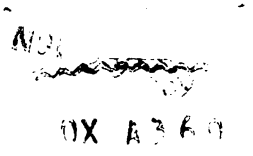

ASPECTS OF HEME BIOSYNTHESIS IN FREE-LIVING AND SYMBIOTIC *RHIZOBIUM JAPONICUM*

By

Yael Avissar

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ABSTRACT

ASPECTS OF HEME BIOSYNTHESIS IN FREE-LIVING AND SYMBIOTIC *RHIZOBIUM JAPONICUM*

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I. The activity of δ-aminolevulinic acid synthase (ALAS) in free-living *Rhizobium japonicum* and in bacteroids was assayed. The assay for this first step in heme biosynthesis was based on a colorimeteric determination of the reaction product δ-aminolevulinic acid (ALA). The procedure developed involved partial purification of the ALA on a sulfonic acid resin, condensation with 2,4-petadione and reaction of the resulting pyrrole with the Ehrlich reagent.

Using this assay, the requirements for enzyme activity in cell free extracts were determined. The requirements were shown to be similar to those of ALAS in other bacterial and mammalian systems.

Developmental studies of soybean root nodules showed that there was a correlation between the ALAS

activity in the nodule bacteroids and the concentration of heme in the nodule. ALAS appeared to be a regulatory enzyme so far as its activity seemed to be rate limiting for heme synthesis in *Rhizobium* as in all other cells and tissues known.

II. Cell multiplication and the activation of tetrapyrrole formation were studied in free-living *Rhizobium japonicum* under conditions of restricted aeration. When the cells were grown in a fermentor on a yeast extract-mannitol medium with vigorous aeration, the culture reached a cell density of approximately 100 Klett units (0.200 Absorbance units) before a stationary phase was attained. At the end of this stationary phase, there was also some decline in cell viability. This decline was reversed by discontinuing the aeration of the culture. In the new growth phase, which followed, there was a three- to five-fold increase in the numbers of viable cells and in the total amount of cell protein.

The reduced air supply also caused approximately ten-fold increases in the activities of the first two enzymes of the pathway of heme synthesis, ALAS and δ-aminolevulinic acid dehydrase (ALAD). The increase in enzymic activities in the free-living cells was accompanied by a similar increase in the level of heme accumulating in the cells. At the same time, there was

a large increase in the amounts of porphyrins released into the medium. The excreted porphyrins were identified on the basis of their chromatographic and spectrophotmetric properties as protoporphyrin and coproporphyrin. The cytochrome complement of the "microaerobic" free-living cells resembled that of symbiotic bacteroids in that cytochrome a-a_3 was absent and a CO-binding cytochrome 552 was present.

These observations suggest that oxygen tension may play a role in inducing a differentiation process which, in a nodule, can lead to bacteroid formation.

DEDICATION

To Jacob

ACKNOWLEDGMENTS

I wish to thank my major advisor, Dr. Kenneth Nadler, Jr., for his friendly, helpful guidance and constructive criticism at all stages of my research. I am also very grateful to the other members of my guidance committee, Drs. Philip Filner, Robert Scheffer, and Peter Wolk, for advice and support and for their stimulating discussions concerning my work.

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Finally and from the bottom of my heart I want to thank my husband Jacob and my son Oded for their unconditional love and support and for their unfailing patience in living with a part-time wife and mother.

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LIST OF ABBREVIATIONS

ALA	δ-aminolevulinic acid
ALAD	δ-aminolevulinic acid dehydrase (EC 4.2.1.24)
ALAS	δ-aminolevulinic acid synthase (EC 2.3.1.37)
COPR	coproporphyrin
COPR'gen	coproporphyrinogen
Lb	leghemoglobin
PBG	porphobilinogen
PROTO	protoporphyrin
PROTO'gen	protoporphyrinogen
URO	uroporphyrin
URO'gen	uroporphyrinogen
YM	yeast-mannitol

INTRODUCTION

The Role of Leghemoglobin in Nitrogen Fixation

The red pigment of legume nodules was first recognized as a hemoglobin which is able to bind O_2 reversibly by Kubo in 1939 (69), and was consequently named leghemoglovin (Lb). Subsequent spectral (6), amino acid sequence (44) and sequence alignment studies (58) emphasized the similarities among the structures of lehemoglobin, myglobin and the monomeric subunits of animal hemoglobins. Several components of soybean Lb have been identified and labeled Lb a, Lb b and Lb d (2). All the leghemoglobins are characterized by extremely high affinity for oxygen ($p_{\frac{1}{2}}o_2$ values o.04 to 0.08 mm Hg), as compared to mammalian hemoglobins with $p_{\frac{1}{2}}$ values mostly in the range between 4 to 14 mm Hg (2, lll). This extremely high O_2 affinity and the observation that Lb is partly oxygenated in living tissues (2,ll4) suggested the hypothesis that the function of leghemoglobin *in vivo* is to facilitate the inward diffusion of oxygen through the nodule cortex into the endosymbiotic bacteroids (59,

62, 112).

Leghemoglobin is present in all legume root nodules, but absent from non-leguminous nitrogen-fixing nodules. Lb is clearly associated with the central, N_2 fixing, parenchymal tissue. This tissue is composed of swollen plant cells with vesicles containing bacteroids. The precise location of Lb within the cells is still in dispute. Evidence from x-ray microprobe analysis of fixed sections points to Lb being located in the plant cell cytoplasm, outside the vesicles (32), as does the immunochemical method of Verma and Bal (108), while optical and electron microscopy of DAB (oxidized 3,3'-diaminobenzidine)-stained tissue indicate that it is inside the vesicles (15). Autoradiography of serradella nodules also points to the Lb iron being inside the vesicles (39).

Calculations based on the degree of oxygenation of Lb in air, assuming that Lb *in vivo* has the same properties as pure Lb, indicate that the average free O_2 tension in soybean symbiotic tissue might be no more than 0.006 mm Hg (or 11 nM dissolved O_2), (6). Bacteroids isolated from nodules show half maximal respiration at a very low free O_2 tension, and in a nodule they appear to be respiring at less than their maximal rate (7). In fact the rate of O_2 uptake by

bacteroids liberated by grinding nodules is about ten times that of the intact nodule. It seems reasonable to suppose that the rate of oxygen supply to the bacteroids via leghemoglobin is the limiting factor in their rate of respiration. The observation that the concentration of dissolved oxygen is very low in the symbiotic tissue in the nodule has considerable importance in relation to the stability of bacteroid nitrogenase and its protection from inactivation by O_2. According to Smith and Tjepkema (100, 104), the cortical layer of the nodule offers considerable resistance to oxygen penetration, which can explain the fact that only about 20% of the Lb in the nodule is oxygenated (7) and the fact that CO inhibits nodule respiration only to a limited extent (104). Other authors (14, 101) claim that the nodule cortex cannot constitute a barrier to O_2 penetration since it contains a network of air-conducting tubules interconnected with similar tubules in the central tissue. Bergersen and Goodchild (14) locate the barrier to oxygen diffusion at the parenchyma cells. This latter proposal is acceptable if the leghemoglobin is in fact confined to the bacteroid-containing membrane sacs, since the cytoplasm of the parenchyma cells then could be a major barrier.

Parallel measurements of nitrogenase activity and O_2 consumption (18) and of ATP formation indicate an increase in the efficiency of bacteroidal oxidative phosphorylation is brought about by the presence of oxidized Lb. This increase in oxidative phosphorylation can account for the increase in bacteroidal nitrogenase activity. Measurements of nitrogenase activity and O_2 uptake conducted with dense bacteroidal suspensions showed that increasing the concentration of oxygenated Lb from 0 to 1 mM caused only a 30% increase in O_2 consumption, but acetylene reduction increased from 0 to 2 μ moles per assay (7). The role of leghemoglobin in delivering free, dissolved oxygen is further confirmed by experiments in which other O_2 carrier proteins, such as hemoproteins with high affinity for O_2 from other phyla or even hemerythrin and hemocyanin (oxygen carrying molecules from a worm and a lobster, respectively, which contain no heme!) could somewhat substitute for Lb in enhancing the O_2 uptake and rates of acetylene reduction by isolated bacteroids (112). Based on the above and similar experiments Appleby *et al.* (7) arrived at the conclusion that Lb acts in delivering dissolved oxygen by facilitated diffusion — that is, transport of oxygen brought about by random translational displacement of carrier (Lb) molecules. This type of diffusion will occur whenever certain requisite conditions prevail:

1.) the carrier molecule must be free to move in the solution,

2.) the carrier molecule (Lb) must bind O_2 in some part of the system,

3.) there must be a gradient of O_2 pressure and of fractional saturation of the Lb across the distance through which facilitation is occurring.

The last requisite can be reworded as meaning that this is a steady-state system, in which O_2 is continuously supplied at one end of the system and continuously removed at the other. All of the above requirements are met in the nodule and it seems reasonable to suppose that Lb acts as a facilitating agent for diffusion of O_2. In bacteroid suspensions supplemented with Lb (0.1 mM) and in intact nodules, the concentration of Lb-bound O_2 exceeds that of free O_2 more than 1000-fold (7). This means that essentially all of the O_2 flux across the bulk of the solution is in the form of LbO_2.

A consequence of leghemoglobin-facilitated O_2-diffusion is that free O_2 is delivered to the bacteroid surface at a greater partial pressure than would be obtainable in the absence of leghemoglobin (assuming equal O_2 uptake). Calculations based on a model system yield an estimated of O_2 pressure at the bacteroid

surface as approximately 0.04 mm Hg near the half saturation level of Lb (7). Lb-facilitated diffusion of O_2 stimulates oxidative phosphorylation, as mentioned previously, with a resulting increase in the ATP/ADP ratio which, in turn, is directly related to bacteroidal nitrogenase activity (7).

Stokes' mathematical analysis suggests that the O_2 buffering properties of leghemoglobin could protect bacteroid nitrogenase against relatively rapid fluctuations in the tension of delivered O_2, which would be produced if instaneous O_2 demands were satisfied. Such fluctuations at their low point would result in low phosphorylating efficiency, while at the high point sufficient O_2 might be present to inactivate bacteroid nitrogenase (7).

The general conclusion of the above is that leghemoglovin — by facilitating the supply of free, dissolved O_2 to the bacteroid surface — allows adequate O_2 flux at a moderately low free O_2 tension, which is nevertheless adequate for the operation of high-efficiency bacteroid oxidative phorphorylation. The resultant ATP, delivered at a high ATP/ADP ration, is necessary for bacteroid nitrogenase activity.

The Biosynthesis of Leghemoglobin

Leghemoglobin is a product characteristic of the legume *Rhizobium* symbiosis, since neither cultured free living rhizobia nor uninoculated legumes are capable of producing it. During nodulation there is a dramatic increase in heme formation coordinated with the synthesis of the apoprotein. Topologically, leghemoglobin is probably either situated in the vesicles, outside both the bacteroids and the plant tissue or more likely, in the plant cytoplasm according to recent evidence (108). Since Lb is obviously a product of functional symbiosis because neither the bacteria nor the plants it produce in their free-living state, it has been suggested that a different part of the molecule is probably produced by each symbiont (27, 28, 38).

The apoprotein of Lb is produced by the plant host. This was proven by experiments which demonstrated that leghemoglobin specificity depends on the identity of the plant host and not on the identity of the *Rhizobium* species (29, 38). It was also shown by the actual isolation of soybean leghemoglobin mRNA from root nodules and its *in vitro* translation (108, 109). The isolated mRNA seemed to be of eukaryotic origin since it contained poly A and was isolated in the form of polysomes containing 80s ribosomes.

On the other hand the heme prothetic group appears to be of bacterial origin (28, 48), since bacteroids incorporate a cariety of precursors including glycine (92), organic acids (60, 93) and ALA (28, 48, 60) into heme, while the plant fraction of the nodule does not produce appreciable amounts of heme.

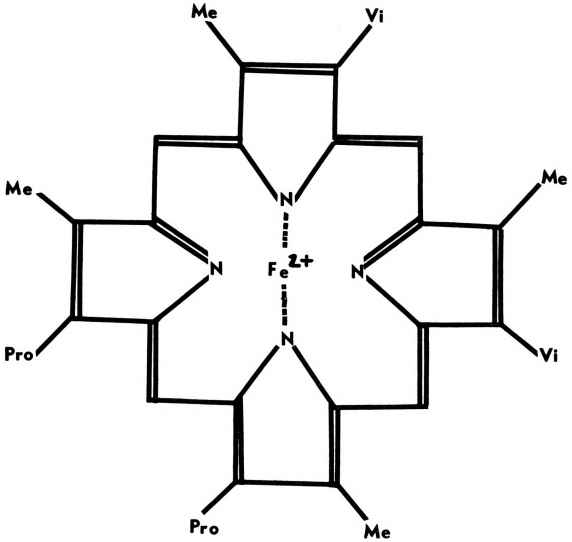
The Biosynthesis of Porphyrins and Heme

Heme is essential as the prosthetic groups of hemoglobin, myoglocin, cytochromes and several enzymes. It is a cyclic tetrapyrrole with a central bivalent iron atom held by four coordination bonds (Figure 1). The four ligand groups of the porphyrin form a square-planar complex with the iron in the center, the remaining fifth and sixth coordination points of the iron are perpendicular to the plane of the porphyrin ring. When the fifth and sixth positions of the iron are occupied, the resulting structure is a hemochrome of hemochromogen. In the heme proteins myglobin and hemoglobin, the fifth position is occupied by an imidazole group of histidine residue while the sixth position is either unoccupied (deoxyhemoglobin) or occupied by oxygen or other ligands, such as carbon monoxide. In nearly all cytochromes, on the other hand, both the fifth and the sixth positions are occupied by R-groups of specific amino acid residues

Figure 1. The structure of the heme molecule.

Abbreviations: Vi = vinyl ($-CH=CH_2$)
Me = methyl ($-CH_3$)
Pro = propionic acid ($-C_2H_4COOH$)

HEME



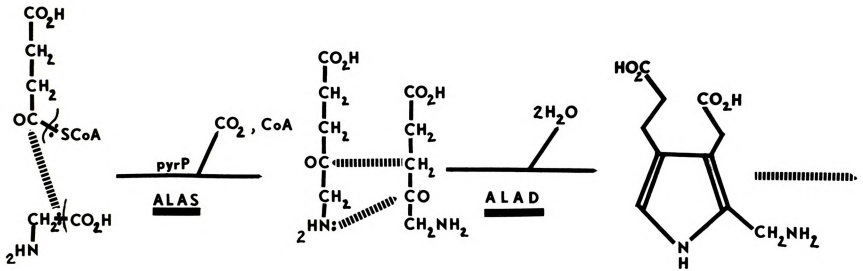
of the proteins. These cytochromes therefore cannot bind with ligands like oxygen, carbon monoxide and cyanide, with the important exception of cytochrome a_3 which normally binds oxygen in its biological function.

The biosynthetic pathway of prophyrins is an especially important pathway because of the central role of the porphyrin nucleus in both heme and chlorophyll. The tetrapyrroles are constructed from four molecules of the monopyrrole derivative porphobilinogen (PBG), which is synthesized in the steps shown in figure 2. The pathway in animal tissues was largely deduced from isotopic tracer and enzyme studies. D. Shemin (98) and Neuberger (90) established that glycine and succinate contributed the nitrogen and carbon atoms to protoheme of hemoglobin and the same is generally assumed to hold for the heme groups of cytochromes.

The overall scheme (Figure 3) and the main intermediates of protoheme biosynthesis have been established (72, 98, 102). Glycine first reacts with succinyl CoA to yield enzyme-bound α-amino-β-keto adipic acid, which is then decarboxylated to yield δ-aminolevulinic acid. This reaction is catalyzed by the pyridoxalphosphate requiring enzyme δ-aminolevulinic acid synthase (ALAS). Two molecules of δ-aminolevulinic acid (ALA) then condense to form porphobilinogen (PBG), through the

Figure 2. The biosynthesis of porphobilinogen.

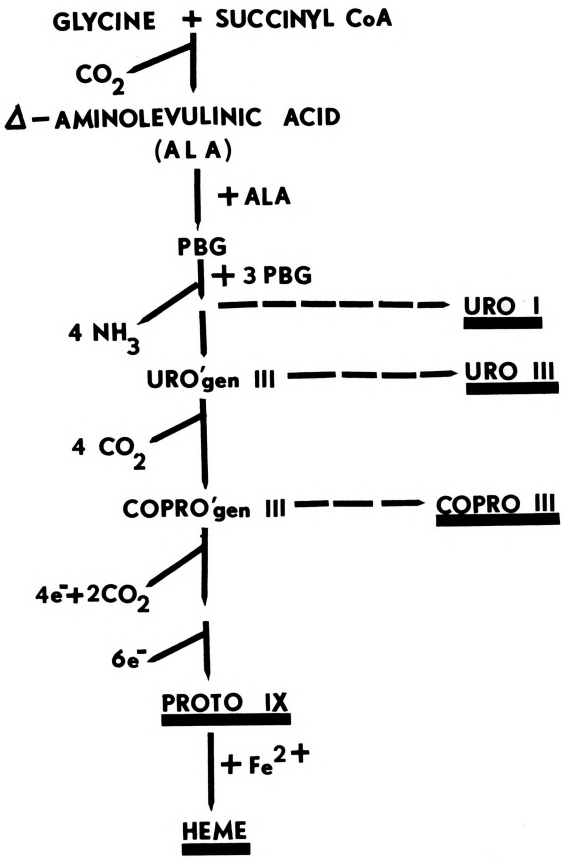
The dotted arrows between two molecules indicate the direction of the nucleophilic attack (pyr P is pyridoxal phosphate).



SuccinylCoA + Glycine —(ALA Synthase)→ Δ-AminoLevulinic Acid —(+ALA, ALA Dehydratase)→ PorphoBilinoGen

Figure 3. The heme biosynthetic pathway. (Pigmented compounds are underlined.)

Abbreviations:
PBG = porphobilinogen
URO = uroporphyrin
URO'gen = uroporphyrinogen
COPRO = coproporphyrin
COPRO'gen = coproporphyrinogen
PROTO = protoporphyrin


GLYCINE + SUCCINYL CoA
CO_2
Δ – AMINOLEVULINIC ACID
(A L A)
+ ALA
PBG
+ 3 PBG
URO I
4 NH_3
URO'gen III
URO III
4 CO_2
COPRO'gen III
COPRO III
$4e^- + 2CO_2$
$6e^-$
PROTO IX
+ Fe^{2+}
HEME

action of δ-aminolevulinic acid dehydrase (ALAD). The mechanism of this unusual and complex reaction involves the intermediate formation of a Schiff's base between the keto group of one molecule of δ-aminolevulinic acid and a ε-amino group of a lysine residue of the enzyme (Figure 2). Four molecules of porphobilinogen now serve as precursors of the cyclic tetrapyrrole protoporphyrin which is synthesized through a series of complex reactions. In the presence of two enzymes, PBG-deaminase and uroporphyrinogen III co-synthetase, four molecules of PBG condense to form a hexahydroporphyrinogen, uroporphyrinogen III. Uroporphyrinogen III is subsequently decarboxylated to coproporphyrinogen III which undergoes oxidative decarboxylation to protoporphyrin IX. This step is catalyzed by coproporphyrinogen oxidase. The last step in heme synthesis is the incorporation of ferrous iron catalyzed by the enzyme ferrochelatase.

This pathway of heme synthesis has been found to operate in bacteria as well as in animal cells (72). Photosynthetic bacteria form bacteriochlorophyll by a pathway which is identical to the heme pathway up to the point of protoporphyrin IX formation.

The regulation of the pathway has been investigated both in heme-producing systems (mainly developing red blood cells) and in bacteriochlorophyll producing

systems (*Rhodopseudomonas* species). End product inhibition and repression of ALA synthesis has been invoked as a principal regulatory step in the formation of heme and bacteriochlorophyll (21, 67), though some evidence points to a regulatory step later in the pathway (30).

Several studies were conducted investigating ALAS induction and its suppression by oxygen in photosynthetic bacteria, where the major role of this enzyme is in the production of bacteriochlorophyll (50, 57, 73). Those studies also demonstrated the high turnover rate of the enzyme.

In animals, most of the biosynthesis of heme takes place in the mitochondria, but several intermediate steps are carried out in the cytoplasm (96). A similar type of compartmentalization could possibly exist between bacteroids and the plant cell cytoplasm.

Specific Problems of Heme Biosynthesis and Its Regulation in Soybean Root Nodules

Our investigation was related to the theory that the bacteroid is the site of synthesis of the large amounts of heme necessary for leghemoglobin production. The bacteroid had been indicated as the site of heme synthesis for leghemoglobin on the basis of experiments

in which the incorporation of labeled aminolevulinate (^{14}C-ALA) into heme by various fractions obtained from nodules was assayed (28, 48). The experiments established that bacteroids were capable of incorporating ALA into heme to a far greater extent than the nodule cytoplasmic fraction. However, since ALA was shown to limit the formation of heme precursors by *Rhizonium japonicum* (45) and the source of ALA for heme synthesis was unknown, the bacteroids could not be considered as being solely responsible for the synthesis of heme, though Richmond and Salomon (93) suggested that bacteroids could form ALA. The first enzyme of the heme pathway, ALAS, had never been reproducibly demonstrated in either the cytoplasmic fraction of the bacteroid fraction of soybean root nodules (47), before the study reported here was undertaken. The present research was directed towards developing an assay procedure for the ALA-producing enzyme in root nodules. If the enzyme was indeed of bacterial origin, it could be expected to be similar to δ-aminolevulinic acid synthase of other bacteria. On the other hand there was a distinct possibility that the first step the pathway takes place in the plant cytoplasm in which case the enzyme would probably be quite different, since higher plants contain an ALA synthesizing enzyme which catalyzes the incorporation of the entire carbon skeleton of either α-KG or

glutamate into ALA (11, 87).

In view of the fact that all heme-producing systems investigated are primarily regulated on the level of ALA synthesis (69, 77), it seemed most important to identify the enzyme responsible for ALA synthesis in soybean root nodules and to localize it in either the bacteroid or the plant tissue.

An assay for measuring ALAs activity has in fact been developed. This assay enabled us to investigate the requirements for enzyme activity *in vitro* and to investigate the changes in the enzyme levels in soybean root nodules during their development. We were also able to demonstrate that a bacteroid enriched fraction contained ALAS activity and that this activity was correlated with the changes in the levels of accumulated heme. The increased heme production by bacteroids is only one indication of the altered physiological state of a bacteroid from the state of a free-living *Rhizobium*.

The factors responsible for the differentiation process could be approached with relative ease if the conditions prevailing in a nodule could be reproduced in a laboratory culture. Free-living rhizobia are normally grown either aerobically or anaerobically with nitrate as the terminal electron acceptor (31). Since the immediate surroundings of the bacteroid in a nodule are characterized by very low oxygen tension (30), it

seemed reasonable to attempt the creation of "nodule-like" surroundings by reducing the oxygen supply to the culture (such conditions lead to N_2 fixation in free living *Rhizobium*). The free living rhizobia reacted to a sudden decrease in oxygen supply of the culture by starting a new growth phase which was maintained for several days and resulted in a three-fold increase in cell numbers. This "microaerobic" growth indicated that a new physiological state had been attained which might be similar to that of bacteroids.

When the cells grown with restricted aeration had been collected they appeared to be similar to bacteroids in size and form and in the high concentration of heme in the cells. Subsequent investigation revealed that the microaerobically grown cells also resembled bacteroids in the high activity of the heme biosynthetic pathway as well as in their altered cytochrome composition.

Microaerobic growth of *Rhizobium* appears at present to offer a convenient model system for investigating certain aspects of the development of nodules.

MATERIALS AND METHODS

Media

Yeast extract-manitol medium (YM) contained in grams per liter: mannitol, 10; yeast extract (Difco),1; K_2HPO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 0.2; NaCl, 0.1; $CaSO_4 \cdot 2H_2O$, 0.1. Media were autoclaved for 20 minutes per liter medium at 15 p.s.i.

Nitrogen-free legume nutrient medium was prepared as described by Johnson *et al.* (62), and contained the following macronutrients in grams per liter: K_2SO_4, 0.34; KH_2PO_4, 0.12; K_2HPO_4, 0.01; $MgSO_4 \cdot 7H_2O$, 0.49; $CaSO_4 \cdot 2H_2O$, 1.03; $CaCl_2 \cdot 2H_2O$, 0.06; and the following micronutrients in milligrams per liter: ferric citrate, 6.25; H_3BO_3, 1.47; $MnSO_4 \cdot 7H_2O$, 0.78; $ZnSO_4 \cdot 7H_2O$, 0.12; $CuSO_4 \cdot 5H_2O$, 0.05; $Na_2MoO_4 \cdot 2H_2O$, 0.025; $CoCl_2 \cdot 6H_2O$, 0.11.

Growth Conditions

Rhizobium japonicum strain 3I1b-110 was kindly supplied by D. C. Weber, Beltsville, MD; strain 11927 was purchased from the American Type Culture Collection.

Bacterial strains were maintained on YM agar (1.5%) plates and were transferred every three months. Liquid cultures, in 100 ml YM medium, were started from slants. When outgrowth occurred, two-liter flasks containing one liter YM medium were inoculated with five milliliters of culture and were aerated by agitation (70 cycles per minute) on a reciprocal shaker for two or three days. Cells were harvested by centrifugation at 6000 g for 15 minutes, washed and resuspended in nitrogen-free nutrient medium and used immediately to inoculate soybean seeds.

Fifty milliliters of liquid culture in YM broth, started from slants, were used for inoculation of the fermentor (Virtis, bench top, model 43-1000), which contained ten liters of YM medium. The culture was grown at 30 C to stationary phase with aeration (11 liters air per minute) and paddle agitation (250 r.p.m.). For microaerobic growth, during which the fermentor remained open to the air through plugged drying tubes, forced aeration was discontinued while agitation was maintained. Samples of the culture were forced out under slight pressure of N_2 gas. Cells were collected and washed by centrifugation (6000 g for 15 minutes).

Soybean seeds (Glycine max. cv. Hark) were surface disinfected for 20 seconds in 1% NaOCl, washed in running tap water, and planted in a vermiculite-perlite bed.

The bed was inoculated with a suspension of *R. japonicum* in nitrogen free medium (no nodulation was observed in uninoculated controls). Aerated N-free nutrient medium was circulated through the bed of germinating seeds for one week. The beds were illuminated with two 40W Grolux fluorescent lamps (Westinghouse Electric Corp., Bloomfield, NJ), at a distance of about 60 cm. Inoculated seedlings were transferred to pots (5-6 per pot) containing a mixture of vermiculite and perlite (1:1 by volume), and grown in a greenhouse with supplementary illumination from four 40W Grolux fluorescent lamps at a distance of 35-45 cm (depending on the height of the plant). Seedlings were watered with nitrogen-free medium. Every third watering was with tap water to remove accumulated salts. Under these inoculation conditions, nodules form predominantly at the crown of the root (88). Only nodules at the crown were harvested for these experiments (a few smaller nodules formed on the roots during growth periods longer than 20 days).

Analytical Methods

Protein concentrations were determined by the method of Lowry *et al.* (80) and were confirmed by independent determinations by the Biuret method (76), using bovine serum albumin as standard. (We have

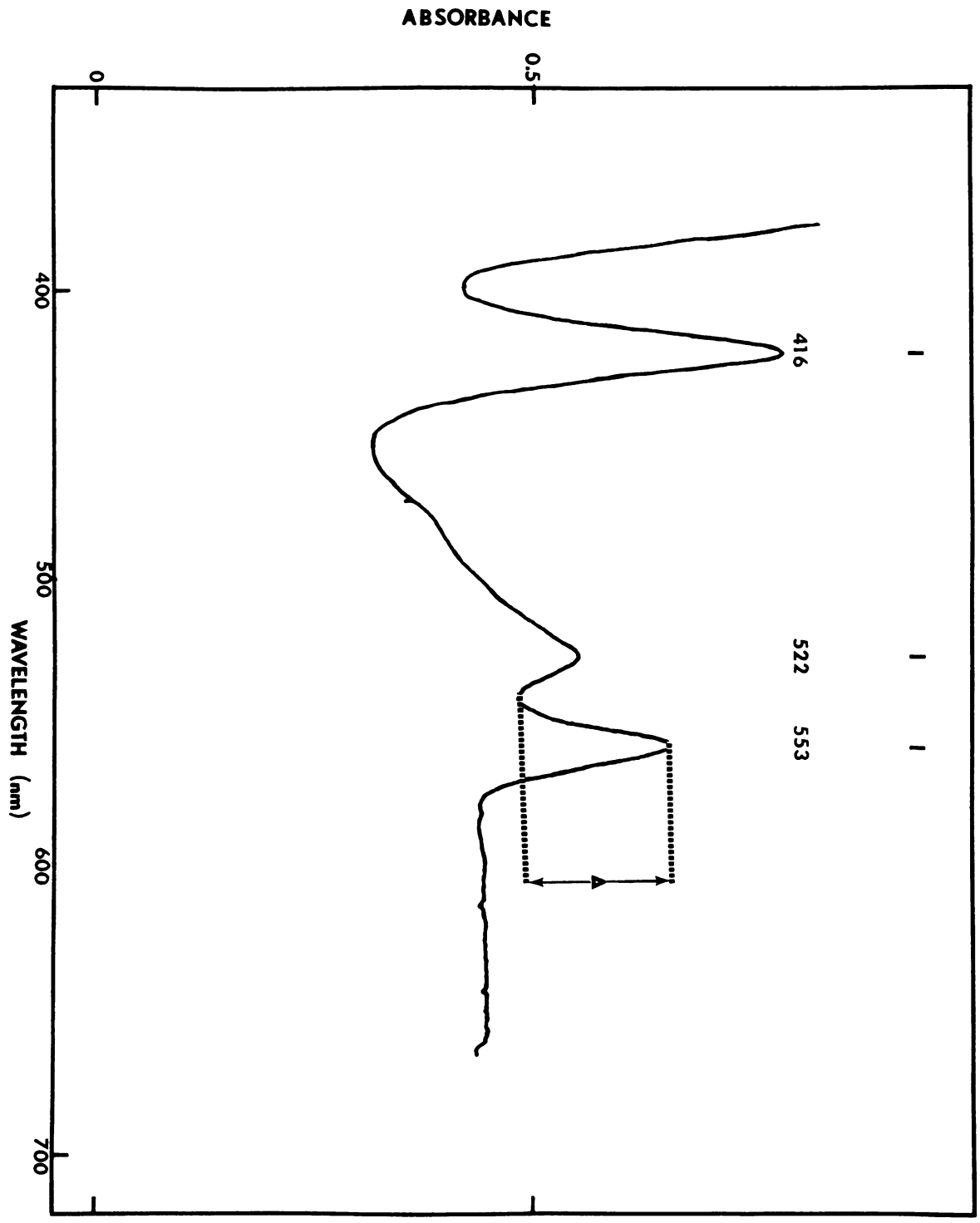
observed, but cannot explain the fact that HEPES buffer does not interfere with protein determinations by the method of Lowry *et al.* in the presence of *R. japonicum* cell extracts, whereas, as is well known, HEPES buffer is Lowry-positive and interferes badly with the determination of other proteins such as bovine serum albumin in aqueous solution.)

Heme was determined from the difference spectrum of the reduced minus oxidized pyridine hemochrome (46), assuming a millimolar extinction coefficient of 20.7 (Figure 4). The hemochrome is a complex formed by the coordination of two molecules of pyridine with heme. This complex has a characteristic and sharp α-band which can be helpful in heme identification. The difference spectrum is obtained in aqueous alkaline pyridine solution by recording with a Cary 15 recording spectrophotometer the absorbance of a sample after reduction with sodium dithionite versus a sample which has been oxidized by potassium ferricyanide (Figure 4). The difference in absorbance between the peak at 553 mm and the trough at 540 nm, marked Δ in the figure, is proportional to the concentration of heme.

Figure 4. Pyridine hemochrome difference spectrum of the reduced minus oxidized bacteroid extract.

Δ marks the absorbance difference which is used to compute the concentration of heme. This absorbance difference is measured between the 553 peak and the low point of the adjacent trough at 540 nm.

The millimolar extinction coefficient of the difference is assumed to be 20.7 (46).


ABSORBANCE
0
0.5
400
500
600
700
WAVELENGTH (nm)
416
522
553

Porphyrin Absorption Spectra

Porphyrins appeared in the culture medium ("soluble" porphyrin) as well as in a gelatinous brown-purple precipitate on the walls of the fermentor ("insoluble" porphyrin). Soluble porphyrins were adsorbed on a 1 cm thick pad of neutral alumina (Fisher Scientific Co., Brockman activity I, 80-200 mesh) equilibrated with 3% acetic acid. The alumina was washed with distilled water and the porphyrins were eluted with 1N HCl, to obtain the absorption spectrum of the acid form, or with methanol-H_2SO_4 (20:1, v/v) to obtain the porphyrin methyl esters for the determination of the absorption spectrum of the neutral form (41), and for subsequent identification the porphyrin methyl esters.

Insoluble porphyrins from the brownish precipitate were solubilized and methylated overnight by shaking in methanol-H_2SO_4 as described above. Insoluble porphyrins in the acid form were obtained by shaking overnight with ethyl acetate-acetic acid (3:1, v/v) and isolated by partitioning as described by Dresel and Falk (42). No porphyrins, as determined by red fluorescence under UV light, were washed out of the organic phase by aqueous sodium acetate. Porphyrins were transferred from ethyl acetate to 3N HCl; the absorption spectra of the aqueous phases (diluted to 1N HCl) were recorded

as before.

Chromatography of Porphyrins and Porphyrin Esters

Porphyrin methyl esters were analyzed by thin layer chromatography with the following three systems:

a.) benzene-ethylacetate-ethanol, 190:20:75 (v/v) on silicagel G plates (Brinkman), (41);

b.) water-acetone-dioxane, 2:7:1 (v/v) reverse phase chromatography on silicon impregnated Eastman Chromatogram Sheets (26);

c.) petroleum ether-paraffin oil-chloroform, 1:1:10 (v/v) on Eastman Chromatogram Sheets, a modification of the method of Chu and Chu (26).

Spots were located by their red fluorescence under UV light. Porphyrin methyl esters (Sigma) were used as standards.

Cytochrome Absorption Spectra

Cells were washed and resuspended in 0.1 M sodium phosphate buffer, pH 6.8. Cells were reduced with several grains of sodium dithionite or were oxidized by passing oxygen gas through the suspensions for ten minutes. CO-binding pigments were detected after passing CO through the reduced cell suspensions by the appearance of a trough in the difference spectrum.

The absorption spectra of dense cell suspensions were obtained using a single beam spectrophotometer similar to that described by Butler (22), on line with a Hewlett-Packard 2108 mX minicomputer. The sample (1.4 ml) was contained in a cylindrical cuvette. The path length of the vertical measuring beam through the sample was 0.23 cm. For taking low temperature spectra the cuvette was placed in a small Dewar vessel with a transparent bottom, which contained liquid nitrogen. Absorbance values were measured every 0.4 mm and recorded by the computer. The fourth derivative was obtained using finite differentiating intervals of 3.2, 3.6, 4.0 and 4.8 nm. The fourth derivative analysis was used only to indicate the precise wavelength of the absorption maxima and minima, since no quantitative meaning can be assigned to the fourth derivatives (23, 24).

The wavelength accuracy was caliberated to 0.1 nm using emission lines from a mercury lamp. The absorbance was calibrated by using a neutral density filter.

The curves presented in figure 15 are direct photographic reproductions of the spectral data plotted by the computer via an x-y recorder.

Oxygen Concentration Measurements

Samples were collected by siphoning out the desired sample from the fermentor at a steady, slow flow rate, through a glass tube inserted to the bottom of the collecting vessel, a 50 ml erlenmeyer flask. The flow of the culture medium was uninterrupted, and the collecting vessel was allowed to overflow approximately 100 ml before the vessel was removed and sealed immediately with a rubber serum cap — taking care to exclude air bubbles. The concentration of the dissolved oxygen in the sample was measured immediately by the Winkler method (1). Using this method the dissolved oxygen is qualitatively replaced by molecular iodine, which is then titrated with sodium thiosulfate to a fine end point visualized by the disappearance of the blue color, due to the color reaction between the iodine and a small volume of starch solution. The titrating solution was prepared fresh every time from a refrigerated, 20-times concentrated stock solution which was calibrated by titrating measured amounts of potassium iodate (dried at 100 C for three to four hours). The chemical reactions involved are the following:

a.) $MnSo_4 + KOH \rightarrow Mn(OH)_2 + K_2SO_4$ (white precipitate formed);

b.) $2Mn(OH)_2 + O_2 \rightarrow 2MnO_2 + 2H_2O$ (brownish precipitate formed in the presence of dissolved oxygen);

c.) $2MnO_2 + 2H_2SO_4 \rightarrow Mn(SO_4)_2 \_ 2H_2O$ (precipitate dissolved in acid);

d.) $Mn(SO_4)_2 + 2KI \rightarrow MnSO_4 + K_2SO_4 + I_2$ (molecular iodine released);

e.) $2Na_2S_2O_3 + I_2 \rightarrow Na_2S_4O_6 + 2NaI$ (molecular iodine titrated).

This method is capable of detecting a minimum of 0.1 ppm dissolved oxygen. The accuracy of the measurement is $\pm$ 0.2 ppm.

Preparation of Cell-Free Extracts

Bacteroid extract

One to three grams of nodules were homogenized in a prechilled mortar with 2 ml per of 0.1 M HEPES-NaOH buffer (pH 8.0) containing 2 mM β-mercapto ethanol and 1 mM $MgCl_2$ ("extraction buffer"). The nodule brei was squeezed through two layers of cheesecloth and one layer of Miracloth. Bacteroids were collected from the filtrate by centrifugation at 6000 g for 15 minutes. The resulting clear supernatant solution ("plant fraction") was decanted and used directly for the determination of Lb heme and plant enzymic activities. The pelleted bacteroids were washed and resuspended in 5 ml extraction buffer and the resuspended bacteroids were broken by cavitation using three 30-second bursts from a Sonifier-Cell-Disruptor (Model W185, Heat

Systems Ultrasonics Inc.) at power setting 5. The sonicate was clarified by centrifugation for ten minutes at 20,000 g. Bacteroid enzyme activities were assayed in the resulting clear supernatant solution ("bacteroid extract"). Contamination of the bacteroid extract by plant material is considered minimal (below 5 nmoles per ml), since no Lb was detected in the bacteroid extract by the pyridine-hemochromogen method (46).

Free-living rhizobia extract

Vegetative *Rhizobium* cells were harvested in batches of approximately 500 ml. The cells were collected by centrifugation at 6000 g for 15 minutes. The pellet was washed and resuspended in 5 ml extraction buffer. The following steps were similar to the ones employed for obtaining the bacteroid extract.

Enzyme Assays

δ-aminolevulinic acid synthase (ALAS)

1.) Three milliliters final volume of reaction mixture contained the following in μmoles: HEPES buffer (pH 8.0), 300; β-mercaptoethanol, 1.5; $MgCl_2$, 50; sodium succinate, 300; glycine, 300; ATP (disodium salt from a freshly prepared stock), 21; CoA, 0.9; and pyridoxal phosphate, 0.9.

The reaction was initiated by adding bacteroid extract containing 3 mg protein or less to the reaction mixture (unless otherwise stated). The test tubes containing the complete reaction mixture were placed in a shaker in a water bath at 30 C and incubated for three hours (unless otherwise stated). Incubation was terminated by the addition of 0.6 ml ice cold 20% trichloracetic acid saturated with $HgCl_2$. At the same time the tubes were removed from the water bath and stored on ice for several hours (the duration of storage up to 24 hours did not affect the amount of ALA recovered). The accumulated precipitate was removed by centrifugation at 20,000 g for ten minutes and the supernatant solution was applied to a 3.5 ml column of Dowex-50-W-X8, 200 to 400 mesh resin prepared in disposable 5 ml syringes and equilibrated with 0.2 M (in sodium) sodium citrate buffer (pH 3.07). ALA was eluted by 0.2 M (in sodium) sodium citrate buffer pH 5.10 as described by Beale *et al.* (11). The eluted δ-aminolevulinate (ALA) was converted to ALA-pyrrole by condensation with 2,4 pentadione (85) for colorimetric determination with the modified Ehrlich reagent of Urata and Granick (107).

2.) An alternative method of assaying the ALA produced was adopted at a later stage in this work and was found to give results identical to those obtained

by the already described method. This alternative dispenses with the chromatographic separation of ALA and uses a different way of producing the ALA pyrrole (Figure 5). The samples, obtained after removal of the protein precipitate as above, were neutralized by the addition of concentrated NaOH (7N) and diluted two-fold with 1 M sodium phosphate buffer (freshly prepared stock) pH 6.8. Ethyl aceto-acetate (0.1 ml) is added to the sample and the ALA-pyrrole is formed by boiling the sample for ten minutes as described by Mauzerall and Granick (85). The Ehrlich-positive material, the removal of which necessitated the introduction of the chromatographic step, does not form in this alternative assay method.

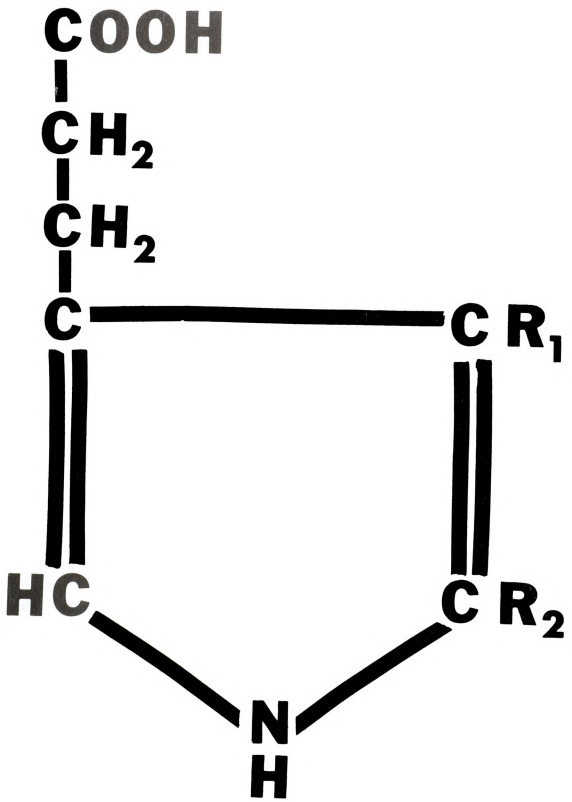
One unit of ALAS activity is defined as the activity which produces one nanomole of ALA per hour.

δ-aminolevulinic acid dehydrase (ALAD)

The reaction mixture contained in a final volume of 1 ml the following μmoles: Tris-HCl (pH 8.5), 50; (subsequently if was found that Na-phosphate buffer, pH 7.5 increased the activity by 18%); $MgSO_4$, 5; β-mercaptoethanol, 12.5; ALA (Sigma chemical), 2.5; and bacterial extract or plant fraction containing less than 1 mg protein. The complete reaction mixture was incubated for two hours at 30 C in a water bath. The reaction was terminated by the addition of 0.25 ml

Figure 5. The structures of three substituted pyrroles which are assayed by the Ehrlich reaction in the procedures described for measuring ALAS and ALAD activities.

PBG	$R_1 = —CH_2COOH$; $R_2 = —CH_2NH_2$
ALA-acetylacetone pyrrole	$R_1 = —COCH_3$; $R_2 = —CH_3$
ALA-ethylacetate	$R_1 = —COCH_2CH_3$; $R_2 = —CH_3$


COOH
CH2
CH2
C
C R1
HC
C R2
N
H

ice-cold 20% (w/v) trichloroacetic acid saturated with $HgCl_2$. The precipitate was removed by centrifugation at 20,000 g for ten minutes. The resulting supernatant solution was assayed spectrophotometrically for the porphobilinogen (PBG) formed, using the color reaction of PBG with Ehrlich reagent (85, 107). One unit of ALAD activity is defined as the activity which forms one nanomole of PBG per hour.

Succinyl thiokinase

Succinyl thiokinase (STK) activity was measured by the rate of succinic hydroxamate formation — a modification of the method by Burnham and Lascelles (21). Each milliliter of final reaction mixture contained, in μmoles: sodium succinate, 100; coenzyme A (Sigma chemical), 1.4; ATP (disodium salt, freshly prepared stock), 5; β-mercaptoethanol, 10; neutral NH_2OH (freshly prepared stock), 800; and 200 μl bacterial extract. The complete reaction mixture was incubated for 30 minutes in a 28 C water bath. One unit of STK activity is defined as the activity which forms one micromole of succinic hydroxamate per hour. Specific activity is units per mg protein.

Nitrogenase-catalyzed acetylene reduction

Acetylene reduction was assayed by measuring ethylene produced in an atmosphere of 6% C_2H_2 using

30 µl of gas sample with a Varian Aerograph Model 1200 gas chromatograph (Varian Instruments Division, Palo Alto, CA) after ten to 30 minutes incubation with the sample at room temperature. When very low concentrations of C_2H_2 had to be detected, 1 ml samples were analyzed with a Varian Aerograph series 2400 gas chromatograph after several hours (or days) of incubation at room temperature. This instrument was equipped with a 45 x 0.32 cm column containing Al_2O_3 and was operated at 80 C with N_2 as the carrier gas.

Preparation of Succinyl-CoA

Succinyl CoA was prepared by a method modified from that of Simon and Shemin (99). The procedure was carried out in ice-cold distilled water with a continuous stream of N_2 gas bubbling through the reaction vessel. Seventeen mg (22 µmoles) of CoA were dissolved in 3 ml of ice-cold water. The pH was kept at 7.0 - 7.5 by adding solid $NaHCO_3$ when necessary. Approximately 1.5 mg succinic anhydride was added gradually until no more free SH-groups were left (as assayed on a drop plate by nitroprusside reagent). At completion the pH was dropped to 2 - 3 with the addition of 1N HCl. The acidified solution was frozen in 0.3 ml aliquots and stored at -20 C until needed.

Methods for Assaying the Growth of Cells in Culture

1.) Absorbance of the culture was determined using a Klett colorimeter with a standard red filter. Absorbance units were expressed in Klett units or the corresponding absorbance units: 1 Klett unit = 0.002 absorbance unit.

2.) Protein was assayed in a precipitate obtained from 100 ml culture by centrifugation at 6000 g for 15 minutes. The pellet was washed, diluted as required and assayed for protein content by the Lowry method for sparingly soluble proteins (80).

3.) Samples of the culture were diluted with 20 mM sterile buffer Na-phosphate pH 6.8 and 0.1 ml of the final dilution was plated on three sterile plates with YM agar. The plates were incubated at 30 C for four days before the colonies were counted. The number of viable cells in the culture sample was calculated according to the counts.

RESULTS

δ-Aminolevulinic Acid Synthase (ALAS) Activity in Bacteroids and in Free-Living *Rhizobium japonicum* Cells

Since extracts of soybean root nodule bacteroids and of free living rhizobia contain a material which interferes with the colorimetric assay for ALA, a partial purification of the ALA produced in the incubation mixture is necessary before the conventional assay for ALA can be used. The purification procedure employed involved the absorption of the ALA in the incubation mixture onto a sulphonic acid resin at pH 3.07 and its subsequent elution at pH 5.10. In the absence of this purification step the absorption of the colored compound formed from ALA pyrrole and the Ehrlich reagent (absorption maximum 553 nm; see Methods) is obscured by the absorption of another colored substance (extinction maximum 520 nm) also formed with the Ehrlich reagent, but not involving ALA. After the ion exchange procedure outlined, this interfering substance ceases to be a problem and the ALA formed by the bacteroids *in vitro* can be readily determined in the column eluates

by standard colorimetric procedures (85). The efficiency of the ion exchange purification stem can be estimated in Figure 6 by comparing the ration of the absorbance at 520 nm and 553 nm before and after purification with the ratio obtained when synthetic, standard ALA is used. (These ratios are: A. crude incubation mixture, 0.36; B. column eluate from incubation mixture, 0.72; C. synthetic ALA, 0169.)

In addition to the insertion of the ion exchange step, the standard method for assaying ALAS activity (20) was modified by increasing 20-fold the concentration of the buffer in the "extraction buffer" and by changing both the buffering agent (HEPES instead of Tris-HCl) and the pH (8.0 instead of 7.5). The need for increasing the buffering capacity became apparent when the pH of the bacteroid-suspension medium was checked both before and after sonication. During sonication the storage product poly-β-hydroxy butyrate is known to depolymerize, which results in a sharp pH drop. HEPES buffer was employed in preference to Tris-HCl since its pH (7.55) was closer to the required pH (8.0) than that of Tris (7.0), and therefore its buffering capacity in that range was superior. Tris buffer was also known to interfere with the activity of pyridoxal phosphate requiring enzymes.

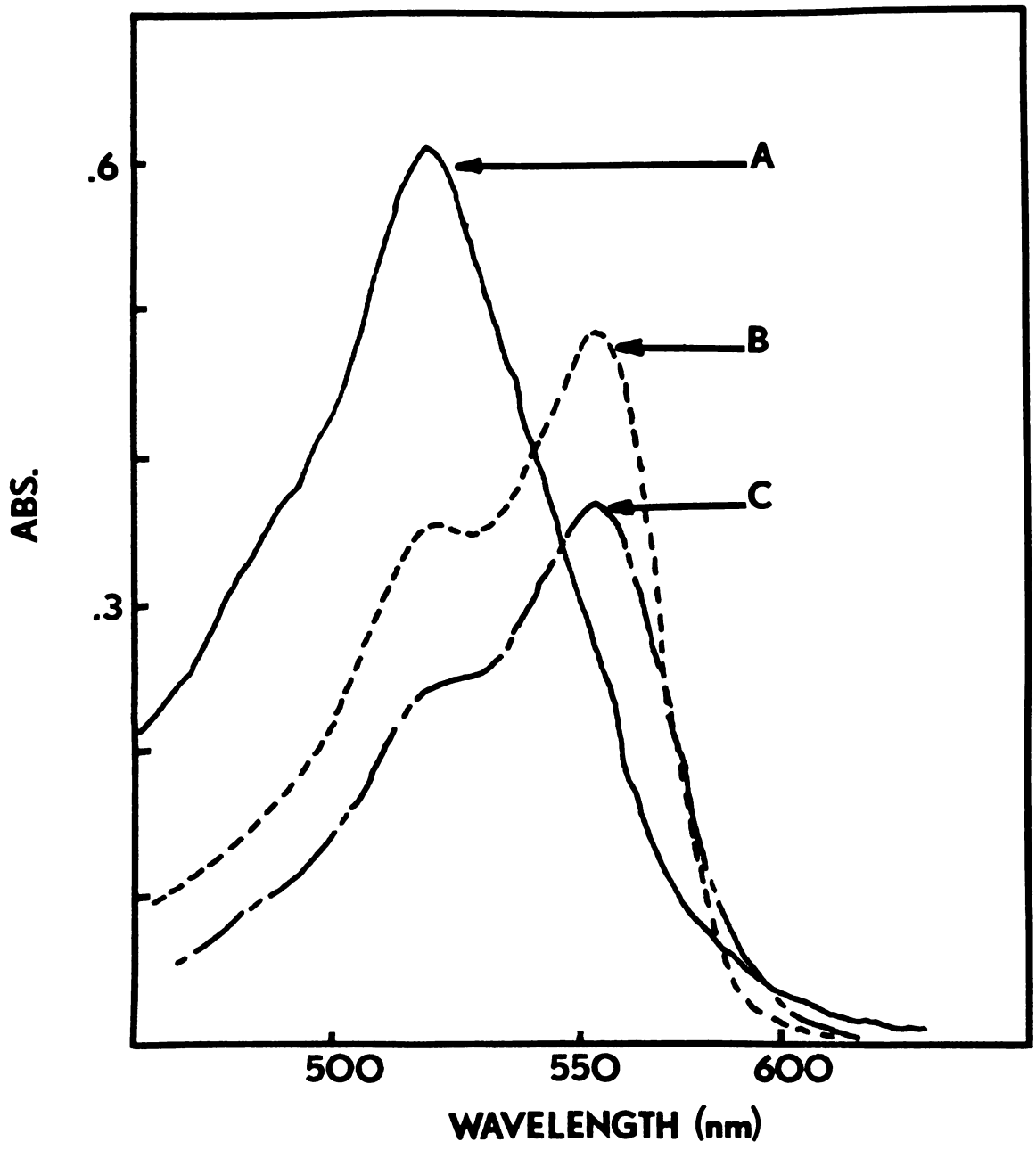
Figure 6. Absorption spectra of the color compounds produced with Ehrlich's reagent showing the effect of purification by ion exchange chromatography of ALA produced by bacteroid extracts *in vitro*.

A. Crude bacteroid extract after addition of TCA and clarification by centrifugation.

B. Same as A — after chromatography on Dowex 50W-X8 (note: absorbance x 10).

C. Synthetic ALA.

Samples containing ALA were treated as described in MATERIALS AND METHODS to form ALA-pyrrole which was then mixed with equal volumes of modified Ehrlich reagent. Absorption spectra of the resulting color compounds were recorded after 15 minutes incubation at room temperature, with a Cary 15 recording spectrophotometer.


.6
.3
ABS.
A
B
C
500
550
600
WAVELENGTH (nm)

Using this modified assay procedure ALAS activity was readily measured in the bacteroid fraction whereas no activity was detected in the plant fraction of soybean root nodule extracts.

ALAS activity in cell free extracts of soybean root nodule bacteroids was constant and apparently stable for at least six hours in the reaction mixture employed (Figure 7). The addition of penicillin G (100 units per ml) to the reaction mixture did not result in a significant change in measured activity, indicating that the measured activity after long periods of incubation was almost certainly not due to bacterial contamination.

The pH optimum for ALAS activity in crude extracts was above 7.5 (Figure 8). ALAS from other bacteria or mitochondria has also pH optima of 7.5 - 8.0.

ALA production is completely dependent on the presence of the substrates glycine and succinate and is somewhat less dependent on exogenous CoA and on pyridoxal phosphate (Table 1). The limited dependence on added cofactors is probably due to the presence of some CoA and some pyridoxal phosphate in crude bacteroid extracts. The apparent formation of ALA in the absence of added ATP is somewhat artifactual; in the absence of added ATP, a compound accumulates in cell free extracts which co-elutes with ALA from the cation exchange resin and

Figure 7. ALAS activity of bacteroid extracts.

A. Reaction mixture contained 1.25 mg protein and was incubated for the periods indicated in a water bath at 30 C.

B. Reaction mixture contained various amounts of bacteroid protein as indicated and was incubated for four hours at 30 C in a water bath.

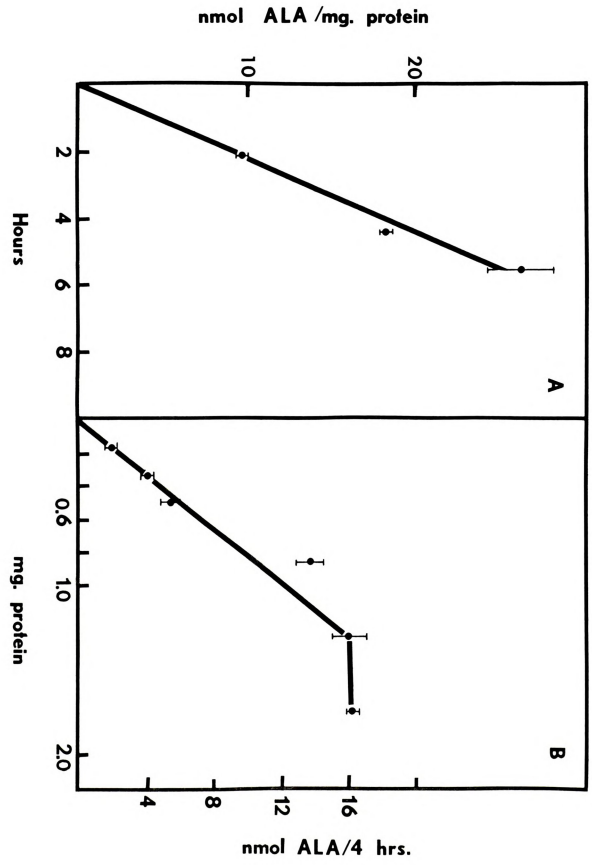

nmol ALA /mg. protein
10
20
2
4
6
8
Hours
A
B
0.6
1.0
2.0
mg. protein
4
8
12
16
nmol ALA/4 hrs.

Figure 8. Effect of pH on ALAS activity in cell-free extracts of bacteroids.

The buffering agent was 0.1 M Na-phosphate in the range 5.7 - 7.5 and 0.1 M HEPES-NaOH in the range 7.5 - 8.5. Equal values were obtained for ALAS activity in both buffers at pH 7.5.

The reaction mixture contained 1 mg/ml bacteroidal protein, and 0.1 M buffer. Incubation was for 2.5 hours at 30 C.

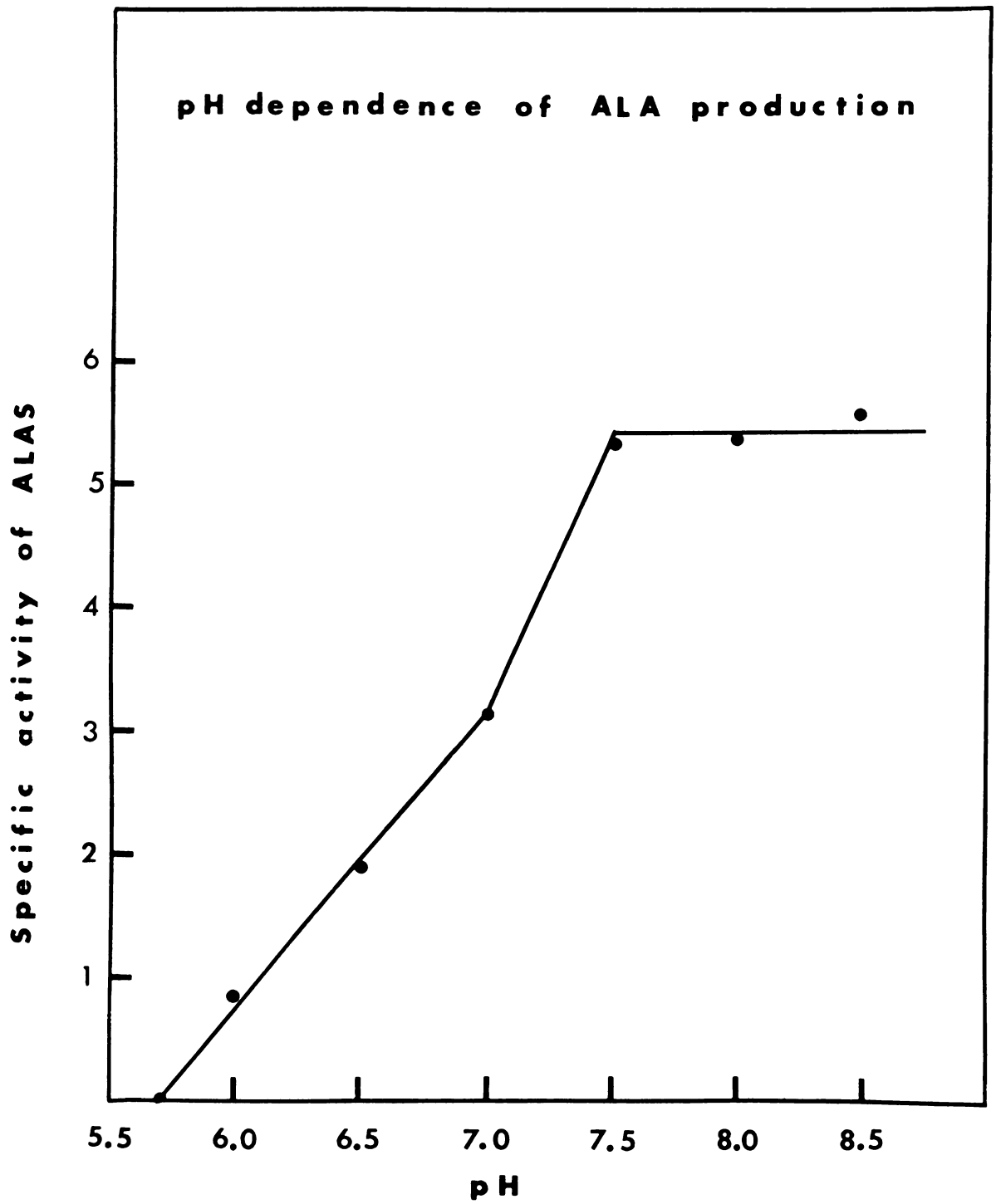

pH dependence of ALA production
Specific activity of ALAS
6
5
4
3
2
1
5.5
6.0
6.5
7.0
7.5
8.0
8.5
pH

Table 1. Requirements for bacteroid ALAS activity *in vitro*.

System	nmol ALA/mg Protein/hr	% Complete
complete	7.3 ± 0.1	100
-glycine	0.1 ± 0.03	1
-succinate	0.3 ± 0.03	4
-ATP	2.6 ± 0.03	36
-Coenzyme A	1.6 ± 0.09	22
-pyridoxal phosphate	1.9 ± 0.03	26
+ sodium levulinate, 300 µmoles	6.3 ± 0.1	86
+$MgCl_2$, 30 µmoles	6.5 ± 0.1	89

Bacteroid ALAS activity was determined as described in MATERIALS AND METHODS. Assay mixtures containing 3 mg protein in 3 ml final volume were incubated at 28 C for 200 minutes. Omitted sodium succinate or ATP (disodium salt) were replaced by equimolar amounts of NaCl; in other cases omitted substances were replaced with equal volumes of distilled water.

forms a pyrrole-like derivative with acetyl acetone; this pyrrole forms a 520 nm absorbing colored compound with the Ehrlich reagent. Levulinic acid, a competitive inhibitor of ALAD is not required, probably because ALAD is not active under the conditions of this reaction. The Mg^{++} ion concentration supplied in the extraction buffer is probably sufficient, therefore no further addition of $MgCl_2$ is required.

ALA synthesis in this *in vitro* system is dependent upon the production of the immediate substrate succinyl CoA by another enzyme (succinyl thiokinase) in the extract. The activity of this enzyme, however, is probably not a limiting factor in ALAS activity because its activity in the bacteroid and in the vegetative cell extracts is 300 to 1000 times higher than the activity of ALAS (see also 47). Succinate, CoA and ATP can be replaced by chemically prepared succinyl-CoA, but the resulting enzyme activity is considerably lower, about ten percent of the activity obtained with the standard reaction mixture. The lowered activity can probably be ascribed to the combination of the instability of succinyl-CoA in solution (which might be due to the presence of an active thioesterase in the crude extract) and the long duration of the incubation (usually three hours).

ALAS activity in cell-free extracts prepared from free-living rhizobia was lower by one order of magnitude than the activity of bacteroid ALAS. In all other respects (such as stability, substrate, cofactor requirements, pH optimum and protein saturation value) the characteristics of the enzyme activity from the free-living cells were similar to the characteristics observed in bacteroids as described in the previous section. Incubating the extracts in a boiling water bath for five minutes resulted in a complete loss of ALAS activity.

Storing the extracts in a frozen state, with or without glycerin (20%), resulted in a 50% loss of activity overnight, but no additional losses were observed during more prolonged storage. Freezing the harvested bacteria (in extraction buffer) or the nodules prior to extraction proved to be a better method for storage, since the enzyme activities there appeared to be unaffected even after several weeks of storage.

Changes in ALAS Activity During the Development of Nodules

Nodules become apparent on the roots of soybean plants at about 12 - 14 days after inoculation. Nodules develop fairly rapidly after this time, and reach

maximum size at about 38 - 42 days after inoculation. However the amount of bacteroid protein extractable from the nodules increased only during the first 26 days after inoculation and is steady for the following 24 days (the same is true of extractable "plant protein"). All experiments were conducted with plants within 50 days after inoculation. Since inoculation is performed at the start of germination, the time elapsed after inoculation is the same as the time after germination (Figure 9).

ALAS activity and heme accumulation were assayed during various stages of nodule development. The observed variations in bacteroid ALAS activity were clearly correlated with the variations in heme content (Figure 10). The activity of ALAS was calculated in this case on the basis of nodule fresh weight since a correlation with extrabacterial heme concentration was sought. ALAS activity could not be detected in very young primordia which also did not contain measureable amounts of heme. Thereafter, nodule heme content and bacteroid ALAS activity increased in parallel, both reaching a maximum six weeks after inoculation. As nodules senesce and their heme is degraded, bacteroid ALAS activity declined. The plant fraction of the nodules was tested unsuccessfully for ALAS activity

Figure 9. Growth of soybean root nodules and *Rhizobium japonicum* in the nodules.

Seeds were germinated and inoculated with *Rhizobium japonicum* on perlite-vermiculite beds and transferred to pots (six per pot) after one week. All the nodules harvested were crown nodules.

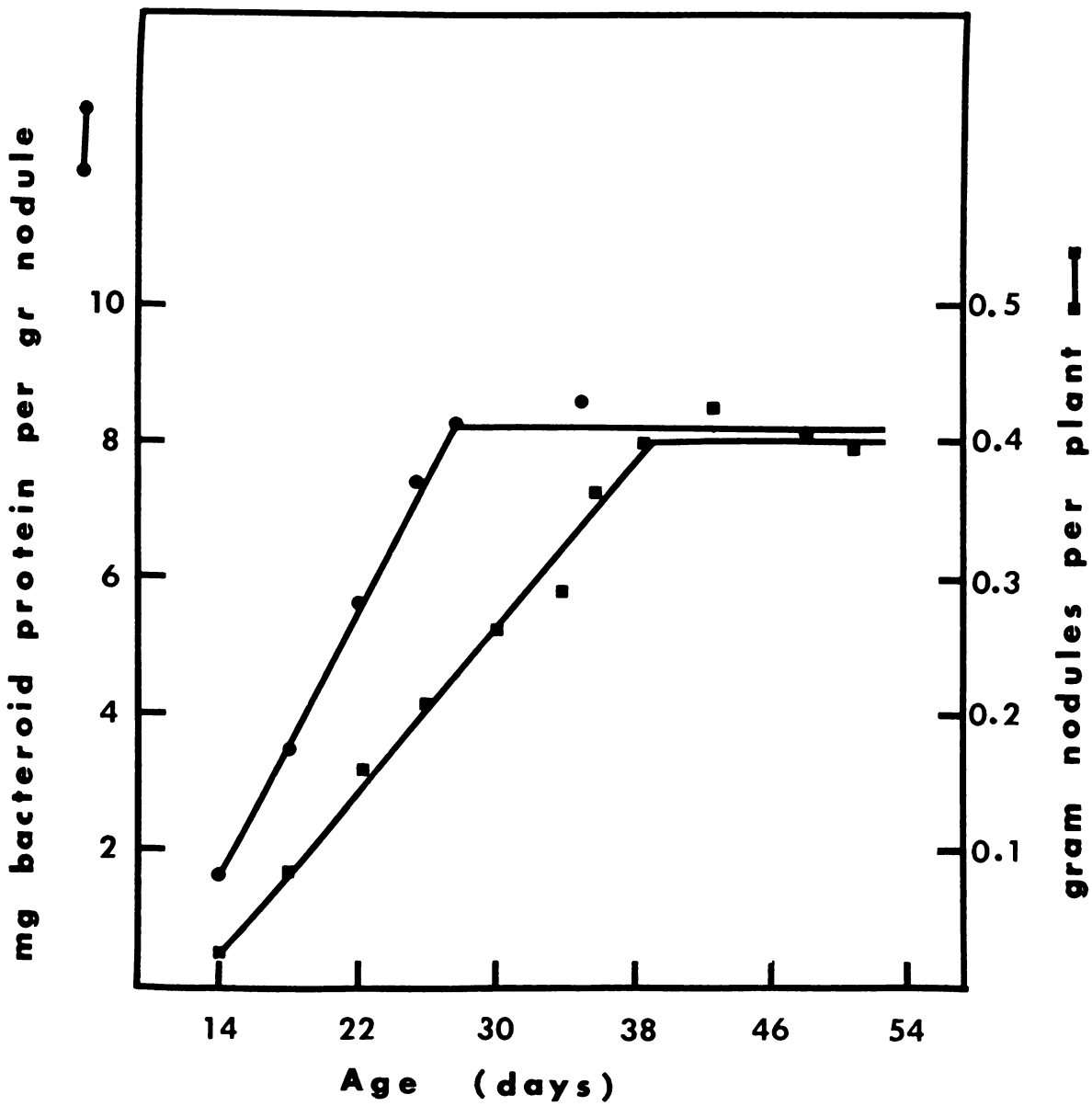
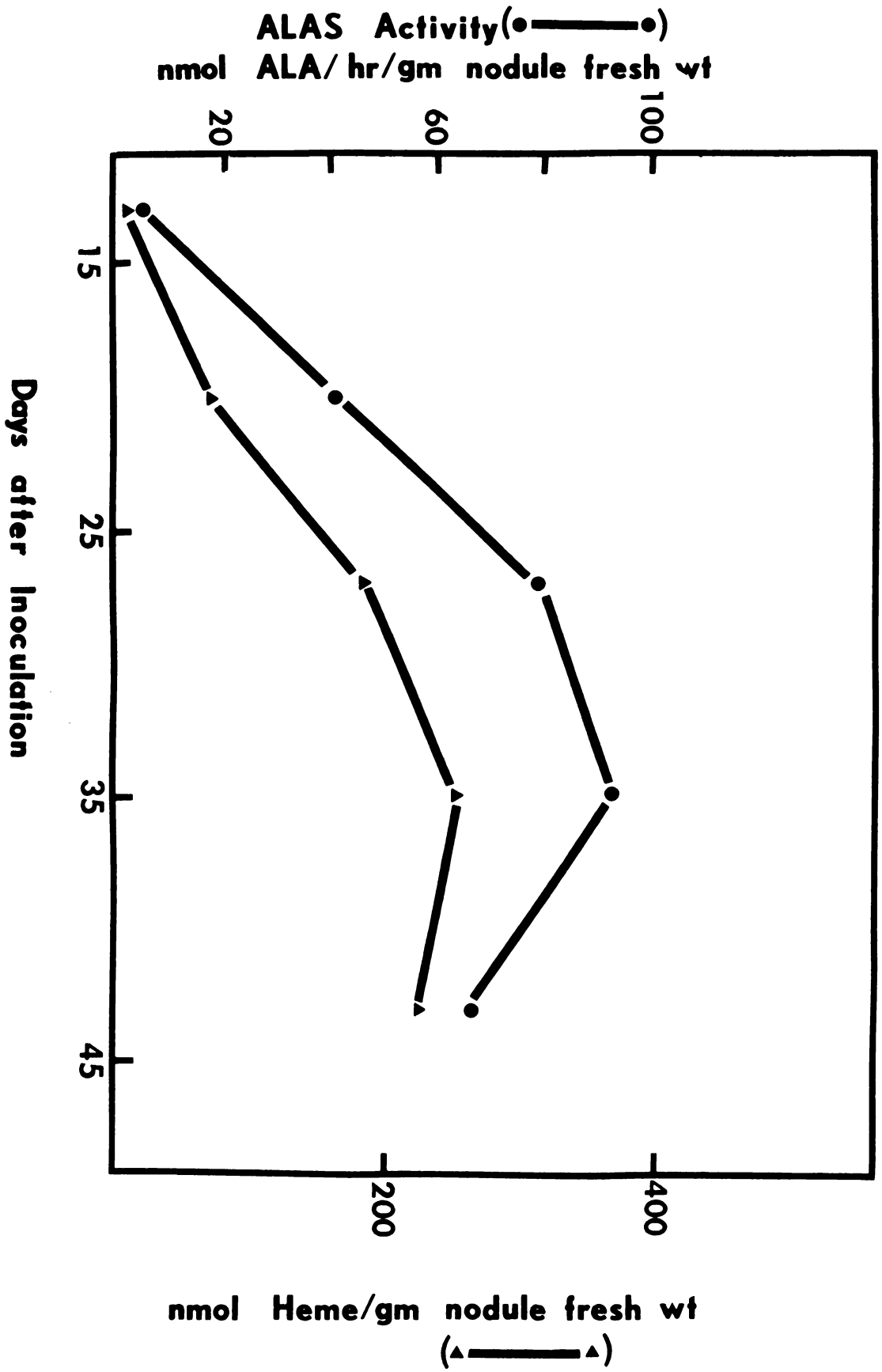

mg bacteroid protein per gr nodule
gram nodules per plant
10
8
6
4
2
0.5
0.4
0.3
0.2
0.1
14
22
30
38
46
54
Age (days)

Figure 10. Bacteroid ALAS activity and Lb heme content of developing nodules.

Nodules formed on "Hark" soybeans by *Rhizobium japonicum* 3Ilb-110 were harvested on the days indicated after inoculation. Lb heme is determined in the plant fraction as the pyridine hemochromogen; bacteroid ALAS activity was assayed as described in MATERIALS AND METHODS.


ALAS Activity(●——●)
nmol ALA/ hr/gm nodule fresh wt
20
60
100
15
25
35
45
Days after Inoculation
200
400
nmol Heme/gm nodule fresh wt
(▲——▲)

during all stages of development.

When inoculated with *R. japonicum* strain 11927, soybean (cv. Hark) plants produce inefficient nodules, unable to fix nitrogen and lacking leghemoglobin (88). In our studies it was found by microscopic observation that such nodules contained bacteroids, although in considerably smaller numbers than in effective nodules. Extracts of bacteroids isolated from such inefficient nodules lacked detectable ALAS activity. This observation supports the correlation between Lb accumulation and bacteroid ALAS activity. Therefore bacteroid ALAS activity appears to be the limiting factor in Lb formation.

Growth of Free-Living *Rhizobium japonicum* 3I1b-110 with Restricted Aeration

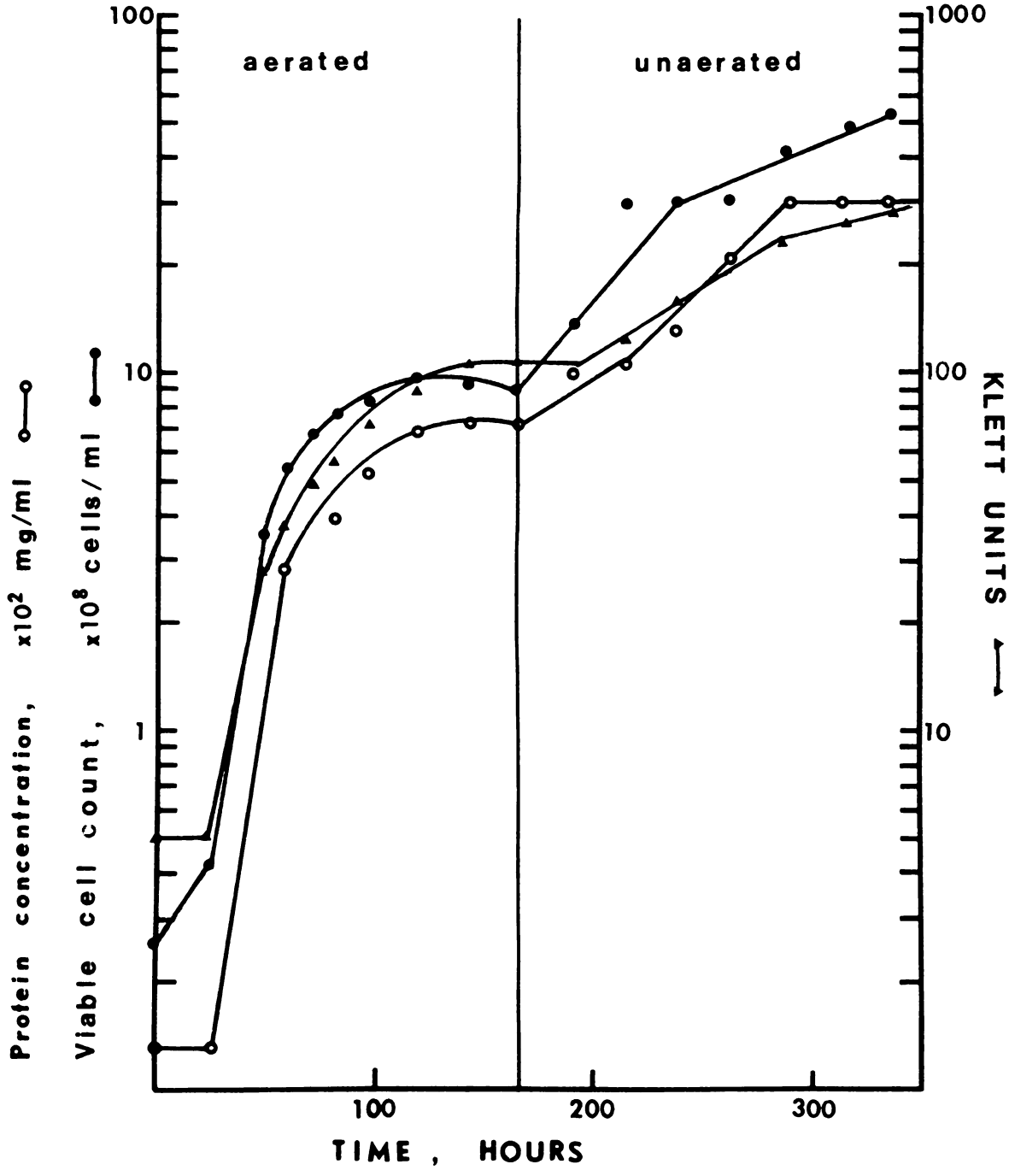
Rhizobium japonicum cells were grown on yeast-mannitol (YM) medium in a vigorously aerated, mechanically agitated fermentor (see Methods). When the cell density had stopped increasing the forced aeration was discontinued while the mechanical agitation continued. During this second, "microaerobic" phase the only source of oxygen for the cells was the oxygen remaining in the gas phase above the culture medium and any oxygen that could diffuse in through the aeration tubes.

The fully aerobic stage of growth was characterized by a very short log phase with a generation time of eight hours followed by a longer period of gradually slowing growth (Figure 11). The culture reached a stationary phase when its density was about 100 Klett units (0.20 Absorbance units). Towards the end of the stationary phase cell viability began to decline. It was at this time that forced aeration of the culture was discontinued, whereupon there was a rapid drop in the concentration of dissolved oxygen. The dissolved oxygen was about 6 ppm during the period of forced aeration but dropped to 0 (or below 0.1 ppm) as measured by the Winkler method (1), within three days. During this period of declining oxygen concentration, and following it, the bacteria started growing again, though at a slower rate than in the early stages of fully aerobic growth (generation time about 24 hours). A second stationary phase followed occurring a density of about 300 Klett units (0.6 Absorbance units), or about four times more viable cells per milliliter culture and about four times more protein in the cell pellet than during the first, aerobic stationary phase.

Although the dissolved oxygen concentration was below the level of detection after the third day under restricted aeration, the cells still required oxygen.

Figure 11. Growth of *Rhizobium japonicum* initially with, and subsequently without, forced aeration.

See MATERIALS AND METHODS for description of the system. Protein was measured by the Lowry (80) procedure for sparingly soluble proteins.


aerated
unaerated
100
10
1
1000
100
10
100
200
300
TIME , HOURS
Protein concentration, x10² mg/ml
Viable cell count, x10⁸ cells/ml
KLETT UNITS

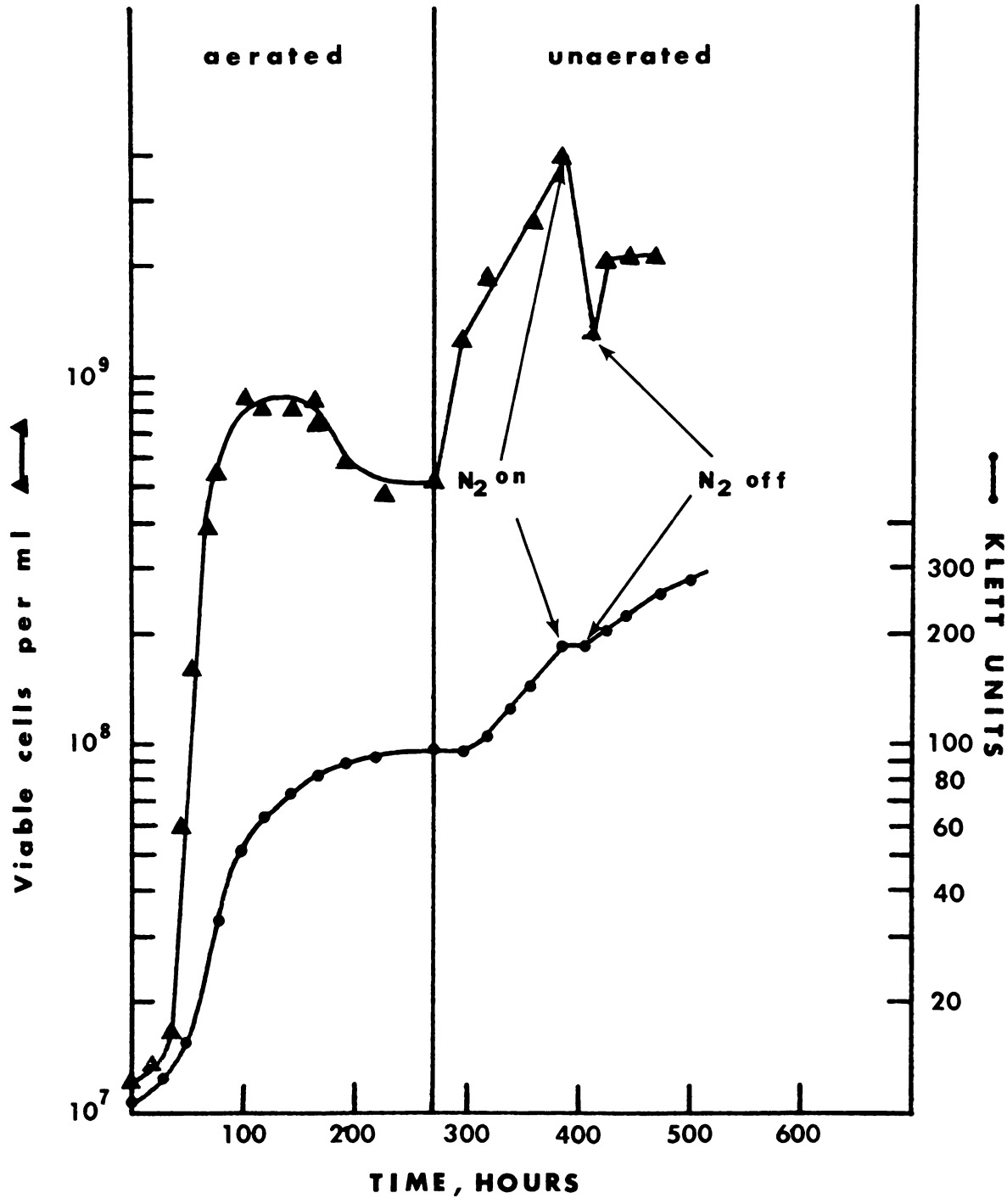
Sparging the culture with nitrogen at this stage (Figure 12) resulted in an immediate drop in cell viability. Growth was resumed after the nitrogen sparging was discontinued. (The cell viability did not reach the level attained in the absence of nitrogen sparging, probably because the N_2 sparging was effected in a relatively late phase of the second growth stage.) This indicates that a low level of oxgyen vital for the support of growth is diffusing into the culture as a result of the agitation.

These "microaerobically" grown rhizobia, produced as a result of the limited aeration, did not fix nitrogen during their renewed growth phase as indicated by their failure to reduce acetylene. No detectable amount of acetylene was produced, even after prolonged incubation of the cells with 6% acetylene in either a 1% or a 2% oxygen-containing argon atmosphere. This indicates that any levels of acetylene reduction which were undetected would have to be less than 0.1 nanomoles per hour in a flask containing 30 ml of dense culture (∿200 Klett units). This would be about 2% of the reported values for nitrogenase activity in cultured rhizobia (17).

Rhizobium japonicum also grows "microaerobically" without a preceeding period of forced aeration. When the cells were inoculated directly into the fermentor

Figure 12. The effect of sparging with nitrogen on growth and cell viability of *Rhizobium* cells with limited aeration.

See MATERIALS AND METHODS for description of the system.


aerated
unaerated
10^9
10^8
10^7
N_2 on
N_2 off
Viable cells per ml
KLETT UNITS
300
200
100
80
60
40
20
100
200
300
400
500
600
TIME, HOURS

without forced aeration, there was a brief log phase of growth (generation time 18 hours), followed by a second, slower phase (generation time 42 hours) which continued for five days. In this case the fermentor culture reached a density of 320 Klett units (0.64 Absorbance units).

In an attempt to elucidate the factors responsible for the renewed growth with limited aeration, we tried to determine the factors limiting growth during the first, aerobic stationary phase. Rhizobia were grown aerobically with various amounts of carbon source (mannitol) and nitrogen source (yeast extract), (see Table 2). The results indicate that the major factor in causing a stationary phase at low cell densities is possibly the depletion of the carbon source (mannitol). Increasing the concentration of the nitrogen source (yeast extract) resulted in a 50% increase in the optical density of the stationary phase. It seems that the initial growth rate is limited by the concentration of the yeast extract (N-source), whereas the final yield is limited by the concentration of mannitol (C-source). This indicates that in the microaerobic growth phase cells might utilize a different carbon source (produced during the aerobic phase) or perhaps utilize mannitol more efficiently.

Table 2. Effect of variations in the level of carbon and nitrogen sources on the aerobic growth of *Rhizobium japonicum*. Growth was measured by changes in absorbance (Klett units).

grams per liter		Time after Inoculation (days)									
Yeast Extract	Mannitol	1	2	3	4	5	6	7	8	9	10
1.0	1.0	6	27	48	51	52	51	52	54	53	54
1.0	5.0	5	29	52	60	64	65	66	67	66	66
1.0	10.0	6	32	71	86	97	103	110	110	109	110
1.0	20.0	5	29	90	130	160	190	211	230	248	260
0.1	10.0	1	8	19	23	26	28	30	34	39	40
0.5	10.0	3	13	34	41	46	51	52	54	55	57
1.0	10.0	5	34	73	90	100	105	107	105	107	106
2.0	10.0	8	71	126	151	155	153	150	152	153	154

Activation of Tetrapyrrole Biosynthesis by Restricted Aeration

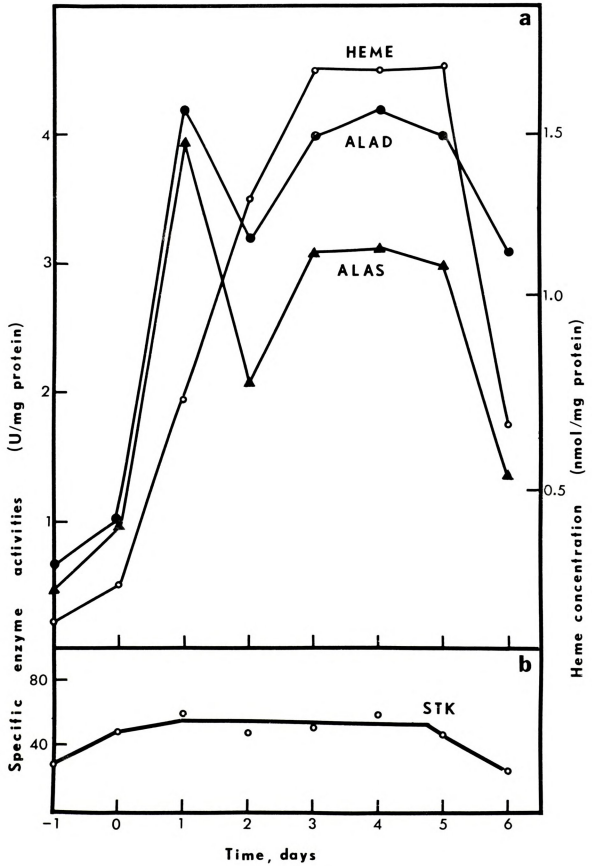
Cell pellets from cultures incubated with restricted aeration had a pink-red coloration. Cell extracts from such microaerobic cultures contained about ten times more heme than the aerated, stationary phase cells (Figure 13). During the same period there was also an approximately ten fold increase in the activities of both ALAS and ALAD.

The increase in the enzyme activities was virtually complete within one day after the termination of forced aeration, whereas the heme content continued to increase for several days. The elevated levels of ALAS and ALAD activities, as well as the high heme content persisted for several days. Experiments of mixing extracts of "microaerobic" cells with extracts of aerobic cells did not indicate the presence of an inhibitor of either ALAS or ALAD in the extracts of aerobic cells. Succinyl thiokinase activity did not increase significantly during the same period (Figure 13b). Since succinyl thiokinase activity, unlike ALAS or ALAD is not associated primarily with heme synthesis, this indicates that the increases in the activities of these enzymes of the heme synthesis pathway are not part of a general increase in enzyme activities provoked by the

Figure 13a. Changes in the intracellular heme content and the activities of the first two enzymes of the heme pathway (ALAD and ALAS) during "microaerobic" growth.

-1 days refers to a late log phase aerated culture one day before the cessation of the forced aeration.

Figure 13b. Changes in the activities of succinyl thiokinase (STK) during the same period.


a
HEME
ALAD
ALAS
4
3
2
1
1.5
1.0
0.5
Specific enzyme activities (U/mg protein)
Heme concentration (nmol/mg protein)
b
STK
80
40
−1
0
1
2
3
4
5
6
Time, days

changed conditions. The increased heme accumulation during the restricred aeration, therefore, appears to be the result of a specific activation of heme biosynthesis.

Identification of the porphyrins excreted by *Rhizobium japonicum* during restricted aeration

During unaerated incubation, a brownish-purple "insoluble" material was deposited on the walls of the fermentor, while a material which fluoresced red under UV light accumulated in the culture medium. These pigments have been partially purified. Their spectrophotometric and chromatographic properties are presented in Figures 14a to 14d and in tables 3 and 4. The visible absorption spectra of these pigments indicated that they were prophyrins. Both the "soluble" and the "insoluble" porphyrins had a strong absorption band around 400 nm (Soret band), characteristic of the aromatic porphyrin macrocycle. In neutral solvents, such as chloroform (Figures 14a and 14c) the methyl esters of both porphyrins had absorption spectra with four bands in the 500-640 nm region, the intensities of the absorption bands steadily decreasing towards the red ("eito-type" spectra). "Etio-type" spectra are characteristic of porphyrins with methylene carbons adjacent to the macrocycle in six of the eight peripheral positions (46). In aqueous acid (Figures 14b

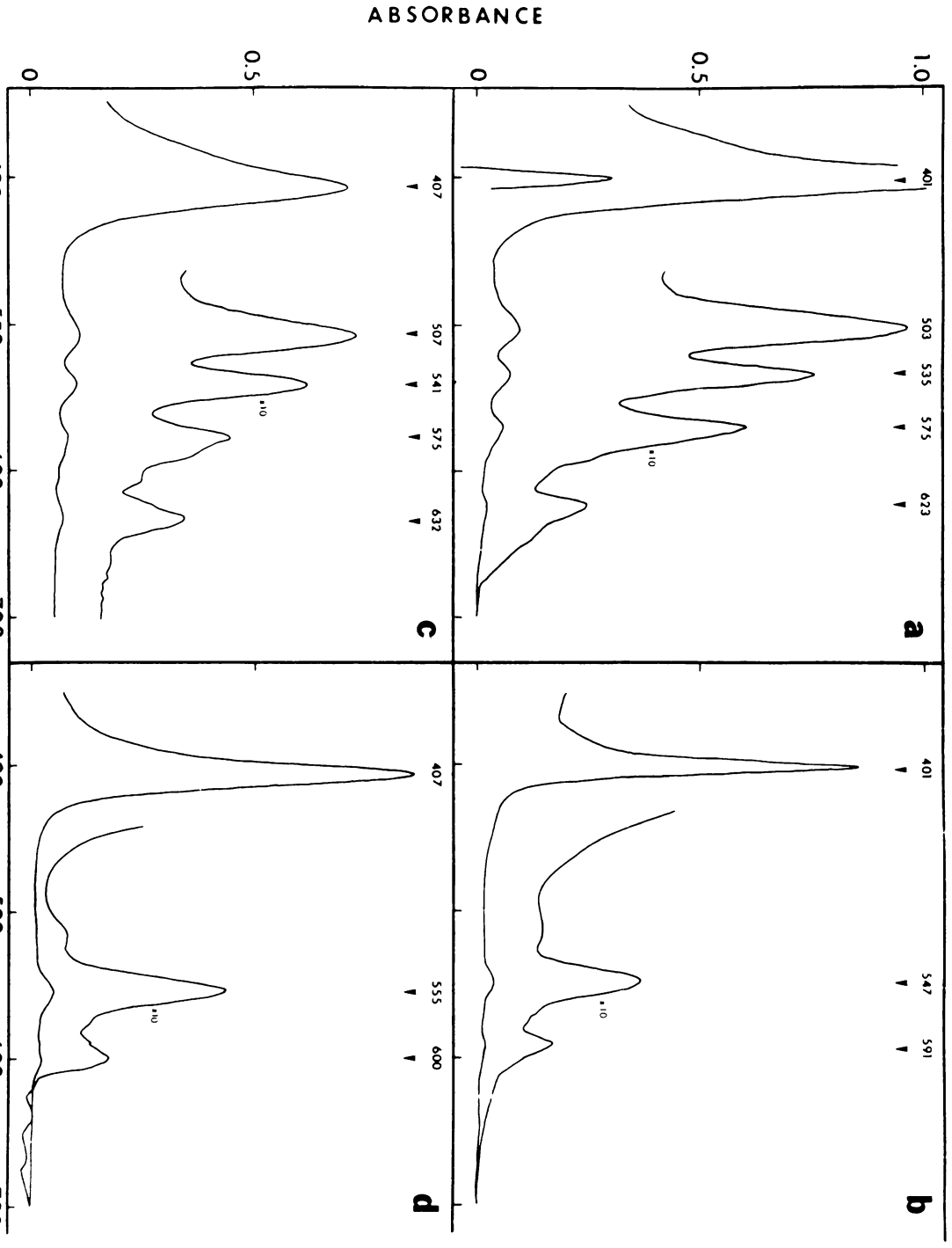
Figure 14. Absorption spectra of porphyrins excreted by *Rhizobium japonicum* microaerobic conditions and the methyl esters obtained from them.

a. "Soluble" porphyrin ester in chloroform.

b. "Soluble" porphyrin in 1N HCl.

c. "Insoluble" porphyrin ester in chloroform.

d. "Insoluble" porphyrin in 1N HCl.


ABSORBANCE
1.0
0.5
0
401
503
535
575
623
a
•10
401
547
591
b
•10
407
507
541
575
632
c
•10
407
555
600
d
•10
400
500
600
700
WAVELENGTH (nm)

and 14d), the Soret band of both "soluble" and "insoluble" porphyrins was narrower and absorption spectra, typical of porphyrin di-cations were observed with two bands in the 540-600 nm region. The "insoluble" porphyrin was tentatively identified as protoporphyrin IX on the basis of absorption spectra in the 540-600 nm region (Table 3). The methyl esters of the "insoluble" porphyrin co-chromatographed with standard protoporphyrin IX dimethyl ester in three systems (Table 4). On the basis of absorption spectra, the "soluble" porphyrin(s) appeared to be a mixture of coproporphyrin with some protoporphyrin (Table 3). Thin-layer chromatography of the methyl esters (Table 4) confirmed this identification, and indicated the presence of trace amounts of other porphyrins, with three and more than four carboxyl groups.

The amounts of accumulated coproporphyrin and protoporphyrin were crudely estimated to be 500 nm per liter and 100 nm per liter respectively on the basis of the intensity of the Soret band in the absorption spectrum. This is likely to be an underestimate due to the fact that the collection of the precipitate was not complete. No significant amounts of extracellular heme were found.

Table 3. Absorption maxima of porphyrins and of their methyl esters in acid and neutral solutions.[c]

Porphyrin[a]	Absorption maxima (nm)							
	in 1N HCl			in chloroform				
	Soret	II	I	Soret	IV	III	II	I
URO[b]	406	552	593	406	502	538	572	627
COPRO[b]	401	548	591	400	498	532	566	621
"soluble"[d]	401	547	591	401	503	535	570	623
PROTO[b]	408	556	600	407.5	505	541	575	630
"insoluble"[d]	407	555	600	407	507	541	575	632

[a]Abbreviations: URO = uroporphyrin; COPRO = coproporphyrin; PROTO = protoporphyrin.

[b]From: J.H. Furhop and K.M. Smith (ed). 1975. p. 784 & p. 872 in *Porphyrins and Metalloporphyrins*. Elsevier Scientific Publishing Co., Amsterdam.

[c]Absorption spectra of porphyrins in HCl, and of their methyl esters in chloroform.

[d]See MATERIALS AND METHODS.

Table 4. Chromatographic properties of the methyl esters of the major porphyrins excreted by cells incubated without aeration.

System	Rf Values				
	URO I	COPRO I + III Standard	"soluble" porphyrin	PROTO IX Standard	"insoluble" porphyrin
I	0.4	.25	.25 (.44)[a]	.44	.44
II	0.7	.36	.36 (.50)	.50	.50
III	—	.55	.53 (.27)	.27	.27

Systems: I = benzene - ethyl acetate - ethanol 190/20/7.5 v/v.
II = petroleum ether - paraffin oil - chloroform 1/1/10 v/v.
III = water - acetonitrile - dioxan 2/7/1 v/v.

Abbreviations: URO = uroporphyrin; COPRO - coproporphyrin; PROTO = protoporphyrin.

[a]Parentheses refer to Rf value of minor component.

Cytochromes of Unaerated Cells

Since heme biosynthesis is generally activated in response to changes in the cellular hemeoprotein content (3, 4, 31), the cytochrome pattern of the free-living *Rhizobium japonicum* cells grown in the fermentor under the above described conditions of limited aeration has been studied (Figures 15 and 16). The difference between spectra of dithionite-reduced and oxygen-oxidized unaerated cells (Figure 15e) was similar to that of bacteroids (Figure 15c), but not similar to that of aerobic, exponentially growing cells (Figure 15a). The broad band at 603 nm which is the α-band of cytochrome a-a_3 was present in aerobic, log phase cells (Figures 15a and 15b) but missing from the unaerated cells (Figures 15e and 15f) and bacteroids (Figures 15c and 15d). The band at 635 nm which is also characteristic of aerobic cells and may represent a d-type cytochrome (77) is absent from unaerated cells and from bacteroids. The absence of cytochromes a-a_3 in unaerated cells was confirmed by CO difference spectra (Figure 16). No trough was observed in the 600 nm region, even after 30 minutes exposure to CO, indicating the absence of CO-binding cytochrome oxidase in unaerated cells. The presence of a c-type cytochrome 552 with a high affinity for CO was indicated by the trough at 552 nm in the CO

Figure 15. Difference spectra of reduced minus oxidized whole *Rhizobium japonicum* 3I1b-110 cells, and the fourth derivative[1] of each spectrum.

a. Aerobic log phase cells.
b. Fourth derivative of a.
c. Bacteroids from soybean root nodules.
d. Fourth derivative of c.
e. Unaerated stationary phase cells.
f. Fourth derivative of e.

Note the absence of any peaks above 600 nm in the spectra of unaerated cells and bacteroids, in contrast with the peaks observed here in the spectrum of log-phase cells.

[1]The fourth derivative is used to indicate the exact location of the various peaks, but cannot be used for quantitative comparisons. Each peak in the fourth derivative spectrum corresponds to a peak or shoulder in the spectrum.

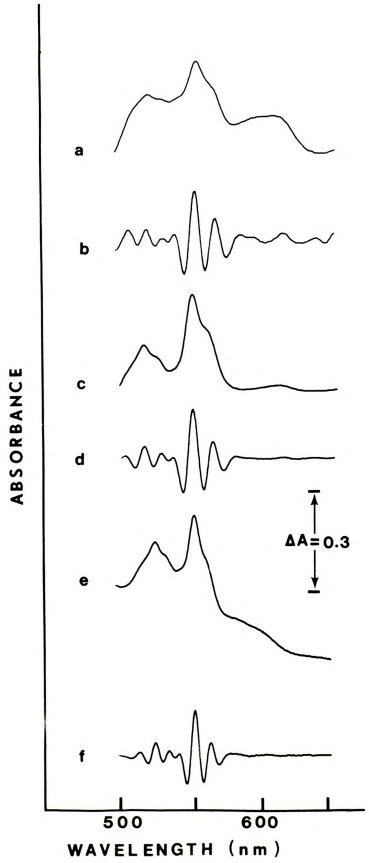

a
b
c
d
e
f
ABSORBANCE
ΔA=0.3
500
600
WAVELENGTH (nm)

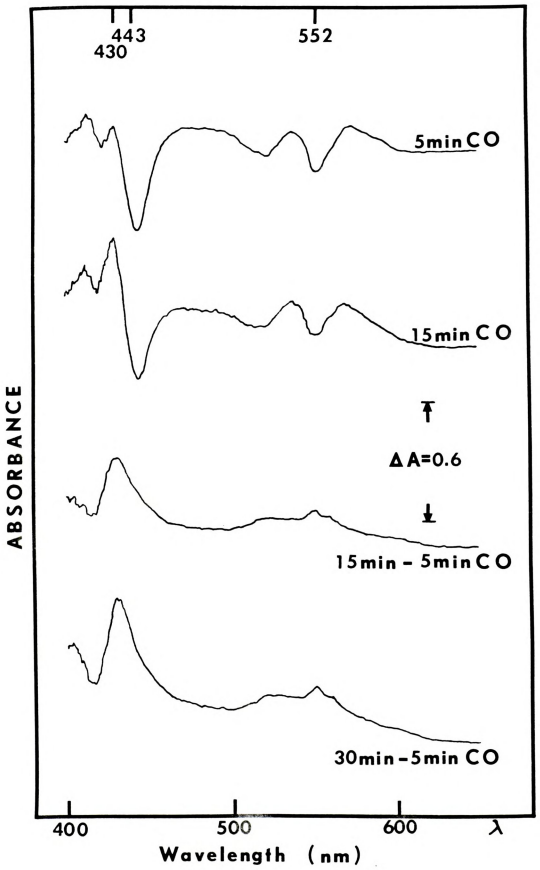
Figure 16. Difference spectra due to CO binding of microaerobic *Rhizobium japonicum* 3I1b-110 cells.

Samples were reduced with sodium dithionite. The spectrum of the reduced state was obtained by a modified Cary 15 spectrophotometer and stored in the computer connected to it (see MATERIALS AND METHODS), (I). Subsequently the sample was bubbled with CO after 30 minutes. Readings of the spectra were taken and stored as above, during the period of the bubbling, after five (II), 15 (III) and 30 (IV) minutes.

The computer performed the subtraction of the spectra as indicated below. The spectra presented are the resulting difference spectra:

5 min CO:	5 min. in CO minus the original reduced sample (II - I).
15 min CO:	15 min. in CO minus the original reduced sample (III - I).
15 min - 5 min:	15 min. in CO minus 5 min. in CO (III - II).
30 min - 5 min:	30 min. in CO minus 5 min. in CO (IV - II).

Note the presence of a CO binding pigment at 552 nm and at 443 nm (troughs), and the absence of a CO binding pigment in the absorption area of cyt a-a_3 (595 nm to 605 nm), (25).

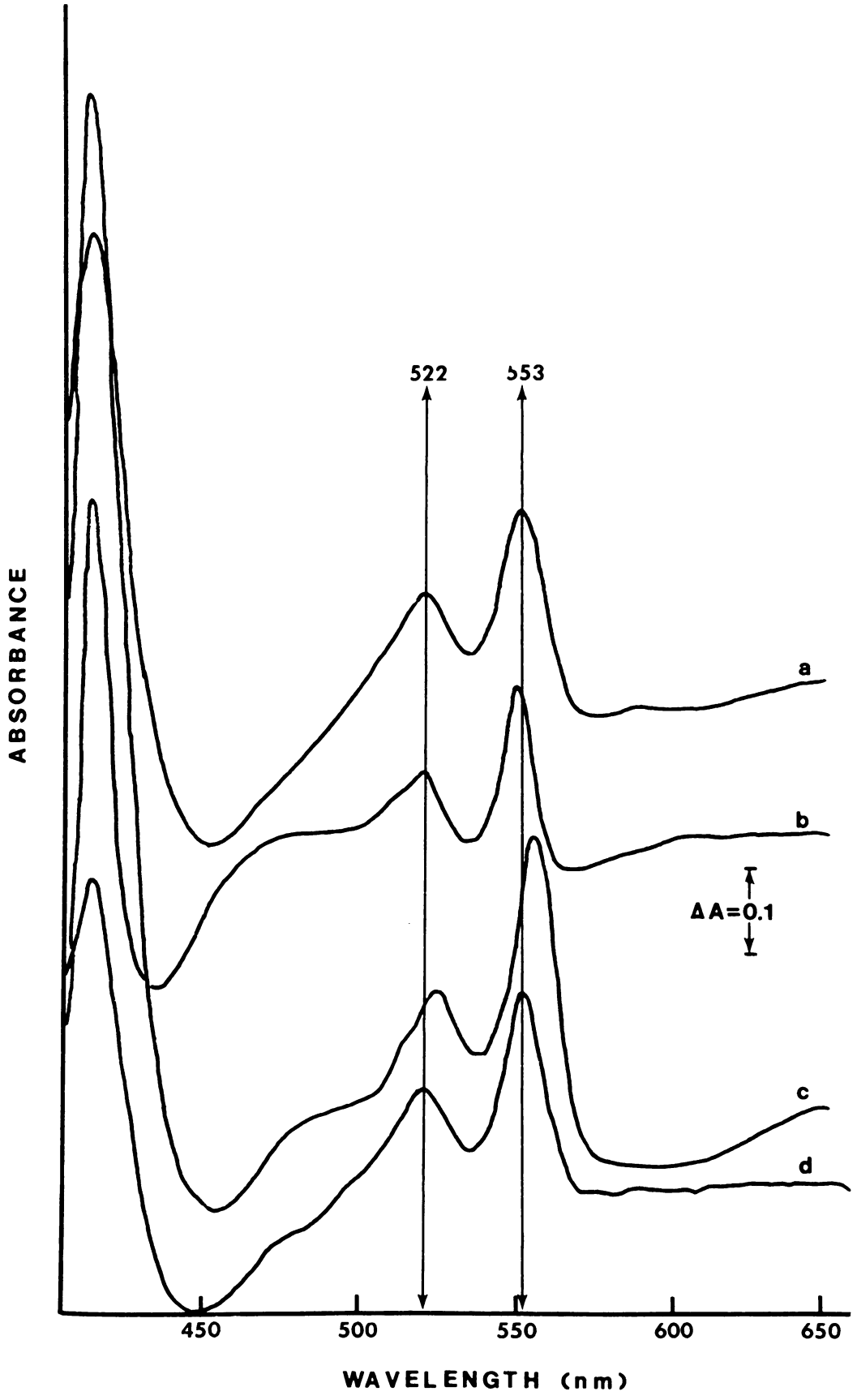

443
430
552
5min CO
15min CO
ΔA=0.6
15min - 5min CO
30min - 5min CO
ABSORBANCE
400
500
600
λ
Wavelength (nm)

difference spectrum after five minutes bubbling with CO. Cytochrome 552 continued to bind CO slowly in the next 30 minutes. The presence of another, low-affinity CO binding pigment was indicated by the slow appearance of the absorption of the CO complex at 430 nm during 30 minute exposure to CO. Unaerated cells and bacteroids appeared to contain only b-type (α-band at 560) and c-type (band at 551-552) cytochromes while aerobic cells had the usual combination of a b and c-type cytochromes (peak at 603), (4). No cytochrome P-450 was observed in CO-difference spectra of unaerated cells or bacteroids in contrast to the report of Appleby (3), (Figure 16).

The difference spectra of the reduced minus oxidized pyridine hemochromes of the extracts of unaerated cells also indicated the presence of a mixture of b-type and c-type cytochromes (Figure 17). The dithionite reduced minus ferricyanide oxidized difference spectra of extracts of unaerated cells and bacteroids had peaks at 553 nm and 415-416 nm. The difference spectrum of a mixture of c-type (peaks at 418 nm and 557 nm) cytochromes would have peaks at intermediate values (416 nm and 553-555 nm), similar to those observed in extracts of unaerated cells.

Figure 17. Pyridine hemochrome difference spectra.

a. Bacteroid extracts.
b. Cytochrome c (bovine heart).
c. Leghemoglobin.
d. Extracts of microaerobic cells.


522
553
a
b
ΔA=0.1
c
d
ABSORBANCE
450
500
550
600
650
WAVELENGTH (nm)

DISCUSSION

I. The heme pathway in *Rhizobium* has been investigated in an effort to improve our understanding of leghemoglobin biosynthesis. Most of the enzymes of the pathway have been identified before in various organisms but in this study ALAS activity has been identified and localized for the first time in a bacteroid-enriched fraction of soybean root nodules. It seems clear that cell-free extracts of *Rhizobium* bacteroids from soybean root nodules and cell-free extracts from free-living "microaerobic" *Rhizobium japonicum* cells have abundant activity of the first enzyme of the heme biosynthetic pathway, δ-aminolevulinic acid synthase (ALAS), whereas extracts of free-living aerobic cells have much lower, but still measurable activity. Early investigators who chalimed either no ALAS activity or very low and variable ALAS activity in root nodules (47) were possibly mistaken. They were probably misled by the large amount of Ehrlich positive material which is present in bacteroids even in the absence of ALA — hence the erratic appearance of spurious "ALA".

Partial purification of the ALA in the bacteroid reaction mixture by ion exchange before the use of colorimetric ALA assay, or replacing the acetylacetone reagent with ethyl acetoacetate before the colorimetric assay, combined with the use of a much more effective buffer, eliminated these assay problems. As a result, we can state with some confidence that the average activity of ALAS *in vitro* is about 40 nanomoles of ALA produced per gram nodules per hour. Inasmuch as the nodules produce heme (in leghemoglobin during the linear phase of growth at a rate of about 0.5 nanomoles per gram of nodules per hour requiring 4.0 nanomoles of ALA), it is clear that the *in vitro* activity of the enzyme is about 40 times greater than is needed for synthesis of the heme part of leghemoglobin in the nodules. However, we have no information about the activity of ALAS *in vivo* in bacteroids. Clearly the activities *in vivo* are less than the maximum activity *in vitro*, probably because the substrate and co-factors are all in excess *in vitro*, and the feedback inhibition exerted by heme probably operating *in vivo* is absent.

The changes in ALAS activity during nodule development (Figure 10) are paralleled by the changes in leghemoglobin accumulation; which suggests that ALAS activity may limit the formation of Lb. The fact that a great increase in ALAS (and ALAD) activity immediately

preceeds a similar increase in heme production in "micoaerobic" free-living *Rhizobium* cells points in the same direction. Nevertheless, this circumstantial evidence is not conclusive and a considerable amount of work is still required to establish that ALA synthesis is in fact the regulatory step in heme formation for leghemoglobin synthesis. Although heme and bacteriochlorophyll synthesis seem to be regulated by the activity of ALAS (51, 52, 71, 74, 79, 103), and chlorophyll formation in greening algae and higher plants is also characterized by increased ALA formation (8, 9, 10), however there is evidence pointing to additional or alternative regulatory reactions later in the pathway. Cutting and Schulman present evidence that heme synthesis in *Rhizobium* is subject of negative control by heme and positive control by the apoprotein of leghemoglobin (30). Other investigators demonstrated the regulatory role of the iron insertion step, which is catalyzed by ferrochelatase (43, 81). Various steps in the pathway may become rate limiting in the absence of a terminal electron acceptor (e.g. anaerobic conditions), (61).

ALAS activity was detected exclusively in the bacteroid enriched fraction of root nodules. The absence of activity from the plant cytoplasm fraction

was not due to the presence of an inhibitor in the plant fraction, since the addition of "plant extract" to "bacteroid extract" did not inhibit the enzyme activity in the latter, and also indicates that loss of ALA through the action of ALAD (which is quite active in the plant fraction) is not a problem. The failure to find ALAS activity in the plant cytoplasm fraction of the nodule is certainly consistent with the idea that the heme part of leghemoglovin is produced by the bacteroids. However, our failure and the failure of others to detect ALAS activity in the plant fraction of root nodules poses its own problems. Roots produce cytochromes, that necessitates heme synthesis, and therefore they presumably must produce ALA. Our failure to detect ALAS activity in the plant cytoplasm might be explained in several ways:

a.) After the bacteroids start producing large amounts of ALA (and heme) the plant enzyme could be deactivated or repressed. Cytochrome production would be, in that case, dependent upon bacteroidal ALA production and secretion of part of the ALA produced by symbiotic bacteria. In this case one would expect to detect some ALAS activity in non-nodulated root extracts — but such activity has not been found.

b.) It is possible that ALAS in the plant cells is compartmentalized (e.g. in mitochondria as in animal cells), (95), and active only in an intact compartment. The grinding methods employed were likely to rupture

fragile organelles and such a hypothetical compartment would probably have been destroyed. Other investigators also failed to detect plant ALAS activity (47).

c.) ALA produced by the plant fraction might have been rapidly metabolized by other enzymes in the extract (106) and therefore escape detection. However, in the experiments in which the two extracts were mixed this phenomenon would probably have shown itself as an apparent inhibition of ALA synthesis.

d.) The activity of an ALA-synthesizing enzyme in the plant fraction could be too low to be detected by the colorimetric method employed (below one nanomole per mg protein per hour) and still be sufficient to produce the minute amounts of ALA required to support mitochondrial cytochrome synthesis.

e.) It is quite possible that ALA is formed in the host plant cell via the recently proposed five-carbon pathway (10, 11, 87), in which case this activity would have escaped detection, because the required substance (glutamate or α-KG) was not supplied or because conditions *in vitro* were not optimal for its activity.

The coordination of the regulation of heme and apoprotein synthesis is a very intriguing problem. While the regulation of globin synthesis by heme in animal systems is fairly well established (54, 55, 78) the mechanism of the regulation of leghemoglobin production has never been identified. The regulation of apoprotein synthesis by heme in reticulocytes is mediated by a cascade of changes in various enzyme

activities resulting in a greatly amplified effect on general protein synthesis. This type of regulation is well suited for a cell specialized for producing essentially a single type of protein, such as a red blood cell. One would expect more specificity of a regulatory system operating in a living, functional plant cell which, probably, produces a multitude of proteins, while only the synthesis of hemoproteins might be expected to be regulated by heme.

Oxygen plays a central role in the regulation of cytochrome biosynthesis. In yeast, molecular oxygen is required for the synthesis of cytochrome (53) and in mammalian cell cultures intermittent anaerobiosis leads to a marked decrease in cytochrome oxidase activity (68). Heme apparently regulates the biosynthesis of cytochrome oxidase in some mammalian tissues (113), that is probably not the case in *Rhizobium* because the levels of cytochrome oxidase are lowest under conditions of increased heme synthesis (31 and present study).

The biosynthesis of cytochrome in higher organisms is somewhat similar to leghemoglobin synthesis in legume root nodules in the sense that in both cases the heme and the apoprotein are produced by different compartments. In thecase of cytochromes the initial and final stem of heme biosynthesis take place in the

mitochondria (several intermediate steps in the pathway take place in the cytoplasm), (96), while the apoprotein is synthesized in the cytoplasm. The assembly takes place in the cytoplasm most probably though this point is still debated (66). At any rate the transfer of the apoenzyme or the holoenzyme does take place across the mitochondrial membrane (34, 35, 64, 92). The biosynthesis of various cytochromes, especially mammalian cytochrome c has been extensively investigated (12, 13, 36, 49, 65, 94, 95) and can possibly serve as a model for leghemoglobin biosynthesis.

The coordination of the regulation of heme and apoprotein synthesis in soybean root nodules has additional interesting aspects due to the fact that regulation in this case is effected by an interaction between two different organisms. Leghemoglobin is probably the only known example of a symbiotically produced molecule and elucidation of the mechanisms regulating its synthesis will inevitably contribute to a better understanding of symbiosis.

II. The fact that rhizobia are able to start a new growth phase in an apparently depleted medium (Figure 11) as a result of a sudden decrease in oxygen supply merits further investigation. The data presented

in Table 2 seem to indicate that the aerobic stationary state is reached when the carbon source (mannitol) becomes yield limiting. At least the amount of growth achieved before the stationary phase is reached depends on the amount of mannitol originally present. This is surprising since the mannitol content of the medium is very high (10g/l) and the total mass of bacteria at the end of both growth phases is less than 1 g/l. It seems reasonable to suppose that the new growth phase results from a change in metabolism involving the utilization of different carbon (e.g. products of mannitol catabolism excreted or accumulated during aerobic growth) or nitrogen sources, although it is also possible that under "microaerobic" conditions some depleted minor growth factor ceases to be essential. It is interesting to note that while increasing the amount of yeast extract in the medium results in a relatively small increase in the cell density reached before stationary phase, the yeast extract concentration has a pronounced effect on the initial rate of growth (see day two in table 2). This suggests that the availability of nitrogen is the factor limiting the initial rate of growth while the availability of carbon determines the final yield. Characterization of the role of various macro and micro nutrients in the

attainment of the stationary phase would be best done in a chemostat on a defined medium.

The "microaerobic" conditions capable of inducing the new growth phase are also poorly defined. Since the dissolved oxygen concentration is very low (less than 0.1 ppm) in the later stages of "microaerobic" growth, it would be desirable to measure the rate of diffusion of oxygen into the culture. This rate must be equal to the rate of oxygen uptake into the cells once the oxygen concentration of the medium has reached a steady-state level (apparently about zero ppm). Since the rate of oxygen uptake by cells seems to be the major factor determining the characteristics of the new growth phase, once the rate of uptake is determined the "microaerobic" conditions could probably be reproduced in smaller vessels. At present investigation of the ill defined "microaerobic" state is hindered by the need to reproduce it with the large fermentor in which it was originally observed. A precise definition of the microaerobic state will make a detailed comparison between the microaerobic conditions inducing nitrogen fixation and those inducing heme production possible.

III. The discovery of the ability of rhizobia to grow under restricted aeration provides us with a novel

approach to the investigation of the metabolism of *Rhizobium*. *Rhizobium* has long been known to exist in nature in either one of two forms: an aerobic, free-living soil bacterium or a symbiotic bacteroid in the root nodules of legumes, where the levels of oxygen are known to be low. The free-living, "microaerobic" state constitutes a condition which is intermediate between vegetative cells and bacteroids in many respects. Rhizobia supplied with low levels of oxygen resemble bacteroids in appearance and in several of their physiological characteristics. Both bacteroids and "microaerobic" free-living cells are enlarged, nonmotile or sluggish cells with refractile granules which are composed of a sudanophylic material (poly-β-hydroxy-butyrate), (110). We have demonstrated that "microaerobic" rhizobia produce large amounts of heme (Figure 13) and that the activities of ALAS and ALAD, the first two enzymes of the heme biosynthesis pathway are about as high in these cells as in bacteroids. This activation appears to be specific to the enzymes of heme biosynthesis, since succinyl thiokinase — an enzyme which is not exclusively (or, indeed, primarily) associated with the pathway, does not undergo a similar increase (Figure 13b). A marked increase in the rate of synthesis of ALAS and ALAD as a result of

decreased aeration also has been observed in *Rhodopseudomonas spheroides*, where these enzymes which catalyze the first steps of bacteriochlorophyll synthesis are induced by a shift from dark to light without aeration (71, 74).

The fact that an increase in the activity of ALAS correlates with a proportional increase in heme accumulation and porphyrin excretion suggests that ALAS activity is a limiting factor in heme synthesis in cultured rhizobia as it seems to be in other tetrapyrrole forming systems (21, 51, 71, 74).

Cytochrome a-a_3 appears to be drastically reduced in the free-living cells supplied with low levels of oxygen, so that the cytochrome spectrum is similar to that of bacteroids and markedly different from the spectrum of aerobic cells in the 600 nm region (Figure 15). It is interesting to note that increased heme formation in *Rhizobium* is associated with a concomitant loss of cytochrome a-a_3. Bacteroids which are producing heme for Lb (3) and *R. japonicum* grown anaerobically with nitrate (31) both have an increased cytochrome content, but lack cytochrome a-a_3. In contrast, aerated free-living cells have cytochrome a-a_3, but produce relatively less heme (4). A similar correlation has been indicated in the activation of bacteriochlorophyll synthesis by low oxygen (83) in

Rhodopseudomonas. These results might suggest that cytochrome oxidase has some role in this oxygen effect. However, in a mutant of *Rhodopseudomonas capsulata*, which lacks cytochrome oxidase activity but retains the regulation of bacteriochlorophyll synthesis by oxygen, it is clear that cytochrome oxidase is not such a mediator (84). The relationship between the loss of cytochrome a-a_3 and the activation of tetrapyrrole synthesis in rhizobia remains to be explored.

There is a similarity between the conditions that induce nitrogen fixation and the conditions that induce heme synthesis in *Rhizobium*. Bacteroids in root nodules start producing large amounts of heme for leghemoglobin synthesis and start fixing nitrogen in a late stage of nodulation (89). Similarly restricted oxygen supply, which induces heme and porphyrin synthesis is free-living *R. japonicum*, also induces nitrogen fixation in "cowpea" strains of rhizobia (70, 86, 91, 105).

Although heme synthesis and nitrogenase activity could be corrdinately regulated in *Rhizobium*, there is also evidence pointing to the possibility of independent regulation. A mutant strain of *R. japonicum* produces small ineffective nodules (lacking Lb and incapable of fixing nitrogen); however, the same strain can be induced to fix nitrogen in culture (82).

The fact that a number of characteristics, previously believed to be present exclusively in the symbiotic state, were reproducible in culture has double significance:

a.) Reduced oxygen tension, capable of inducing numerous "bacteroid-like" characteristics, might possibly play a similar, inductive role in bacteroid development in root nodules. The role of reduced oxygen tension in regulating nitrogenase activity in nodules (16, 82) and in free-living rhizobia (17, 19) is being intensely investigated, but it seems possible that nitrogenase activation is only one aspect of a complex change in physiological state and metabolism, involving a change in the nutrient requirements, respiratory rate, growth rate and increased production of hemoproteins and free heme.

b.) Careful regulation of growth conditions, especially the oxygen tension and the nitrogen supply may result in the development of a method for growing "free-living bacteroids" capable of fixing nitrogen and excreting heme simulataneously. Exogenous supply of apoprotein might be necessary for heme excretion. Such a "synthetic-nodule" could be a valuable means for investigating the physiology of the symbiotic state and clarifying the relative importance of the interactions between the symbionts.

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