BBA 45746

ELECTRON TRANSPORT SYSTEMS OF *RHIZOBIUM JAPONICUM*

I. HAEMOPROTEIN P-450, OTHER CO-REACTIVE PIGMENTS, CYTOCHROMES AND OXIDASES IN BACTEROIDS FROM N_2-FIXING ROOT NODULES

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(Received August 5th, 1968)

SUMMARY

1. This work characterizes some cytochromes, oxidases and CO-reactive pigments of *Rhizobium japonicum* bacteroids isolated from N_2-fixing soybean root nodules, and considers their functions in electron transfer or processes related to symbiotic nitrogen fixation.

2. In common with cultured (non N_2-fixing) Rhizobia, bacteroids contain non-autoxidizable cytochrome *c* (550, Rhizobium) and cytochrome *b*. They lack the oxidases, cytochromes a–a_3 and *o*, found in cultured cells. Exclusively present in bacteroids are the soluble, autoxidizable CO-reactive pigments, cytochrome *c* (552, Rhizobium) and the haemoprotein P-450. Bacteroids also contain the pigments P-420 and P-428 tentatively identified by the major absorption peaks of their CO complexes. All pigments are biologically reducible and none appear to be artifacts.

3. The succinate-oxidase pathway of aerobically-prepared bacteroids is inhibited by CN^- and EDTA but not by CO; it may include a CN^--sensitive pigment with absorption peak (when reduced) at 502 nm.

INTRODUCTION

Whereas *Rhizobium spp.* grown in pure culture are unable to fix N_2, bacteroids isolated from nodules on legume roots inoculated with the same *Rhizobium spp.* are able to catalyse an O_2-dependent reduction of N_2 to ammonia[1]. Since N_2 fixation in extracts of these bacteroids requires strict anaerobiosis, added chemical reductant and an ATP-generating system[2], then O_2 respiration of whole bacteroids must be coupled to energy-supplying systems essential for N_2 fixation.

Earlier work[3–5] has described differences in the cytochrome pattern of these two Rhizobium forms, with the cytochrome *a* of cultured cells missing from bacteroids. This paper further characterizes some of the haemoproteins and other components of

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; Q_{O_2} (N), μl O_2-uptake/h per mg protein nitrogen.

the bacteroid electron transport system, with emphasis given to CO-reactive pigments; N_2 fixation in intact nodules, bacteroids or bacteroid extracts[6] is known to be inhibited by CO. The accompanying paper[7] presents a corresponding study with cultured Rhizobium, so enabling the identification of electron transport components and CO-reactive pigments exclusively present in the bacteroids and possibly related to the N_2-fixation process.

MATERIALS AND METHODS

Preparation of bacteroids

Lincoln strain soybeans were grown in a glasshouse at 25–30° in soil inoculated with *Rhizobium japonicum* strain 505 (Wisc.). Plants were harvested at 5 weeks, the washed roots placed in cold water and root nodules rapidly picked into 0.1 M phosphate (pH 6.8) at 0°. All subsequent manipulations were carried out at 0–4°. These nodules were washed, resuspended in 3–4 vol. of 0.1 M phosphate (pH 6.8) and ground in a Waring blendor for about 1 min. The brei was squeezed through fine cheese cloth to remove nodule cortex tissue and centrifuged at 200 × *g* (10 min) to remove starch granules and remaining cortex. Bacteroids were sedimented at 6000 × *g* and at least three times resuspended in 0.1 M phosphate (pH 6.8) and recentrifuged until spectrophotometric examination showed the washings to be free of leghaemoglobin. Bacteroids were finally suspended to about 25% (wet wt./v) in 0.1 M phosphate (pH 6.8) for immediate use or storage at —15° or —196°.

Cell rupture and fractionation

Crystalline deoxyribonuclease (1 mg) and $MgCl_2$ (to 0.1 mM) were added to 50 ml of bacteroid suspension and the mixture passed twice through a modified French press[8] at 15000 lb·inch^{-2} pressure. This disrupted-cell suspension, whose viscosity decreased rapidly due to deoxyribonuclease action, was centrifuged at 10000 × *g* (30 min) to precipitate large fragments of cell wall, plasma membrane, and poly β-hydroxybutyrate granules. Recentrifugation of this turbid supernatant at 144000 × *g* (120 min) or 190000 × *g* (60 min) gave a translucent amber particle pellet and a clear amber supernatant. The particles were washed twice by resuspending and recentrifuging and finally made to 25% (wet wt./v) in 0.1 M phosphate (pH 6.8). Electron microscopy of OsO_4-fixed particle sections showed them to be small fragments with a double-membrane structure. Since comparable sections of whole bacteroids showed no double-membrane structures other than plasma membrane or cell wall, it was concluded that these particles were fragments of one or both of these membrane structures.

Spectrophotometric techniques

A Cary model 14R spectrophotometer equipped with 650-W tungsten–iodine light source, alternative 0–1, 1–2 A or 0–0.1, 0.1–0.2 A slidewires, and model 1462 scattered transmission accessory with Dumont 7664 photomultiplier, was used for recording absolute and difference spectra, generally at 20°. Spectrophotometer slit widths were generally <0.1 mm and pen noise <0.001 absorbance (A). Unless otherwise indicated, difference spectra were measured in 10-mm light-path cuvettes; this

permitted the use of diluted suspensions which were more convenient than concentrated suspensions for gas equilibration.

To record difference spectra at −196°, the single-freeze technique of BONNER[9] with dilutions in 0.1 M phosphate (pH 6.8) was used. The cuvette assembly (2 mm light path) was cooled by liquid N_2 contained in an oval vessel hollowed out of polyurethane foam and fitted with evacuated double-glass windows.

Photo-dissociation spectra. Parallel-focussed light from a 650-W tungsten–iodine lamp was passed through 4.5 cm of 1.0 M $CuSO_4$, a Corning 3-69 glass filter and a Balzer's (Lichtenstein) broad-band interference filter type K 4. The transmitted light (525–575 nm band width) was directed through a 6-mm diameter glass-fibre light pipe onto the side of the sample cuvette (10 mm light path, 3 mm liquid width) to give $2.6 \cdot 10^5$ ergs·cm^{-2}·sec^{-1}. The photomultiplier was protected from actinic light by a Corning type 5-58 glass filter having 360–480-nm transmission. Thus, haemoprotein CO-complexes were excited in the region of their α- and β-absorption bands, and dissociation spectra measured in the Soret region.

Sample preparation for spectrophotometry. Dilutions of bacteroid fractions were made in 0.1 M phosphate (pH 6.8) and other reagents added as powders or minimal volume of concentrated solution at pH 6.8, giving the following final concentrations: sodium dithionite, 0.5 mg/ml; $K_3Fe(CN)_6$, 100 μM; NADH or NADPH, 1.0 mM; sodium succinate, 20 mM; KCN, 3 mM. 1-min equilibration with CO (Matheson C. P. grade) added as a steady stream of small bubbles at 20° and an atmospheric pressure of 720 mm Hg produced a 1 mM solution. Lower CO concentrations were achieved by injection of a 1 mM solution into a liquid-filled cuvette fitted with a rubber ampoule cap, and containing a plastic-coated stirring magnet. Other gases were added by bubbling for 1 min.

O_2 equilibration always preceded CN^- addition, to allow the possibility of CN^- combination with oxidized pigment. A 10-min equilibration was generally allowed with reagents other than O_2 before adding the succeeding reagent or recording the difference spectrum. Cuvette contents were layered with degassed paraffin oil whenever necessary to maintain anaerobic conditions.

Quantitative calculation of pigment concentrations. The approximate concentration of each cytochrome or CO-reactive pigment in bacteroid fractions was calculated from (reduced *minus* oxidized) or (reduced + CO *minus* reduced) difference spectra respectively, by measuring ΔA between a wavelength pair corresponding to an absorption peak and trough (or plateau) of that component or related component in pure solution. Thus, for cytochrome *c* (550, Rhizobium), $\Delta A_{mM} = 23.2$, (550–536 nm)[10] and for cytochrome *b*, $\Delta A_{mM} = 17.9$, (559–580 nm), *cf. Escherichia coli*[11]. For the CO complex of cytochrome *c* (552, Rhizobium), $\Delta A_{mM} = 17.6$, (536–551.5 nm)[10] and for the CO complex of P-450, $\Delta A_{mM} = 87$, (448–490 nm)[12].

An indication of the concentrations of the P-420–CO and the P-428–CO complex in any fraction was obtained by firstly calculating the concentrations of the P-450–CO and the cytochrome *c*-552–CO complex as described above. Knowing the CO difference spectra of these two purified pigments[10,12], the absorption due to their combined presence was calculated at 1–2-nm intervals and these values subtracted from the original CO difference spectrum. The position of the remaining absorption peak (between 420 and 428 nm) was assumed to indicate the relative concentrations of the other two CO-reactive pigments, tentatively named P-420 and P-428 (see text). Their

approximate combined concentration was estimated by measuring ΔA ($\lambda_{max.}$ — 480 nm) and arbitrarily assuming the value of $\Delta A_{mM} = 111$ as found for the microsomal P-420–CO complex[13].

With the general procedures described above, bacteroid particle and supernatant fractions showed negligible enhancement of specific absorption due to multiple internal reflection (*cf.* ref. 14), but unfrozen suspensions of whole or disrupted bacteroids gave at least 50 % enhancement. For this reason, percent distributions of pigments between fractions are not presented.

O_2 respiration

This was measured in a Braun type VL 85 photosynthetic Warburg apparatus (5-cm stroke, 110 strokes/min) at 25°. For CO_2 absorption, centre wells of the 15-ml reaction flasks contained 0.1 ml of 1 M KOH, or 0.5 ml of $Ca(CN)_2$–$Ca(OH)_2$ solution[15] during CN^--inhibition experiments. Final liquid volume in the main compartment was 2.4 ml, and buffer was 0.1 M phosphate (pH 6.8). Inhibitors were added to this compartment before temperature equilibration and substrate (sodium succinate to 50 mM final concentration) added from a sidearm after equilibration. For experiments on CO inhibition, flasks were flushed with CO–O_2 (95:5, v/v) and controls with N_2–O_2 (95:5, v/v). After 15 min equilibration in the dark, (CO-inhibited) O_2 uptake was measured in alternate 15-min dark and light periods. The 40-W incandescent lamps of this apparatus gave $4 \cdot 10^5$ ergs·cm^{-2}·sec^{-1} on the bottom surfaces of reaction flasks.

In other experiments the gas phase was air, and for all experiments bacteroid concentration was adjusted to give about 50 μl O_2 uptake/15 min in the absence of inhibitors, with succinate as substrate.

Protein estimations

The method of LOWRY *et al.*[16], for proteins insoluble in cold alkali, was used for protein examinations on all Rhizobium fractions. Crystalline bovine serum albumin (Armor) was used as a standard.

Reagents

Sodium dithionite was purchased from May and Baker, England, deoxyribonuclease from the Sigma Chemical Co., U.S.A., and enzymically-reduced NADH and NADPH from Calbiochem., U.S.A. Other chemicals used were reagent grade. Phosphate buffers were prepared by mixing and diluting 0.1 M Na_2HPO_4 and 0.1 M KH_2PO_4 to the stated phosphate molarity and pH.

RESULTS

Distribution of cytochromes in bacteroid fractions

The cytochrome content of whole cells and particles was conveniently shown in (dithionite-reduced *minus* O_2-oxidized) difference spectra (Fig. 1); in supernatants much cytochrome remained reduced after O_2 equilibration and $Fe(CN)_6^{3-}$ was added to achieve complete oxidation. The results confirmed earlier work[3–5], although the higher resolution of the Cary spectrophotometer permitted some correction of band positions. Thus, whole bacteroids contained cytochrome *c* with α-peak at 551 nm and cytochrome *b* with α-peak near 559 nm but no trace of cytochromes a–a_3 with ab-

sorption near 603 nm. The absence of cytochrome *a* components was confirmed by repeating the difference spectra at low temperature, where a 20-fold enhancement of cytochrome-*b* absorption (with α-peak shifted to 556 nm at —196°) and of cytochrome-*c* absorption (α-peak to 548 nm, —196°) was achieved. The inability to demonstrate splitting of these α-bands at —196° implied the absence of multiple components of cytochromes *b* or *c*. However, on examining their distribution between fractions (Fig. 1; Table I) the α-peak of soluble cytochrome *c* was measured at 550 nm and

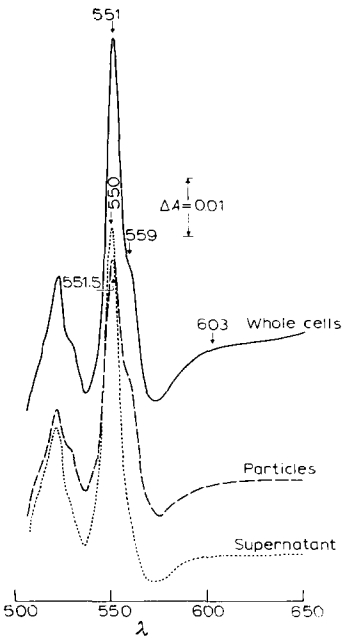

Fig. 1. Dithionite-reduced *minus* oxidized difference spectra, showing total cytochrome content of bacteroid fractions. Protein concns. (mg/ml) were: whole cells, 2.8; particles, 2.0; supernatant 6.0.

TABLE I

HAEMOPROTEIN CONCENTRATIONS IN BACTEROID FRACTIONS

Cytochrome concentrations (μmoles haem/g protein) were calculated from the (reduced *minus* oxidized) spectra of Fig. 1, and CO-reactive pigment concentrations (μmoles haem/g protein) from the CO difference spectra of Figs. 2–4, as described in MATERIALS AND METHODS. 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. Because of 'enhanced absorption', as discussed in MATERIALS AND METHODS the true values for whole cells are probably 50% lower than shown.

Haemoprotein	*Whole cells*	*Particles*	*Supernatant*
Cytochromes *c*-550 and *c*-552	0.94	0.86	0.39
Cytochrome *b*	0.42	0.64	0.11
CO-reactive pigments			
Cytochrome *c*-552	0.24	0.20	0.06
P-450	0.11	0.045	0.06
P-428	+	?	0.02
P-420	++	0.10	?

the remaining particle-bound cytochrome *c* had its α-peak at 551.5 nm. The soluble cytochrome *c* was purified and characterized as cytochrome *c* (550, Rhizobium)[10]. Table I gives the apparent concentration (μmoles per g protein) of cytochromes *b* and *c* in each fraction, but because of the 'enhanced-absorption' phenomenon in intact bacteroids no balance sheet of distribution is presented. Nevertheless it was estimated that about 30 % of total cytochrome *c* and 12 % of total cytochrome *b* was released into solution following cell disruption. The remainder appeared to be tightly bound to the membrane fragments (particles).

Distribution of CO-reactive pigments in bacteroid fractions

Our earlier spectra[3,4] and those of TUZIMURA AND WATANABE[5] apparently showed a pigment with CO complex absorbing near 416 nm in bacteroids from soybean root nodules as well as in cultured cells of the same Rhizobium strain; this pigment was tentatively identified[17] as cytochrome *o*, a bacterial oxidase first described by CASTOR AND CHANCE[18]. The present work, however, shows peaks at 413.5, 538 and 566 nm, with 551-nm trough, in the CO difference spectrum (Fig. 2) of dithionite-reduced whole bacteroids, and these peak and trough positions are several nm lower than those supposedly characteristic of cytochrome *o* (ref. 18). Evidence to be presented elsewhere[10] shows that they are due to the CO complex of a soluble, autoxidizable cytochrome *c* (552, Rhizobium) not identical with or derived from cytochrome *c* (550 Rhizobium).

The earlier CO difference spectra of whole bacteroids[3,4] also had a peak or shoulder at 428–430 nm, thought to be due to the formation of CO complexes of cytochromes a_1 or a_3 (*cf.* ref. 19), even though no trace of these cytochromes was detected in the (reduced *minus* oxidized) difference spectrum (*cf.* Fig. 1) of any bacteroid fraction. However, in the new CO difference spectrum of dithionite-reduced bacteroids (Fig. 2, solid trace) the shoulder at 425 nm could not be identified as the

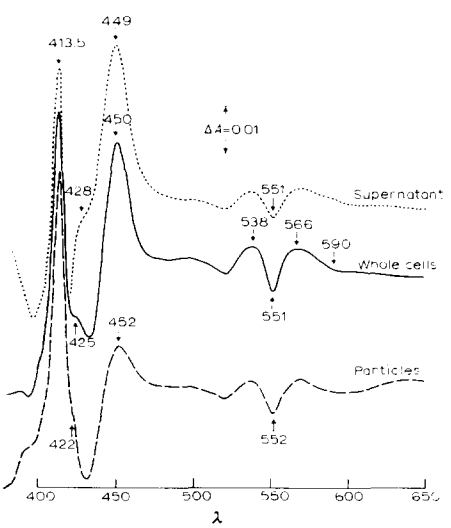

Fig. 2. CO difference spectra of bacteroid fractions. All traces are of (dithionite + CO *minus* dithionite) difference spectra, and protein concns. (mg/ml) were: whole cells, 2.5; particles, 2.3; supernatant, 9.0.

cytochrome a_1–CO or the cytochrome a_3–CO complex. Using the 0–0.1 A slidewire of the Cary-14 spectrophotometer, no absorption peak or shoulder of these CO complexes could be detected near 590 nm but a peak, due to the cytochrome a_3–CO, was readily detectable in the corresponding spectrum of cultured cells[7]. As described below, the 425-nm shoulder in this CO difference spectrum of bacteroids appears to be due to the combined presence of at least two new pigments, one with CO complex absorbing near 428 nm and the other near 420 nm.

A remarkable feature of this CO difference spectrum (Fig. 2) is the appearance of a broad peak at 450 nm. Such a peak, characteristic of the CO complex of haemoprotein P-450 of animal membranes[13], does not appear in the CO difference spectrum of cultured cells[7].

More information about the nature of bacteroid CO-reactive pigments was gained from a study of their distribution between fractions from disrupted cells. The CO-reactive cytochrome *c*-552 was about equally distributed between particles and supernatant (Fig. 2; Table I) but most P-450 was released into the supernatant. This ready solubility of P-450 was quite unexpected as attempts to solubilize the particle-bound P-450 from microsomes[13] had resulted in its degradation to a soluble, low mol. wt. P-420, a conventional protohaemoprotein whose CO complex had an absorption peak at 420 nm. If the bacteroid pigment with CO complex absorbing near 425 nm was similarly derived from bacteroid P-450 one might expect it to be present only in the supernatant, but this was not so.

Identification of P-420 and P-428

The uncorrected CO difference spectrum of a bacteroid supernatant (Fig. 2, dotted trace) showed a shoulder at 428 nm, whereas the corresponding spectrum of particles (Fig. 2, dashed trace) had a just-detectable shoulder near 422 nm. When the latter spectrum was corrected for absorption due to the P-450–CO and cytochrome *c*-552–CO complexes, as described in MATERIALS AND METHODS, there appeared (Fig. 3) the CO difference spectrum of a conventional protohaemoprotein, with peak at 420 nm and troughs at 435 and 560 nm (*cf.* microsomal P-420 (ref. 13), and cytochrome *o* (ref. 20). The pigment responsible is tentatively named P-420 but its nature remains obscure. It is unlikely to be derived from soluble P-450 since it is particle-bound; although its spectrum closely resembles the CO difference spectrum of cytochrome *o* no corresponding CO-sensitive oxidase could be detected in later experiments.

The solid trace of Fig. 4A represents the CO difference spectrum of succinate-reduced whole bacteroids. By comparison with the stable CO difference spectrum of

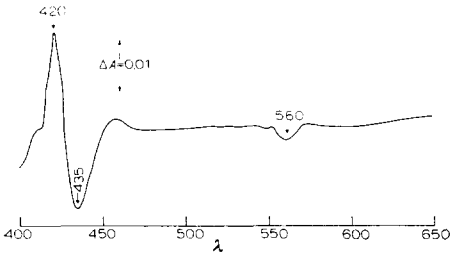

Fig. 3. Computed CO difference spectrum of P-420 in bacteroid particles. The (dithionite + CO *minus* dithionite) difference spectrum of particles, from Fig. 2, was corrected for the cytochrome *c*-552–CO complex and P-450–CO complex absorption as described in MATERIALS AND METHODS.

dithionite-reduced bacteroids (Fig. 2, solid trace) a 428-nm peak is clearly visible, and the responsible pigment tentatively named P-428. The corrected spectrum, obtained by subtracting absorption due to the P-450–CO and cytochrome *c*-552–CO complex is shown as the dotted trace of Fig. 4A, and more closely resembles the 'abnormal' CO difference spectrum of P-450 (ref. 12) than the CO difference spectrum of a classical haemochromogen. In particular, it has a broad trough near 398 nm, possibly representing the absorption peak of reduced P-428 which remains, uncomplexed, in the reference cuvette. Also, this corrected CO difference spectrum is featureless between 590 and 605 nm proving that P-428 is not the CO-reactive cytochrome a_3 (*cf.* ref. 19).

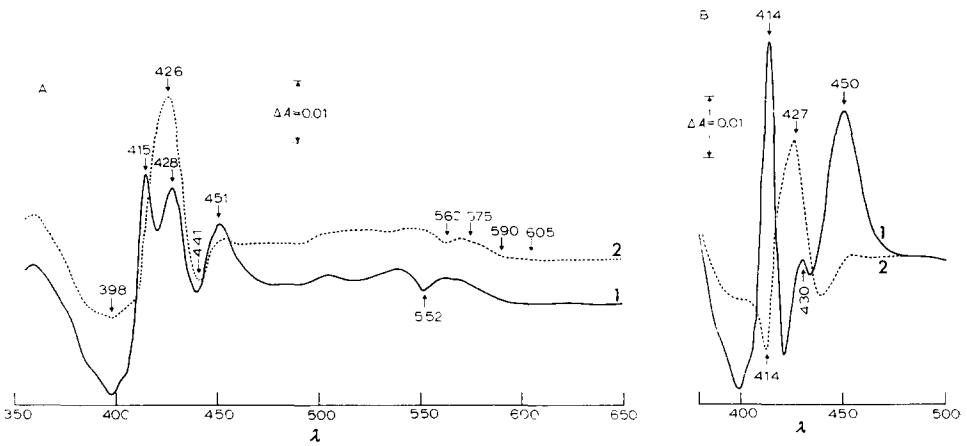

Fig. 4. Appearance of P-428 in computed CO difference spectra. A. Bacteroids. Trace (1) is the CO difference spectrum of succinate-reduced whole bacteroids (7.2 mg protein/ml) recorded 14 min after CO addition. Trace (2) has been corrected for absorption due to the P-450–CO and cytochrome *c*-552–CO complex, as described in MATERIALS AND METHODS. B. Supernatant. Trace (1) is the CO difference spectrum of a dithionite-reduced supernatant solution (8.7 mg protein/ml) from disrupted bacteroids, recorded immediately after CO equilibration. Trace (2) has been corrected for the P-450–CO and cytochrome *c*-552–CO complex absorption.

The relatively slow appearance of a 428–430-nm peak in CO difference spectra of bacteroid supernatants, meant that such spectra were always dominated by the peaks of P-450 and cytochrome *c*-552–CO complexes; this made correction procedures more hazardous. Typically, although the corrected CO difference spectrum of Fig. 4B (trace (2)) shows a peak at 427 nm, suggesting a predominance of P-428 over P-420 in bacteroid supernatants, some irregularity at 414 nm was probably caused by over-correction for the presence of the cytochrome *c*-552–CO complex.

In the corrected CO difference spectra of other supernatants, the Soret peak has appeared between 428 and 422 nm. This is interpreted as showing the release into solution of P-428 together with variable amounts of P-420. Alternatively, variable amounts of the soluble P-450 may have been degraded to give a soluble P-420 (*cf.* ref. 13) not identical with or derived from the particle-bound P-420 (Fig. 3).

Although the CO complexes of peroxidase and modified catalase have absorption peaks near 425 nm (ref. 21), bacteroid fractions containing P-428 and/or P-420 had only slight catalase, cytochrome-*c* peroxidase or guaicol peroxidase activities (*cf.* refs. 22–24). The function of these pigments is not yet known.

Biological reduction of bacteroid pigments

Dithionite was used as a reductant during experiments designed to detect total pigments as it permitted the reduction of those whose natural substrates or reductases were lost during fractionation. This reagent could also have reduced other, denatured pigments with no biological function, particularly CO-reactive haemoproteins. Mammalian cytochrome *c* does not react with CO unless denatured[25] and CO-reactivity is generally assumed to be sufficient evidence for denaturation of other haemoproteins[26]. Conversely, the biological reducibility of cytochromes[25,27] generally indicates their native state, so Table II records some observations on biological reduction of bacteroid pigments. Endogenous respiration of whole bacteroids permitted the substantial reduction of all pigments so far described, CO complex formation being assumed to require prior reduction of the appropriate pigment.

All pigments were autoxidizable or had oxidases located on bacteroid membranes, since washed, substrate-deficient particle suspensions showed complete pigment oxidation after O_2 equilibration. Little or no re-reduction occurred during anaerobic incubation of these oxidized-particle suspensions at 20° (Table II, column 2) but subsequent addition of NADH or NADPH caused the substantial reduction of all pigments except P-450 (Table II, column 3), implying the existence of membrane-bound reductases.

The substantial endogenous reduction of P-450, also cytochrome *b*, cytochrome *c*-552 and P-428 in a bacteroid supernatant (Table II, column 4) implies the presence of soluble reductases for these components. The cytochrome *c*-550 also present in this supernatant is not autoxidizable[10], and since this cytochrome remains reduced following O_2 equilibration it is assumed that the supernatant contains no cytochrome

TABLE II

BIOLOGICAL REDUCTION OF CYTOCHROMES AND CO-REACTIVE PIGMENTS

Sample and reference cuvettes were initially equilibrated with O_2, then percent reduction of cytochromes measured from (reduced *minus* oxidized) difference spectra, 20 min after N_2 equilibration of the sample cuvette to allow endogenous reduction, or 20 min after N_2 *plus* NADH addition. In separate experiments, percent formation of CO complexes was measured 20 min after CO equilibration of the sample cuvette. This had been preceded by 20 min equilibration of both cuvettes with N_2 or N_2 *plus* NADH. In control experiments dithionite (*cf.* Figs. 1 and 2) was assumed to give 100% reduction. The symbols (++) and (+) indicate respectively the estimated high or low percent reduction of pigments whose absorption bands were partly obscured by those of other pigments.

Haemoproteins	*Whole cells: endogenous minus* O_2	*Particles*		*Supernatant: endogenous minus* $Fe(CN)_6^{3-}$
		Endogenous minus O_2	*NADH minus* O_2	
Reduction				
Cytochromes *c*-550 and *c*-552	87	16	24	90
Cytochrome *b*	100	18	30	++
Reduction with CO complex formation				
Cytochrome *c*-552	62	0	76	72
P-450	100	0	0	92
P-428	++	0	?	++
P-420	+	0	++	+

c 552 oxidase. From these experiments it is not possible to make any assumption about the presence or absence of a soluble reductase for cytochrome *c* 550.

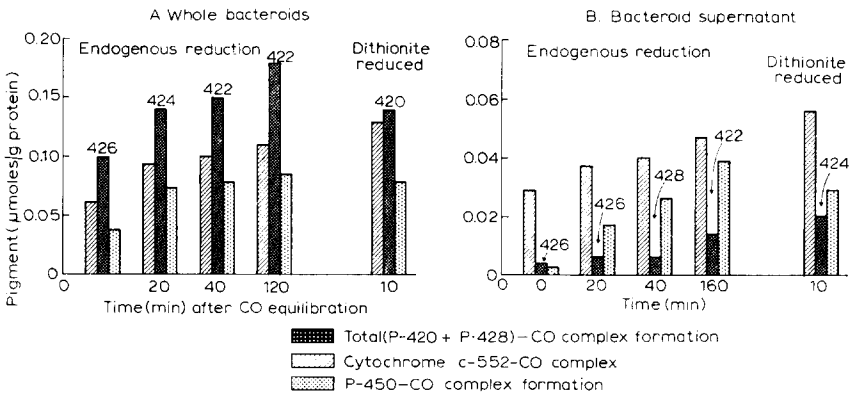

Fig. 5. Time course for formation of CO complexes in bacteroid fractions. The concentrations of the cytochrome *c*-552–CO and P-450–CO complex were measured from difference spectra at the stated times after CO addition to whole bacteroids or supernatants previously reduced by endogenous respiration, or dithionite addition. The concentration of the (P-420 + P-428)–CO complex was measured from spectra corrected between 418–430 nm for the cytochrome *c*-552–CO and P-450–CO complex absorption. Relative concentrations of P-420–CO and P-428–CO complexes may be estimated from the peak wavelength (in nm) of each corrected spectrum, shown above each bar.

The formation of CO complexes in dithionite-reduced bacteroid preparations was complete within 10 min, but significant differences in their relative rates of formation were observed following CO addition to biologically-reduced fractions (Fig. 5). Thus in whole bacteroids, reduced by 10 min endogenous respiration (Fig. 5A), the P-428–CO complex appeared very rapidly and its absorption peak often dominated the CO difference spectrum for first 10 min (*cf.* also succinate reduction, Fig. 4A). The shift with time of the combined absorption peak of the (P-420 + P-428)–CO complex from 426 to 422 nm (Fig. 5A), suggested that the P-420–CO complex was also formed, but more slowly. It is unlikely that P-428 and P-420 represent different complexes of a single pigment since they are differently located within the cell (Figs. 3 and 4). Although P-428 was released into solution when bacteroids were disrupted (Figs. 2 and 4B, also Table I) the relatively-slow formation of the P-428–CO complex in such supernatant solutions (Fig. 5B) suggested that most P-428 reductase, known to be present in whole bacteroids (Fig. 4A), had remained particle bound.

The slow formation of the P-450–CO complex in bacteroid supernatants reduced by endogenous reduction (Fig. 5B) cannot be similarly explained, since washed particles retain no P-450 reductase (Table II, column 3). It is possible that Rhizobium P-450 normally exists in an oxidized or part-oxidized steady state (*cf.* peroxidase[28]) with substantial reduction achieved only when trapping agents such as CO are added. Alternatively, if reduced P-450 is able to form a stable O_2 complex (similar to a proposed functional form of microsomal P-450 (ref. 29)) then O_2–CO competition for the active site could have delayed formation of the CO complex.

When ferric leghaemoglobin[30] (50 μM) was added to a fresh, unaerated 144000 *g* supernatant from ruptured bacteroids, ferrous leghaemoglobin was formed

rapidly, but immediately became and remained fully oxygenated, indicating a residual O_2 concentration of at least 0.1 μM (*cf.* ref. 30). This experiment showed that bacteroid supernatants had the capacity for rapid electron transfer to acceptors other than O_2, a property which would favour the formation of reduced, oxygenated forms of other pigments such as P-450. In contrast, an anaerobic suspension of whole bacteroids reduced ferric leghaemoglobin more slowly, forming ferrous rather than ferrous–O_2 complex, thus emphasizing the O_2-scavenging ability of intact bacteria.

The comparable formation rates of the cytochrome *c*-552–CO complex in naturally-reduced whole bacteroids and supernatants (Fig. 5A,B) discounted any possibility of O_2–CO competition for the active site of this haemoprotein as present in supernatants.

Bacteroids lack the cytochromes a_3 and *o*, which function as oxidases in Rhizobium cultured cells[7], so it was anticipated that some of these newly-described CO-reactive haemoproteins might function as alternative oxidases. However, our earlier results[4] indicating inhibition of succinate respiration by CO, had been challenged by TUZIMURA AND WATANABE[5], so these experiments were repeated (Table III). The considerable stimulation of endogenous respiration by CO made it difficult to prove any inhibition of succinate-induced respiration. The experiments recorded in Table III show CO inhibition of net-succinate respiration in a dark period but not the previous light period; this result would be expected if a photosensitive haemoprotein–CO complex were involved, but it could not be consistently repeated. The demonstration of CO-inhibited respiration in Rhizobium cultured cells was facilitated by the presence of 0.01 M EDTA[7] but addition of this reagent did not reveal any CO inhibition of bacteroid O_2 uptake. Likewise, although photochemical action spectrum determinations for reversal of CO-inhibited cultured-cell respiration consistently revealed the

TABLE III

INHIBITION AND ACTIVATION OF BACTEROID RESPIRATION

All measurements were made in duplicate by Warburg respirometry, using procedures described in MATERIALS AND METHODS. Bacteroid concentrations were adjusted to give about 50 μl O_2 uptake per 15-min period in flasks containing succinate, but no inhibitor or activator. The Q_{O_2} (N) value for 'endogenous' respiration was 150–200, and for 'gross' respiration in presence of succinate, 400–600. 'Net-succinate' respiration = 'gross' respiration *minus* 'endogenous' respiration, arbitrarily assuming no suppression of 'endogenous' respiration by succinate. Results are shown as apparent percent inhibition (−) or activation (+) compared with the untreated control.

Treatment	*Endogenous respiration*	*Net-succinate respiration*
CO		
dark	+18	+16
light	+12	+8
dark	+45	−21
EDTA		
100 μM	−7	−26
1.0 mM	−14	−28
10 mM	−7	−43
40 mM	−7	−50
CN^-		
10 μM	0	−100
100 μM	−67	−100
1.0 mM	−83	−100

oxidase function of cytochromes a_3 and o (ref. 7), identical experimental conditions produced no action spectrum for bacteroids.

The succinate-induced respiration of bacteroids was completely inhibited by 10 μM CN^- and 50% inhibited by 40 mM EDTA (Table III) suggesting that some metalloprotein other than a CO-sensitive haemoprotein could be a component of succinate oxidase. Endogenous respiration was almost unaffected by EDTA but inhibited 83% by 1.0 mM CN^-, suggesting the presence of a separate (or branched) electron transport pathway.

CO affinity of bacteroid haemoproteins and photodissociation of their CO complexes

These properties were investigated to see if high CO affinity, or low photosensitivity, could have prevented the demonstration of a photochemical action spectrum in bacteroids and thereby the implication of their CO-sensitive haemoproteins as oxidases. As measured in a dithionite-reduced supernatant (Fig. 6), cytochrome *c*-552 showed apparent half combination with CO at 1.6 μM, P-450 at 1.1 μM and (P-420 + P-428) at 0.7 μM.

A slope $n = 1$ for log/log plots of CO complex formation against CO concen-

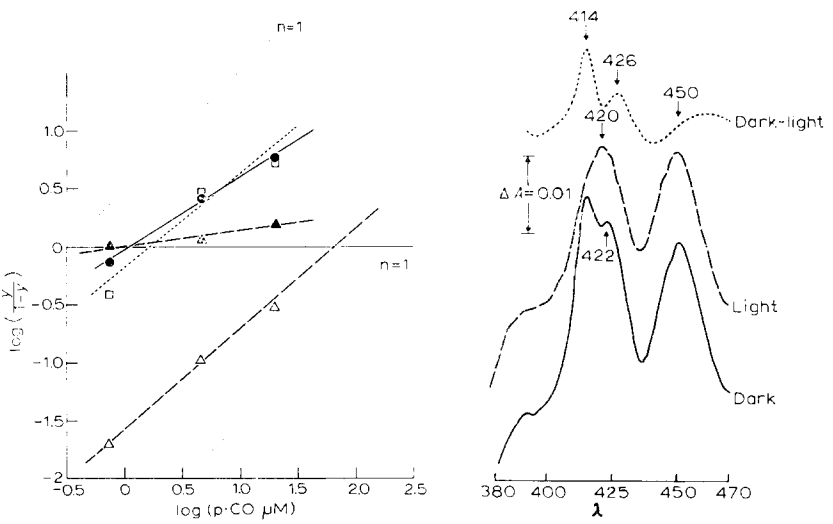

Fig. 6. CO equilibria of bacteroid supernatant pigments. Increasing amounts of CO were obtained by injection of a 1 mM solution (see MATERIALS AND METHODS) to a dithionite-reduced supernatant (7.7 mg protein/ml) and difference spectra recorded 10 min after succeeding injections. Final saturation with CO (1 mM) was assumed to give complete formation of all CO complexes: cytochrome *c*-552–CO, 0.31 μM; P-450–CO, 0.26 μM; (P-420 + P-428)–CO, 0.12 μM. From the Hill equation (*cf.* ref. 31), $y/(1-y) = K.X^n$, where y = fractional combination of each component with CO, K = dissociation constant and X = molarity of uncombined CO; then $\log y/(1-y) = \log K + n \log X$, and so for a plot of $\log y/(1-y)$ against $\log X$, the slope will be n. At half combination with CO, $\log y/(1-y) = 0$. □---□, cytochrome *c*-552–CO complex; ●—●, P-450–CO complex; ▲---▲, (P-420 + P-428)–CO complex; △---△, P-420–CO complex (recalculated).

Fig. 7. Photodissociation of bacteroid haemoprotein CO complexes. The 'Dark' trace is of a CO (10 μM) difference spectrum of a dithionite-reduced bacteroid extract (8.5 mg protein/ml) prepared as described in the text. The 'Light' spectrum was recorded during subsequent side illumination of the sample cuvette (*cf.* MATERIALS AND METHODS) with actinic light (525–575 nm, $2.6 \cdot 10^5$ ergs·cm^{-2}·sec^{-1} measured at the cuvette surface). The 'dark *minus* light' spectrum was obtained by subtraction.

tration as in Fig. 6 would mean the presence of only one type of CO binding site without haem–haem interaction (as in myoglobin); a slope $n < 1$ could indicate the presence of multiple components with differing CO affinities or of independent and different binding sites on a single component[31]. Considering the uncertainties of calculation procedures used to produce Fig. 6, the slopes of log/log plots for the cytochrome *c*-552–CO and P-450–CO complex formation are not sufficiently < 1 to justify any assumption that multiple (differently-reactive) components of these pigments were present in the crude supernatant.

The absorption peak of the (P-420 + P-428)–CO complex appearing in the corrected spectra used to produce Fig. 6 could not, however, be assumed to represent a single CO-reactive species. The slope of the log/log plot was approx. 0.1 (Fig. 6) and the position of this peak shifted from 427 nm, at 0.74 μM CO, to 422 nm at 1.0 mM CO. This implies that P-428 had a much higher CO affinity than did P-420. Making the bold assumption that P-428–CO complex formation was complete, and P-420–CO complex formation negligible at 0.74 μM CO, then increased CO concentration could cause only the formation of the P-420–CO complex. A log/log plot of P-420–CO complex formation, made following these assumptions, shows a slope n of approx. 1 as required for a unimolecular reaction, with half combination occurring at 60 μM CO (Fig. 6).

Photodissociation of these CO complexes was attempted using a 10000 $\times$ *g* (10 min) supernatant from disrupted bacteroids; this was representative of all pigments present in whole cells, but much less turbid. The CO concentration (10 μM) was chosen as a compromise to give partial to complete complexing of all CO-sensitive pigments and a CO difference spectrum recorded in the dark (Fig. 7, 'Dark') showed the formation of 0.21 μM cytochrome *c*-552–CO, 0.21 μM P-450–CO and 0.34 μM (P-420 + P-428)–CO complex. In the 'light' CO difference spectrum the unchanged peak at 450 nm showed that photodissociation of the P-450–CO complex had not been achieved; the remaining dominant 420-nm peak suggested the substantial photodissociation of both the P-428–CO and the cytochrome *c*-552–CO complex. This is shown more clearly in a replotted dark *minus* light spectrum (Fig. 7, dotted trace); photodissociation of the cytochrome *c*-552–CO complex was estimated as 42% and of the (P-420 + P-428)–CO complex as 17%. Since this latter peak in the replotted spectrum appears at 426 nm, it is assumed to represent dissociation of the P-428–CO complex, not the P-420–CO complex.

CN^- effects on bacteroid spectra, and identification of P-502.

The appearance of a 428-nm peak in the CO difference spectrum of succinate-reduced bacteroids (Fig. 4A) had previously suggested[4,5] the presence of cytochrome a_3. However, the (succinate *minus* CN^- + succinate) difference spectrum of bacteroids (Fig. 8, trace (1)) shows no trough at 592 nm, which would have been expected if ferrous cytochrome a_3–CN^- complex had formed in the reference cuvette (*cf.* refs. 32, 33). This observation confirms other evidence in this paper showing that bacteroid P-428 is not cytochrome a_3.

The extra absorption at 551.5 and 419 nm in the (uninhibited) sample cuvette (Fig. 8, trace (1)) was probably due to partial oxidation of cytochrome *c*-552 in the CN^--inhibited reference cuvette. This could mean that cytochrome *c*-552 is a com-

ponent of the CN^--sensitive electron transport pathway, despite the contrary evidence of CO-insensitive respiration (Table III).

Likewise, an unusual peak near 502 nm in this difference spectrum (Fig. 8, trace (1)) could mean the presence of a pigment whose reduced form, in the absence of

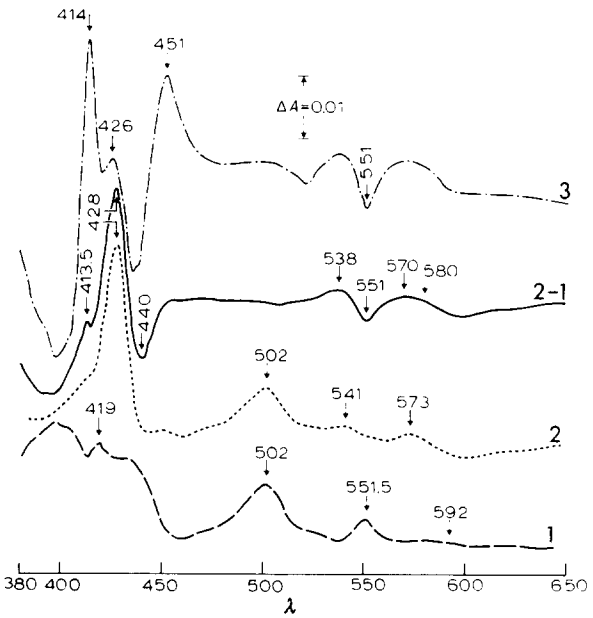

Fig. 8. Appearance of P-502, and of P-428–CO complex in CN^- difference spectra of bacteroids. Trace (1) is the (succinate *minus* CN^- + succinate) difference spectrum and trace (2) the subsequent (succinate + CO *minus* CN^- + succinate − CO) difference spectrum of whole bacteroids (12 mg protein/ml) in 2-mm light-path cuvettes. Trace (2) − (1) was obtained by subtraction, showing essentially the CO difference spectrum of CN^--sensitive pigments (see text). Trace (3), shown for comparison with trace (2) − (1) is the CO difference spectrum of this preparation in the absence of CN^-.

CN^-, had specific absorption at 502 nm. In the reference cuvette (with CN^- present) this pigment could have remained oxidized, possibly as a CN^- complex. This 502-nm peak could not have been due to a (reduced pigment *minus* reduced pigment–CN^- complex) difference spectrum, as it did not appear if dithionite reduction of both cuvettes preceded CN^- addition to the reference cuvette. The new pigment (tentatively named P-502) has little or no affinity for CO, since trace (2) of Fig. 8, which is a (succinate + CO *minus* CN^- + succinate + CO) difference spectrum, shows the 502-nm peak at undiminished intensity. P-502 therefore has the properties of a known component of the succinate-oxidase system, being sensitive to CN^- but not to CO.

By subtracting the (succinate *minus* CN^- + succinate) spectrum from the (succinate + CO *minus* CN^- + succinate + CO) spectrum, as shown in Fig. 8 trace (2)—(1), the CN^- difference spectra of CO-insensitive pigments such as P-502 were eliminated. This trace should show the CO difference spectrum of pigments which could not react with CO in the presence of CN^-, either because their enzymic reduction had been blocked or because their reduced forms were more strongly bound by CN^- than by CO. For comparison, trace (3) of Fig. 8 shows all CO-reactive pigments in these succinate-reduced bacteroids; it was recorded in the absence of CN^-.

The dominating peak in this CO difference spectrum of CN^--sensitive pigments (Fig. 8, trace (2)—(1)) is at 428 nm, compared with 426 nm in the CO difference spectrum of total CO-reactive pigments (Fig. 8, trace (3)). This means that the P-428–CO complex rather than the P-420–CO complex formation was blocked by CN^-. Once again the absence of any 590-nm peak, 610-nm trough (which are present in the CO difference spectrum[32,33] of CN^--sensitive cytochrome a_3) shows that bacteroid P-428, as appearing in Fig. 8, trace (2)—(1) is not cytochrome a_3. The shoulder between 570 and 580 nm in this spectrum is characteristic of P-428 (*cf.* Fig. 4A).

The absence of a 450-nm peak in Fig. 8, trace (2)—(1), shows that P-450 reduction and CO complex formation were not inhibited by CN^- *in vivo*, and the slight peak at 413.5 nm suggests that cytochrome *c*-552–CO complex formation was partly inhibited.

DISCUSSION

The objective of this work was to identify electron transport components exclusively present in N_2-fixing Rhizobium bacteroids and a comparison of Fig. 9, below, with Fig. 10 of the accompanying paper[7] makes this possible. All autoxidizable pigments are shown as possible oxidases, but since turnover numbers are not yet known, some of them, like the pigment 503 of yeast[34] may react only sluggishly with O_2. In particular, kinetic data will be needed to support the identification (Fig. 9) of the CN^--sensitive, CO-insensitive P-502 as a major oxidase of bacteroids.

The CO-sensitive oxidase cytochromes a_3 and *o* of Rhizobium cultured cells[7] are lost during bacteroid formation but the inconsistent demonstration (Table III; refs. 4,5) of CO-inhibited respiration in aerobically-prepared bacteroids suggests that their CO-reactive pigments do not have a major role in O_2 respiration. However, BERGERSEN AND TURNER[6] routinely detect a light-reversible, CO-inhibited O_2 uptake in anaerobi-

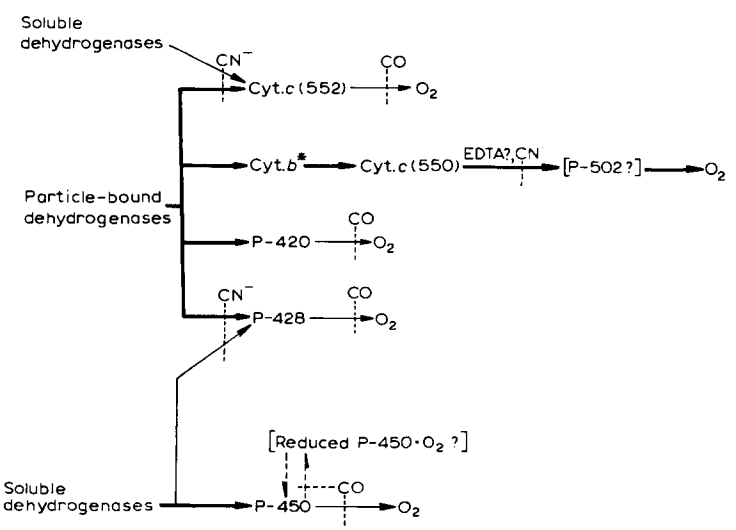

Fig. 9. Electron transport components of bacteroids. Thin solid lines represent possible minor pathways and thick lines major pathways of electron flow. Dotted lines represent sites of inhibitor action. Cyt. b^* is probably identical with cytochrome *b* (556 nm, −196°) found in cultured cells[7].

cally-isolated bacteroids which have retained N_2-fixing ability. Bacteroids used in the present work were isolated aerobically at 0° and had lost N_2-fixing ability. The rapid determination of a photochemical action spectrum on anaerobically-isolated bacteroids[1,6] could reveal the involvement of one or more of their exclusive CO-reactive pigments (Fig. 9; *cf.* Fig. 10 of ref. 7) in a fragile oxidase system which decays as N_2-fixing ability is lost. It is already known (C. A. APPLEBY AND F. J. BERGERSEN, unpublished experiments) that the pattern of CO-reactive pigments in anaerobically and aerobically-prepared bacteroids is almost identical so these pigments themselves do not appear to be artifacts of the aerobic preparation procedure. Their biological reducibility (Table II) also discounts this possibility and the demonstrated photosensitivity of at least cytochrome *c*-552–CO and P-428–CO complexes (Fig. 7) allows the possibility of photochemical action spectrum determinations if these pigments are part of an oxidase system containing other, O_2-sensitive, components.

All the CO-reactive pigments described here have spectroscopic properties which suggest they are haemoproteins; the soluble cytochrome *c*-552 and haemoprotein P-450 are exclusively present in bacteroids (*cf.* cultured Rhizobium[7]) and the CN^--insensitive bacteroid P-420 is not identical with the P-420 (cytochrome *o* ?) of cultured cells. A pigment similar to the soluble P-428 of bacteroids may also be present in cultured cells but the dominance of CO-reactive cytochrome *c* in the latter form makes proof difficult[7].

The membrane-bound P-450 of animal cells[13], which resembles bacteroid P-450[12,35], has many possible biological functions[36], including the anaerobic reduction of azo compounds to amines[37]. The bacteroid P-450 might be similarly involved in N_2 reduction to ammonia. However, it is noted that the major function of animal[36] and plant[38] cell P-450 is in O_2 activation prior to hydroxylation reactions and that in *Bacillus cereus*[39], pigments similar to bacteroid cytochrome *c*-552 and P-428 may be involved as hydroxylases. As yet, none of these bacteroid haemoproteins has been implicated in hydroxylation reactions.

ACKNOWLEDGEMENTS

Some of these experiments were carried out during the tenure of a Rockefeller Foundation Fellowship in the Agricultural Sciences at the Graduate Dept. of Biochemistry, Brandeis University. Dr. D. J. GOODCHILD is thanked for his production and interpretation of electron micrographs, and Mrs. L. GRINVALDS for her skilled assistance.

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