reduced) or (oxidized-CN^- complex *minus* reduced) difference spectra of pigments whose combination with CN^- may be reversed by CO, or which were insensitive to CO. As discussed with reference to Fig. 7 this interference need not be considered for the cytochrome *b*-type pigments, but it is important for the elucidation of cytochrome a–a_3 properties, as presented later.

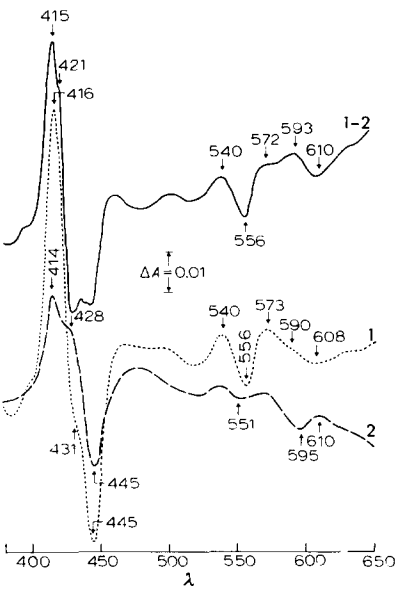

Fig. 8. Effect of CN^- on CO-reactive pigments of Rhizobium cultured cells. Trace (1) is the (succinate + CO *minus* succinate) and trace (2) the (CN^- + succinate + CO *minus* cyanide + succinate) difference spectrum of whole cells (19.5 mg protein/ml, 2-mm light path). Trace (1) depicts all CO-reactive pigments present, and trace (2) only those pigments able to form CO complexes in the presence of CN^-. Trace (1) — (2), obtained by subtraction, shows the CO difference spectra of those pigments which remained oxidized and unable to form CO complexes in the presence of CN^-. As described in the text this latter trace also shows the CN^- difference spectrum of pigments unable to react with CO, or whose CN^- complexes were dissociated by CO.

Apart from absorption due to cytochromes a–a_3 complexes, the outstanding features of this computed spectrum (Fig. 8, trace (1)—(2))were the asymmetric peak at 415 nm with shoulder at 421 nm, the peak at 540 nm, the shoulder at 572 nm and the trough at 556 nm, representing the CO difference spectrum of those CO-reactive pigment(s) whose reduction was blocked by CN^-. The dominating peak (415 nm) must be at least partly due to Rhizobium haemoglobin–CO complex ($Hb^{2+}CO$), whose major peak appears near 416 nm (Fig. 6B); the simplest explanation of the 421-nm shoulder would be that it represents the absorption peak of the cytochrome *o*–CO complex. Unfortunately the photochemical action spectrum (Fig. 4) shows no clear Soret peak for the cytochrome *o*–CO complex, so this complex could conceivably have its peak at 415 nm, with the 421-nm peak being due to a third CO-reactive pigment. This hypothetical third pigment could not be identical with bacteroid P-420, whose reduction and CO complex formation is not inhibited by CN^- (ref. 1).

Unlike the cytochrome *o* components of *R. rubrum*[21] or *S. aureus*[22], which remain oxidized in the presence of 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide and are thereby distinguishable from other cytochromes *b* in low-temperature difference spectra, all cytochromes in whole cells or particles from cultured Rhizobium were completely reducible by succinate respiration following prior equilibration with 8 μM 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide. This observation (repeatable from spectra recorded at 20° or —196°) and also the observation that Rhizobium cytochrome *o* reduction is inhibited by CN^- (*cf.* refs. 21, 22) means that in different microorganisms the cytochrome *o* pigments can have different properties and/or be associated with different electron flow systems.

Nature of cytochromes a–a_3 and their function in the respiratory chain

Besides assisting the elucidation of cytochrome *b*, cytochrome *o* and Rhizobium haemoglobin properties, the spectra of Figs. 7 and 8 yielded much information on the nature of Rhizobium cytochromes a–a_3 complex.

The CN^- difference spectrum of dithionite-reduced bacteria (Fig. 7B, trace (2)—(1)) shows a 594-nm peak and 610-nm trough, as reported[8,23] for mitochondrial cytochrome a_3. The concentration of ferrous cytochrome a_3–CN^- complex formed in this preparation was calculated as 0.42 μM (*cf.* MATERIALS AND METHODS); this was 57 % of the total cytochromes a–a_3 concentration calculated from the (reduced *minus* oxidized) difference spectrum (Fig. 7B, trace (1)). From a CO difference spectrum, not shown, of this same dithionite-reduced preparation, cytochrome a_3–CO complex concentration was calculated as 0.35 μM, or 47 % of total cytochromes a–a_3. These figures support the idea that the cytochromes a–a_3 complex of cultured Rhizobium contains approximately equal amounts of the CO and CN^--reactive component a_3 and the unreactive component a.

Stability of ferrous and ferric cytochrome a_3–CN^- complexes

CN^- was added to cultured-cell suspensions during O_2 equilibration (*cf.* ref. 1), so in the preparation used to record Fig. 7B, ferric cytochrome a_3–CN^- complex formation must have preceded a rapid conversion to ferrous cytochrome a_3–CN^- which followed dithionite addition; the stable spectrum (trace (2)) appeared within 1 min. Others have shown that mammalian ferric cytochrome a_3–CN^- complex, once formed, is very slowly reduced[23], if at all, by dithionite. The CN^- difference spectra of cultured cells, after succinate (Fig. 7A, trace (2)—(1)) or endogenous respiration showed respectively 70 and 30 % formation of ferrous cytochrome a_3–CN^- within 10 min. This means that at pH 6.8 the stability ratio of ferric compared with ferrous cytochrome a_3–CN^- complex must be very much lower for the Rhizobium than for the mitochondrial enzyme.

Difference spectra of Rhizobium cytochrome a_3 complexes and the ultraviolet–visible spectrum of cytochromes a–a_3

In the CO difference spectrum of succinate-reduced whole cells (Fig. 8, trace (1)) the shoulder at 590 nm and trough at 608 nm confirm the formation of cytochrome a_3–CO complex[7,8,23]. This is in contrast with trace (2) of Fig. 8, a (CN^- + succinate + CO *minus* CN^- + succinate) spectrum of the same preparation, where a trough appears at 595 nm and a peak at 610 nm. This spectrum would be expected if mito-

chondrial[8] and Rhizobium cytochrome a_3 complexes have similar spectroscopic properties. Ferrous cytochrome a_3–CN^-, with absorption peak at 595 nm, and present in the reference cuvette (*cf.* previous section) would have a higher specific absorption than ferrous cytochrome a_3–CO complex formed in the sample cuvette by displacement of CN^- by CO. The net result would be an absorption deficiency at 595 nm in this difference spectrum (Fig. 8, trace (2)).

As explained above, the computed spectrum (Fig. 8, trace (1)—(2)) should show the CN^- difference spectrum of pigments whose combination with CN^- is reversed by CO. The appearance of a peak at 593 nm and a trough at 610 nm, characteristic of the CN^- difference spectrum of cytochrome a_3 (*cf.* Fig. 7B, trace (2)–(1), also ref. 8), means that Rhizobium ferrous cytochrome a_3 has a greater affinity for CO than for CN^-.

In the (reduced *minus* oxidized) difference spectrum of mitochondrial cytochromes a–a_3 complex[8], the ultraviolet to visible absorption-peak ratio of cytochrome a_3 has a high value, ([ΔA,(444—465)nm]/[ΔA,(603—625)nm]) = 23.4, whereas this ratio for cytochrome a has a low value, ([ΔA,(445—461)nm]/[ΔA,(605—625)nm]) = 2.9. This should enable one to calculate the relative concentration of these two reduced cytochrome components in other situations, but in preparations from cultured Rhizobium the relatively-high concentration of cytochrome b masks the ultraviolet absorption of and prevents any estimation of the relative amounts of cytochromes a and a_3. However, the resuspended unwashed particles from disrupted cells sometimes retained sufficient substrate to allow the steady-state reduction of cytochromes a–a_3, whereas other cytochromes became substantially oxidized. In Fig. 9, particles resuspended in air-equilibrated buffer at 0° then stored at 20° under N_2 for 30 min, showed complete reduction of cytochromes a–a_3, measured from absorption at 603 nm, about 30–50% reduction of cytochrome c and no reduction of cytochrome b. The Soret absorption peak of cytochromes a–a_3 is clearly shown at 444 nm and the absorption ratio, ([ΔA,(444—480)nm]/[ΔA,(603—630)nm]) of 8.5

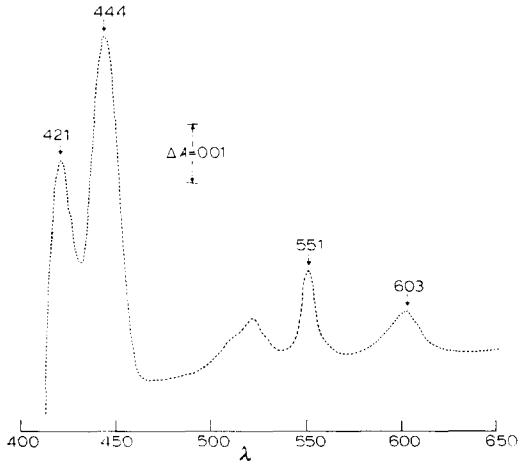

Fig. 9. Appearance of cytochromes a–a_3 and cytochrome c in spectra of unwashed particles. This is a (reduced *minus* O_2-oxidized) difference spectrum in which endogenous reduction of unwashed particles (10 mg protein/ml, 2-mm light path) under N_2 was not preceded by O_2 equilibration (*cf.* Table II).

is consistent with the presence of a mixture of cytochrome components *a* and a_3 having classical spectral properties.

Sequence of electron transport components in cultured cells

As steady-state conditions were achieved in the preparation used to record Fig. 9, the relative levels of cytochrome reduction probably reflect their relative redox potentials. So one could postulate the sequence: substrate → cytochrome *b* → cytochrome *c* → cytochromes a–a_3 → O_2, as also found in animal mitochondria. In this sequence, cytochrome *b* would be the CN^--insensitive cytochrome *b* component (Fig. 2) which has its α-absorption peak at 556 nm (—196°). The cytochrome *b* (559.5 nm, —196°), presumed to include cytochrome *o*, also remained oxidized under the conditions recorded for Fig. 9, so one must presume the redox potential of cytochrome *o* to be lower than that of the other oxidase, cytochrome a_3.

A possible difficulty in identifying cytochrome *b* (556 nm, —196°) as a component of a classical mitochondrial-type electron transfer system and cytochrome *b* (559.5 nm, —196°) as cytochrome *o* is the effect of antimycin A when added to O_2-oxidized particle suspensions. In the presence of 30 μM antimycin A, NADH caused 80% reduction of the cytochrome *b* (559.5 nm, —196°), no reduction of the cytochrome *b* (556 nm, —196°) and 25% reduction of the cytochromes a–a_3 and *c* achieved in an inhibitor-free preparation (*cf.* Table II). In the classical mitochondrial oxidase system, cytochrome *b* stays reduced in the presence of antimycin A (ref. 24) and subsequent components (cytochromes *c*, c_1, *a*, a_3) become oxidized. Conversely, the evidence that *S. aureus* cytochrome *o* (ref. 22) is unaffected by antimycin A does not conflict with the present identification of cytochrome *b* (559.5 nm, —196°) as Rhizobium cytochrome *o*.

DISCUSSION

Fig. 10 lists all the pigments characterized in this paper, the effects of inhibitors on them, and their possible function in oxidase systems. Kinetic evidence is not yet available to support this scheme, but as with bacteroids[1], autoxidizability is accepted as presumptive evidence of an oxidase function.

CN^- and other inhibitor studies allow the existence in cultured cells but not bacteroids of a classical electron transfer chain including the components *b*, *c*, *a*, a_3, although the excess of cytochrome *c* over other components (Table I; Figs. 1 and 7) suggests that it might function in other electron flow pathways as well. However, there is no reason for including cytochrome *c* in the oxidase pathway terminating with cytochrome *o*. Indeed, if the Rhizobium haemoglobin does represent solubilized, functional cytochrome *o* it is noted that cytochrome *c* present in crude supernatants containing this haemoglobin always remains reduced. This would mean that soluble cytochrome *o* is not a cytochrome-*c* oxidase.

The negligible autoxidation rate of Rhizobium haemoglobin, probably isolated as $Hb^{2+}O_2$ in the presence of cytochrome *c*, makes it unlikely that this pigment is any kind of functional oxidase. The slowly-autoxidizable nature of a soluble, assumed cytochrome *o* component of Vitreoscilla[5] was excused by making the analogy with purified mitochondrial cytochrome oxidase, which becomes autoxidizable only in the

presence of cytochrome *c* (ref. 25). The presence of cytochrome *c* makes a similar explanation invalid for the non-functional nature of Rhizobium haemoglobin.

Although a partly-purified, soluble haemoglobin-like pigment of *Acetobacter suboxydans* stimulated the lactate-oxidase activity of particles prepared from this organism[4], the crystalline pigment[26] did not. Likewise, neither of the purified, soluble cytochrome *o* pigments of Vitreoscilla have any oxidase activity[5]. Thus, both of these organisms resemble cultured Rhizobia in having, besides a (presumed) particle-bound cytochrome *o*, soluble haemoglobin-like pigments of unknown function.

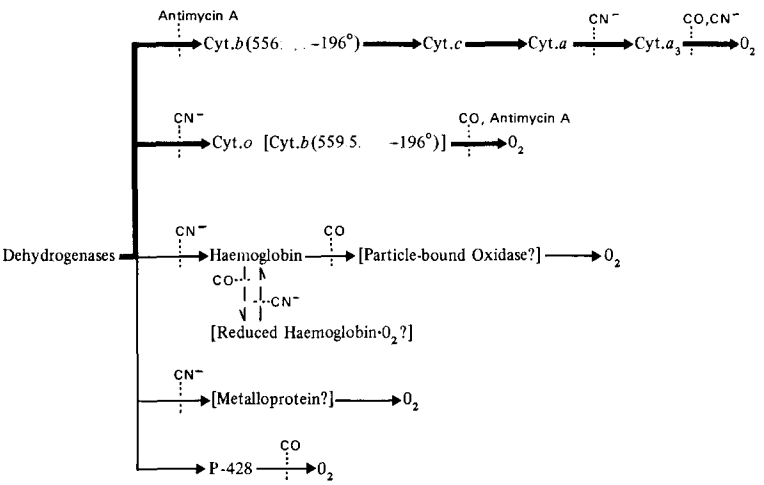

Fig. 10. Electron transport components of cultured Rhizobium. Thin solid lines represent possible minor pathways and thick lines major pathways of electron flow. Dotted lines represent sites of inhibitor action.

Rhizobium haemoglobin appears to be isolated as a stable O_2 complex ($Hb^{2+}O_2$), but the respiratory processes of whole cells can apparently cause its deoxygenation to Hb^{2+}. In whole cells, in the presence of CN^-, its status is uncertain. Fig. 7A, trace (2)—(1) suggests that CN^- has prevented its deoxygenation, possibly by inhibition of all oxidases (Table III; Fig. 10) with consequent decrease of O_2 demand within the cell. This interpretation, if correct, could mean that Rhizobium haemoglobin functions as an O_2 carrier; if Rhizobium haemoglobin as present in unbroken bacteria, is equivalent to cytochrome *o*, it could alternatively mean the demonstration of an O_2 complex of this oxidase. However, the results shown in Fig. 8 are most-readily explained by assuming that in whole cells in the presence of CN^-, Rhizobium haemoglobin exists as the ferric form (Hb^{3+}) rather than as Hb^{2+}, since it is unreactive with CO. In aerated, substrate-deficient particles the Rhizobium haemoglobin (if present) certainly exists as Hb^{3+} even in the absence of CN^-, since CO difference spectra (not illustrated) show no formation of $Hb^{2+}CO$. Consequently Fig. 10 shows Rhizobium haemoglobin as linked to a particle-bound oxidase although this pigment itself might be autoxidizable (and an oxidase) in a native, membrane-bound form.

Also unsatisfactory is the status of a CN^--sensitive 'metalloprotein' oxidase (Fig. 10) whose presence was invoked because cultured-cell respiration, although completely sensitive to CN^- was not blocked by $CO–O_2$ (95:5, v/v) (Table III). Finally,

although cultured cells may contain traces of the soluble, autoxidizable, CO-reactive P-428 also found in Rhizobium bacteroids[1] its function as an oxidase (Fig. 10) remains unproven.

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