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Heme Synthesis in Soybean Root Nodules

I. ON THE ROLE OF BACTEROID δ-AMINOLEVULINIC ACID SYNTHASE AND δ-AMINOLEVULINIC ACID DEHYDRASE IN THE SYNTHESIS OF THE HEME OF LEGHEMOGLOBIN[1]

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KENNETH D. NADLER AND YAEL J. AVISSAR[2]
Department of Botany and Plant Pathology, Michigan State University, East Lansing, Michigan 48824

ABSTRACT

During nodulation of soybean (*Glycine max*) by *Rhizobium japonicum*, variations in the activities of two enzymes of heme biosynthesis, δ-aminolevulinic acid synthase (ALAS) and δ-aminolevulinic acid dehydrase (ALAD) are described. δ-Aminolevulinic acid synthase activity is found in the bacteroid fraction of nodules, but is not detected in the plant fraction. Bacteroid ALAS activity parallels heme accumulation during nodule development. δ-Aminolevulinic acid dehydrase activity is found in both bacteroid and plant cytosol fractions. Bacteroid ALAD activity is constant or increases during nodulation while plant ALAD activity falls.

Bacteroid ALAD activity is found in effective, not in inefficient nodules. Plant ALAD activity is found in both effective and inefficient nodules. Plant ALAD activity falls during development of both types of root nodules.

These results support the contention that it is the bacteroid ALAS and ALAD activities, not those of the plant, that are directly involved in formation of leghemoglobin heme, suggesting that the bacteroid may be solely responsible for formation of leghemoglobin heme in the nodule symbiosis.

Legume root nodules have a deep red-brown pigmentation due to the presence of large amounts of a group of hemoproteins called "leghemoglobins." Lb[3] is essential for symbiotic N_2 fixation in legumes, functioning to deliver O_2 to the aerobic endosymbiotic bacteroids at an O_2 tension sufficiently low so as to not inhibit the O_2-sensitive bacteroid nitrogenase. Lb is formed only during development of the nodule symbiosis; neither the uninfected legume host nor the free living *Rhizobium* bacteria produce these hemoproteins or even large amounts of heme. Evidently during nodulation, a large increase in heme formation is required for Lb synthesis. How is this increased heme synthesis regulated and which symbiotic partner produces the heme for Lb?

Tetrapyrrole formation in a variety of organisms is limited by the rate of formation of ALA, the first compound committed to heme and Chl synthesis. It seems reasonable that heme formation in legume nodules might be so regulated. ALA limits the formation of heme precursors by *Rhizobium japonicum* (9) but the effect of exogenous ALA on heme formation has not been determined. Cutting and Schulman (7), on the other hand, propose that heme synthesis by soybean nodule bacteroids is regulated at a step *subsequent* to ALA formation. We present evidence here that suggests that the activity of ALA-synthase is rate-limiting in heme formation in legume nodules.

The globin portion of Lb has been shown to be produced by the host plant (6, 8), while the site(s) of heme synthesis in the nodule remains unclear. Bacteroids incorporate a variety of precursors including glycine (21), organic acids (14, 21), and ALA (5, 12, 14) into heme, suggesting that the bacteria are the site of heme synthesis in nodules. In contrast, Godfrey *et al.* (11) reported that the major portion of nodule ALAD activity is in the plant cytosol fraction and suggested that in the nodule, heme precursors are exchanged between host and symbiont in a similar fashion to the exchange of heme precursors between the mitochondria and cytosol of animal cells (22). The experiments described here tend to support the contention that the bacteroid can be the exclusive site of synthesis of Lb heme in soybean root nodules.

MATERIALS AND METHODS

Media. Yeast extract-mannitol medium (YM) contains, 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 is autoclaved for 15 min at 15 p.s.i.

N-free legume nutrient medium is prepared as described by Johnson *et al.* (15).

Organisms. *R. japonicum* strains 3I1b-61 and 3I1b-110 were kindly supplied by D. C. Weber, Beltsville, Md.; strain 11927 was purchased from American Type Culture Collection. Bacterial strains are maintained on YM agar (1.5%) slants and are transferred every 3 months. Liquid cultures are started from slants in 2 ml YM medium; when outgrowth occurs, cultures are transferred to 50 ml YM media in 1-liter flasks and are aerated by agitation on a reciprocal shaker for 1 day. Cells are harvested by centrifugation at 6,000*g* for 10 min, washed, and resuspended in N-free legume nutrient medium.

Soybean (*Glycine max.* cv. Hark) seeds are surface-sterilized briefly in 1% NaOCl, washed in running tap water, and planted in a vermiculite-perlite bed. The bed is inoculated with a suspension of the appropriate strain of *R. japonicum*. N-free nutrient medium is aerated and circulated through the beds of germinating seeds for 1 week. The beds are illuminated with two 40 w Grolux fluorescent lights. Inoculated seedlings are transplanted into a vermiculite-perlite mixture in pots in a greenhouse. All experiments reported here were performed during the summer when no supplemental illumination was necessary. Plants are watered with N-free medium as described (15).

Nodules form predominantly at the root crown. Only crown nodules were harvested in these experiments.

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[3] Abbreviations: ALA: δ-aminolevulinic acid; ALAS, δ-aminolevulinic acid synthase (EC 2.3.1.37); ALAD: δ-aminolevulinic acid dehydrase (EC 4.2.1.24); Lb: leghemoglobin; PBG: porphobilinogen; YM: yeast extract-mannitol medium.

Fractionation of Nodules. All operations are performed at 0 to 4 C.

Assay of ALAD Activity. One- to three- g nodules are homogenized in a prechilled mortar with 10 to 15 ml of a 5 mM Na-K phosphate buffer (pH 7.5) containing 1 mM $MgCl_2$ and 1 mM β-mercaptoethanol. The nodule brei is squeezed through two layers of cheesecloth and one layer of Miracloth. Bacteroids are collected from the filtrate by centrifugation at 6,000*g* for 10 min. The resulting clear supernatant ("plant fraction") is decanted and is used directly for determination of Lb heme and plant enzymic activities. The pelleted bacteroids are washed, resuspended in 5 ml buffer, and are broken by sonic disruption with three 30-sec bursts from a Sonifier-Cell Disruptor (model W 185, Heat Systems Ultrasonics Inc.) at power setting 5. The sonicate is clarified by centrifugation for 10 min at 20,000*g*. Bacteroid activities are assayed in the resulting supernatant ("bacteroid extract"). Contamination of the bacteroid extract is considered minimal since no Lb is detected in the bacteroid extract by the pyridine-hemochromogen method (10).

Nodules are similarly fractionated for *assay of ALAS* except that 0.1 M Na-phosphate (pH 7.5) buffer containing 1 mM $MgCl_2$ and 1 mM β-mercaptoethanol is used throughout the procedure.

ENZYME ASSAYS

ALAS Activity. A 3-ml final volume contains, in μmol: Na-phosphate (pH 7.5), 300; β-mercaptoethanol, 1.5; $MgCl_2$, 50; sodium succinate, 300; glycine, 300; ATP, disodium salt, 21; CoA, 0.9; pyridoxal phosphate, 0.9. Reaction is initiated by addition of bacteroid extract containing less than 3 mg protein to the reaction mixture in a 30 C water bath. Incubations proceed for the indicated time and are terminated by the addition of ice-cold trichloroacetic acid (saturated with $HgCl_2$) to a final concentration of 3% trichloroacetic acid. Precipitate is removed by centrifugation and the supernatants applied to a 3.5-ml column of Dowex 50-W-X8, 200 to 400 mesh resin equilibrated with 0.2 N sodium citrate (pH 3.07). ALA is eluted as described by Beale *et al.* (4) and is converted to ALA-pyrrole with acetyl acetone (19) for colorimetric determination with the modified Ehrlich reagent of Urata and Granick (25).

ALAD Activity. A final volume of 1 ml contains, in μmol: tris-Cl (pH 8.5), 50; $MgSO_4$, 5; β-mercaptoethanol, 12.5; ALA, 2.5; less than 0.5 ml bacteroid extract or plant fraction. Mixtures are incubated for 2 hr at 30 C. Reactions are terminated by addition of 0.25 ml ice-cold 20% (w/v) trichloroacetic acid saturated with $HgCl_2$. Precipitate is removed by centrifugation and the porphobilinogen formed is determined colorimetrically (19, 25).

ANALYTICAL METHODS

Heme is determined as the pyridine-hemochromogen (10) assuming a millimolar extinction coefficient of 20.7 for the reduced minus oxidized hemochrome.

Protein is determined by the method of Lowry *et al.* (18) using BSA as a standard.

RESULTS

The ALAS activity of cell-free extracts of soybean root nodule bacteroids can be assayed only after ion exchange chromatography on short cation exchange columns (Fig. 1). In the absence of this chromatographic step, the ALA-pyrrole color compound (extinction maximum at 553 nm) formed with the Ehrlich reagent is obscured by material which forms another color compound with the Ehrlich reagent, absorbing maximally at 520 nm. After chromatography (Fig. 1B), ALA formed by bacteroids *in vitro* is readily determined in the column eluates by standard colorimetric procedures (19). The efficacy of the chromatographic step is indicated by the ratio of the absorbances of the pyrrole-Ehrlich color compound at 525 and 553 nm which, for the eluted ALA, is 0.72 (Fig. 1B) as compared to that for synthetic ALA, which is 0.69 (19).

Using this modified assay procedure, ALAS activity in cell-free extracts of soybean root nodule bacteroids is constant and apparently stable for at least 6 hr under the assay conditions employed (Fig. 2, A and B). ALA formation is completely dependent on added substrates, glycine and succinate and is somewhat less dependent on exogenous CoA and pyridoxal

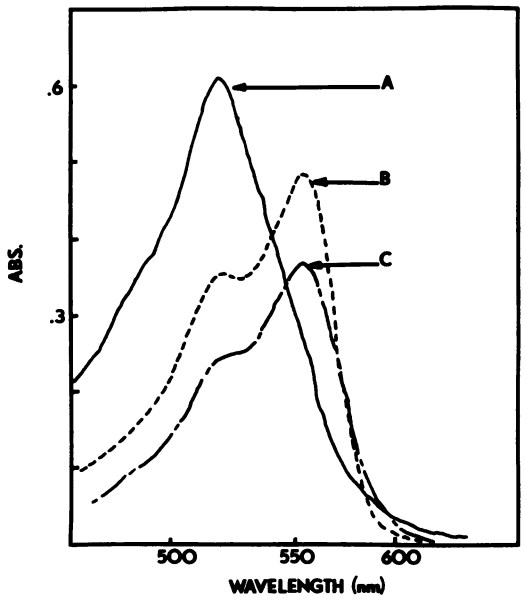

FIG. 1. Purification by ion exchange chromatography of ALA produced by bacteroid extracts *in vitro*. Samples containing ALA are treated as described under "Materials and Methods" to form ALA-pyrrole which is then mixed with equal volumes of the modified Ehrlich reagent (25). Absorption spectra of the resulting color compounds are recorded 15 min later with a Cary 15 recording spectrophotometer. A: Crude bacteroid extracts after addition of trichloroacetic acid and clarification by centrifugation; B: crude bacteroid extracts from (A) after chromatography on Dowex 50-W-X8 columns; absorbance × 10; C: synthetic ALA.

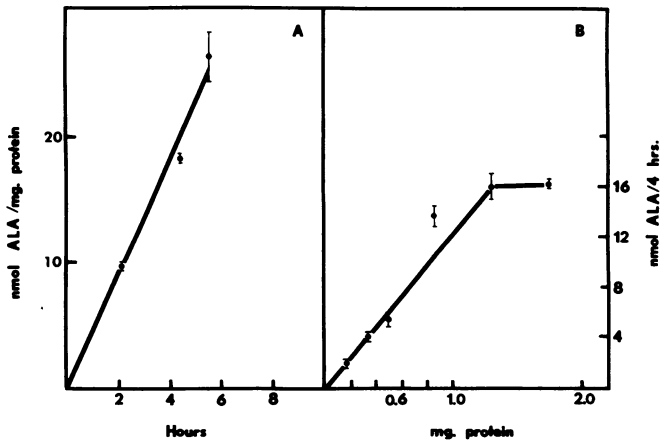

FIG. 2. ALAS activity of soybean root nodule bacteroids. Bacteroids are isolated and disrupted by sonication as described under "Materials and Methods." ALAS activity is determined as described under "Materials and Methods." A: ALAS activity with respect to time. Each ml of final reaction mixture contains 1.25 mg protein. B: ALAS activity with respect to mg bacteroid protein. Reactions are terminated after 4 hr incubation.

phosphate (Table I). The reduced dependence on exogenous cofactors is probably due to presence of CoA and 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 coelutes with ALA from cation exchange resin, and forms a pyrrole-like derivative with acetylacetone; this pyrrole forms a 520 nm-absorbing color compound with the Ehrlich reagent. Penicillin G does not significantly affect ALAS activity in bacteroid extracts (unpublished results), suggesting that ALA formation during incubation *in vitro* is not due to contaminating bacteria or unbroken bacteroids. We have not determined the effects of additions of succinylthiokinase; however, Godfrey *et al.* (11) report and we confirm (unpublished results) the observation that the succinylthiokinase activity in bacteroid extracts is 300 to 1,000 times more active than ALAS activity. This suggests that ALAS activity, not formation of succinyl-CoA may be a limiting factor in ALA formation *in vitro*.

Variations during nodule development in bacteroid ALAS activity suggest that ALAS activity limits heme synthesis in soybean root nodules. On a fresh weight basis, bacteroid ALAS activity correlates with nodule heme content (Fig. 3). ALAS activity cannot be detected in young nodule primordia that have no Lb. Thereafter, nodule heme content and bacteroid ALAS activity increase in parallel, both reaching a maximum 5 weeks after inoculation. As nodules senesce and their heme is degraded, bacteroid ALAS activity declines.

In contrast to the nodule ALAS activity, ALAD activity is found in both bacteroid and plant fractions of effective nodules. Most of the ALAD activity of nodules is found in the plant fraction of rapidly growing nodules (Figs. 4 and 5), confirming a

Table I. Requirements for Bacteroid ALAS Activity in vitro.

Bacteroid ALAS activity is determined as described in Methods. Assay mixtures containing 3 mg protein in 3 ml final volume are incubated at 28 C for 200 min. Deletions of sodium succinate or ATP (disodium salt) are replaced by equimolar amounts of NaCl; other deletions are replaced with equal volumes of distilled water.

System	ALA formed	% complete
	nmol/mg.protein.hr	
complete	7.3 ± 0.1	100
-glycine	0.1 ± 0.03	1
-succinate	0.3 ± 0.03	4
-ATP	2.6 ± 0.03	36
-CoA	1.6 ± 0.09	22
-pyridoxal phosphate	1.9 ± 0.03	26

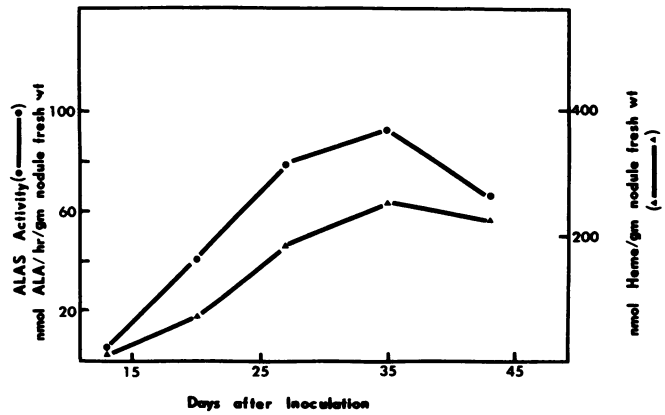

FIG. 3. Bacteroid ALAS activity and Lb heme content of developing nodules. Nodules formed on "Hark" soybeans by *R. japonicum* 3I1b-110 were harvested on the days indicated after inoculation. Lb heme is determined in the plant fraction as the pyridine hemochromogen (10); bacteroid ALAS activity is assayed as described under "Materials and Methods."

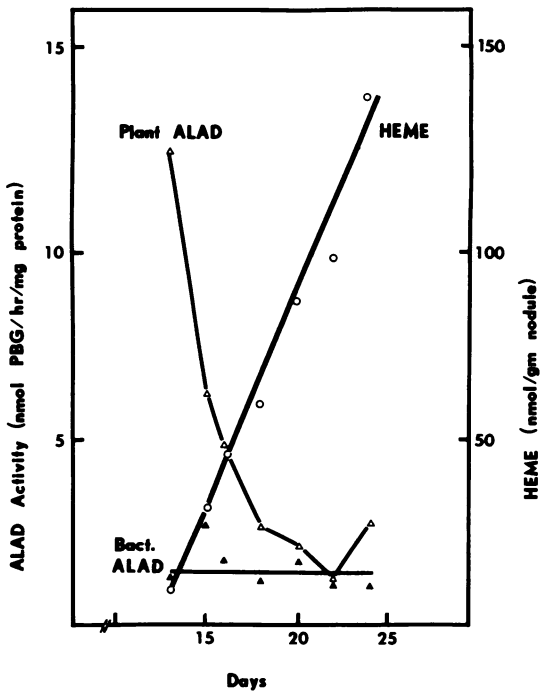

FIG. 4. Plant and bacteroid ALAD activities and Lb heme content of developing nodules. Plant and bacteroid extracts are prepared and ALAD activity, Lb heme are determined as described under "Materials and Methods." Plant ALAD activity (Δ——Δ); bacteroid ALAD activity (▲——▲); Lb heme (O——O). Effective nodulation by strain 3I1b-110.

previous report by Godfrey *et al.* (11). On the basis of this ALAD distribution, these workers (11, 12) proposed that ALA and subsequent precursors of heme are exchanged between the host plant and endosymbiont during nodulation, in analogy to the interchange of heme precursors between the mitochondria and cytosol of animal cells (22). However, as Lb heme is accumulated in the developing nodule, the plant ALAD activity decreases sharply although the bacteroid ALAD activity is unchanged or even increases (Figs. 4 and 5). Even on a fresh weight basis, plant ALAD activity decreases sharply during Lb formation. Mixing experiments do not support the presence of an ALAD inhibitor in the plant fraction of mature nodules. The decrease in plant ALAD activity during the period of rapid heme formation is not consistent with the proposed role for the plant enzyme in Lb synthesis.

Plant and bacteroid ALAD activities can also be compared during formation of effective and inefficient nodules (Fig. 5). As before, a sharp decline in the plant ALAD activity is observed during formation of both effective nodules (formed with *R. japonicum* 3I1b-61) and inefficient (*R. japonicum* 11927) nodules. In contrast, bacteroid ALAD activity is detected only in effective nodules, not in inefficient nodules. Indeed, in the effective nodules formed with strain 3I1b-61, bacteroid ALAD activity is found only when Lb is accumulating. Thus, there is a correlation between Lb accumulation and bacteroid ALAD activity, not with plant ALAD activity.

DISCUSSION

A modified procedure for the assay of ALAS activity in cell-free extracts of soybean root nodule bacteroids is described. Crucial to this procedure is the removal by ion exchange chromatography of substances which obscure the color compound of ALA-pyrrole with Ehrlich reagent. Without the use of an ion exchange step for ALA purification, Godfrey *et al.* (11) reported

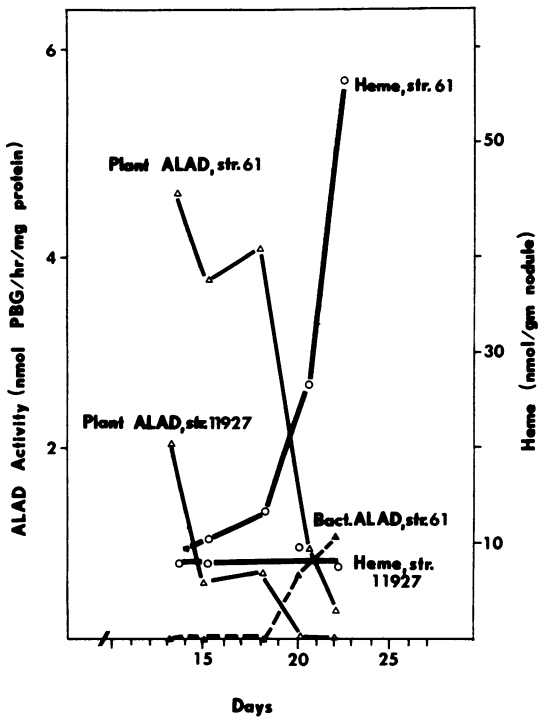

FIG. 5. Plant and bacteroid ALAD activities and Lb heme content of effective *versus* inefficient nodules. Extracts are prepared and determinations made as described under "Materials and Methods." Symbols as in Figure 4. Effective nodulation with strain 3I16-61; inefficient nodulation with strain 11927. Bacteroid ALAD activity in inefficient nodules was below the limits of sensitivity of the assay.

low and variable ALAS activity in cell-free extracts of serradella and lupine nodule bacteroids. The high background and elusive quality of ALAS activity reported by these workers may be due to the presence of materials separated from ALA by ion exchange chromatography in this study. Using this modified assay for ALAS in soybean nodule bacteroids, we have observed a developmental correlation between the Lb content of soybean nodules and the ALAS activity of bacteroids from soybean nodules. Increased ALAS activity is correlated with rapid formation of heme or bacterio-Chl in animal cells (13, 17) and in microorganisms (16, 23). Chl formation in greening algae (1, 2) and higher plants (3) is also characterized by increased ALA formation. The increase in bacteroid ALAS activity during Lb formation (Fig. 3) and the concomitant decrease in nodule ALAD activity (Figs. 4 and 5) may indicate that heme formation in nodules is similarly rate-limited by bacteroid ALAS activity. However, we cannot exclude the possibility that the host plant produces ALA. We have been unable to detect ALAS activity in the plant fraction of effective nodules. Although it is conceivable that ALA is made in the host plant cell via the recently proposed five-carbon pathway (3, 4, 20), or that ALA formed in the plant extract *in vitro* is rapidly metabolized to other amino acids (24), the parallelism between nodule heme and bacteroid ALAS activity suggests a major role for the bacteroid in formation of ALA for synthesis of Lb heme.

Since ALAD is found in both the plant and bacteroid fractions of soybean root nodules, it is more difficult to assign a role in the formation of Lb heme to one of the partners in the nodule symbiosis. Although the major portion of nodule ALAD activity is found in the plant fraction, plant ALAD activity is present both in effective nodules and in nodules that do not form Lb (ineffective nodules). Bacteroid ALAD activity is found only in effective nodules. Bacteroid ALAD activity (1 to 2 nmol PBG formed/hr · mg protein) could support as much as 60 to 120 nmol heme formed/day · g nodule, well in excess of the observed rate of accumulation of Lb heme. The bacteroid ALAD activities reported here are minimum activities since porphyrin fluorescence under UV light is always observed in assays of bacteroid ALAD activity but is not observed in plant ALAD assays (unpublished results). This is consistent with the findings of other workers (5, 12) who had previously noted that the particulate (bacteroid) component of nodules is the principal site of ALA incorporation into heme. These results indicate that bacteroids can be the sole site of Lb heme formation from glycine and succinate. Further experiments are required to demonstrate that Lb heme formation is exclusively a function of the bacterial symbiont.

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LITERATURE CITED

1. BEALE SI 1970 Biosynthesis of δ-aminolevulinic acid in *Chlorella*. Plant Physiol 45: 504-506
2. BEALE SI 1971 Studies on the biosynthesis of δ-aminolevulinic acid in *Chlorella*. Plant Physiol 48: 316-319
3. BEALE SI, PA CASTELFRANCO 1974 The biosynthesis of δ-aminolevulinic acid in higher plants. I. Accumulation of δ-aminolevulinic acid in greening plant tissues. Plant Physiol 53: 291-296
4. BEALE SI, SP GOUGH, S GRANICK 1975 Biosynthesis of δ-aminolevulinic acid from the intact carbon skeleton of glutamic acid in greening barley. Proc Nat Acad Sci USA 72: 2719-2723
5. CUTTING JA, HM SCHULMAN 1969 The site of heme synthesis in soybean root nodules. Biochim Biophys Acta 192: 486-493
6. CUTTING JA, HM SCHULMAN 1971 The biogenesis of leghemoglobin. The determinant in the *Rhizobium*-legume symbiosis for leghemoglobin specificity. Biochim Biophys Acta 229: 58-62
7. CUTTING JA, HM SCHULMAN 1972 The control of heme synthesis in soybean root nodules. Biochim Biophys Acta 261: 321-327
8. DILWORTH MJ 1969 The plant as the genetic determinant of leghaemoglobin production in the legume root nodule. Biochim Biophys Acta 184: 432-441
9. FALK JE, CA APPLEBY, RJ PORRA 1959 Nature, function and biosynthesis of the haem compounds and porphyrins of legume root nodules. Symp Soc Exp Biol 13: 73-86
10. FUHRHOP JH, KM SMITH 1975 *In* KM SMITH, ed, Porphyrins and Metalloporphyrins. Elsevier, New York pp 804-807
11. GODFREY CA, DR COVENTRY, MJ DILWORTH 1975 Some aspects of leghaemoglobin biosynthesis. *In* WDP Stewart, ed, Nitrogen Fixation by Free-living Microorganisms. Cambridge Univ Press, New York
12. GODFREY CA, MJ DILWORTH 1971 Haem biosynthesis from (^{14}C)-δ-aminolaevulinic acid in laboratory-grown and root nodule *Rhizobium lupini*. J Gen Microbiol 69: 385-390
13. GRANICK S, G URATA 1963 Increase in activity of δ-aminolevulinic acid synthetase in liver mitochondria induced by feeding of 3,5-dicarbethoxy-1,4-dihydrocollidine. J Biol Chem 238: 821-827
14. JACKSON EK, HJ EVANS 1966 Propionate in heme biosynthesis in soybean nodules. Plant Physiol 41: 1673-1680
15. JOHNSON GV, HJ EVANS, TM CHING 1966 Enzymes of the glyoxylate cycle in Rhizobia and nodules of legumes. Plant Physiol 41: 1330-1336
16. LASCELLES J 1960 Synthesis of enzymes concerned in bacteriochlorophyll formation in growing cultures of *Rhodopseudomonas spheroides*. J Gen Microbiol 23: 487-498
17. LEVERE RD, A KAPPAS, S GRANICK 1967 Stimulation of hemoglobin synthesis in chick blastoderms by certain 5β androstane and 5β pregnane steroids. Proc Nat Acad Sci USA 58: 985-990
18. LOWRY OH, NJ ROSEBROUGH, AL FARR, RJ RANDALL 1951 Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265-275
19. MAUZERALL D, S GRANICK 1956 Occurrence and determination of δ-aminolevulinic acid and porphobilinogen in urine. J Biol Chem 219: 435-446
20. MELLER E, S BELKIN, E HAREL 1975 The biosynthesis of δ-aminolevulinic acid in greening maize leaves. Phytochemistry 14: 2399-2402
21. RICHMOND JE, K SALMON 1955 Studies on the biosynthesis of hemin in soybean nodules. Biochim Biophys Acta 117: 48-55
22. SANO S, S GRANICK 1961 Mitochondrial coproporphyrinogen oxidase and protoporphyrin formation. J Biol Chem 236: 1173-1180
23. TAIT GM 1973 Aminolaevulinate synthetase of *Micrococcus denitrificans*. Biochem J 131: 389-403
24. TROXLER RF, AS BROWN 1975 Metabolism of δ-aminolevulinic acid in red and blue-green algae. Plant Physiol 55: 463-467
25. URATA G, S GRANICK 1963 Biosynthesis of δ-aminoketones and the metabolism of aminoacetone. J Biol Chem 238: 811-820