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THE EFFECT OF OXYGEN UPON PROTOPORPHYRINOGEN FORMATION IN *RHIZOBIUM JAPONICUM*

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Major professor

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THE EFFECT OF OXYGEN UPON PROTOPORPHYRINOGEN FORMATION IN *RHIZOBIUM JAPONICUM*

By

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ABSTRACT

THE EFFECT OF OXYGEN UPON PROTOPORPHYRINOGEN FORMATION IN *RHIZOBIUM JAPONICUM*

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Enzymic protoporphyrinogen formation in aerobic and anaerobic suspensions of *Rhizobium japonicum* strain 3I1b-110 was investigated. Aerobically incubated suspensions of both free-living and symbiotic bacteria metabolize aminolevulinic acid to uroporphyrinogen, coproporphyrinogen, and protoporphyrin. Suspensions of both free-living and symbiotic bacteria incubated under anaerobic conditions were found to produce reduced amounts of protoporphyrin when incubated in aminolevulinic acid. However, uroporphyrinogen and coproporphyrinogen production was increased suggesting that oxygen is required for coproporphyrinogen oxidation. Coproporphyrinogen oxidative decarboxylase (coproporphyrinogenase) activity in cell-free extracts of free-living and symbiotic bacteria was

also examined. These studies showed that oxygen is the major terminal electron acceptor for coproporphyrinogen oxidation. In the absence of oxygen, no protoporphyrin was formed in an *in vitro* coproporphyrinogenase assay unless a mixture of cofactors (ATP, NADP, Mg^{++}, and L-methionine) were added. In the presence of these cofactors, coproporphyrinogenase activity under anaerobic conditions was 5-10% of that observed under aerobic conditions.

These findings suggest two mechanisms for the oxidation of coproporphyrinogen in *R. japonicum*: an aerobic oxidation in which electrons are transferred to oxygen and an anaerobic process in which electrons are transferred to another oxidant, such as NADP. The significance of these findings with regard to the biosynthesis of heme in the microaerophilic legume root nodule is discussed.

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I would wish to thank Dr. Clifford Pollard and Mr. Philip Petty for their help in editing and proofreading this manuscript. Furthermore, I wish to thank Dr. Pollard and Mr. Petty for their friendship. I will not soon forget their kindness.

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LIST OF ABBREVIATIONS

Lb	Leghemoglobin
ALA	δ-Aminolevulinic Acid
PBG	Porphobilinogen
URO	Uroporphyrin
UROgen	Uroporphyrinogen
COPRO	Coproporphyrin
COPROgen	Coproporphyrinogen
PROTO	Protoporphyrin
PROTOgen	Protoporphyrinogen
COPROgenase	Coproporphyrinogen Oxidative Decarboxylase

INTRODUCTION

The Role of Leghemoglobin in Legume Root Nodules

Bacterial nitrogenase activity in legume root nodules is critically affected by the concentration of dissolved oxygen at the surface of the endosymbiotic bacteroids (1,2). Oxygen irreversibly inhibits nitrogenase activity and represses the synthesis of the enzyme (1). However, the large amounts of ATP required for nitrogenase activity are synthesized via oxidative phosphorylation reactions of aerobic respiration in *Rhizobium*. Leghemoglobins present in nitrogen-fixing legume root nodules appear to deliver oxygen to the bacteroid surface at a high flux but at a low partial pressure, thereby maximizing the simultaneous functioning of nitrogenase and oxidative phosphorylation (3, 4, 5). Furthermore, it has been suggested that leghemoglobin is an essential component of nitrogen fixation in legume root nodules, based upon the observation that nodules lacking leghemoglobin invariably lack nitrogenase activity (6).

Leghemoglobin is the generic name for a class of monomeric legume hemoproteins of molecular weight 15-17,000 daltons containing protoheme IX as a prosthetic

group (7). All leghemoglobins are characterized by their extremely high affinity for oxygen as compared to mammalian hemoglobins. Leghemoglobin is half-oxygenated (half oxyleghemoglobin) at an oxygen pressure of 0.05 mm Hg whereas mammalian hemoglobins are half-oxygenated at oxygen pressures of 4 to 14 mm Hg (5,8). However, leghemoglobins are generally similar in amino acid sequence (9) and spectral properties (10) to myoglobin and animal hemoglobins. Leghemoglobin is unique to legume nodule systems but nodules from differing species of legumes contain unique leghemoglobins differing in isoelectric point and amino acid composition (10). It has been shown that the apoprotein of leghemoglobin is synthesized in the plant cytoplasm and that the genes for globin are located on plant DNA (11,12,13). The *in vitro* synthesis of globin using mRNA isolated from legume nodules appeared to be of eukaryotic origin because the mRNA contained poly(A) and was isolated in the form of polysomes containing 80s ribosomes. While the biosynthesis of globin is largely determined by the plant host the synthesis of the heme appears to be bacterial in origin (14,15).

The Biosynthesis of Heme

Heme (Figure 1) is the prosthetic group required for the formation of cytochromes, hemoglobin, myoglobin, and a number of enzymes. The four ligand groups of the

tetrapyrrole form a square planar complex with the iron (16). The remaining fifth and sixth coordination positions are perpendicular to the plane of the tetrapyrrole. The fifth coordination position of leghemoglobin is occupied by an imidazole group of a histidine residue of the globin apoprotein. The sixth coordination position is unoccupied which allows the hemoprotein to bind oxygen, carbon monoxide, and cyanide.

The major intermediates of the heme biosynthetic pathway (Figure 2) in animals and bacteria have been well characterized (17). The pathway was deduced largely from the analysis of the enzymes of heme biosynthesis in duck erythrocytes and in bacteria. Shemin (18) and Neuberger (19) determined that glycine and succinate contributed nitrogen and carbon atoms to form the protoheme of hemoglobin. In bacteria, succinyl CoA and glycine are condensed to form the first compound committed to heme biosynthesis, δ-aminolevulinic acid (ALA). The pyridoxal phosphate-requiring enzyme, aminolevulinic acid synthase (EC 2.3.1.27) catalyzes the reaction. Two molecules of ALA are condensed to form the monopyrrole, porphobilinogen (PBG) by the enzyme aminolevulinic acid dehydrase (EC-4.2.1.24). Four molecules of PBG are condensed in a complex reaction catalyzed by two enzymes; PBG deaminase and uroporphyrinogen synthase, to yield the first tetrapyrrole intermediate, uroporphyrinogen III (UROgen III). UROgen III, and eight carboxyl hexahydroporphyrin is

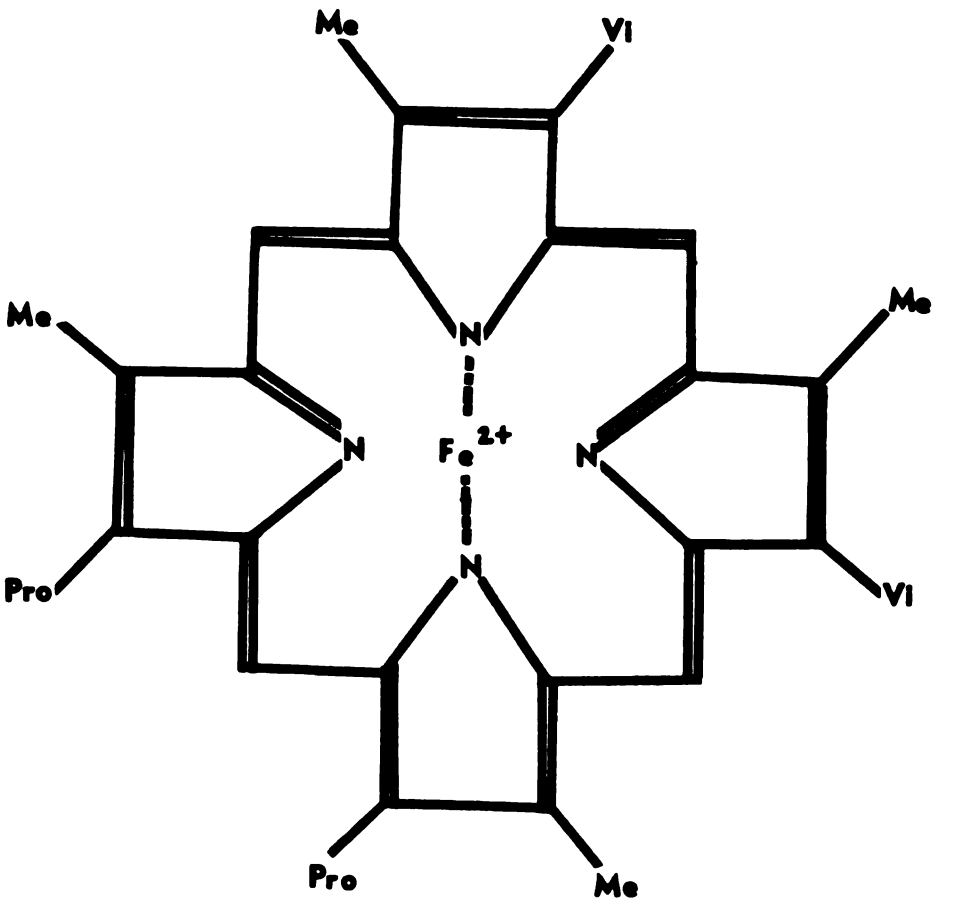


Figure 1. The Structure of the Heme Molecule.

Abbreviations: Vi = vinyl ($-CH=CH_2$)
Me = methyl ($-CH_3$)
Pro = propionic acid ($-C_2H_4CO_2H$)

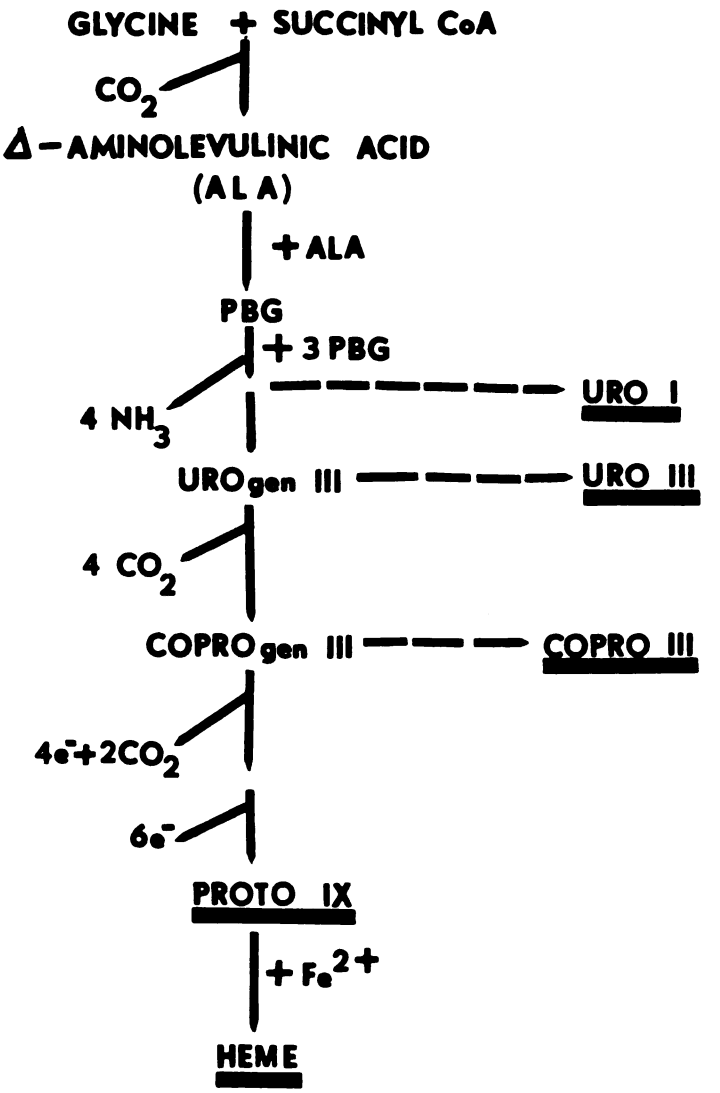


Figure 2. The Heme Biosynthetic Pathway.
(Pigmented compounds are underlined)

Abbreviations: PBG = porphobilinogen
URO = uroporphyrin
UROgen = uroporphyrinogen
COPRO = coproporphyrin
COPROgen = coproporphyrinogen
PROTO = protoporphyrin

decarboxylated by the enzyme UROgen decarboxylase to form coproporphyrinogen III (COPROgen III), a four carboxyl hexahydroporphyrin. COPROgen III is oxidatively decarboxylated by the enzyme COPROgen oxidative decarboxylase (COPROgenase) to form protoporphyrinogen IX (PROTOgen IX), a two carboxyl hexahydroporphyrin. The tetrapyrrole ring of PROTOgen IX is oxidized by the enzyme PROTOgen oxidase to form protoporphyrin IX (PROTO IX). The conversion of COPROgen III to PROTO IX is subsequently described in greater detail. Ferrochelatase catalyzes the insertion of ferrous iron into PROTO IX to form the metalloporphyrin heme. The heme biosynthetic pathway can be regulated by end product inhibition and repression of ALA synthase activity (20,21).

The Oxidation of COPROgen to PROTOgen

The enzymatic conversion of COPROgen to PROTO are the only oxidative steps in the biosynthesis of heme. These reactions involve the oxidative decarboxylation of two propionic acid side chains of COPROgen yielding two vinyl groups and the removal of six tetrapyrrole hydrogens from the PROTOgen formed to yield PROTO (22). The enzymatic conversion of COPROgen to PROTO has been examined in a wide variety of organisms (23, 24, 25). Until recently one enzyme, COPROgenase, was thought to catalyze the oxidation. However, Poulson *et al.* (26) were able to

demonstrate PROTOgen IX oxidation to PROTO IX by a 180,000 dalton enzyme purified from yeast mitochondria. The yeast PROTOgen oxidase did not catalyze the oxidation of COPROgen III or UROgen III. The enzyme was solubilized from mitochondria by sonication in the presence of detergent and thus may be membrane bound *in vivo*. Enzymic PROTOgen IX oxidation was also demonstrated using membrane particles isolated by fractional centrifugation from anaerobically grown *Eschericia coli* (27). Again, neither COPROgen III nor UROgen III was oxidized by the membrane preparation. These experiments suggest that two enzymes, COPROgenase and PROTOgen oxidase, catalyze the conversion of COPROgen III to PROTO IX.

The properties of COPROgenase isolated from animal mitochondria have been extensively investigated. COPROgenase from rat liver mitochondria was purified 60 fold and yielded an electrophoretically homogeneous preparation of molecular weight of about 80,000 daltons (57). The enzyme was found to have an absolute requirement for oxygen, which was determined to be the electron acceptor during the oxidative decarboxylation of the propionic acid residues. Flavin mononucleotides, 1,4-napthoquinone, and hydrogen peroxide were unable to replace oxygen as the electron acceptor for the reaction (57). The enzyme required no cofactors for activity and only COPROgen III served as the biological substrate.

Sano and Granick (23) were first to observe, during

the course of COPROgen decarboxylation, the formation of a tricarboxylic acid porphyrin which was rapidly transformed to PROTOgen. Evidence based upon mass spectrometry of a tricarboxylic acid porphyrin isolated from the harderian gland of the rat (56) indicated that the oxidative decarboxylation of COPROgen takes place in a sequential order (Figure 3). The C-2 propionic acid residue of COPROgen is oxidized first and is followed by the oxidation of the C-4 propionic acid side chain. However, the exact mechanism of the enzymatic decarboxylation of COPROgen is still in dispute.

The apparent absolute requirement for oxygen by COPROgenase isolated from plants and animals stimulated investigations of PROTO formation by the anaerobic bacteria *Rhodopseudomonas spheroides* and *Chromatium D*, which synthesize bacteriochlorophyll during anaerobic growth. PROTO formation by nonphotosynthetic bacteria, particularly *E. coli* and several species of *Pseudomonas* which are known to produce cytochromes during anaerobic growth was also investigated (29). Cell-free extracts of bacteria were examined for oxygen dependent COPROgenase activity (aerobic activity) as well as for COPROgenase activity utilizing alternate electron acceptors under anaerobic conditions (anaerobic activity).

Oxygen dependent COPROgenase activity has been demonstrated in both aerobically and anaerobically grown *E. coli* (29), aerobically grown *P. denitrificans* (29),

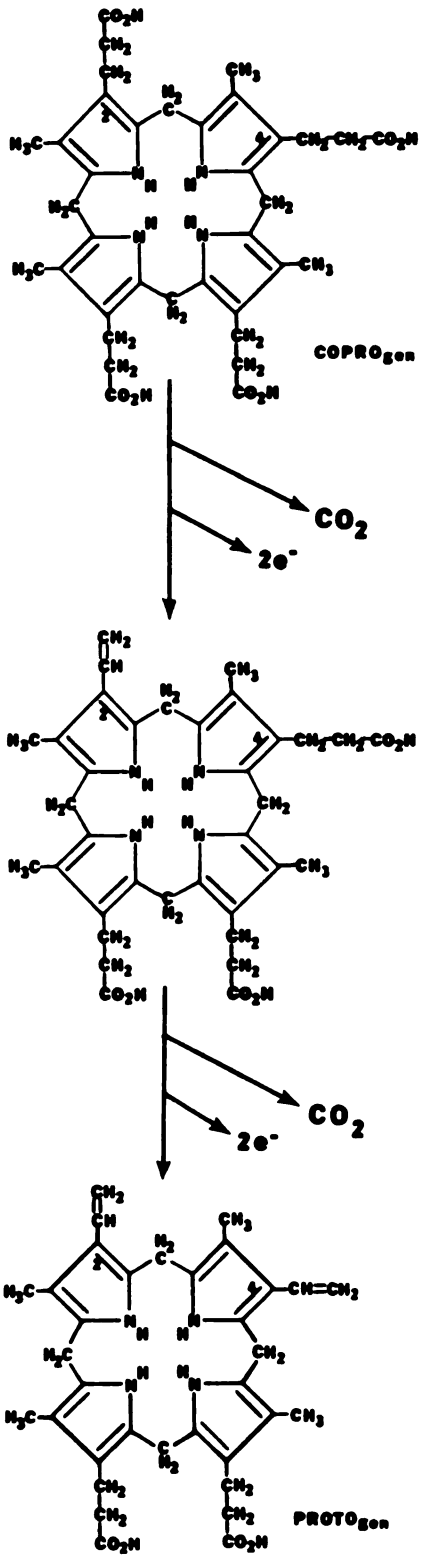


Figure 3. The Sequential Oxidative Decarboxylation of COPROgen III to PROTO IX.

in both aerobically and anaerobically grown *R. spheroides* (30), and in both aerobically and anaerobically grown *Chromatium D* (30,52). COPROgenase from *Chromatium D* has been partially purified (30) and the enzyme appeared to have no cofactor requirement for aerobic activity and was unaffected by iron chelating agents. In general, the properties of the bacterial enzyme were similar to mitochondrial COPROgenase studied by Sano and Granick (23). While an oxygen dependent COPROgenase activity was demonstrated in many bacteria, the corresponding anaerobic activity has been demonstrated in only a limited number of bacteria and the reaction mechanism is poorly understood.

Ehteshamuddin (32) reported that extracts of aerobically grown *Pseudomonas sp.* could convert COPROgen to PROTOgen under both aerobic and anaerobic conditions. Other workers, however, were unable to demonstrate anaerobic COPROgenase activity in *Pseudomonas*. Tait (30) was able to demonstrate anaerobic COPROgenase activity in extracts of photosynthetically grown *R. spheroides* only if the extracts were supplemented with ATP, magnesium, L-methionine, and NADP. Iron chelating agents, 2,4-dinitrophenol, and flavin markedly inhibited anaerobic COPROgen oxidation. Extracts of *R. spheroides* subjected to centrifugation at 105,000xg for 90 minutes exhibited little or no COPROgenase activity under anaerobic conditions when the resulting supernatant fluid and pellet were

assayed separately. However, recombination of the two fractions resulted in the recovery of approximately 75% of the anaerobic COPROgenase activity originally present in the crude extract. In contrast, oxygen dependent COPROgenase activity was located only in the supernatant fluid of extracts subjected to high speed centrifugation. Based upon these data, Tait (30) suggested that the electrons removed from the propionic acid side chains of COPROgen may be donated to NAD(P) in an energy requiring step involving members of the electron transport chain (Figure 4). Tait (30) also demonstrated COPROgen oxidation under anaerobic conditions in extracts of *Chromatium D* which were supplemented with NADP,ATP, Mg^{++}, and S-adenosylmethionine (SAM). The role of SAM in the reaction is unclear. Tait suggested that SAM acts as an allosteric effector because there was no evidence that the conversion of COPROgen to PROTOgen requires preliminary methylation of the enzyme or of some other macromolecule.

Enzymic COPROgen oxidation under anaerobic conditions was not demonstrated in either *E. coli* or several species of *Pseudomonas* (29). These organisms, however, produced cytochromes when grown under conditions of nitrate respiration in the presence of ALA. Jacobs *et al.* (31) suggested that these organisms may possess an oxygen independent mechanism for COPROgen oxidation, but one that is inactivated during cell disruption by sonication.

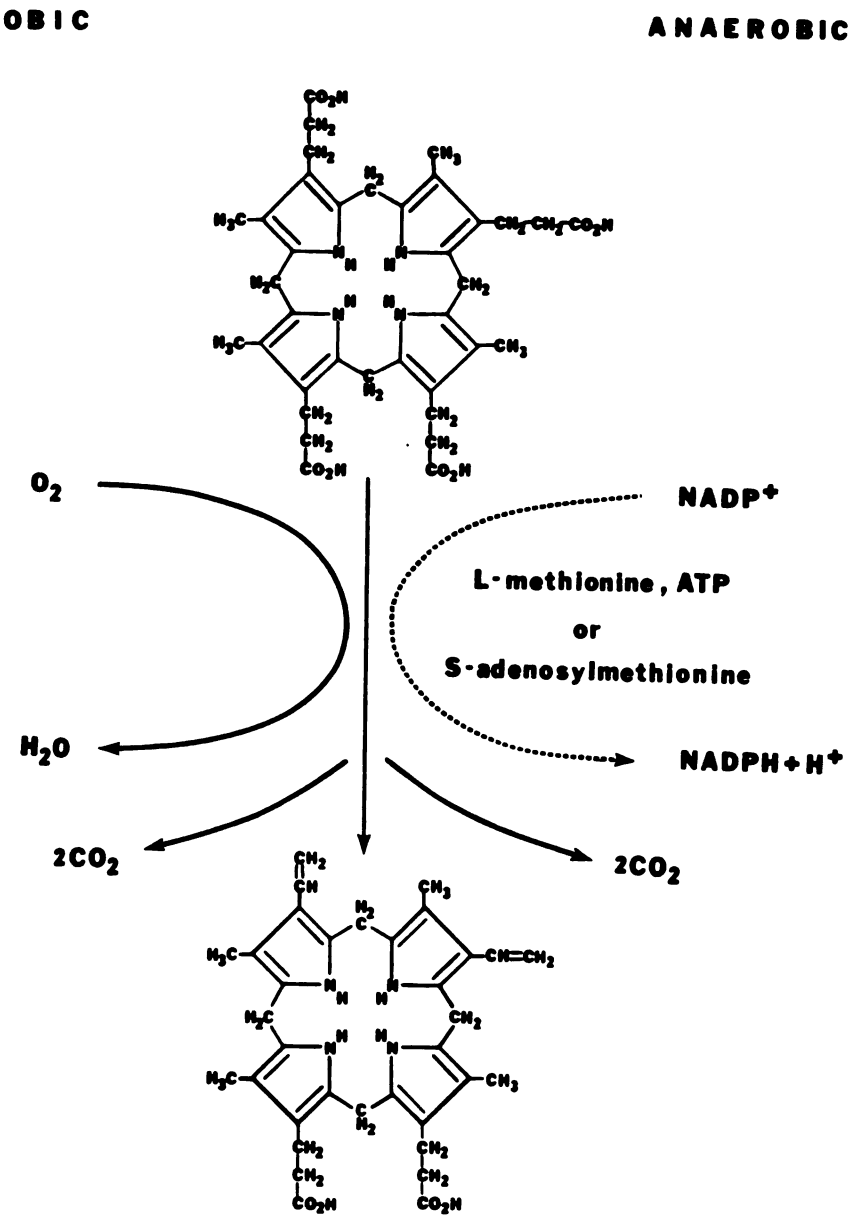


Figure 4. The Oxidative Decarboxylation of COPROgen to PROTOgen by Bactera.

Oxygen dependent aerobic mechanism shown on right.

Postulated anaerobic mechanism shown on left.

Specific Problems of COPROgen Oxidation in *Rhizobium*

Cutting and Schulman (14) demonstrated that heme synthesis in soybean root nodules is principally in the bacteroids. Furthermore, Avissar and Nadler (33) have shown that ALA dehydrase activity in mature nodule tissue is located in the bacteroids. These experiments suggest that the large amounts of heme required for leghemoglobin biosynthesis in nitrogen-fixing legume nodules is bacterial in origin. The characterization of the COPROgenase present in bacteroids is of considerable importance because symbiotic bacteria appear to synthesize heme under the extremely low oxygen tensions present in legume nodules.

In this study, we found that aerobic and anaerobic suspensions of free-living or symbiotic *R. japonicum* produce large amounts of porphyrins from ALA. PROTO accumulation from ALA was used to qualitatively determine the COPROgenase activities of free-living and symbiotic *R. japonicum*. PROTO accumulation in suspensions of free-living bacteria was strongly inhibited by anaerobiosis while PROTO production by symbiotic bacteria was similar during aerobic and anaerobic incubation conditions. These findings suggest that symbiotic bacteria possess a mechanism for oxidizing COPROgen under anaerobic conditions. Cell-free extracts of free-living and symbiotic bacteria were also examined for COPROgenase activity under aerobic and anaerobic conditions. COPROgenase activity in

extracts of symbiotic bacteria incubated under aerobic and anaerobic conditions was about four times higher than COPROgenase activity in extracts of free-living bacteria. These findings support the postulated existence of an anaerobic mechanism for COPROgen oxidation in facultative aerobic bacteria.

MATERIALS AND METHODS

Media

Bacteria were grown on a synthetic glucose-casein hydrosylate (BgluCh) liquid medium (55) containing in grams per liter: K_2HPO_4, 3.6; KH_2PO_4, 0.4; $MgSO_4 \cdot 7H_2O$, 0.05; $(NH_4)_2SO_4$, 1.0; NaCl, 0.5; glucose, 5.0; biotin, 0.0025; casein hydrosylate (Difco), 1.0. Minimal salts medium was adjusted to pH 7.4 with 5N KOH and was sterilized under 15 p.s.i. at 120 C for 20 minutes per liter medium. Glucose, biotin, and casein hydrosylate were autoclaved separately for 20 minutes per liter solution at 15 p.s.i. Complete liquid medium was prepared by the aseptic addition of sterile supplements to the sterile minimal salts medium.

Nitrogen-free legume nutrient medium was prepared as described by Johnson *et al.* (34), 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; $CaSO_4 \cdot 2H_2O$, 1.3; $CaCl_2 \cdot 2H_2O$, 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 (kindly supplied by Dr. D. Weber, Beltsville, MD) was maintained on BgluCh agar (1.5%) slants and was transferred every three months. Liquid cultures, in 100 ml liquid BgluCh were prepared from slants. When outgrowth occurred, one-liter flasks containing 500 ml BgluCh medium were inoculated with 5 ml of culture and were aerated by agitation on an orbital shaker (130 r.p.m.) for four to five days. Cells were harvested by centrifugation at 10,000xg for 20 minutes, washed, and resuspended into either buffered growth medium (as described subsequently) and were used immediately.

Soybean seeds (*Glycine max* var. *Hark*) were surface sterilized for one minute by immersion into a solution of 1% NaOCl, rinsed in running tap water, and were soaked in water for three hours before being planted in a Vermiculite-Perlite (1:1, v/v) bed. The bed was inoculated with a suspension of *R. japonicum* 110, in 500 ml nitrogen-free medium. Aerated nitrogen-free medium was circulated through the bed of seeds for one week. The beds were illuminated by two 40 W Grolux fluorescent lamps (Westinghouse Elec. Corp., Bloomfield, NJ), at a distance of 60 cm from the bed surface. Inoculated seedlings were transplanted into pots (5 to 7 seedlings per pot) containing an inert planting medium consisting of equal parts of Vermiculite and Perlite and were grown in a greenhouse.

Seedlings were watered with nitrogen-free medium. Once a week the pots were flushed with tap water to remove accumulated salts. Under these inoculation and growth conditions nodules formed predominately at the crown of the root (35). Crown nodules were harvested 25-35 days after inoculation.

Methods for Determining Growth of Bacteria

Growth of free-living *R. japonicum* in liquid culture was determined using a Klett colorimeter equipped with a standard red filter. Absorbance units were expressed in Klett units.

Aliquots of sterile culture were diluted with sterile 0.1 M NaCl and 0.1 ml of the final dilution was plated onto three BgluCh agar (1.5%) plates. The plates were incubated at 30 C for five days before the colonies were counted. The number of viable cells in the original culture was calculated according to the plate count.

Isolation of Bacteroids

One to three grams of nodules from 25-35 day old plants were homogenized with a chilled mortar and pestle at 0-4 C in 15 ml 0.1 M HEPES-NaOH, ph 7.4 containing 0.25 M pentaerythrytol and 1.0 mM $MgCl_2$. The brei was filtered through four layers miracloth and the crude filtrate was centrifuged at 150xg to remove plant debris.

The supernatant fluid was centrifuged at 12,000xg. The crude bacteroid pellet was washed in fresh extraction buffer and was again centrifuged at 12,000xg. The bacteroid pellet was resuspended into the subsequently described assay buffers supplemented with 0.25 M pentaerythrytol. Contamination of the bacteroid preparation by leghemoglobin was considered to be minimal because no leghemoglobin was detected in subsequent bacteroid cell-free extracts determined by the pyridine-hemochromogen method (41).

Analytical Methods

Bacterial protein concentrations were determined by the Coomassie Blue dye binding method of Bradford (36) using bovine gamma globulin as the standard.

Production of Porphyrins and PBG by Dense Cell Suspensions

Bacterial cells were harvested by centrifugation as previously described and were resuspended into BgluCh medium supplemented with 0.1 M potassium phosphate, pH 7.4 and 1.0 mM ALA. The controls contained no ALA. Cells were resuspended to a final protein concentration of 0.8 to 1.0 mg protein per ml. Suspensions of bacteroids were prepared using the assay buffer described above supplemented with 0.1 mM sodium succinate. Cells were incubated either aerobically in open tubes (5 ml cells in 20 ml

test tubes) or anaerobically in filled, stoppered test tubes at 28 C on a reciprocal shaker (60 c.p.m.). After 10 minutes incubation the dissolved oxygen concentration present in the closed tubes was measured using a Clark type oxygen electrode (Yellow Springs Inst., Yellow Springs, OH). The experimental values obtained were compared to a zero point dissolved oxygen control using the sodium dithionite method according to Cooper (54). After four to six hours the accumulated PBG and porphyrins were determined as subsequently described.

PBG Determination

Two ml samples of cell suspensions were added to 0.1 ml 100% trichloroacetic acid (TCA). The samples were mixed and were centrifuged in a clinical centrifuge at power setting 6 to remove precipitated cell debris. The concentration of PBG present in the supernatant fluid was determined spectrophotometrically at 555 nm using the color reaction of PBG ($\epsilon = 6.1 \times 10^4$) with modified Ehrlich's reagent (42).

Quantitation of Porphyrins by Fluorescence

Samples of cell suspensions were added to a 10 fold excess of methanol:sulphuric acid (20:1, v/v) to precipitate cellular protein and to methylate the extracted porphyrins. Porphyrin fluorescence was measured in a Turner Filter Fluorometer, Model III, equipped with a

primary filter of 405 nm (Turner no. 110-812) and a secondary filter of 595 nm (Turner no. 110-820). Tubes were placed in the semimicro holder and fluorescence values were recorded using the 3X sensitivity setting.

Florisil Column Chromatography of Porphyrins

Samples of cell suspensions were acidified with 15% HCl to a final concentration of 3% HCl and were further disrupted by cavitation using three 30-second bursts from a Sonifier-Cell Disruptor (Model W185, Heat Systems Ultrasonics, Inc.) at power setting 4. The sonicate was allowed to stand in weak light for 20 minutes to oxidize the porphyrinogens present. The crude extract was centrifuged to remove precipitated proteins and samples of the supernatant were layered onto a 1.5 X 8 cm column of activated Florisil (100-200 mesh, Sigma) that had been equilibrated to 5% HCl (39). The Florisil was activated with water to a final concentration of 10% water as described by Radin (53). Porphyrins were differentially step eluted as follows: uroporphyrin, in 1.0% NH_4OH; coproporphyrin, in 0.1% NH_4OH:acetone (12:1,v/v); protoporphyrin, in 0.1% NH_4OH:ethanol (1:3, v/v). Porphyrins in the eluted fractions were analyzed by thin layer chromatography (see page 21) and were found to contain only one porphyrin. Samples of each fraction were acidified to a final concentration of 3% HCl and the porphyrins were quantitated fluorometrically as previously described.

Coproporphyrin concentrations were determined using a standard coproporphyrin curve. Uroporphyrin and protoporphyrin concentrations were determined from the coproporphyrin curve using the correction factors of 0.75 and 1.25, respectively, according to Schwartz (39).

Porphyrin Chromatography

Samples of cell suspensions were added to a 20 fold excess of methanol:sulphuric acid (20:1, v/v) and were placed in the dark for at least 24 hours to methylate the extracted porphyrins. Precipitated proteins were removed by centrifugation and the methanol:sulphuric acid extract was brought to pH 5-6 with 3% $NaHCO_3$. Porphyrins were partitioned into chloroform until no porphyrins, as determined by red fluorescence, were present in the aqueous phase. The organic phase was washed three times with 3% NaCl and was subsequently evaporated to dryness. The porphyrin methyl ester residue was taken up in 100 microliters ethyl acetate and the solution was used directly for thin layer chromatography of the porphyrin methyl esters.

Porphyrin methyl esters were separated by TLC using the following two solvent systems:

A.) Benzene-ethyl acetate-ethanol, 190:20:7.5 (v/v) on silica gel G1 plates (Brinkman) (37).

B.) Water-acetonitrile-dioxane, 2:7:1 (v/v) reverse phase chromatography on Whatman reversed phase KC_{18} plates.

Porphyrins were located by their characteristic red fluorescence under long wave UV light. Commercially prepared porphyrin methyl esters (Sigma) were used as standards.

Incorporation of ^{59}Fe into Heme by Dense Cell Suspensions

The rate of heme synthesis was determined according to the method of Dailey and Lascelles (40). Cells were incubated in BgluCh medium supplemented with 0.1 M HEPES-NaOH, pH 7.4. $[^{59}Fe]Cl_3$ was added to the cell suspensions to a final activity of 1.0 microcurie per ml suspensions. Cells were incubated in open and closed tubes for 30 minutes to one hour. ^{59}Fe incorporation was terminated by the addition of 1.0 ml 0.2 N HCl and 2.0 ml methyl ethyl ketone per ml reaction mixture. The phases were mixed and were allowed to stand overnight at 4 C. After centrifugation the lower, aqueous phase was removed, and 1.0 ml fresh methyl ethyl ketone and 1.0 ml 0.1 N HCl were added per ml original reaction mixture. After partitioning, the tubes were placed on ice for two hours. After centrifugation, 1.0 ml of the methyl ethyl ketone phase was added to 10 ml scintillation counting cocktail (ACS, Amersham). Vials were counted in the combined ^{3}H-^{14}C-^{32}P window of a Beckman Scintillation Counter, Model LS100C for one minute. Samples of the methyl ethyl ketone phase were evaporated to dryness and the residue was taken up in 100 microliters pyridine. Ten microliters pyridine were spotted onto a

Silica Gel 60 plate and were developed in 2,6-lutidine: water:NH_3 (10:3, v/v) against a protohemin standard (Sigma) according to Falk (38).

Preparation of Cell-Free Extracts

Cells harvested by centrifugation were resuspended into 10 ml 1.0 M HEPES-NaOH, pH 7.5 containing 0.25 M pentaerythrytol and 10 mM dithiothreitol. The cells were broken by cavitation using twelve 15-second bursts from a Sonifier-Cell Disruptor at power setting 4. The crude sonicate was centrifuged for 8 minutes at 25,000xg. The resulting clear supernatant fluid was used immediately or was stored at -20 C for future use. No loss of enzymatic activity occured during storage for seven days.

Preparation of Sodium Amalgam

Sodium amalgam (3%) was freshly prepared in small batches according to Mann and Sounders (43). The prepared amalgam was stored at room temperature in a vacuum dessicator and was stable for several weeks. The amalgam was ground to a fine powder in a mortar immediately before use.

Preparation of Coproporphyrinogen

Coproporphyrinogen was prepared by the addition of 10 mg finely powdered amalgam to 10 ml of 2 X 10^{-4} M

deoxygenated coproporphyrin in 0.01 M KOH. The solution was allowed to stand in the dark for 15-30 minutes until no visible pink color was observed in solution. The solution was filtered through a sintered glass funnel and the filtrate was titrated to pH 8 with 40% H_3PO_4. The filtered solution was gassed with Ultra-pure Argon gas (Matheson) and was used immediately.

COPROgenase Assay

The COPROgenase assay was performed under aerobic conditons in 15 ml test tubes and the standard incubation mixture contained 2 ml cell-free bacterial extract and 0.5-0.75 ml freshly prepared COPROgen. Controls were prepared using 0.1 M HEPES-NaOH, pH 7.5 in place of bacterial extract. The open tubes were shaken vigorously (180 r.p.m.) in the dark at 38 C for 90 minutes in a water bath shaker (American Optical). The reaction was stopped by the addition of 8 ml ethylacetate:acetic acid (3:1, v/v) and the tubes were allowed to stand in weak light for 30 minutes to oxidize the porphyrinogens present. The resultant porphyrins were differentially extracted by the increasing HCl number method of Sano and Granick (23). The concentrations of porphyrins recovered were determined in a Cary no. 15 Spectrophotometer (Varian Inst.). Absorbancy was measured for COPRO at 401 nm ($\epsilon = 4.7 \times 10^{-5}$), for PROTO at 408 nm ($\epsilon = 2.4 \times 10^{-5}$), and for a three carboxyl porphyrin at 403 nm (est. $\epsilon = 3.6 \times 10^{-5}$). Porphyrins were further

characterized by thin layer chromatography on Silica Gel 60 plates developed in 2,6-lutidine:H_2O:NH_3 (10:3, v/v) according to Falk (38). Commercial porphyrins were used as standards and the chromatographed porphyrins were located as red fluorescent areas under UV light.

Enzyme preparations were incubated under anaerobic conditions in Thunberg tubes sparged with Ultra-pure argon gas (oxygen contamination less than 2 ppm, Matheson). The standard reaction mixture contained 2 ml bacterial extract, 0.5-0.75 ml COPROgen, and the following cofactors (in micromoles): ATP, 2; NADP, 2; $MgSO_4$, 10; L-methionine, 0.5. Control tubes were prepared as described for the aerobic assay. All tubes were exhaustively sparged with argon gas and were evacuated prior to incubation at 38 C in the dark for 90 minutes. The reaction was stopped by the addition of 10 ml ethylacetate:acetic acid (3:1, v/v) and the porphyrins were extracted as previously described for the aerobic assay.

RESULTS

Free-living *R. japonicum* cells incubated aerobically in a buffered growth medium containing ALA produced PBG and porphyrin at a linear rate over a period of six hours (Figure 5). Porphyrin production was saturated at ALA concentrations greater that 1.0 mM (Figure 6). Furthermore, concentrations of cells equivalent to protein concentrations greater than 1.0 mg protein per ml of cell suspension did not increase porphyrin synthesis from ALA (Figure 7). In contrast, PBG production from ALA was linear with time, with increasing ALA concentrations, and with increasing bacterial protein concentrations (Figures 5, 6, and 7) under all conditions tested. These findings indicate that neither the uptake of ALA, which presumably occurs in bacteria by the L-lysine transferase system, nor the activity of ALA dehydrase limits the production of porphyrins from ALA in free-living *R. japonicum*. In the absence of ALA, free-living bacteria accumulate only one to five picomoles porphyrin per milligram protein when incubated aerobically in a buffered growth medium. The values for porphyrin production shown in Figures 5, 6, and 7 represent the amount of porphyrin formed from ALA minus the small amount of porphyrin formed in the absence of ALA.

Figure 5. Production of PBG and Porphyrins by Suspensions of Laboratory Cultured *R. japonicum* Incubated Aerobically in 1.0 mM ALA.

Cells were harvested by centrifugation and were resuspended into buffered growth medium containing 1.0 mM ALA. Cells were incubated at 28 C for the time periods indicated.

PBG was determined colorimetrically as previously described.

Porphyrins were determined fluorometrically. 1.0 relative fluorescence unit = 1.5 pmole COPRO.

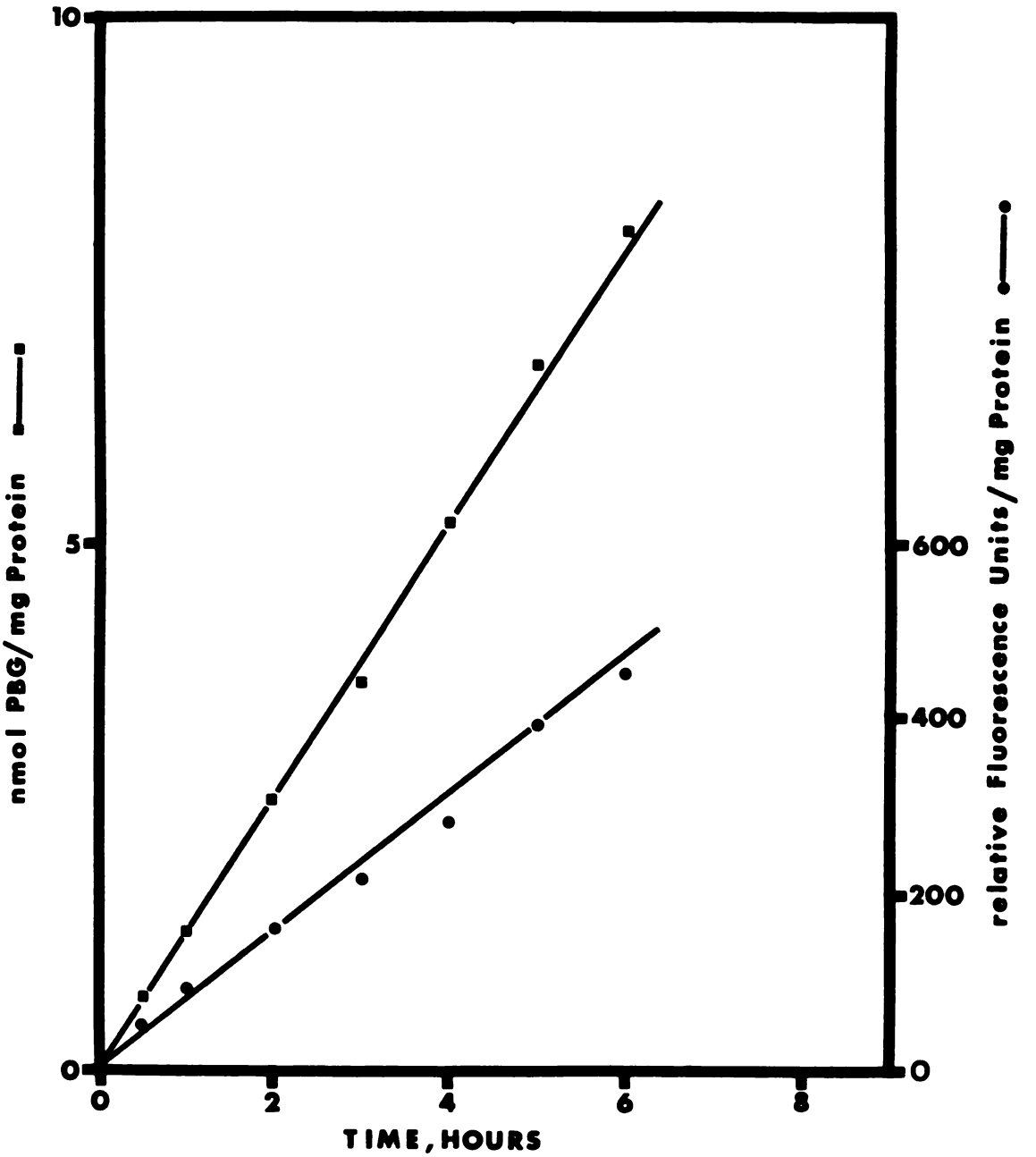

10
5
0
nmol PBG/mg Protein
600
400
200
0
relative Fluorescence Units/mg Protein
0
2
4
6
8
TIME, HOURS

Figure 6. Production of PBG and Porphyrins by Dense Cell Suspensions of *R. japonicum* Incubated Aerobically in Increasing Concentrations of ALA.

Cells were resuspended to 0.8 to 1.0 mg cell protein per ml buffered growth medium containing the indicated concentrations of ALA.

Cells were incubated at 28 C for 4 hours.

PBG was determined colorimetrically as previously described.

Porphyrins were determined fluorometrically as previously described.

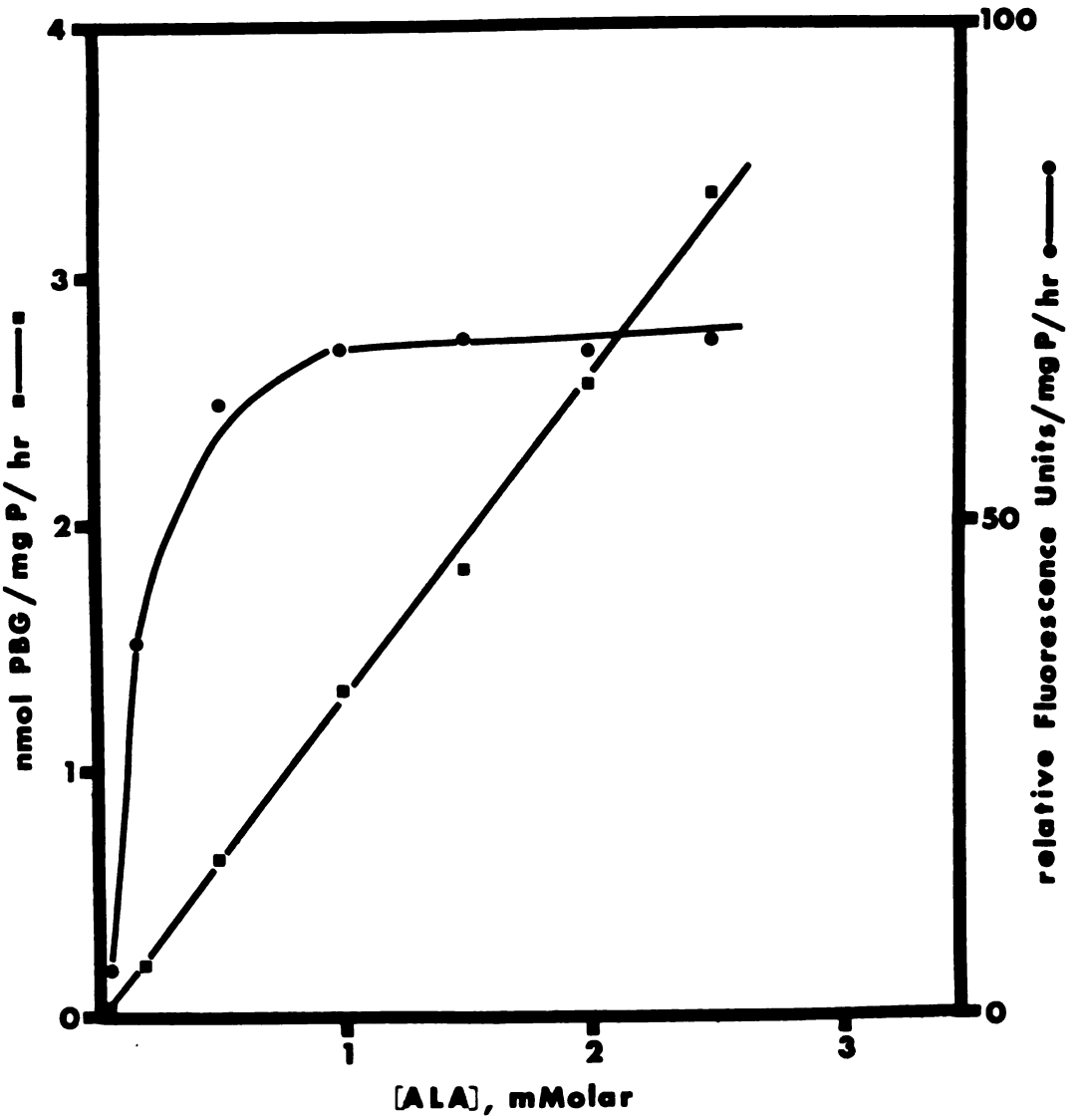

4
3
2
1
0
100
50
0
1
2
3
nmol PBG / mg P / hr
relative Fluorescence Units / mg P / hr
[ALA], mMolar

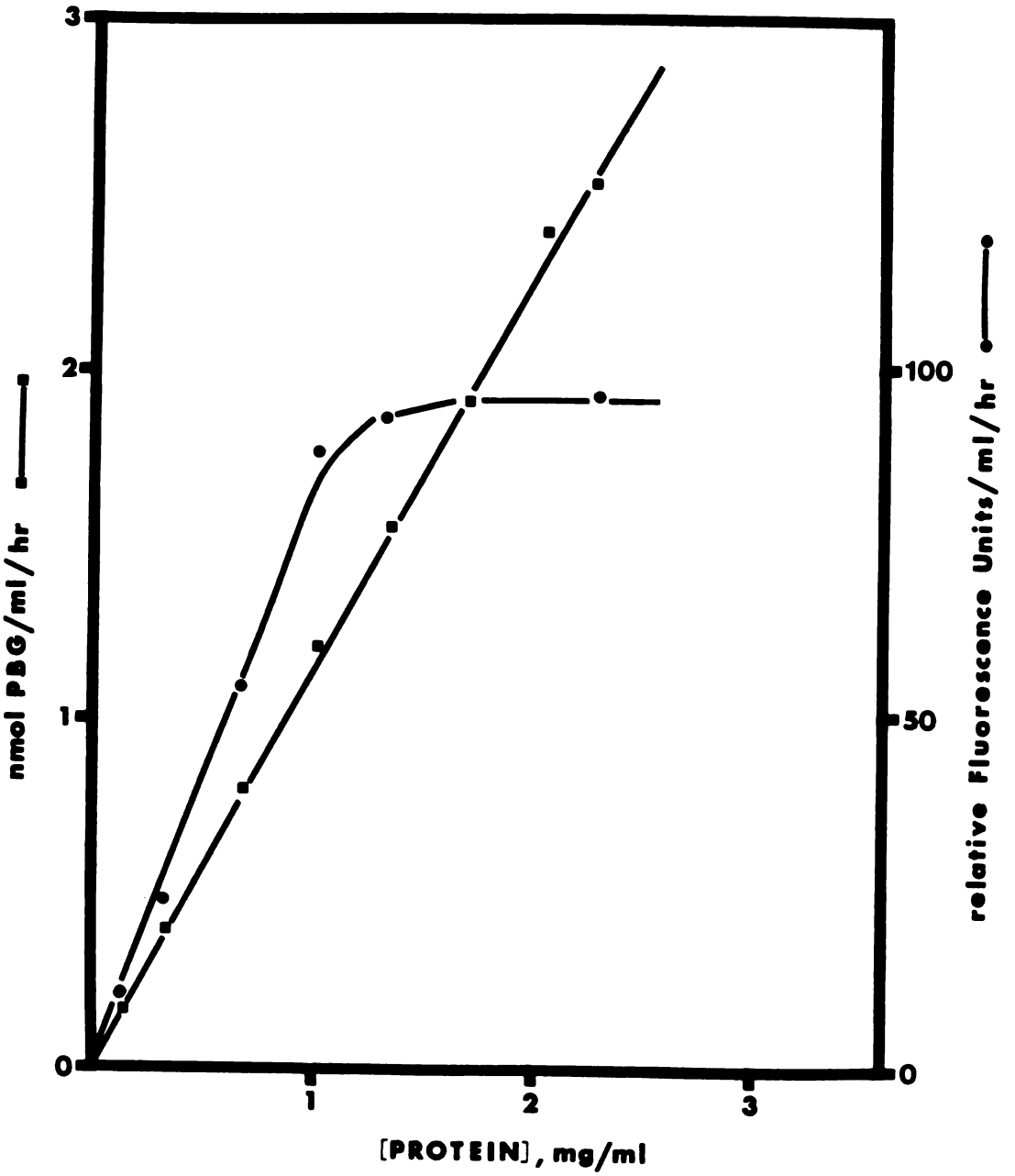
Figure 7. Production of PBG and Porphyrins by Suspensions of *R. japonicum* Incubated Aerobically in Increasing Concentrations of Bacteria.

Cells were resuspended into buffered growth medium to the final protein concentrations indicated.

Cells were incubated at 28 C for 4 hours.

PBG was determined colorimetrically as previously described.

Total porphyrins were determined fluorometrically as previously described.


3
2
1
0
nmol PBG/ml/hr
100
50
0
relative Fluorescence Units/ml/hr
1
2
3
[PROTEIN], mg/ml

The relationship of porphyrin synthesis to the length of time at which bacteria were grown aerobically in batch culture was examined in *R. japonicum* (Figure 8). The rate of porphyrin synthesis by free-living bacteria cultured for different periods of time appeared to be the same when cell suspensions were incubated in open tubes. However, the formation of porphyrin from ALA by cell suspensions incubated in closed tubes varied with the age of the culture. The greatly increased porphyrin production observed in cell suspensions incubated in closed tubes may reflect an increase in the highly oxygen labile UROgen precursors that are available for porphyrin formation (46,47). The rates of porphyrin formation by *R. japonicum* isolated from 25 to 35 day old soybean nodules were similar to those produced by laboratory cultured bacteria (Table 1). Again, porphyrin production in cells incubated in closed tubes was observed to be greater than porphyrin production by cells incubated in open tubes.

In contrast to porphyrin synthesis, PBG accumulation by cells incubated in closed tubes was significantly lower than that observed for cells incubated in open tubes (Table 3). This observation suggests, again, that porphyrin synthesis is dependent upon the efficient formation of oxygen labile UROgen precursors (47). The levels of PBG formed from ALA by bacteroids incubated in open and closed

Figure 8. Production of Porphyrins by Dense Cell Suspensions of Free-Living *R. japonicum* Incubated in Open Tubes and Closed Tubes.

Cells were harvested by centrifugation and were resuspended into buffered growth medium to a final cell protein concentration of 0.8 to 1.0 mg protein per ml resuspension. Cells were incubated in open tubes and in filled stoppered tubes for 4 to 6 hours at 28 C.

Porphyrins were determined fluorometrically as previously described.

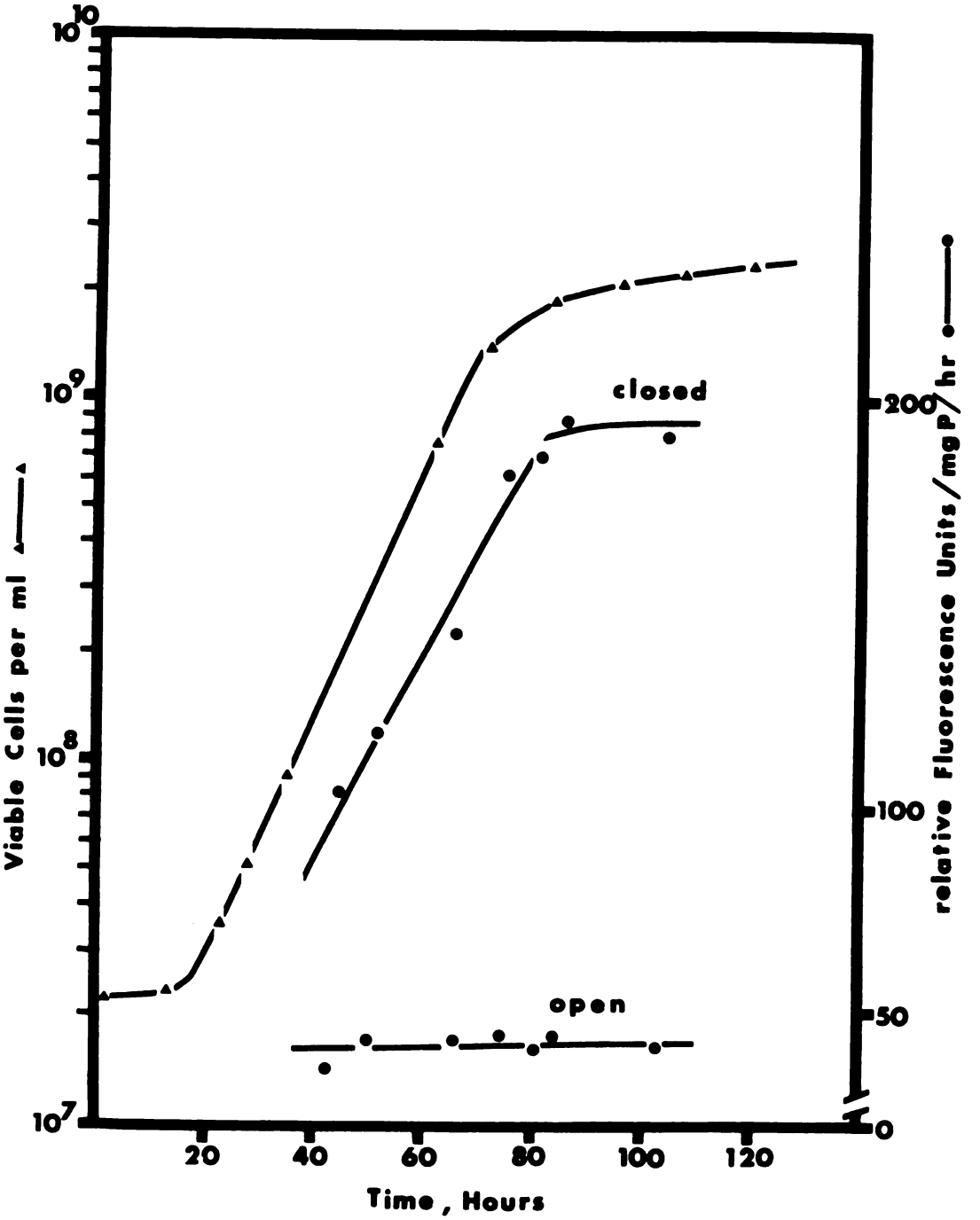

10^{10}
10^{9}
10^{8}
10^{7}
Viable Cells per ml
closed
open
200
100
50
0
relative Fluorescence Units/mgP/hr
20
40
60
80
100
120
Time, Hours

Table 1. Total Porphyrins Produced by *R. japonicum* Bacteroids

Incubation Conditions	Total Porphyrins relative fluorescence units / mg protein / hour
aerobic	57 ± 12
anaerobic	147 ± 23

Bacteroids were isolated aerobically by centrifugation as described in Methods and Materials.

Cells were incubated in a buffered bacterial growth medium supplemented with 0.25 M pentaerythrytol, 1.0 mM succinate, and 1.0 mM ALA.

Cells were incubated for 4 hours at 28 C.

Porphyrins were determined fluorometrically.

tubes were similar to the levels of PBG produced by free-living bacteria (Table 3).

The major bacterial porphyrins produced from ALA were identified by thin layer chromatography of the porphyrin methyl esters. A comparison of the R_f values of the major porphyrins formed from ALA with authentic porphyrin standards is shown in Table 2.

The major bacterial porphyrins produced from ALA were also identified by Florisil column chromatography of the free porphyrins (Table 3). The results were obtained using free-living bacteria harvested during late exponential growth and symbiotic bacteria isolated from nodules harvested from plants that were 25 to 35 days old. Suspensions of laboratory grown *R. japonicum* incubated under aerobic conditions produced PROTO from ALA (TAble 3). The bacteria produced greater amounts of URO and COPRO than PROTO. Suspensions of laboratory grown bacteria incubated in closed tubes produced significantly more URO and COPRO than cells incubated in open tubes. However, the cells incubated in closed tubes produced reduced amounts of PROTO. Since the dissolved oxygen concentration in the closed tubes was less than 2 to 3 ppm (as measured with an oxygen electrode) after 8 to 9 minutes incubation at 28 C, the small amount of PROTO formed in the closed tubes (during a 4 to 6 hour incubation period) suggests that COPROgen oxidation in *R. japonicum* is largely, but not absolutely, dependent upon the presence of molecular oxygen. The amount of

Table 2. Chromatographic Separation of the Methyl Esters of the Porphyrins Accumulated by Laboratory Cultured *R. japonicum* and *R. japonicum* Bacteroids.

System	URO III Standard	Bacterial "URO"	COPRO III Standard	Bacterial "COPRO"	PROTO IX Standard	Bacterial "PROTO"
I	0.07	0.08	0.29	0.30	0.45	0.47
II	0.91	0.9	0.37	0.37	0.22	0.21

Systems: I Benzene-ethyl acetate-ethanol (190:20:7.5, v/v)

II Water-acetonitrile-dioxan (2:7:1, v/v)

Abbreviations: URO=Uroporphyrin, COPRO=Coproporphyrin, PROTO=Protoporphyrin

Table 3. Heme Synthesis and the Rate of Porphyrin Accumulation[d] by *R. japonicum*.

Form of Bacteria	Incubation Conditions	PBG[c] nanomoles PBG / mg Protein/ hour	URO	COPRO picomoles / mg Protein/ hour	PROTO	HEME[e] counts per minute / mg Protein/ hour
Free-living[a]	aerobic	1.2 ± 0.2	14 ± 3	17 ± 2	7 ± 2	3098 ± 207
Free-living	anaerobic	0.2 ± 0.1	59 ± 7	91 ± 12	2 ± 1	1790 ± 171
Symbiotic[b]	aerobic	1.2 ± 0.2	22 ± 4	25 ± 7	11 ± 2	4320 ± 207
Symbiotic	anaerobic	0.2 ± 0.1	64 ± 6	67 ± 5	9 ± 3	1790 ± 171

a) Cells were incubated in buffered growth medium supplemented with 1.0 mM ALA.

b) Cells were incubated in buffered growth medium supplemented with 1.0 mM ALA, 0.25 M pentaerythrytol, and 1.0 mM succinate.

c) PBG quantitated colorimetrically as previously described.

d) Porphyrins quantitated fluorometrically after separation by florisil column chromatography.

e) Heme synthesis estimated as the rate of incorporation of radioactive iron into heme by cells resuspended into buffered growth medium supplemented with 10 nanomoles ALA per ml suspension.

intracellular PROTO present in the cell suspension at time zero was negligible and it is unlikely that all the PROTO formed during a 6 hour incubation period was formed during the first 9 minutes, under aerobic conditions. Therefore, these data suggest that an oxygen deficiency greatly reduces the overall rate of PROTO formation, although a small amount of PROTO is formed in an anaerobic environment.

Early attempts to demonstrate porphyrin biosynthesis in bacteroids isolated from legume nodules (48) resulted in a failure to demonstrate PROTO production from ALA. It is known that free-living rhizobia undergo numerous cell wall modifications during bacteroid formation to produce a cell wall that is thin and pliable (49, 50). These modifications possibly render the bacteroid osmotically sensitive. Therefore, in the present investigation bacteroids were utilized that were routinely suspended into buffered growth medium containing 0.25 M pentaerythrytol, in an attempt to protect the cells against osmotic shock. The addition of succinate to the buffered growth medium was also required in order to demonstrate porphyrin production from ALA by symbiotic *R. japonicum*. The addition of succinate to the buffer ensured linear porphyrin formation in bacteroids for up to eight hours incubation.

Aerobically incubated suspensions of symbiotic *R. japonicum* produced porphyrins from ALA in amounts

similar to those produced by free-living bacteria (Table 3). However, bacteroids incubated in closed tubes produced a larger amount of PROTO than was produced by free-living bacteria. This may indicate the presence of greater COPROgenase activity in bacteroids as compared to free-living bacteria, perhaps reflecting a higher level of enzyme present in bacteroids than is present in free-living bacteria.

The rate of heme synthesis was measured qualitatively by the rate of ^{59}Fe incorporation into heme in suspensions of whole cells. Ninety-eight percent of the radioactive material that was extracted into methyl ethyl ketone ("heme" extract) migrated at the same rate as authentic protohemin in several TLC systems. The rate of incorporation of radioactive iron into heme by aerobically incubated free-living bacteria greatly exceeded the rate observed in bacterial suspensions incubated in closed tubes (Table 3). The rate of ^{59}Fe incorporation into heme by aerobic suspensions of bacteroids was also significantly greater than the rate observed in bacteroid suspensions incubated in closed tubes (Table 3). These data indicate that the reduced rate of PROTO formation in anaerobic suspensions of *R. japonicum* is not the result of an increased rate of ferrochelatase activity in cells incubated under anaerobic conditions. PROTO is a substrate for heme synthesis. Therefore any reduction in the amount of PROTO that is

available for heme synthesis would reduce the rate of radioactive iron incorporation into heme.

The results of the experiments that examined porphyrin synthesis from ALA by *R. japonicum* suggest that in the genus *Rhizobium* there exists both an aerobic and anaerobic mechanism for the oxidation of COPROgen. Therefore, COPROgenase activity was determined in cell-free preparations of free-living and symbiotic *R. japonicum*. The identity of the porphyrins formed was confirmed by their absorption spectra in the Soret region (Figure 9) and by thin layer chromatography of the HCl extracts (Figure 10). Oxygen-dependent COPROgenase activity was demonstrated in unsupplemented crude enzyme extracts from free-living and symbiotic bacteria (Tables 4 and 5). Neither fumarate nor nitrate additions to the reaction mixture enhanced PROTO formation under aerobic conditions. Strikingly, the crude extracts prepared from bacteroids appeared to be approximately four times more active than crude extracts prepared from free-living bacteria.

COPROgenase activity under anaerobic assay conditions in unsupplemented enzyme extracts of both free-living and symbiotic bacteria could not be demonstrated and neither nitrate nor fumarate could replace oxygen as an electron acceptor in the unsupplemented enzyme extracts. However, small amounts of enzyme activity were demonstrated under anaerobic conditions in the crude extracts if the following cofactors were present:

ATP, NADP, Mg^{++}, and L-methionine. The effect of iron chelators upon enzyme activity and the effectiveness of NAD rather than NADP were not determined. The activities of anaerobic bacteroid extracts were again greater than the enzyme activities observed in anaerobic extracts of free-living bacteria. These findings support the suggested existence of two mechanisms of enzymatic COPROgen oxidation in some groups of heterotrophic bacteria.

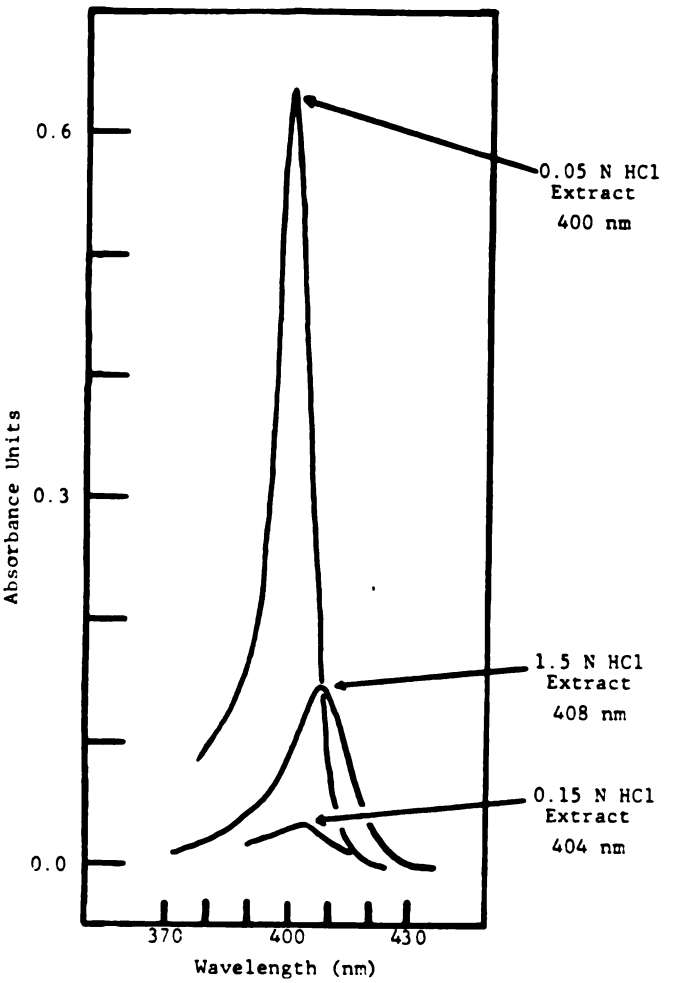


Figure 9. Soret Maxima of Porphyrins Recovered After Aerobic Incubation of Coproporphyrinogen in the Presence of *R. japonicum* Cell-Free Extracts.

COPROgenase activity determined as described in Materials and Methods.

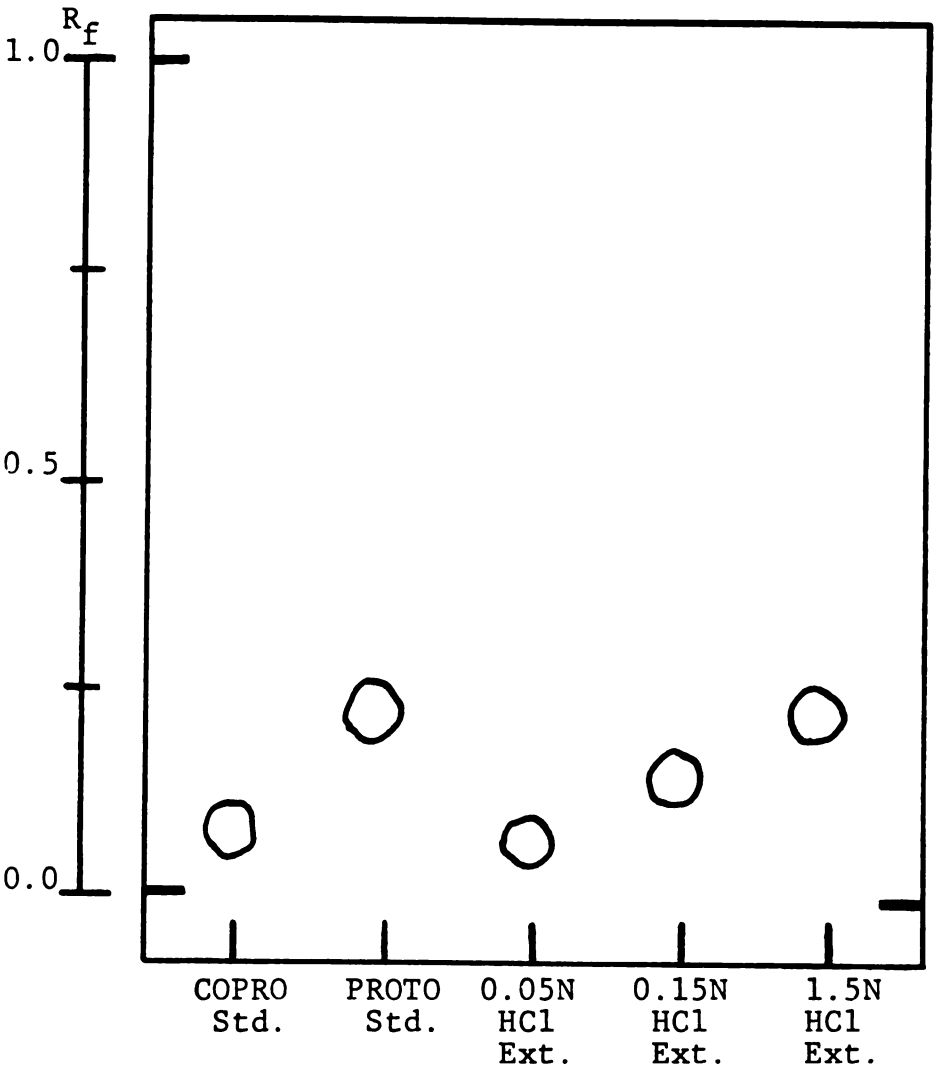


Figure 10. Chromatographic Separation of Porphyrins Recovered After Aerobic Incubation of COPROgen in the Presence of Bacterial Extracts.

Plate: Silica Gel 60
System: 2,6-Lutidine:H_2O:NH_3 (10:3, v/v)

Table 4. Coproporphyrinogenase Activity in Extracts of Laboratory Grown *R. japonicum.*

Conditions of Assay	Expt.	Supplements to Assay	Extract Protein (mg Protein)	Initial Substrate (nmoles)	Porphyrins Recovered (nmoles) COPRO[c]	PROTO
Aerobic[a]	1	none	10.2	43.9	33.8	6.9
	2	none	10.6	70.2	65.4	7.7
Anaerobic[b]	1	none	10.2	43.9	39.4	0
	2	none	12.0	35.4	26.3	0
Anaerobic	1	ATP, NADP, Mg^{++}, L-methionine	12.0	35.4	23.4	0.41
	2	ATP, NADP, Mg^{++}, L-methionine	10.4	52.1	45.8	0.35

a) Aerobic incubations were shaken vigorously in the dark at 38 C for 90 minutes.

b) Anaerobic incubations were performed in evacuated Thunburg tubes without shaking in the dark at 38 C for 90 minutes.

c) Included to indicate recovery of substrate.

Table 5. Coproporphyrinogenase Activity in Extracts of *R. japonicum* Bacteroids.

Conditions of Assay	Expt.	Supplements to Assay	Extract Protein (mg Protein)	Initial Substrate (nmoles)	Porphyrins Recovered (nmoles) COPRO[c]	PROTO
Aerobic[a]	1	none	4.5	43.9	29.3	12.5
	2	none	4.7	52.1	27.9	14.1
Anaerobic[b]	1	none	5.2	52.1	36.9	0
	2	none	4.5	43.9	40.9	0
Anaerobic	1	ATP, NADP, Mg^{++}, L-methionine	5.2	52.1	42.8	1.7
	2	ATP, NADP, Mg^{++}, L-methionine	4.5	50.2	48.9	1.1

a) Aerobic incubations were shaken vigorously in the dark at 38 C for 90 minutes.

b) Anaerobic incubations were performed in evacuated Thunbrug tubes without shaking in the dark at 38 C for 90 minutes.

c) Included to indicate recovery of substrate.

DISCUSSION

The oxidative decarboxylation of COPROgen was investigated in free-living and symbiotic *R. japonicum* in order to better understand the mechanism of symbiotic heme biosynthesis in legume root nodules. The production of PROTO from ALA in aerobic and anaerobic cell suspensions is circumstantial evidence that COPROgenase activity is present in *R. japonicum*. The production of PROTO from ALA by suspensions of symbiotic bacteria was shown to be generally unaffected by anaerobiosis whereas the absence of oxygen in suspensions of free-living bacteria generally inhibited PROTO formation from ALA. These findings suggest that there exists two mechanisms for the oxidation of COPROgen in *R. japonicum*. The inhibition of PROTO formation from ALA observed in suspensions of free-living bacteria incubated under anaerobic conditions would suggest that rhizobia oxidize COPROgen utilizing an obligately oxygen-dependent COPROgenase. However, the recovery of roughly equal amounts of PROTO from either aerobic or anaerobic suspensions of symbiotic bacteria suggests that an alternate mechansim for COPROgen oxidation may operate in the absence of oxygen. COPROgenase activity in cell-free extracts of *R. japonicum* was also

examined and the results suggest that the mechanism of anaerobic COPROgen oxidation may be quite similar to the postulated mechanism of anaerobic COPROgen oxidation present in *R. spheroides* and *Chromatium D* (30).

The detection of COPROgenase activity in extracts of *R. japonicum* incubated under anaerobic conditions is particularly relevant to the study of leghemoglobin biosynthesis in mature legume nodules. The average free oxygen tension in soybean nodule tissue has been calculated to be no more than 11 nM dissolved oxygen (10), yet the COPROgenase activity under aerobic conditions in cell-free extracts of *Chromatium D* was determined to be half oxygen saturated at 1.0 mM dissolved oxygen (52). Assuming that the properties of COPROgenase of *R. japonicum* are similar to the properties of the *Chromatium* enzyme, it would seem unlikely that an oxygen dependent COPROgenase activity could account for the 0.5 nanomoles of heme per gram nodule per day that are required for leghemoglobin biosynthesis during the linear phase of nodule development (35). However, the average activity of COPROgenase in extracts of symbiotic *R. japonicum* incubated under anaerobic conditions was calculated to be about 2.0 nanomoles PROTOgen produced per gram nodule per hour. Thus, it is clear that the *in vitro* activity of the enzyme under anaerobic conditions can account for the PROTOgen needed to synthesize the heme necessary for leghemoglobin biosynthesis in legume root nodules. It seems reasonable

to assume, however, that the *in vivo* activity of COPROgenase is much lower than the activity *in vitro*, probably because the substrate and co-factors are all in great excess *in vitro*.

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