Haem Biosynthesis from [^{14}C]- δ -Aminolaevulinic Acid in Laboratory-grown and Root Nodule *Rhizobium lupini*

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SUMMARY

Both laboratory-cultured bacteria and the bacteroid forms of *Rhizobium lupini* (strain wu8) isolated from serradella root nodules synthesize haem from [^{14}C]- δ -aminolaevulinic acid (ALA) and release the product into the incubation medium. Laevulinic acid, an inhibitor of haem synthesis in other organisms, effectively inhibits this synthesis in *Rhizobium*. When compared on the basis of fresh weight, protein or numbers, the laboratory-grown bacteria are more active in the incorporation of [^{14}C]-ALA than are the bacteroid forms, either when intact or when disrupted by sonication. A synergistic effect between cytoplasmic (plant) and particulate (bacteroid) extracts from serradella nodules is observed which is greater than that for soybean nodules. It is proposed that the haem synthesis for leghaemoglobin in serradella root nodules is a co-operative effort between plant and bacteroid.

INTRODUCTION

The bacteroid fraction of soybean root nodules is more competent in incorporating [^{14}C]- δ -aminolaevulinic acid (ALA) into haem than is the plant fraction (Cutting & Schulman, 1969). This observation suggested that the bacteroid is the site of haem synthesis for leghaemoglobin (LHb) *in vivo*. However, the formation of ALA is known to be the rate-limiting step in most organisms including bacteria (Doss & Philipp-Dormston, 1971), while cells which are inactive in haem synthesis can incorporate ALA into porphyrins and haems (Lascelles, 1964; Bogorad, 1965). Also, the comparison of haem synthesis in intact bacteroids and disrupted plant cells (Cutting & Schulman, 1969) neglects possible effects of disruption on levels of ALA incorporation.

If the incorporation of ALA into haem represents the level of active haem synthesis, then one would expect the laboratory-grown rhizobia, which possess relatively low levels of haem (Smith, 1949; Appleby, 1969*b*), to have a markedly lower activity than the bacteroids isolated from the nodule. We have therefore examined the capacities of laboratory-grown *Rhizobium lupini* and bacteroids of predominantly the same strain to incorporate [^{14}C]-ALA into haem, both before and after disruption by sonication. The distribution of this activity between the cytoplasmic (plant) and particulate (bacteroid) fractions of serradella root nodules is also presented.

METHODS

Organisms and medium. *Rhizobium lupini*, strain wu8 (Parker & Oakley, 1963) was maintained on 2 % agar slants at 28°. The medium (YP3 + P) for both broth culture and agar gels consisted of (g./l.): sucrose, 5; mannitol, 5; K_2HPO_4 , 0.3; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1; CaCO_3 ,

0.1; NH_4Cl , 0.05; urea, 0.03; NaCl , 0.1; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.01; yeast extract (Difco), 2.5; and potato extract from 200 g. of fresh potato autoclaved for 15 min. at 15 p.s.i. and filtered through cheesecloth. After autoclaving, 2 l. flasks containing 500 ml. of broth were inoculated and maintained at 28° on a reciprocating shaker for 4 days. Bacteria were harvested by centrifugation at 5000 g for 20 min. and after washing in the incubation buffer once, were used immediately. Samples were examined by phase-contrast microscopy and streaked on to YM3+P agar plates in order to detect contaminating organisms. A minimum of 1000 bacteria were counted under dark-field illumination at a magnification of 800 in a Hawksely-Thoma counting chamber.

Serradella (*Ornithopus sativus* Brot.) seed was inoculated with strain WU8 and sown in the field in sandy soils. Nodules were removed about 60 days after emergence while they were still pink. Fifty nodules were squashed and tested against WU8 antiserum using the technique of Parker & Grove (1970). The proportion of WU8 positive nodules was variable in the range 50 to 70 %.

Preparation of extracts. Nodule fractions were isolated according to Cutting & Schulman (1969). Bacteria were disrupted by sonication for 20 min. in a Raytheon Sonicator (9 kcyc.) with the sample chamber cooled to 0° .

Incubations and isolation of haem. Wet bacteria (0.5 to 1.0 g.) or extract equivalent to 5 g. of nodule tissue was suspended in the Honda-type medium of Cutting & Schulman (1969) increased to 10 mM in tris.HCl. The incubation was started in 1 mM-ALA after addition of δ -aminolaevulinate-4- ^{14}C (53 mCi/mM). These suspensions were incubated in flasks on a shaking water bath at 28° for the required length of time. Incubation was stopped by cooling the culture in ice and centrifuging at 10,000 g for 20 min. Carrier haem (500 $\mu\text{g.}$) was added to the supernatant and haem was isolated using the methods of Jackson & Evans (1966). The final column eluate was rotary evaporated to dryness and the residue was suspended in 1 ml. 0.15 N- NH_4OH which was stored at -20° . Radioactivity was shown to be exclusively located in the haem by thin-layer chromatography (Miyauchi, 1968) and scintillation counting of Cab-O-Sil (Ott, Richmond, Trujillo & Foreman, 1959) suspensions of gel removed from the plate.

Analytical procedures and reagents. Soluble protein was determined by the Lowry reaction (Lowry, Rosebrough, Farr & Randall, 1951) using lysozyme as a standard (Sigma Chemical Co, London.). Aminolaevulinic acid was assayed by the method of Moore & Labbe (1964). Haem samples were decolourized (Adler & George, 1965) before scintillation counting in a Triton X-100 cocktail (Patterson & Greene, 1965) on a Packard 3320 Tri Carb counter. Disintegrations per minute (d.p.m.) were calculated from the scintillation counts and the counting efficiency (70 %) as determined by internal standards. Values were not corrected for haem yields in the isolation procedure which were 40 %. [^{14}C]- δ -Aminolaevulinic acid was supplied by the Radiochemical Centre, Amersham, Buckinghamshire. Unlabelled δ -aminolaevulinate. HCl from Fluka AG, Buchs, Switzerland; haemin from George T. Gurr Ltd, London; and laevulinic acid from the Sigma Chemical Co., St Louis, Missouri, U.S.A., were used. Acids were neutralized with KOH prior to addition to the medium. Other solvents and reagents were of analytical grade.

RESULTS

Rhizobia very efficiently removed ALA and radioactivity from the medium during the first 30 min. of incubation (Fig. 1a) after which the distribution of ^{14}C between the organism and medium did not change more than a few per cent. The extracellular radioactivity, however, was no longer in the form of ALA. The faint colouring of the medium indicated that the ALA was only partially converted to oxidized porphyrins.

Fig. 1. (a) The changes in extracellular ALA (○—○) and ^{14}C (△—△) during incubation of laboratory-grown *Rhizobium*. (b) The synthesis of extra-particulate haem during the incubation. Bacteria were suspended at the rate of 0.75 g. wet organisms per 10 ml. of medium containing 1 μCi of [^{14}C]-ALA as described in Methods. Samples were removed at intervals and the required assays were performed in duplicate.

Release of haem from the bacteria into the medium occurred linearly (Fig. 1b) from an internal pool of ALA and its products, perhaps even haem, established early during the incubation. Values of Lowry-positive substances were $230 \pm 50 \mu\text{g.}/10 \text{ ml.}$ throughout the incubation, indicating that lysis was not occurring.

Laevulinic acid is a competitive inhibitor of aminolaevulinate dehydratase (5-amino-laevulinate hydro-lyase, EC 4.2.1.24) in several organisms (Nandi & Shemin, 1968; Beale, 1970). In the laboratory-grown bacteria, laevulinic acid did not significantly affect the uptake of ALA, but there was 85% inhibition of incorporation into the extra-particulate haem (Table 1). It inhibited 70 to 75% in sonicates of bacteroids (Table 2) demonstrating that the major effect was not on the release of the haem into the medium. When cultures were first flushed with nitrogen and incubated in a sealed container at a reduced shaking rate, the synthesis of haem was severely inhibited and there was less uptake of ^{14}C into the bacteria (Table 1). These effects were primarily the result of the reduced oxygen tension since the agitation was sufficient to keep the bacteria suspended and the solution thoroughly mixed.

Although particulate extract from serradella was more active than the cytoplasmic extract, there was a significant synergistic effect when the two were recombined (Table 2). This

Table 1. *The effects of laevulinic acid (LA) and reduced oxygen tension on the synthesis of extra-particulate haem by laboratory-grown rhizobia*

Each result is for 1.0 g. of rhizobia (wet wt) suspended in 10 ml. of medium containing 2.0 μCi of [^{14}C]-ALA as described in Methods. Based on the specific activity of the added ALA, each nmole of newly synthesized haem is equivalent to 3520 d.p.m.

Treatment	Haem d.p.m./ 6 h./g. rhizobia (wet wt)	Control (%)	^{14}C remaining in medium (%)
Aerobic	8800	100	15
+ 10 mM-LA	1230	14	23
Anaerobic	1320	15	69

synergism is more dramatic in serradella, but it is also observed in soybean nodule extracts (Cutting & Schulman, 1969).

The particulate fraction appears to be the dominant site of haem synthesis based on ALA incorporation. Laboratory-grown rhizobia, however, were more active than the bacteroids in their capacity to synthesize haem from ALA (Table 3). When the results were expressed per organism, a reduced difference appeared due to the increased content of protein in the bacteroids, but in either case there was no increase in activity apparent in the bacteroid. Disruption of either type of bacterial cell did not decrease their relative rate of haem synthesis, although about 30 % more incorporation into haem was found in both types of sonicated extracts.

Very young nodules were not examined, but mature nodules were most active prior to greening. The results recorded for serradella bacteroids (Table 3) are of the most active preparation obtained. The field-grown serradella nodules were only partially of the WU8 strain, but there was no indication that this had any effect on activity. Lupin (*Lupinus luteus*) bacteroids, all of the WU8 strain, were lower in activity (70 to 120 d.p.m./ 10^{10} rhizobia) than serradella preparations.

DISCUSSION

The results demonstrate that *Rhizobium lupini* (strain WU8) is capable of haem synthesis when supplied with exogenous ALA. The question remains, however, whether this capacity reflects activity of haem synthesis *in vivo*.

Both laboratory-cultured bacteria and bacteroids contain very low levels of haem when compared to the nodule content of LHB haem (Smith, 1949; Appleby, 1969*a, b*). If the bacteroid is the site of haem synthesis for LHB, one would expect a dramatic increase in the activity of the pathway. We have not observed such an increase in the pathway from ALA

Table 2. *The incorporation of [14 C]-ALA into haem by the cytoplasmic and particulate fractions of serradella nodules*

Extract equivalent to 5 g. of nodule tissue was incubated in 10 ml. medium as described in Table 1 and Methods.

Extract	Treatment	Haem d.p.m./ 6 h./4 g. nodule	Control (%)
Cytoplasmic + particulate	—	11,600	100
Cytoplasmic	—	1,070	9
Particulate	—	3,470	30
Particulate	Sonicated	4,670	40
Particulate	Sonicated + 10 mM-LA	1,330	11

Table 3. *The comparison of the incorporation of [14 C]-ALA into haem by intact and sonicated preparations of laboratory-grown rhizobia and the particulate fraction of serradella nodules*

Laboratory-grown rhizobia (0.5 g.) and particulate fraction (0.5 g.) were suspended in medium as described in Table 1 and Methods.

Rhizobium	Treatment	Haem d.p.m./6 h.		
		Per g. wet cells	Per mg. protein	Per 10^{10} cells
Laboratory-grown	Intact	13,000	810	410
Nodule	Intact	6,700	300	200
Laboratory-grown	Sonicated	16,200	1,010	—
Nodule	Sonicated	9,000	400	—

to haem. Instead, laboratory-grown bacteria are more active than bacteroids. The reduced specific activity in bacteroids could be attributed to increased protein content per organism relative to an unaltered level of enzymes for haem synthesis. Increases in protein per organism during development have been recorded for bacteroids of the same strain isolated from *Lupinus luteus* nodules (Dilworth & Williams, 1967). Disruption of the rhizobia increases their synthesis of haem relative to the plant extract and this comparison is the better for measuring the competence of each symbiont in ALA incorporation.

Although many nodule parameters such as turnover rates of haem are unknown, a tentative calculation based on average nodule age, haem content and rates of ALA incorporation suggests that the level (± 40 $\mu\text{mole haem/h./g. nodule}$) of synthesis in both types of rhizobia may be adequate to produce the required amounts of LHb haem. Moreover, strains of *Rhizobium melioli* accumulate porphyrins in culture medium under rather specific conditions (Hendry & Jordan, 1969). It is clear, though, that in our laboratory-grown rhizobia under the conditions of plentiful ALA, an excess of product is being formed which is released into the medium. This may also be true of the bacteroids although their activity is lower. In soybean nodules, for example, ALA is incorporated at least tenfold more readily into haem than is any precursor of ALA (Jackson & Evans, 1966). Similar responses to exogenous ALA have been observed in other bacteria (Doss & Philipp-Dormston, 1971).

The considerable synergistic effect between cytoplasmic and particulate fractions suggests that the plant occupies a role in the synthesis from ALA, be it as a source of enzymic activity, cofactors or as an acceptor for the haem produced. The reduced activity of the bacteroids and the synergism could both be explained if one of the steps in haem synthesis from ALA is repressed in the bacteroid and carried out by the plant. Bacteroids of soybean have lost much of their capacity to form porphobilinogen from ALA when compared to the laboratory-grown *Rhizobium japonicum* (Falk, Appleby & Porra, 1959).

Based on our results and those of others (Falk *et al.* 1959; Cutting & Schulman, 1969), there does not appear to be a 'site' of haem synthesis in the nodule, but rather a co-operative effort between plant and bacteroid which must be analysed in another way. As in many organisms, the regulation of haem synthesis may be at the level of ALA synthesis in *Rhizobium* and the legume root nodule. In the latter case, ALA synthesis could still reside in or be controlled by the plant. Elucidation of this control is frustrated, however, by the repeated failure to demonstrate the mechanism of ALA synthesis in any plant tissue.

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