as a high molecular weight compound) was separated on a Sephadex G-75 column from a small amount of xanthommatin which behaved as expected.

Ajami and Riddiford used gel electrophoresis as an additional method to identify their "pigment-protein". We have not been able to repeat their results with extracts from *Drosophila* heads. We have never observed pigment associated with protein bands after electrophoresis under a variety of conditions. Similarly, in sucrose gradients, no pigments, oxidized or reduced, entered the gradients, although cytochrome *c*, used as a control, entered as expected.

We conclude that there is no evidence for an ommochrome–protein complex in *Drosophila* heads under the conditions described, and it is reasonable to suppose that the same is true in saturniid moths. Of course, since the methods used always included detergents, one cannot rule out the possibility that these reagents disrupt a pigment–protein complex. However, in view of the insolubility of xanthommatin and dihydroxanthommatin (and the more complex ommins), it may well be that the association of these pigments with proteinaceous material in pigment granules is essentially nonspecific.

References

Ajami, A., & Riddiford, L. (1971) *Biochemistry 10*, 1455–1460.

Butenandt, A., Schiedt, U., Biekert, E., & Cromartie, R. Jan. T. (1954) *Justus Liebigs Ann. Chem. 590*, 75–90.

Ephrussi, B., & Herold, J. L. (1944) *Genetics 29*, 148–175.

Fuge, H. (1967) *Z. Zellforsch. Mikrosk. Anat. 83*, 468–507.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Oliver, D. V., & Phillips, J. P. (1970) *Drosophila Inf. Serv. 45*, 58.

Phillips, J. P., & Forrest, H. S. (1973) *Genetics 73*, 45–56.

Ryall, R. L., & Howells, A. J. (1974) *Insect Biochem. 4*, 47–61.

Shoup, J. R. (1966) *J. Cell Biol. 29*, 223–249.

Leghemoglobin Biosynthesis in Soybean Root Nodules. Characterization of the Nascent and Released Peptides and the Relative Rate of Synthesis of the Major Leghemoglobins†

Desh Pal S. Verma,* Stephan Ball,‡ Claude Guérin, and Laura Wanamaker

ABSTRACT: There are two major forms of leghemoglobin (Lb) in soybean root nodules. In mature nodules, LbF (the electrophoretically fast-moving component) exists in larger quantity than LbS (slow moving), in a ratio of about 1.5:1. Analysis of their relative rates of synthesis both in vivo and in vitro indicates that in mature (>3-week-old) root nodules LbS is made at a higher rate than LbF, but in young nodules (<12 days) LbF synthesis predominates. Analysis of Lb synthesized in vitro using DEAE-cellulose chromatography showed that LbF can be resolved into two components, identical with those designated as Lbc_1 and Lbc_2 for unlabeled preparation of Lb by Appleby et al. [Appleby, C. A., Nicola, N. A., Hurrell, J. G. R., & Leach, S. J. (1975) *Biochemistry 14*, 4444]. However, Lbb, a minor component, appears to be an artifact of protein isolation and fractionation or generated due to some ligand binding. There is no precursor–product relationship between Lb components. LbF can be detected by in vitro translation of nodule polysomes 6 days after infection with *Rhizobium* and is followed by the appearance of LbS and nitrogenase activity on the 7th and 10th days, respectively. Accumulation of LbF over LbS during nodule development appears to be due to different half-lives of the two components. In vitro synthesis of Lb is initiated by methionine which is cleaved before completion of the peptide chain. The completed and released peptide of LbS has valine as its amino terminus, which corresponds to its known sequence. Lack of a precursor and its vectorial discharge into membrane vesicles suggests that Lb does not cross the membrane envelope enclosing the bacteroids and remains in the host-cell cytoplasm after translation.

The presence of leghemoglobin (Lb)[1] in legume root nodules, developed due to the symbiotic association of legume roots with *Rhizobium*, is directly correlated with their effectiveness in fixing atmospheric nitrogen (Virtanen et al., 1947; Smith, 1949; Jordan & Garrard, 1951; Graham & Parker, 1961; Johnson & Hume, 1973). The role of Lb in facilitating the diffusion of oxygen at a relatively low oxygen tension (Appleby, 1969, 1974; Bergersen et al., 1973; Wittenberg et al., 1974; Bergersen & Turner, 1975) is well established. Owing to such oxygen tension, Lb may prevent the rapid diffusion of oxygen to the bacteroids where nitrogenase, an oxygen-sensitive enzyme, is located (see Verma et al., 1978).

Most legumes have several chromatographically distinguishable leghemoglobins in their root nodules. In soybean there are two major forms, LbS and LbF, which are distinguishable electrophoretically (Ellfolk, 1972). However, on DEAE-cellulose chromatography, while LbF can be resolved into two subcomponents, three other minor species have been

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[1] Abbreviations used: Lb, leghemoglobin; LbS and LbF, electrophoretically slow-moving and fast-moving components of Lb on polyacrylamide gels (these components correspond to Lba and Lbc_1 + Lbc_2 of Appleby et al., 1975); DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)aminomethane; poly[A(+)] RNA, RNA containing poly(adenylic acid); $NaDodSO_4$, sodium dodecyl sulfate; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; δ-ALA, δ-aminolevulinic acid; Cl_3AcOH, trichloroacetic acid; $K_3Fe(CN)_6$, potassium ferricyanide.

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detected (Appleby et al., 1975). The ratio of the two major forms of Lb in soybean root nodules has been shown to change during development (Fuchsman et al., 1976). Although their primary structure (Ellfolk & Sievers, 1971; Hurrell & Leech, 1977; Sievers et al., 1977) suggests that they are distinct gene products, part of the Lb multiplicity could be caused by oxidation/reduction states of Lb components and their binding to ligands (Appleby et al., 1973a,b). It is yet to be determined if these distinct forms have different functions in root nodules.

Little is known about the biosynthesis of Lb in legumes and the control and/or contribution of each symbiotic partner to the relative rates of synthesis of various Lb components, their assembly, and their stabilities in the nodules. We have shown (Verma & Bal, 1976; Verma et al., 1978) that in soybean apo-Lb is synthesized on free polysomes in the host-cell cytoplasm where it has been localized. Since bacteroids synthesize large quantities of heme in nodules (Cutting & Schulman, 1969; Godfry & Dilworth, 1971; Nadler & Avissar, 1977), it is assumed that this heme is used by newly synthesized apo-Lb in the assembly of a functional molecule. If Lb is present in the space between the bacteroid and the membrane envelope enclosing them (Dilworth & Kidby, 1968; Bergersen & Goodchild, 1973; Gourret & Fernandez-Arias, 1974), apo-Lb must cross the membrane enclosing the bacteroids. Accordingly Lb may be synthesized as a precursor molecule similar to other secretory proteins (Blobel, 1977), and its assembly with heme may take place within the space between the membrane envelope and the bacteroids (Appleby, 1974).

We present evidence that (1) apo-Lb is synthesized as a discrete molecule, with methionine as an initiator, and (2) the released peptide, with an N-terminal valine (LbS) or glycine (LbF), does not cross the membrane envelope enclosing the bacteroids. Its assembly with heme (synthesized and secreted by bacteroids) appears to take place in the host-cell cytoplasm where the functional molecule is principally located (Verma & Bal, 1976; Verma et al., 1978). The relative rates of biosynthesis of LbS and LbF change during root-nodule development; LbF synthesis predominates in young nodules while LbS predominates in mature. The two Lb components appear to turn over with different rates, resulting in the accumulation of LbF, and thus they may have different roles in root-nodule symbiosis. Both LbS and LbF are detected before the appearance of nitrogenase activity during development of root nodules.

Materials and Methods

Isolation of Polysomes and mRNA. Soybean (*Glycine max*, var. Prize) was obtained from Strayer Seed Farms, Hudson, IA, and germinated in vermiculite at 27 °C. Three-day-old seedlings were inoculated with *Rhizobium japonicum* (strain 61A76) and grown as described earlier (Verma et al., 1974; Verma & Bal, 1976). Nodules were harvested and frozen immediately in liquid nitrogen and stored until used. Total polysomes were isolated (Verma et al., 1974) or separated into free and membrane-bound fractions (Verma & Bal, 1976). Polysomes were resuspended in 20 mM KCl, 1 mM $Mg(OAc)_2$, and 5 mM mercaptoethanol and used immediately for translation studies or frozen as pellets in liquid nitrogen. Polysomal RNA was extracted with chloroform–phenol–isoamyl alcohol (50:50:1 v/v) and fractionated on an oligo(dT)-cellulose column as previously described (Verma et al., 1974) with the exception that $NaDodSO_4$ was omitted from the binding buffer and RNA was recovered by centrifugation at 50000 rpm for 16 h in a Beckman 65 rotor. RNA was resuspended in doubly distilled water and frozen at −70 °C.

In Vitro Translation. Polysomes and RNA fractions were translated in a wheat germ cell-free system prepared according to Marcu & Dudock (1974) and treated with micrococcal nuclease (150 units/mL) for 10 min at 20 °C (Pelham & Jackson, 1976) in order to reduce endogenous (mRNA) activity (Verma & Ball, 1977). The translation reaction contained in 100 μL the following: appropriate concentrations of polysomes or mRNA, 15 μL of micrococcal nuclease-treated wheat germ S-23, 1 mM ATP, 8 mM creatine phosphate, 4 μg of creatine phosphokinase, 25 μM GTP, 2 mM dithiothreitol, 90 mM potassium acetate, 2.5 mM $Mg(OAc)_2$, 2 μg of wheat germ tRNA, 40 μM spermidine tetrahydrochloride (neutralized), 10 μCi of [4–5-^{3}H]leucine (sp act. 120 Ci/mmol, Amersham Corp.), 50 μM each of 19 unlabeled amino acids, and 20 mM Hepes buffer (pH 7.6). The reaction mixture was incubated for 90 min at 25 °C. After incubation the sample was diluted (1:1) with distilled water and centrifuged for 60 min at 50000 rpm in a Beckman 65 rotor. Released peptides were separated from nascent material which pelleted with the ribosomes. An aliquot (5–10 μL) was precipitated with hot Cl_3AcOH (Verma et al., 1974) to determine total incorporation.

Analysis of Translation Product. Three major species of leghemoglobin, LbS(a), LbF(c_1), LbF(c_2), and a minor component, Lbb, were isolated from 100 g of soybean nodules by DEAE-cellulose chromatography as described by Appleby et al. (1975). Their purity was checked by rechromatography on DEAE-cellulose and electrophoresis on disc gel (Davis, 1964) at pH 8.9. An aliquot of the translation product was analyzed on disc gel after mixing with 10 μg each of LbS and LbF, 5 μg of hemin (Sigma Chemical Co.) dissolved in ethylene glycol (Pelham & Jackson, 1976), 100 μg of $K_3Fe(CN)_6$ (in excess of molar equivalent of Lb), 10% glycerol, and 2 μL of bromophenol blue (water-saturated solution). The gel (7.5% acrylamide) was prepared as a 1.5-mm thick slab with stacking gel photopolymerized on the top in a Bio-Rad slab gel electrophoresis apparatus. Electrophoresis was carried out at 20 mA of constant current/slab. Care was taken not to exceed the maximum voltage of 200 V in order to minimize heating. After electrophoresis, gels were stained with Coomassie blue (0.5% in 10% acetic acid and 25% methanol), and they were destained in the above solvent and fluorographed (Bonner & Lasky, 1974) with prefogged X-ray film (Kodak XR-1). Plates were stored at −70 °C during the exposure period. To determine the LbF/LbS ratio, the negatives were scanned with a densitometer and gravimetric analysis was performed.

In order to analyze translation products by DEAE-cellulose chromatography, released peptides were mixed with crude homogenate from nodules and ammonium sulfate (up to 50% saturation) was added. The resulting pellet was discarded and the supernatant was made 80% saturated with ammonium sulfate. The precipitate thus obtained (containing Lb) was dissolved in 1 mL of Tris-acetate, pH 9.00, and 2 mg of $K_3Fe(CN)_6$ was added. The mixture was passed over a column (0.7 × 20 cm) of Sephadex G-25 (Pharmacia Fine Chem.) equilibrated in 0.01 M sodium acetate buffer, pH 5.2. The pooled Lb peak fractions were applied to a Whatman DEAE-52 cellulose column (0.9 × 25 cm). The column was packed in 0.5 M acetate buffer (pH 5.2) and washed with distilled water until the effluent conductivity was that of 0.01 M acetate buffer. The column was eluted with a linear gradient of 10–50 mM acetate buffer, pH 5.2 (100 mL). The eluate was monitored with an ISCO-UA-5 UV monitor and about 2-mL fractions were collected. An aliquot (100–200

μL) of each fraction was mixed with 6 mL of Aquasol (New England Nuclear Corp.), and the radioactivity was determined in a Beckman LS-333 scintillation counter. The peak fractions were concentrated with a UM-10 Diaflo membrane (Amicon Corp.) after raising their pH to 9.0 (Verma & Bal, 1976) with Trizma base (Sigma Chemical Co.) and electrophoresed on disc gel at pH 8.9 as above.

Determination of the Sequence of [^{3}H]Valine-Labeled Product. A 9S poly(A)-containing mRNA fraction was prepared from total poly[A(+)] mRNA by two cycles of sucrose gradient centrifugation (Verma & Ball, 1977). This mRNA was translated in the nuclease-treated wheat germ translation system using 10 μCi of [2,3-^{3}H(N)]valine (sp act. 14.5 Ci/mmol; New England Nuclear). The completed and released peptides were mixed with 100 μg of pure LbS and precipitated with acid-acetone (0.006 N HCl in acetone) at −20 °C for 14 h. The pellet was resuspended in 20% acetic acid and 150 nmol of myoglobin was added (as a carrier for sequencing). The sample was subjected to microsequencing on a Beckman 890B automatic sequencer. Sequencing was carried out by using a quadrol program (Crine et al., 1977). In the first cycle no coupling reagent (phenyl isothiocyanate) was added and the fraction collected was considered as "wash". In subsequent cycles thiazolinones collected in butyl chloride were analyzed directly for radioactivity in a toluene-based scintillation mixture (Crine et al., 1977). Fifteen cycles of sequencing were carried out to detect the presence and position of valine in LbS synthesized in vitro.

Analysis of the Product Formed with Pactamycin. Translation of 9S mRNA was carried out as above in the presence of pactamycin (5×10^{-6} M) or fusidic acid (300 μg/mL) for 30 min at 25 °C using 20 μCi of [^{35}S]methionine (sp act. 510 Ci/mmol; Amersham Corp.). Ribosomes were isolated from the incubation mixture by centrifugation at 50 000 rpm for 60 min in a Beckman 65 rotor. Ribosome-associated peptides were released by incubation with 40 mM NaOH for 30 min at 37 °C and were analyzed on paper electrophoresis (Kappen & Goldberg, 1976). Standards were run in parallel and stained with ninhydrin (0.3% in acetone containing 10% acetic acid). Radioactivity in 1-cm strips was determined in 10 mL of Omnifluor (New England Nuclear) in toluene.

Removal of Heme from Lb and Its Reconstitution. Heme was removed from purified LbS (0.2 mg in 0.3 mL) twice by HCl–acetone. The resulting pellet was dissolved in 0.2 mL of H_2O and divided into two parts and lyophilized. To one sample 50 μL of hemin dissolved in ethylene glycol was added, and ethylene glycol (50 μL) was added to the other sample. To both samples 100 μL of 0.1 M Tris-HCl, pH 8.00, was added and the samples were left at room temperature for 2 h. Just prior to electrophoresis on disc gels, sample buffer (Tris–glycine, 10% glycerol, 100 μg of $K_3Fe(CN)_6$, and 2 μL of bromophenol blue) was added to each sample. They were electrophoresed on a disc gel at pH 8.9 and stained with Coomassie blue as described above.

Removal of heme from Lbb was accomplished by the silver sulfate method used for cytochrome *c* (Falk, 1964). To Lbb (1 mg in 0.4 mL of acetate buffer, pH 5.2) was added 0.6 mL of distilled H_2O and 0.3 mL of glacial acetic acid. One milliliter of silver acetate (0.024 M) was added to the above mixture and the mixture incubated for 80 min at 60 °C. After incubation, the sample was cooled and passed through a column of Sephadex G-25 equilibrated in 0.1% NH_4HCO_3. Fractions containing apo-Lbb were collected and lyophilized. To ascertain that the tenacity of heme binding with Lbb was not due to poor removal of nicotinic acid, the method for removal of nicotinic acid and oxidation of Lb (Appleby et al., 1975) was repeated.

Measurement of the Vectorial Discharge of Lb Peptide. Translation of mRNA from free polysomes was carried out in the presence of "stripped" membranes (Blobel & Dobberstein, 1975) prepared from 12-day-old nodules to determine if any of the nascent material synthesized in response to this mRNA is vectorially discharged into membranes and thereby passes through the membrane envelope enclosing the bacteroids. As a control experiment, membrane-bound poly[A(+)] mRNA from 12-day-old nodules, which were actively synthesizing membrane proteins (unpublished data), was translated in the presence of stripped membranes. Total membranes were prepared from 12-day-old root nodules by homogenizing 2 g (fresh weight) of tissue in 6 mL of 0.1 M Tris, pH 8.00, 0.4 M sucrose, 5 mM mercaptoethanol, and 0.1 mM $MgCl_2$. The particulate material sedimenting between 5000- and 100000*g* (30 min) was resuspended in 0.05 M Tris, pH 8.0, 10 mM EDTA, and 5 mM mercaptoethanol and was layered on a sucrose gradient (Shore & Maclachlan, 1975). The fraction sedimenting at a density of 1.11 g/cm^3 was collected. It was diluted (1:1) with water, centrifuged at 20000*g* for 10 min, and resuspended in 20 mM KCl, 1 mM $MgCl_2$, 5 mM mercaptoethanol, 20 mM Hepes, and 20% glycerol. These membranes (20–50 μg) were used in the translation assay. After translation, one aliquot was treated with 0.2% Triton X-100 and directly centrifuged in an Eppendorf microcentrifuge, while two aliquots were treated with trypsin (0.4 mg/mL) with and without Triton X-100 for 60 min at 32 °C before centrifugation. The radioactivity in the supernatant and pelleted fractions was determined after precipitation with hot Cl_3AcOH.

Determination of Lb Concentration and Nitrogenase Activity during Root-Nodule Development. The concentration of Lb in the postmitochondrial supernatant (23000*g*) was determined by the pyridine hemochromogen method (deDuve, 1948) using E_{mM} at 557 nm of 34.6 (Paul et al., 1953). The concentration of total Lb (including apo-Lb which may be present in young nodules) was determined by rocket immunoelectrophoresis (Axelson et al., 1973) by use of monospecific antisera to Lb. Gels (1% agarose in 0.04 M Barbital buffer III, Bio-Rad Lab., pH 8.6) containing 4–5 μL of antiserum/cm^2 of gel were cast on a 10 × 10 cm glass plate. Electrophoresis was carried out at 200 V for 2.5 h by use of the barbital buffer. Purified Lb was used as a standard. Gels were washed in 0.1 M NaCl, pressed with filter papers, and stained with Coomassie blue (0.2%) in 50%:10% methanol–acetic acid. The height of the rockets were proportional to the Lb concentrations.

In order to measure the translatable Lb mRNA in nodules, polysomes were prepared at different stages of development and translated in vitro, and the product was analyzed on disc gels, which was then fluorographed as described above.

Nitrogenase activity was measured by incubating root nodules (attached to the roots) in a serum-stoppered glass tube in an atmosphere of 20% C_2H_2, 10% O_2, and 70% He (Hardy et al., 1968). Reduction of C_2H_2 to C_2H_4 was measured on a gas chromatograph (Hewlett-Packard, 5720A) by use of a Porasil C/phenyl isocyanate column (132 × 4 mm) (Chromatographic Specialties Ltd.).

Results

In Vitro Synthesis of Lb. Between the two major forms of Lb in soybean root nodules, LbF always exists in a larger quantity than LbS (a ratio of >1.5:1) in nodules of different

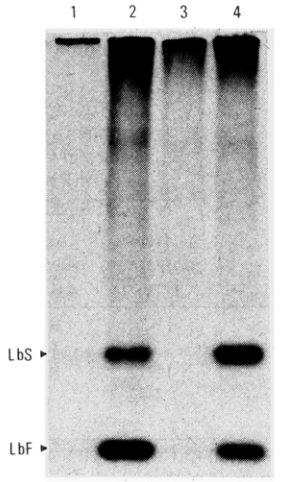

FIGURE 1: Disc gel electrophoresis of the in vitro translation product synthesized by poly[A(−)] and poly[A(+)] mRNA from 12-day (lanes 1 and 2) and 21-day (lanes 3 and 4) root nodules. Completed released peptide chains were prepared as described under Materials and Methods, mixed with heme, and oxidized with $K_3Fe(CN)_6$. Purified LbS and LbF were electrophoresed in a parallel slot as markers. Gels were stained, dried, and fluorographed on prefogged X-ray films (see Materials and Methods for details).

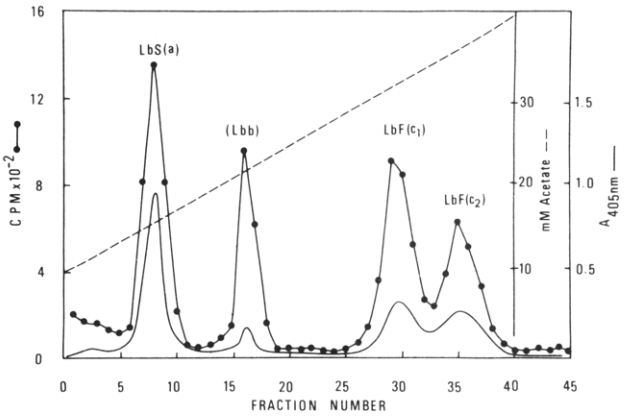

FIGURE 2: Elution profile of soybean leghemoglobins cochromatographed with the in vitro translation product on DEAE-52 cellulose column. Column was eluted with a linear gradient of acetate buffer, pH 5.2 at 2 °C, and optical density was measured by monitoring the effluent through an ISCO UV monitor. Radioactivity associated with each fraction was measured in Aquasol (New England Nuclear). Peak fractions were pooled, concentrated, and analyzed on disc gels to establish their identity.

ages (Fuchsman et al., 1976). However, biosynthetic data, both in vitro (Verma et al., 1974) and in vivo (Verma, (1976), suggested that LbS is the predominant product of translation in mature nodules. The possibility of a precursor–product relationship between LbS and LbF was excluded by the lack of transfer of radioactivity from LbS to LbF during pulse-chase experiments (Ball, 1978). From analysis of the in vitro translation product of Lb mRNA on nondenaturing disc gels it appeared that the newly synthesized product was aggregating (see Figure 5, Verma et al., 1974). Furthermore, since this analysis was performed without added heme, depending upon the availability of residual heme in the wheat germ translation system, it is possible that most of the product may represent apo-Lb (which moves slower than Lb), giving a broad peak corresponding to LbS. By adding extra heme to the in vitro translation product and oxidizing it with excess $K_3Fe(CN)_6$ (Appleby et al., 1975), we obtained two tight bands of Lb on disc gels (Figure 1) corresponding to the purified LbS and LbF markers. Even under these stringent conditions, the translation product of poly[A(+)] mRNA from mature nodules (>3-weeks-old) showed that LbS is made in quantities larger than LbF (Figure 1, slot 4). Similar experiments performed on young nodules (<12 days) revealed that LbF is the major product of translation at this earlier stage of root-nodule development (Figure 1, slot 2). These differences are not due to any differential rate of translation since both polysomes (initiated in vivo) and mRNA (initiated in vitro) gave similar results.

Translation of poly[A(−)] mRNA fractions showed that the bulk of the LbS and LbF mRNA activity is present in poly[A(+)] fractions, and its distribution does not change during root-nodule development (Figure 1, slots 1 and 3). Although Lb was a major product of translation in poly[A(+)] mRNA, it represented less than 2% of the total poly[A(−)] mRNA activity (cf. Verma et al., 1974).

Analysis of soybean Lb on a DEAE-cellulose column has shown (Appleby et al., 1975) that, besides LbS (Lba) and LbF (which can be resolved into two components, c_1 and c_2, existing in almost equal concentrations), there are three other minor forms of leghemoglobin, Lbb, Lbd_1, and Lbd_2. In order to determine whether these latter forms are true products of translation or are formed due to any posttranslational modifications, we analyzed the in vitro translation products of nodule polysomes under the conditions described by Appleby et al. (1975). Total polysomes were isolated from 2-week-old root nodules and translated in vitro in the nuclease-treated wheat germ translation system as described under Materials and Methods. The released peptide chains were collected, mixed with a crude Lb fraction, and analyzed on a DEAE-cellulose column. Figure 2 shows a typical profile of Lb as obtained by Appleby et al. (1975). The radioactivity profile corresponds with fraction a, b, c_1, and c_2. The identity of each peak was confirmed by disc gel electrophoresis. No radioactivity was present beyond fraction number 40 of Figure 2 where d_1 and d_2 are eluted from the column (data not shown). Whereas Lbb represented only about 8% of the total Lb, the radioactivity associated with this fraction was much higher (17.5%) and varied in different preparations. This fraction is not detectable in direct analysis of the product on disc gels after treatment with heme and $K_3Fe(CN)_6$ (see Figure 1).

Characterization of Lbb. In an effort to understand the nature of Lbb, the purified Lbb fraction was analyzed for dissociation and reassociation with heme. While heme can be easily removed from LbS (Lba) and LbF (Lbc_1 + Lbc_2) by the classical acid–acetone procedure, this treatment does not appear to remove a significant amount of heme from Lbb. However, it could be removed by the silver sulfate treatment effective for cytochrome *c* (Falk, 1964). Apo-Lbb obtained after silver sulfate treatment migrates very close to apo-LbS on nondenaturing gels (data not shown). The tenacity of heme binding to Lbb is not due to nicotinic acid since repeated attempts to remove it failed to facilitate heme dissociation.

Reassociation of heme with apo-LbS (Lba) showed (Figure 3) that, while most of the apo-LbS is converted into holo-LbS, a band comigrating with the position of Lbb on disc gels is formed. The intensity of this band is variable in different preparations and increases significantly in crude extracts stored for several days at 4 °C. These data suggest that Lbb may be an artifact of isolation/fractionation procedures or it may be formed due to the binding of some other ligands, the concentrations of which vary in the extract. Ligand binding influences the dissociation of heme (Bunn & Jandl, 1968). In any event there are three major products of translation of Lb mRNA: LbS (Lba) and LbF, the latter consisting of Lbc_1 and Lbc_2 in almost equal quantities.

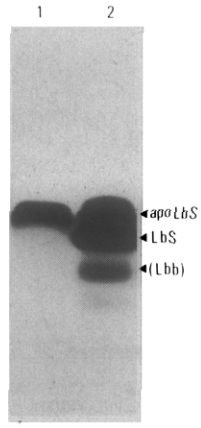

FIGURE 3: Disc gel electrophoresis of apo-LbS (lane 1) after reconstitution with heme and oxidation with $K_3Fe(CN)_6$ (lane 2). Position of apo-LbS, LbS, and Lbb run on the parallel slot is marked by the arrows.

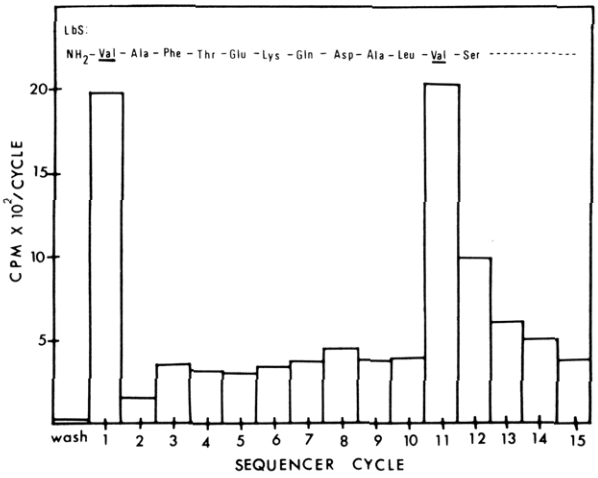

FIGURE 4: Sequence analysis of the in vitro translation product of 9S mRNA labeled with [^{3}H]valine. Analysis on sodium dodecyl sulfate gels showed that Lb is the predominant product (>96%) of translation by this mRNA preparation, which was also confirmed by immunoprecipitation with Lb antibodies (data not shown). The completed released peptides were precipitated with HCl–acetone and processed for microsequencing as described under Materials and Methods. Fifteen cycles of sequencing were carried out. The known sequence of LbS up to 15th position is marked (Ellfolk, 1972).

Sequence Analysis of the Amino Terminus of Newly Synthesized Product. Since apo-Lb appears to utilize heme synthesized by *Rhizobium* during symbiosis (Cutting & Schulman, 1969; Godfrey & Dilworth, 1971; Nadler & Avissar, 1977), and if Lb is principally localized in the space between bacteroids and the membrane envelope enclosing them (Dilworth & Kidby, 1968, Bergerson & Goodchild, 1973; Bourret & Fernandez-Arias, 1974), apo-Lb must cross the membrane enclosing the bacteroids and thus may be synthesized as a precursor molecule (Blobel, 1977). To test this possibility, a purified 9S mRNA fraction which synthesized predominantly Lb in vitro (Verma & Ball, 1977) was translated in the nuclease-treated wheat germ translation system in the presence of [^{3}H]valine. This amino acid exists on the first and eleventh positions of LbS (Ellfolk, 1972). Microsequencing of the released translation product shows (Figure 4) that radioactivity is eluted at the first and eleventh cycles of the sequenator corresponding to the known positions of valine in LbS. Since LbF is very similar to LbS in its sequences (Nicola, 1975; Hurrell & Leech, 1977; Sievers et al., 1977), it is unlikely that it would be synthesized as a precursor. Lack of a precursor is consistent with the preferential translation of Lb on free polysomes (Verma & Bal, 1976).

Table 1: Vectorial Discharge of the Nascent Peptides Synthesized by mRNA from Free and Membrane-Bound Polysomes of Root Nodules and Rabbit Reticulocytes[a]

	incorporation of nascent peptides into membrane vesicles in the presence of mRNA from		
treatment	free polysomes (cpm)	membrane-bound polysomes (cpm)	rabbit reticulocytes (cpm)
untreated	1926	9275	2687
Triton X-100	791	1235	307
trypsin	1502	8223	2602
Triton X-100 + trypsin	142	330	222

[a] Translation was carried out using mRNA from free and membrane-bound polysomes of root nodule (Verma & Bal, 1976) and rabbit reticulocytes as described under Materials and Methods in the presence of membrane vesicles (20 μg of protein) prepared from 12-day-old nodules. Rabbit globin mRNA was prepared from reticulocyte lysate (Gibco Co.) as for Lb (Verma et al. 1974). After translation, aliquots containing 100 000 cpm from each sample were treated as above with Triton X-100 (0.2%) and trypsin (0.4 mg/mL) in 20 mM KCl, 1 mM $MgCl_2$, 5 mM mercaptoethanol, 20 mM Hepes (pH 7.6), and 20% glycerol. They were incubated for 1 h at 32 °C and centrifuged for 10 min at 14000*g* to obtain a membrane pellet. The pellet was washed with the above buffer and dissolved in 0.1 M NaOH. Hot Cl_3Ac-OH-insoluble radioactivity was determined (see Materials and Methods).

Since some secretory proteins such as ovalbumin are not synthesized as precursors (Palmiter et al., 1978), we investigated the possibility of segregation of nascent Lb chains into membrane vesicles cotranslationally. The data in Table I show that, while membrane-bound mRNA can discharge some of its product vectorially into membrane vesicles (line 3), very little product directed by free mRNA was found in membrane vesicles (see also rabbit globin, control). It is the latter fraction which translates Lb preferentially though both mRNA have similar activity (Verma & Bal, 1976). These results suggest that, since Lb does not have any "signal peptide" and does not segregate into membrane vesicles, it may not cross the membrane envelope enclosing the bacteroids.

Initiation of Lb Translation in Vitro. The translation of most eucaryotic proteins is initiated with methionine. Completed and released peptides of Lb do not contain any methionine. Thus, if Lb were also initiated with methionine, it must be cleaved before completion of the peptide chain. We translated Lb mRNA in vitro in the presence of pactamycin, an inhibitor which allows formation of the first dipeptide (Kappan & Goldberg, 1973). Translation was carried out in the presence of [^{35}S]methionine and the product was analyzed on paper electrophoresis. Figure 5 shows that a peak corresponding to the Met-Val marker is present in the translation product of 9S mRNA. The second peak migrates very close to Met-Gly, the first dipeptide in LbF, although some activity in this peak was obtained without added mRNA, which may represent endogenous activity. In the absence of Lb mRNA in the translation system and in the presence of fusidic acid which inhibits the translocation reaction (Cundliffe, 1972), the formation of both dipeptides is prevented (Figure 5). We are not certain when methionine is cleaved from the Lb nascent chain; however, in analogy with hemoglobin synthesis, it may be removed when the nascent chain is about 15–20 amino acids long (Jackson & Hunter, 1970). Due to the lack of large

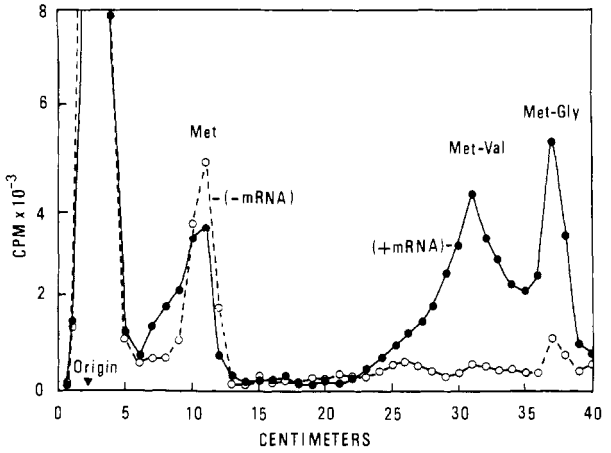

FIGURE 5: Electrophoretic analysis of the product synthesized by 9S mRNA of soybean root nodules in presence of Pactamycin. Translation was carried out as described under Materials and Methods using 20 μCi of [^{35}S]methionine and 50 μg each of unlabeled amino acids. The inhibitors were added at the beginning of the reaction. Presence of fusidic acid in the reaction gave similar results as without added mRNA (data not shown). Standards (20 μg each) of methionine, Met-Val, and Met-Gly were run in parallel.

Table II: Ratio of LbF/LbS during Development of Soybean Root Nodules

age of nodules (days after infection)	rate of synthesis (LbF/LbS) in vivo[a]	rate of synthesis (LbF/LbS) in vitro[b]	total accumulation (LbF/LbS)
12	1.74	1.9	*c*
14	1.44		1.75
21	0.95	0.86	1.5
28	0.74		1.46

[a] Measured by in vivo labeling of nodules with [^{3}H]leucine, followed by isolation of Lb and analysis on disc gels as described under Materials and Methods. [b] Total polysomes were isolated and translated in vitro in wheat germ translation system. The product was analyzed on disc gel as above. [c] Based upon the absorbance at 410 nm this ratio could not be determined accurately at this stage of root-nodule development.

quantities of Lb mRNA, we are currently unable to determine precisely these events.

Synthesis of Lb in Relation to the Appearance of Nitrogenase Activity in Root Nodules. There is a close relationship between the presence of Lb and the development of nitrogenase activity in nodule bacteroids. We determined the appearance of Lb by using three methods differing in sensitivity: direct measurement of hemochromogen, the use of antibodies against Lb, and analyses of the translation product of nodule polysomes during nodule development. Figure 6 shows that Lb measured as hematin appeared on the same day as the nitrogenase activity. However, the presence of apo-Lb can be detected 3 days and the in vitro translation product 4 days prior to nitrogenase activity. Nitrogenase activity is detected on the 10th day after infection. Of the two Lb, LbF appears first and is followed by LbS (see Figure 6, inset).

The accumulated ratio of LbF/LbS declines during root-nodule development (Table II; see also Fuchsman et al., 1976). The biosynthetic data both in vivo and in vitro show an even greater decline in the synthesis of LbF as the nodules mature. These data suggest that the two Lb components have different half-lives and as a result LbF accumulates over LbS even though the latter is synthesized at a higher rate in mature nodules. Since Lb is a relatively stable molecule (Lb half-life in lupin nodule is about 18 days; Dilworth & Coventry, 1977), their differential rate of synthesis during root-nodule development suggests that these are different gene products and may have different functions in root-nodule symbiosis.

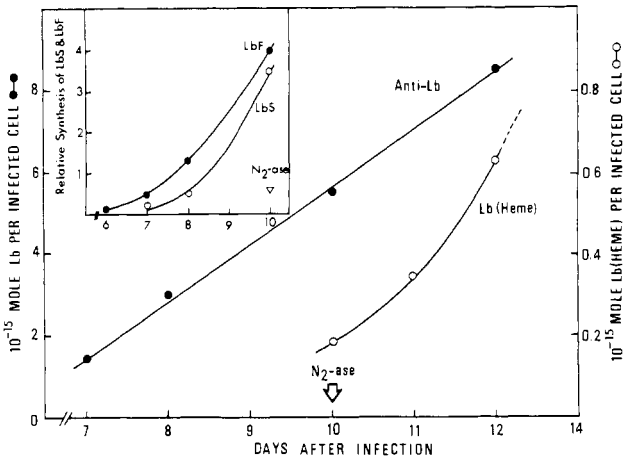

FIGURE 6: Appearance of Lb in relation to nitrogenase activity during root-nodule development. Total Lb was determined by hemochromogen (Lb-heme), by rocket immunoelectrophoresis (anti-Lb), and by in vitro translation of polysomes in wheat germ translation system (inset) (see Materials and Methods). Relative rates of synthesis of LbS and LbF were measured by electrophoresis of the translation products on disc gels and densitometric analysis of the fluorographs. Nitrogenase was assayed by reduction of acetylene into ethylene. The data are presented per infected cell which were counted in 10-μm thick sections after staining with hematoxylin (Fisher). Volume of the nodules was calculated according to Bergerson & Goodchild (1973). Interstitial cells were excluded from the infected zone.

Discussion

Synthesis of Lb in Vitro. We have shown (Verma et al., 1974) that Lb is encoded by a 9S mRNA which is preferentially translated on free polysomes (Verma & Bal, 1976) in the host-cell cytoplasm. Analysis of the in vitro translation product under nondenaturing conditions, where LbS and LbF can be resolved, showed (Verma et al., 1974) that LbS was a predominant product of translation. Reexamination of the in vitro translation product of mRNA from mature nodules (>21 days) under more stringent conditions, i.e., by adding heme to the translation product and oxidizing it with ferricyanide before electrophoresis, showed that LbF and LbS are well resolved although their ratio is still far lower (0.86) than their steady-state level (1.5) at this stage of development (Figure 1). A reverse picture is obtained by analyzing young nodules (<12 days after infection) where LbF is found to be the predominant product of translation. These differences are due neither to the lack of poly(A) on mRNA (Figure 1), to methylation of mRNA (similar inhibition of LbS and LbF by 7-methylguanosine 5′-monophosphate; Hickey et al., 1976), nor to differential translatability as judged by changing the ionic conditions of incubation (data not shown); they must reflect the concentrations of their respective mRNAs in the total RNA population.

Since soybean Lb could be resolved by chromatography on DEAE-cellulose into three major and three minor components (Appleby et al., 1975), we attempted to analyze the initial translation product with this method in order to determine whether these Lb components are primary gene products or whether they arise through posttranslational modifications. Four radioactive peaks, corresponding to Lb a, b, c_1, and c_2, are obtained (Figure 2). The amount of radioactivity corresponding to Lbb is highly variable in each experiment and is almost absent if the product is analyzed directly on a disc gel after treatment with heme and $K_3Fe(CN)_6$ (Figure 1). Furthermore, since Lbb can be generated in vitro during

reconstitution of apo-LbS with heme, it appears that its presence in the translation product (Figure 2) may not reflect an in vivo condition. On DEAE chromatography, we could not detect any radioactivity beyond fraction c_2 where fractions d_1 and d_2 of Appleby et al. (1975) are eluted. Thus there appear to be three major forms of Lb which are synthesized in vitro by polysomes or mRNA. These data along with the observation that addition of heme or any other tissue-specific factor has no influence on in vitro translation of Lb (Verma et al., 1974) suggest that these three forms of Lb must be coded for by three different mRNA populations, the relative levels of which appear to change during root-nodule development. The primary sequences of LbS(a) (Ellfolk & Sievers, 1971), LbF(c_1) (Nicola, 1975), and LbF(c_2) (Hurrell & Leach, 1977), although very similar, have significant differences, suggesting that they are distinct gene products. This is also borne out by the fact that the ratio of their synthesis changes during root-nodule development (Table II) and they appear to be turning over with a different rate, resulting in the accumulation of LbF in mature nodules.

Nature of the Primary Translation Product and Its Association with Heme. Although Lb has been localized in the host-cell cytoplasm (Verma & Bal, 1976; Verma et al., 1978), we investigated the possibility that apo-Lb may cross the membrane envelope enclosing bacteroids in order to associate with heme synthesized by bacteroids (Appleby, 1974). Most proteins which cross the membrane barrier are either synthesized as precursor molecules with a hydrophobic end (Blobel, 1977) or are blocked at their amino terminus (Palmiter, et al. 1978). Analysis of the newly completed peptide chain, synthesized in vitro with 9S Lb mRNA, showed that it is synthesized as a discrete molecule with valine being the amino terminal for the LbS component. Like most eucaryotic proteins, translation of Lb is initiated with methionine which is cleaved before the peptide chain is released. Furthermore, apparent lack of segregation of Lb peptides into membrane vesicles (Table I) is consistent with the notion that this molecule does not cross the membrane barrier and is localized exclusively in the host-cell cytoplasm (see Verma & Bal, 1976, Verma et al., 1978).

Most of the available evidence suggest (Cutting & Schulman, 1969; Godfrey & Dilworth, 1971; Nadler & Avissar, 1977) that apo-Lb utilizes the heme synthesized and secreted by bacteroids. This is consistent with the observation that most ineffective strains do not synthesize and secrete significant amounts of heme (Falk et al., 1959, Nadler & Avissar, 1977). The data in Table I suggest that apo-Lb may not cross the membrane enclosing the bacteroids and heme synthesized and secreted by bacteroids must associate with apo-Lb in the host-cell cytoplasm. Bacteroids enclosed in the membrane envelope (Verma et al., 1978) can synthesize labeled heme with [^{14}C]-δ-ALA and secrete it into the medium (unpublished data). Since apo-Lb may not distinguish between heme synthesized by mitochondria or bacteroids, the utilization of heme is made simply on the basis that much of the heme synthesized in this tissue is bacteroidal. This heme has been shown to be associated with apo-Lb (Cutting & Schulman, 1969). It is not known how the coordination, if any, of the heme synthesis with that of apo-Lb is regulated during root-nodule development.

Intracellular Site of Lb. The controversy regarding the intracellular site of Lb in root nodules originated due to the use of indirect methods for its localization, where the presence of Lb was correlated with heme or Fe (Dilworth & Kidby, 1968; Bergersen & Goodchild, 1973; Gourret & Fernandez-Arias, 1974). Using monospecific antibodies to Lb we demonstrated (Verma & Bal, 1976) that Lb is present in the host-cell cytoplasm where it is synthesized. We could not detect any immunoreactive material inside the isolated membrane envelopes enclosing the bacteroids (Verma et al., 1978). Robertson et al. (1978) showed by isolating the bacteroids enclosed in the membrane envelope from Lupin nodules in the presence of ferritin that these envelopes do not open and reseal during fractionation, thereby leaking all their Lb contents. These data, along with analyses of the primary translation product of Lb mRNA, conclusively prove that Lb is a cytoplasmic protein of the host cell and it does not cross the membrane envelope enclosing the bacteroids (see Verma & Bal 1976, Verma et al., 1978). Its proposed function, i.e., to regulate diffusion of oxygen (Appleby, 1969, 1974; Bergersen et al., 1973; Wittenberg et al., 1974; Bergersen & Turner, 1975), must be performed by its being in the host-cell cytoplasm. Lb does not need to be in direct contact with the bacteroids (Appleby, personal communication); however, it may require some mechanism, not yet understood, which might act as a terminal oxidase for bacteroids (see Appleby, 1974).

Synthesis of Lb in Relation to Nitrogenase Activity in the Nodules. A correlation between the presence of Lb and the effectiveness of nodules in fixing nitrogen has been observed in several legumes (Virtanen et al., 1947; Smith, 1949; Jordan & Garrard, 1951; Graham & Parker, 1961; Johnson & Hume, 1973). However, it is not certain which of the two molecules, Lb or nitrogenase (N_2-ase), appears first during nodule development. This problem is further compounded by the fact that the sensitivities of the two assays used to detect the presence of Lb and N_2-ase are very different. In soybean, Bergerson & Goodchild (1973) observed the appearance of Lb 2 days prior to the development of N_2-ase activity. By using three different assay procedures which differ vastly in their sensitivities, we detected the synthesis of Lb 4 days prior to N_2-ase activity (Figure 6). Among the two major forms of Lb, LbF appears first, followed by LbS and N_2-ase induction. It is not certain what factors control the sequence of these events. Mutations both in host and in bacteria may lead to ineffectiveness of the nodules and all ineffective nodules examined to date contain little or no Lb. Using a pure cDNA probe to Lb mRNA we have directly demonstrated that Lb sequences are present in soybean genome (Baulcombe & Verma, 1978); however, expression of these genes appears to be controlled by *Rhizobium*.

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References

Appleby, C. A. (1969) *Biochim. Biophys. Acta 188*, 222–229.
Appleby, C. A. (1974) in *The Biology of Nitrogen Fixation* (Quispel, A. Ed.) Chapter 11.5, pp 521–554, North-Holland Publishing Co., Amsterdam.
Appleby, C. A., Wittinberg, B. A., & Wittinberg, J. B. (1973a) *Proc. Natl. Acad. Sci. U.S.A. 70*, 564–568.
Appleby, C. A., Wittinberg, B. A., & Wittinberg, J. B. (1973b) *J. Biol. Chem. 248*, 3183–3187.

Appleby, C. A., Nicola, N. A., Hurrell, J. G. R., & Leach, S. J. (1975) *Biochemistry 14*, 4444–4450.
Axelson, N. H., Knoll, B., & Weeke, B., Eds. (1973) *A Manual of Quantitative Immunoelectrophoresis: Methods and Applications*, Univeritetsforlaget, Oslo.
Ball, S. (1978) M.Sc. Thesis, McGill University, Montreal, Canada.
Baulcombe, D., & Verma, D. P. S. (1978) *Nucleic Acids Res. 5*, 4141–4153.
Bergersen, F. J., & Goodchild, D. J. (1973) *Aust. J. Biol. Sci. 26*, 741–756.
Bergersen, F. J., & Turner, G. L. (1975) *J. Gen. Microbiol. 89*, 31–47.
Bergersen, F. J., Turner, G. L., & Appleby, C. A. (1973) *Biochim. Biophys. Acta 292*, 271–282.
Blobel, G. (1977) *Int. Cell. Biol., Pap. Int. Congr., 1st, 1976*, 318–325.
Blobel, G., & Dobberstein, B. (1975) *J. Cell. Biol. 67*, 852–862.
Bonner, W. M., & Lasky, R. A. (1974) *Eur. J. Biochem. 46*, 83–88.
Bunn, H. F., & Jandl, J. H. (1968) *J. Biol. Chem. 243*, 465–475.
Crine, P., Benjannet, S., Seidah, N. G., Lis, M., & Chretien, M. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 1403–1406.
Cundliffe, E. (1972) *Biochem. Biophys. Res. Commun. 46*, 1794–1801.
Cutting, J. A., & Schulman, H. M., (1969) *Biochim. Biophys. Acta 192*, 486–493.
Davis, B. J. (1964) *Ann. N. Y. Acad. Sci. 121*, 404–427.
DeDuve, Ch. (1948) *Acta Chem. Scand. 2*, 264–280.
Dilworth, M. J., & Kidby, D. K. (1968) *Exp. Cell Res. 49*, 148–159.
Dilworth, M. J., & Coventry, D. R. (1977) in *Recent Developments in Nitrogen Fixation* (Newton, W., Postgate, J. R., & Rodriguez-Barraeco, C., Eds.) pp 431–442, Academic Press, London.
Ellfolk, N. (1972) *Endeavor 31*, 139–142.
Ellfolk, N., & Sievers, G. (1971) *Acta Chem. Scand. 25*, 3532–3534.
Falk, J. E. (1964) *Porphyrins and Metalloporphyrins*, pp 181–183, Elsevier, Amsterdam and New York.
Falk, J. E., Appleby, C. A., & Porra, R. J. (1959) *Symp. Soc. Exp. Biol. 13*, 73–86.
Fuchsman, W. H., Barton, C. R., Stein, M. M., Thompson, J. T., & Willet, R. M. (1976) *Biochem. Biophys. Res. Commun. 68*, 387–392.
Godfrey, C. A., & Dilworth M. J. (1971) *J. Gen. Microbiol., 69*, 385–390.
Gourret, J.-P., & Fernandez-Arias, H. (1974) *Can. J. Microbiol. 20*, 1169–1181.
Graham, D. H., & Parker, C. A. (1961) *Aust. J. Sci. 23*, 231–232.
Hardy, R. W. F., Holston, R. D., Jackson, E. K., & Burns, R. C. (1968) *Plant Physiol. 43*, 1185–1207.
Hickey, E. D., Weber, L. A., & Baglioni, C. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 19–23.
Hurrell, J. G. R., & Leach, S. J. (1977) *FEBS Lett. 80*, 23–26.
Jackson, R., & Hunter, T. (1970) *Nature (London) 227*, 672–676.
Johnson, M. S., & Hume, D. J. (1973) *Can. J. Microbiol. 19*, 1165–1168.
Jordon, D. C., & Garrard, E. H. (1951) *Can. J. Bot. 29*, 360–372.
Kappen, L. S., & Goldberg, I. M. (1973) *Biochem. Biophys. Res. Commun. 54*, 1083–1091.
Kappen, L. S., & Goldberg, I. M. (1976) *Biochemistry 15*, 811–818.
Marcu, K., & Dudock, B. (1974) *Nucleic Acids Res. 1*, 1385–1397.
Nadler, K. D., & Avissar, Y. J. (1977) *Plant Physiol. 160*, 433–436.
Nicola, N. A. (1975) Ph.D. Thesis, University of Melbourne.
Palmiter, R. D., Gagnon, J., & Walsh, K. A. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 94–98.
Paul, K. G., Thesrell, H., & Akesson, A. (1953) *Acta Chem. Scand. 7*, 1284–1287.
Pelham, H. R. B., & Jackson, R. J. (1976) *Eur. J. Biochem. 67*, 247–256.
Robertson, J. B., Warbunton, M. P., Lyttleton, P., Fordyce, A. M., & Bullivant, S. (1978) *J. Cell Sci. 30*, 151–174.
Shore, G., & Maclachlan, G. A. (1975) *J. Cell Biol. 64*, 557–571.
Sievers, G., Huhtala, M. L., & Ellfolk, N. (1977) *Acta Chem. Scand., Ser. B 31* (no. 8), 723–724.
Smith, J. D. (1949) *Biochem. J. 44*, 591–598.
Verma, D. P. S., (1976) *Colloq. Int. CNRS, No. 261*, 319–325.
Verma, D. P. S., & Bal, A. K. (1976) *Proc. Natl. Acad. Science U.S.A. 73*, 3843–3847.
Verma, D. P. S., & Ball. S. (1977) in *Translation of Synthetic and Natural Polynucleotides* (Legocki, A., Ed.) pp 211–216, Poznan Agriculture University Press, Poland.
Verma, D. P. S., Nash, D. T., Schulman, M. (1974) *Nature (London) 251*, 74–77.
Verma, D. P. S., Kazazian, V., Zogbi, V., & Bal, A. K. (1978) *J. Cell Biol. 78*, 919–936.
Virtanen, A. I., Jorma, J., Linkola, H., & Linnasalmi, A. (1947) *Acta Chem. Scand. 1*, 90–111.
Wittenberg, J. B., Bergersen, F. J., Appleby, C. A., & Turner, G. L. (1974) *J. Biol. Chem. 249*, 4057–4066.