IDENTIFICATION OF "NODULE-SPECIFIC" PLANT PROTEINS (NODULINS) FROM SOYBEAN ROOT NODULES

by

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IDENTIFICATION OF "NODULE-SPECIFIC" PROTEINS OF SOYBEAN

Anyone who imagines that all fruits ripen at the same time as the strawberries knows nothing about grapes.

Paracelsus

ABSTRACT

Infection of legume roots with Rhizobium species results in the development of a root nodule structure in which the bacteria form an intracellular symbiosis with the plant. It is reported here that the infection of soybean (Glycine max L.) roots with Rhizobium japonicum results in the synthesis by the plant of at least 18-20 polypeptides other than leghemoglobin during the development of root nodules. Identification of these "nodule-specific" host polypeptides (referred to as nodulins) was accomplished by two-dimensional gel analysis of the immunoprecipitates formed by a "nodule-specific" antiserum with in vitro translation products of root nodule polysomes that are free of bacteroidal contaminations. Nodulins account for 7-11% of the total ^{35}S-methionine-labeled protein synthesized in the host cell cytoplasm, and the majority of them are of 12,000-20,000 molecular weight. These proteins are absent from the uninfected roots, bacteroids and free-living Rhizobium, and appear to be codedfor by the plant genes that may be obligatory for the development of symbiosis in the legume root nodules. Analysis of nodulins in ineffective (unable to fix nitrogen) nodules developed due to Rhizobium strains SM5 and 61A24 showed that their synthesis is reduced and their expression differentially influenced by mutations in rhizobia.

Apart from the low molecular weight nodulins, a 35,000 MW polypeptide present in the nodule cytoplasm was also identified as "nodule-specific". This protein, referred to as nodulin-35, represents about 4% of the total cytoplasmic protein in root nodules, and its appearance is not affected by mutations in several nodulating strains of Rhizobium. Nodulin-35 was not detected in uninfected soybean, bacteroids or free-living Rhizobium, and it appears to be synthesized by the plant during the formation of root nodules.

Whereas the transformation of free-living Rhizobium into bacteroids is accompanied by substantial changes within the population of cytoplasmic proteins, the majority of plant polypeptides from nodules are also present in uninfected (non-nodulated) roots. Hence, to further identify and isolate the "nodule-specific" proteins, it was essential to develop several immunological procedures, including a preparative adsorption of antibodies with anti-

gens, the multiple immunoreplica technique, and isolation of a single-copy mRNA by immunoprecipitation of the nascent peptide-polysome complex, which are described in this thesis.

In addition, two polypeptides of bacterial origin were found to be cross-reactive with the "nodule-specific" antiserum, suggesting that they are secreted into the host cell cytoplasm during symbiotic nitrogen fixation.

RESUME

L'infection des racines de légumineuses par des espèces de Rhizobium cause le développement d'une structure nodulaire sur la racine dans laquelle les bactéries forment une symbiose intracellulaire avec la plante. Nous avons trouvé que l'infection des racines de soja (Glycine max L.) par la bactérie Rhizobium japonicum cause la synthèse par la plante d'au moins 18 à 20 polypeptides, autres que la léghémoglobine, durant le développement d'un nodule. L'identification de ces polypeptides hôtes "spécifiques aux nodules" (les nodulines) fut réalisée par l'analyse sur gel bi-dimensionnel des immunoprécipitats formés par un antisérum spécifique aux nodules avec les produits de la traduction in vitro des polysomes de nodules non-contaminés par les bactéroides. Les nodulines représentent de 7 à 11% des protéines marquées par la ^{35}S-méthionine et synthétisées par le cytoplasme de la cellule hôte. Le poids moléculaire de la majorité de ces protéines se situe entre 12,000 et 20,000. Ces protéines sont absentes des racines non-infectées, des bactéroides et du Rhizobium non-associé et semblent être codées par des gènes de la plante nécessaires au développement de la symbiose. L'analyse des nodulines, produites par des nodules non-efficients (incapables de fixer l'azote) et développées par les souches de Rhizobium SM5 et 61A24, démontra une réduction de la synthèse de ces protéines et l'influence différentielle de leur expression par des mutations dans les rhizobia.

Outre les nodulines à poids moléculaires réduits, un polypeptide de 35,000, trouvé dans le cytoplasme des nodules, fut identifié comme "spécifique aux nodules". Cette protéine, nommée noduline-35, représente environ 4% du total des protéines cytoplasmiques dans les nodosités et sa présence n'est pas affectée par des mutations dans les souches de Rhizobium produisant des nodules. Cette protéine végétale ne fut pas détectée dans le soja non-infecté, dans les bactéroides ou le Rhizobium non-associé. Il semble qu'elle soit synthétisée par la plante durant la formation des nodules.

Bien que la transformation des Rhizobia non-associés en bactéroides soit accompagnée par des changements importants parmi la population des

protéines cytoplasmiques, la majorité des polypeptides végétaux des nodules sont présents dans les racines non-infectées. Ainsi, pour continuer à identifier et isoler les protéines "spécifiques aux nodules", il était essentiel de développer plusieurs procédés immunologiques, dont l'adsorption préparative des anticorps par des antigènes, la technique d'immunoreplication multiple (multiple immunoreplica technique) et la préparation d'une copie simple de mRNA par l'immunoprécipitation du complexe peptide-polysome naissant. Ces techniques sont décrites dans ce travail.

De plus, on a trouvé que deux polypeptides d'origine bactérienne démontraient une interaction croisée avec l'antisérum "spécifique aux nodules", ce que nous mène à croire qu'ils sont sécrétés par des bactéroides de Rhizobium dans le cytoplasme de la cellule hôte durant la fixation de l'azote par symbiose.

Traduit par Vahé Sarafian

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TABLE OF CONTENTS

Table of Contents (cont'd)

Table of Contents (cont'd)

Table of Contents (cont'd)

LIST OF FIGURES

PAGE

LIST OF TABLES

ABBREVIATIONS

Ab	Antibody
ATP	Adenosine 5'-triphosphate
B	Free-living bacteria
Bd	Bacteroids
BSA	Bovine serum albumin
cDNA	Complementary deoxyribonucleic acid
DEAE	Diethylaminoethyl
d(T)	Deoxythymidilate
DMSO	Dimethylsulfoxide
DTT	Dithiothreitol
EDTA	Ethylenediaminotetraacetate
EGTA	Ethylene glycol-bis (B-amino-ethyl ether) N,N^1-tetraacetic acid
GTP	Guanosine 5'-triphosphate
HEPES	N-2-hydroxyethylpiperazine-N^1-2-ethanesulfonic acid
IEF	Isoelectric focusing
kB	Kilobase(s)
kD	Kilodalton(s)
Lb	Leghemoglobin
LBF	Electrophoretically fast-moving component of leghemoglobin
LbS	Electrophoretically slow-moving component of leghemoglobin
LPS	Lipopolisaccharide
Md	Megadalton(s)

metLb	Metleghemoglobin
mRNA	Messenger ribonucleic acid
MW	Molecular weight
N-35	Nodulin-35
ND-PAGE	Non-denaturing polyacrylamide gel electrophoresis
nif	Nitrogen fixation genes
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
poly A(+) RNA	Polyadenylated ribonucleic acid
S23	Supernatant after 23,000 x g
SDS	Sodium dodecyl sulphate
SOD	Superoxide dismutase
TCA	Trichloracetic acid
TEMED	N,N,N',N-tetramethylene diamine
Tris	Tris (hydroxymethyl) amino methane
tRNA	Transfer ribonucleic acid

PREFACE

Ten thousand years ago, man learned the rudiments of agriculture; yet, despite the advances in genetics and in the technologies of planting and harvesting during those millenia, agriculture has not kept pace with the increase in human population. It has now become clear that if the production of food does not grow by a higher factor, the hunger that exists in the world will increase in a frightening way. Scientists have both the privilege and responsibility of being aware of this problem more than others. I trust that the search for truth and dignity of mankind must continue to be the highest ideals of their work. I am convinced that within the next decade or two, biologists will possess the capability of drastically enlarging our supplies of food and possibly preventing the tragedy of hunger. It is very encouraging to believe that the revolutionary development of sophisticated technologies in molecular biology of recent years is being directed by rather simple, but genuine, humanitarian goals.

INTRODUCTION AND LITERATURE REVIEW

1. BIOLOGICAL NITROGEN FIXATION

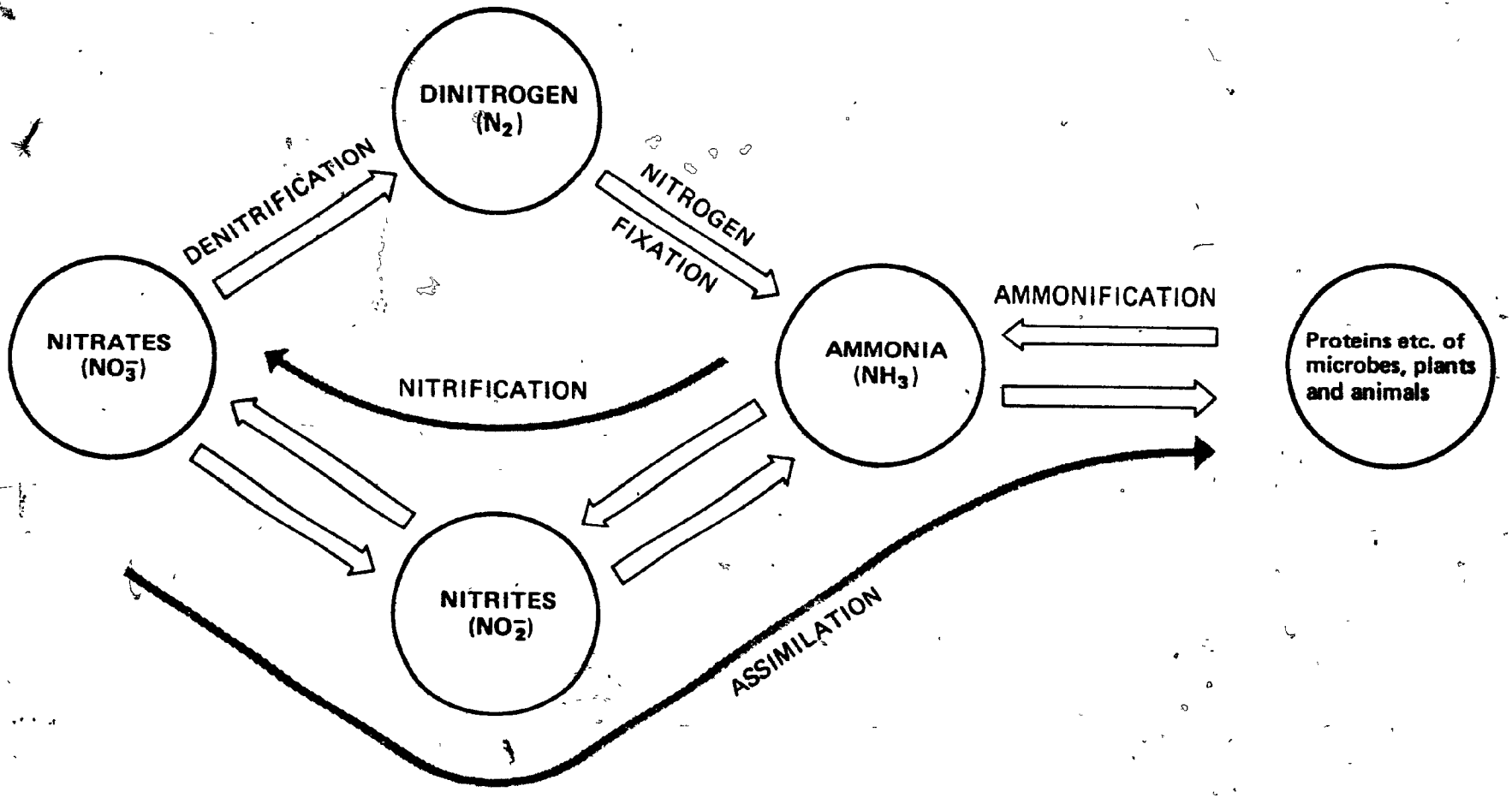
1.1 The Role of Nitrogen Fixation

Nitrogen is an essential constituent of all living organisms. As a major biological element, along with carbon, oxygen and sulphur, it is subject to cyclic processes of great importance from both ecological and economic viewpoints. The nitrogen cycle, shown in Fig. 1 , begins upon the synthesis of nitrogenous living matter (mainly protein) from inorganic nitrogen compounds (nitrate, nitrite and ammonium ions) during growth of plants and their consumption by animals, followed by their return to the soil as a result of decay and putrefaction of organic matter. The loss of nitrogen to the atmosphere from nitrates is returned to the cycle by the process known as nitrogen fixation. Recent estimates suggest that approximately 175 MM tons per annum of nitrogen is being fixed biologically, with 90 MM tons occuring in agricultural soil (Hardy, 1976), whereas only about 40 MM tons is produced chemically (Ewell, 1972).

The great importance of nitrogen in agriculture is well illustrated by the fact that, provided adequate phosphorus and potassium levels, 45 kg/ha of added nitrogen increases the corn yield from about 2195 kg to about 3763 kg/ha, corresponding to 35 kg of corn produced per kg of nitrogen added (Pimentel, 1976). Similarly, by adding 30 kg of nitrogen per hectare, rice yields increase from 3061 kg/ha to about 4542 kg/ha, or about 49 kg per kilogram of nitrogen added per hectare (Pimentel, 1976). While the importance of nitrogen in agriculture has been well established, the process of industrial fixation of nitrogen remains

FIGURE LEGEND ON OTHER SIDE

Fig. 1. Nitrogen cycle according to Postgate (1975).


DINITROGEN
(N_2)
DENITRIFICATION
NITROGEN
FIXATION
NITRATES
(NO_3^-)
NITRIFICATION
AMMONIA
(NH_3)
AMMONIFICATION
Proteins etc. of microbes, plants and animals
NITRITES
(NO_2^-)
ASSIMILATION

principally unchanged since 1913. The original Haber-Bosch process of synthesizing nitrogen gas with hydrogen under a high temperature and pressure has been slightly modified, but not changed. Since the primary resources (natural gas, naphtha and oil) for ammonia synthesis are the basis for about 88% of all ammonia produced (Sweeney, 1976), the recent energy shortage has a major impact on the ammonia industry. Dr. Raymond Ewell, world authority on fertilizer needs, recently said: "The current world fertilizer shortage will continue indefinitely, perhaps for the rest of human history" (Lovvorn, 1976). In the U.S., approximately 80 gallons of gasoline are being used to produce an acre of corn. With fuel shortages and high prices, it is doubtful if developing countries will be able to afford such technologies in the near future.

These long-standing and more recent limitations of nitrogen fertilizer intensify a search for alternative technologies. Recent developments indicate that the main impact of progress in this field is linked to a thorough study of the biological, and not industrial, nitrogen fixation. A comprehensive research in biochemistry of nitrogen fixation during the past decade, as well as the establishment of genetic engineering in plants, have recently formed a novel vision of resolving the problems related to plant productivity.

1.2. Taxonomy and Classification of Nitrogen-Fixing Organisms

Unlike most other fundamental reactions in nature, nitrogen fixation is not a characteristic of plants but is almost haphazardly distributed through a broad spectrum of microorganisms which have little in common other than their ability to reduce nitrogen. Higher plants utilize this resource by forming an association with nitrogen-fixing

TABLE 1A

SOME COMMON NITROGEN-FIXING ORGANISMS

Description	Family and incidence of N_2-fixation (genera)	Selected genus	Reference
Bacteria	Thiorhodaceae (14)	Chromatium	Arnon et al., 1961
	Athiorhodaceae (3)	Rhodopseudomonas	Lindstrom et al., 1951
	Chlorobacteriaceae (7)	Chlorobium	Lindstrom et al., 1950
	Spirillaceae (11)	Desulfovibrio	Postgate, 1970
	Azotobacteriaceae (5)	Azotobacter	Coty, 1967
	Enterobacteriaceae (10)	Klebsiella	Mahl, 1965
	Bacillaceae (3)	Bacillus	Grau and Wilson, 1963
	Mycobacteriaceae (2)	Mycobacterium	Biggins and Postgate, 1971
Blue-green algae	Chroococcaceae (1)	Gleocapsa	Gilchrist et al., 1972
	Nostocaceae (31)	Anabaena	Gorkom and Donze, 1971
	Rivulariaceae (5)	Calothrix	Schneider et al., 1960
	Scytonemataceae (3)	Scytonema	Laloraya and Mitra, 1970
	Oscillatoriaceae (3)	Oscillatoria	Mayse et al., 1957
	Stigonemataceae (6)	Fischerella	Pankow, 1964
Non-legume angiosperm symbioses[a]	Coriariaceae (12)[b]	Coriaria	All listed non-legume angiosperms after Bond (1967), and Burns and Hardy (1975)
	Myricaceae (11)[b]	Myrica	
	Betulaceae (25)[b]	Alnus	
	Casuarinaceae (14)[b]	Casuarina	
	Elaeagnaceae (9)[b]	Elaeagnus	
	Rhamnaceae (30)[b]	Ceanothus	

[a] The most commonly observed microorganisms in the nodules are actinomycetes, eg. Frankia (see text)

[b] Species known to form nodules

TABLE 1B

SOME COMMON NITROGEN-FIXING ORGANISMS, cont'd.[c]

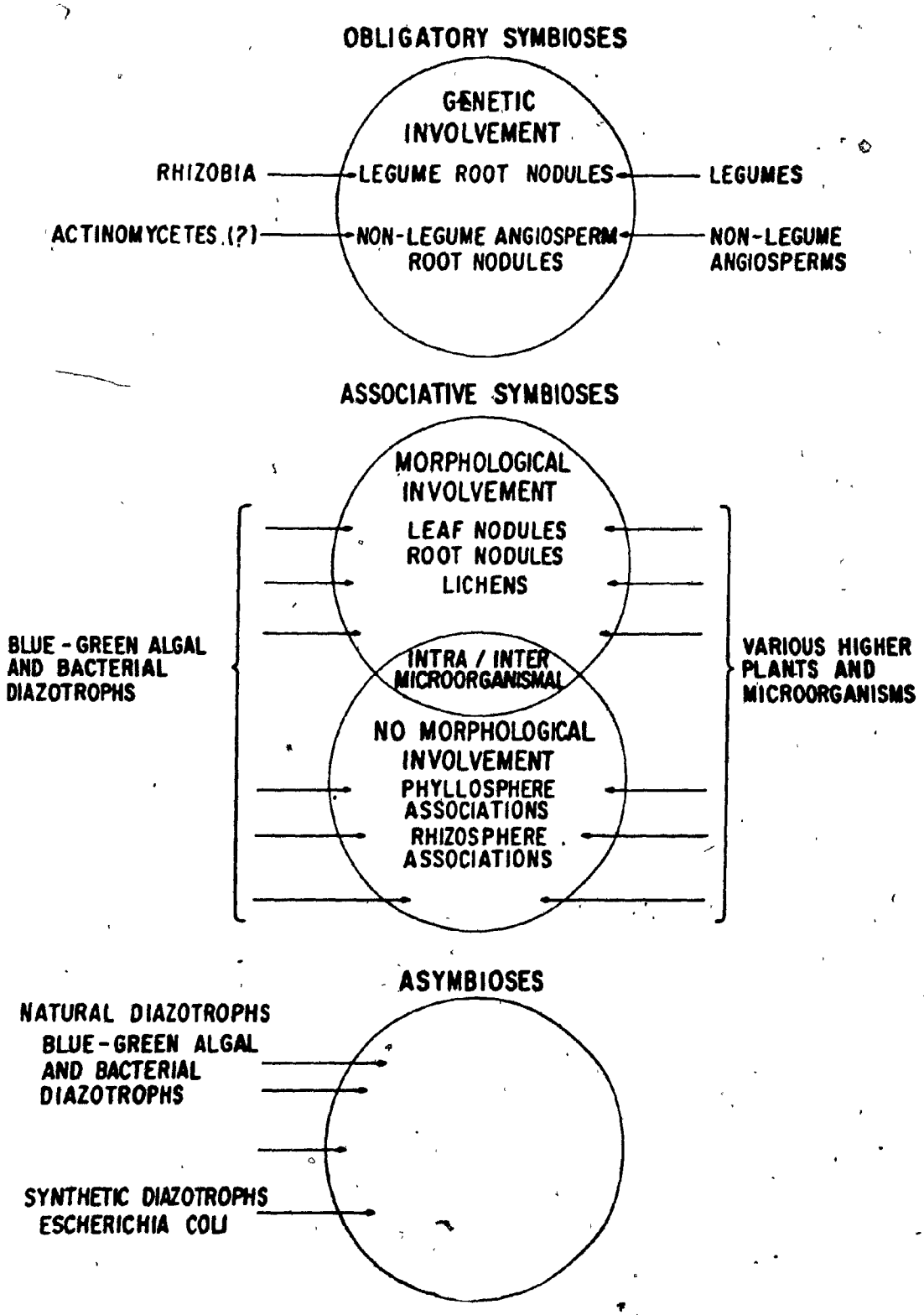
Description	Subfamily	Genera total No.	No. examined	Nodulated (%)	Species No. examined	Nodulated (%)
Rhizobium-legume symbiosis	Mimosoideae	30	19	95	130	92
	Caesalpinioideae	91	31	48	97	34
	Papilionatae	308	154	97	969	94

[c] After Burns and Hardy (1975)

microorganisms in a symbiotic fashion. The failure to obtain a suitable in vitro assay for nitrogen fixation restricted early investigations of the organisms capable of fixing nitrogen. The demonstraton in 1960 by Carnahan et al. (1960a, 1960b) of nitrogen reduction with extracts of Clostridium pasteurianum provided the foundation for analyses of nitrogen-fixing organisms. A direct consequence of this was the discovery of nitrogen fixation in the aerobe, Azotobacter vinelandii (Bulen et al., 1964), followed by similar studies in Bacillus polymyxa (Grau and Wilson, 1963; Witz et al., 1967), Rhodospirillum rubrum (Bulen et al., 1965; Burns and Bulen, 1966), Klebsiella rubiaterarum (Bulen, 1965), Klebsiella pneumoniae (Mahl, 1966), soybean nodules (Bergersen, 1966a; Bergersen, 1966b; Bergersen and Turner, 1967) and a number of other systems (Table 1). The nitrogen-fixing organisms, referred to as diazotrophs (Burns and Hardy, 1975), derived from a wide evolutionary spectrum without any taxonomical relationship (Table 1), are often categorized according to their nitrogen fixing habitat rather than evolutionary origin. Figure 2 illustrates the major biological nitrogen-fixing relationships assembled in three classes: asymbiotic (free-living) and symbiotic (associative and obligatory), as proposed by Burns and Hardy (1975). The inclusion of aerobic, facultative, anaerobic and photosynthetic organisms among the diazotrophs, attests to their physiological and metabolic diversity; however, some genetic bridges and similarities do appear to exist among them. Among the nitrogen-fixing organisms, the bacterial diazotrophs represent about 26 genera, blue-green algae 23, non-legume angiosperms 14, and legumes 650-700 genera (Burns and Hardy, 1975).

FIGURE LEGEND ON OTHER SIDE

Fig. 2. Classification of possibly all diazotrophs in nature (Burns and Hardy, 1975). The complexity and type of nitrogen fixation are unrelated to evolutionary origin of diazotrophs.


OBLIGATORY SYMBIOSES
GENETIC INVOLVEMENT
RHIZOBIA
LEGUME ROOT NODULES
LEGUMES
ACTINOMYCETES (?)
NON-LEGUME ANGIOSPERM ROOT NODULES
NON-LEGUME ANGIOSPERMS
ASSOCIATIVE SYMBIOSES
MORPHOLOGICAL INVOLVEMENT
LEAF NODULES
ROOT NODULES
LICHENS
INTRA / INTER MICROORGANISMAL
NO MORPHOLOGICAL INVOLVEMENT
PHYLLOSPHERE ASSOCIATIONS
RHIZOSPHERE ASSOCIATIONS
BLUE-GREEN ALGAL AND BACTERIAL DIAZOTROPHS
VARIOUS HIGHER PLANTS AND MICROORGANISMS
ASYMBIOSES
NATURAL DIAZOTROPHS
BLUE-GREEN ALGAL AND BACTERIAL DIAZOTROPHS
SYNTHETIC DIAZOTROPHS
ESCHERICHIA COLI

(i) Loose Symbiotic Associations

The class of associative symbioses (see Fig. 2) assembles a great variety of loose associations between free-living diazotrophs and other organisms. Although the biology and mechanisms of these associations remain largely unknown, there are three apparent classes of the loose associative symbioses described in literature: interactions among microorganisms, phylloplane and rhizosphere associations. Interactions among microorganisms often involve organisms capable of decomposing polysaccharides and stimulating nitrogen fixation by associated Azotobacter (Fedorow, 1960), Beijerinckia (Jensen, 1940) and Clostridium (Jensen, 1965), possibly by supplying utilizable carbon sources. The stimulation of nitrogen fixation by bacterial diazotrophs associated with certain blue-green algae (Pankratova, 1970; Shtina and Pankratova, 1970) may occur for the same reason. Nitrogen fixation by Mycobacterium appears to be stimulated by such organisms as Bacterium, Pseudomonas (Fedorow and Kalininskaya, 1961) and Flavobacterium (Kalininskaya and Ilina, 1965). Rhodopseudamonas capsulata, requiring anaerobic conditions to fix nitrogen in pure cultures, fixes nitrogen aerobically in mixed cultures with Bacillus megatarium (Kobayashi, 1970). An interbacterial association has been suggested in the case of Methanobacillus omelianskii, which was initially thought to be a single organism, but was subsequently shown to be a form of symbiotic association of two bacteria (Reddy et al., 1972).

The second class of the loose associative symbioses, phylloplane, assembles free-living diazotrophs associated with the aerial portion of higher plants. The presence of microbes on the surface of leaves (phyllosphere) has been described in some temperate crops as well as in

a variety of tropical plants (Ruinen, 1974). Studies of tropical grasses showed that they exude biologically significant concentrations of carbohydrates, but little or no nitrogenous nutrients, thus providing a suitable habitat for diazotrophs (Ruinen, 1970; Ruinen, 1971). Rhizosphere associations involve a number of different diazotrophic microorganisms loosely associated with or near root systems of higher plants. Among these microbes are Azotobacter (Mishustin, 1970), Klebsiella and Aerobacter (Evans et al., 1972), Bacillus and Clostridium (Rovira, 1963), Beijerinckia (Hilger, 1965) and Derxia (Jensen et al., 1960). In association with these microorganisms, significant nitrogenase activity was observed in rhizospheres of sugar cane (Döbereiner, 1961), corn (Dammergues et al., 1973), rice (Yoshida and Ancajas, 1971) and grasses (Döbereiner, 1969; Moore, 1963). Since a high incidence of nitrogen fixation was found with root systems heavily populated with fungal mycelia (Richards and Voigt, 1964), and since there is no evidence for mycorhizal fixation of nitrogen, it appears that these associations are particularly effective in promoting nitrogen fixation by bacteria and/or algae (Burns and Hardy, 1975).

Whereas the loose associative symbioses described above do not involve any structural or morphological accomodation of the nitrogen-fixing organisms, there are also a number of organisms categorized among associative symbionts, characteristic of morphological accomodations and modifications (Burns and Hardy, 1975; see also Fig. 2). An outstanding example of bacterial participation in this more intimate type of association is the leaf-nodule symbiosis, in which high concentrations of the nitrogen-fixing facultative anaerobe, Klebsiella, occur

extracellularly in the subepidermal leaf of a tropical plant, Psychotria (Lersten and Horner, 1967). These bacteria appear to be involved in the hormonal as well as nitrogen metabolisms of the plant (Silver et al., 1963). Among the bryophytes, Nostoc species were found in symbiosis with the liverworts Blasia and Cavicularia, where they occupy cavities in the underside of the thallus (Stewart, 1966). Similarly, several Anabaena species and the water fern Azolla form a symbiosis in which the alga is housed in leaf pores (Lang, 1965). Interestingly, a form of root nodule symbiosis occurs between certain tropical gymnosperms of the family Cycadaceae and blue-green algae identified as Anabaena or Nostoc (Bond, 1967; Stewart, 1970). Since the nodules appear to form even in the absence of endophyte, and since these symbioses are associative rather than obligatory, this type of root nodule symbiosis is very different from that in angiosperms. Perhaps the most intimate involvement of free-living diazotrophs with other organisms was found in the lichens. For example, Lichina (Stewart, 1970), Peltigera and Collema (Henriksson and Simu, 1971), Leptogium and Colbema (Bond and Scott, 1955) were shown to contain the phycobiont, Nostoc, and to fix nitrogen.

(ii) Obligatory Symbiotic Associations

Many non-legume angiosperms are also capable of symbiotic associations with microorganisms, leading to the formation of root nodules and effective fixation of nitrogen. This symbiosis, referred to as "older-type" (Burns and Hardy, 1975), shares many characteristics with that of legumes, but possesses unique qualities which clearly warrant separate classification (Table 1A). One of them is the difference in the endophyte, where the most commonly observed

microorganisms in the nodules are considered to be actinomycetes. These microbes are known to differentiate into three morphological forms upon symbiosis: hyphae, vesicles and bacteria-like cells (Becking, 1970; Gatner and Gardner, 1970). The nodule initiation and development processes in the "older-type" symbioses follow a pattern very similar to that described for legumes, including root hair curling, entry by infection thread through the root hair, and the eventual appearance of bacteroid-like bodies (Bond, 1974). Despite these developmental analogies to the establishment of symbiotic process in legumes there are several distinct differences, including those in nodule anatomy (Bond, 1974; Bergersen, 1974), the lack of leghemoglobin, and the differentiation of endophyte (Bond, 1974).

Among all nitrogen-fixing organisms, the most abundant (Table 1B) and evolutionary coherent group are legumes (family Leguminoseae). Infection of legume roots with Rhizobium, the free-living soil saprophyte growing on combined nitrogen, establishes a true endo-symbiosis in root nodules.

1.3. Root Nodule Morphogenesis in Legumes

Leguminous root nodules represent a type of abnormal but highly organized growth, leading to the formation of a new structure, often considered an organ sui generis (Libbenga and Bogers, 1974). The morphogenesis of root nodules is accomplished through a series of complex processes according to the following chronology: 1. infection of the host root cell by Rhizobium; 2. development of an infection thread into the cortex tissue of the host; 3. proliferation of cortex tissue to form a nodule; 4. deposition of rhizobia into the host cell

and formation of membrane envelope; 5. differentiation of rhizobia to the bacteroid state. In the normal infection sequence, the root hair curls in response to the rhizobial population which aggregates at its surface. The curling reaction is possibly induced by a rhizobial extracellular polysaccharide (Hubbell, 1970) and indole-acetic acid (IAA; Bulard et al., 1963), as well as by the presence of both IAA (Pate, 1958; Dullaart, 1970) and gibberelin (Dixon, 1969) of host origin. Torrey (1961) and Libbenga and Torrey (1973) suggested that auxins and cytokinins are also involved in the early induction of cell divisions in nodule initiation.

There appears to be a defined host range in the Rhizobium-legume recognition prior to infection of roots in that a single strain of the bacterium can only infect certain species, or even certain varieties within a species, of legume (Allen, 1971). This specificity is the basis for species differentiation in Rhizobium as well as the cross-inoculation groups in legumes (Mishustin and Shil'nikova, 1972; Buchanan and Gibbons, 1974) in the Rhizobium - legume symbiosis. It was suggested that the basis for the specificity of Rhizobium is governed by the ability of the bacterium to induce polygalacturonase specifically in the roots of compatible hosts (Ljunggren and Fabraeus, 1971), thus promoting the cell wall lysis of root hairs and the invasion by the microsymbiont. It should be noted, however, that others (Lillich and Elkan, 1968; Solhain and Raa, 1971) failed to demonstrate enhanced polygalacturonase or pectinase production in several legumes inoculated with infective or non-infective strains of Rhizobium. Recent reports suggest that legume lectins may play a part in the specificity of Rhizobium - legume root nodule symbiosis (Bhuvaneswari et al., 1977;

Wolpert and Albersheim, 1976; Pueppke et al., 1978). Bohlool and Schmidt (1974) showed that the lectin from soybean binds specifically with 22 strains of Rhizobium japonicum which nodulate soybean, whereas it does not bind to any of 23 other strains of Rhizobium which do not nodulate soybean. These data, along with the observation that the lectin from white clover roots appears to be localized at or near the tips of the root hairs (Dazzo and Brill, 1977), suggest that lectins may interact specifically with a polysaccharide on the surface of Rhizobium. This conclusion is further substantiated by the fact that legume lectins specifically interact with an O-antigen moiety of Rhizobium lipopolysaccharide (LPS) (Wolpert and Albersheim, 1976), and by the apparent relationship between the lack of O-antigen and the inability of nodulation by mutant strains of R. japonicum (Maier and Brill, 1978) and R. leguminosarum (Sanders et al., 1978).

It has also been observed that a leguminous plant causes accumulation of its own particular nodule bacterium in the rhizosphere (Purchase and Nutman, 1957; Rovira, 1961), and that before nodulation the root surface is covered with a bacterial matrix (Dart and Mercer, 1964). Due to the selective character of this phenomenon, governed by compatibility of the host and Rhizobium, it may be considered the first specific event in the root nodule symbiosis. Currier and Strobel (1976) showed that Rhizobium spp. reveal a high degree of chemotaxis to root exudates in legumes. Although the incidence of nodulation in 10 different legumes examined seems to coincide with chemotaxic interactions between root exudates and various Rhizobium spp., several non-legumes, which never nodulate, were also shown to produce root exudates capable of attracting Rhizobium (Currier and Strobel, 1976).

Since roots excrete a number of substances, eg. vitamins, carbohydrates and amino-acids (Rovira, 1956a and 1956b), it remains unclear what compounds cause the chemotaxis of Rhizobium in the rhizosphere of legumes. Preliminary results with R. japonicum (Currier and Strobel, 1976) indicate that it is attracted to some simple sugars.

The entry of Rhizobium into the host cells of root hairs, the invagination of the host cell wall (Nutman, 1956) and formation of the infection thread, appear to be associated by increased levels of pectinase and cellulase activities (Verma and Zogbi, 1978). While significant amounts of cellulase were shown to be secreted from legume roots in response to phytohormones of Rhizobium (Duulart, 1967), free-living Rhizobium show little cellulase or pectinase activities in pure cultures (Ljunggren, 1969). However, a marked increase in pectinase activity, with no significant cellulase activity, was observed in R. trifolii upon incubation with clover seedlings in vitro (Ljunggren, 1969). The elevated quantities of pectinase in bacteroids and cellulase in host cells measured in root nodules of soybean (Verma and Zogbi, 1978), along with the above observations, indicate the host origin of cellulase and bacterial origin of pectinase. It also suggests that to develop a symbiotic association between these two organisms, a cooperative action of cell wall hydrolases in early stages of rhizobial infection is a pre-requisite. The initial steps of infection were also shown to require calcium (Lowther and Loneragan, 1968), although its role in the formation of nodules is obscure.

(i) Membrane Envelope

The presence of a membranous structure enclosing Rhizobium upon entry into the host cells, called membrane envelope, was first observed by Bergersen and Briggs (1958). There are three hypotheses regarding the origin of this membrane: 1. that Rhizobium cells are enclosed by the plasma membrane of the host cell due to endocytosis (Bergersen and Briggs, 1958; Goodchild and Bergersen, 1966; Dixon, 1967; Tu, 1974; Newcombe, 1976; Verma et al., 1978); 2. that it is derived from the host endomembrane system, eg. nuclear envelope (Prasad and De, 1971) or endoplasmic reticulum (Jordan et al., 1963), and 3. that it is synthesized de novo (Dart and Mercer, 1963a; Dart and Mercer, 1964; Dart and Mercer, 1966). Verma et al., (1978) described a procedure for isolation of membrane envelope from soybean nodules and presented some further biochemical as well as structural evidence for the host origin of this membrane. Although there is an apparent continuity of the plasma membrane around the initial infection thread (Sahlman and Fahraeus, 1963; Dixon, 1964; Newcomb, 1976), it is not certain whether the same membrane continues to proliferate during later stages of infection or if other endocellular membranes (Jordon et al., 1963; Karnovsky, 1965) participate in this process. Since most of the membrane envelope is synthesized in the early stages of infection and since bacterial proliferation ceases in mature nodules (Gunning, 1970), it is believed that the development of this structure is essential for the structural establishment of the root nodule symbiosis. The presence of membrane envelope also seems to condition the effectiveness of the Rhizobium-legume symbiosis. If the membrane envelope is not formed, the rhizobia may become parasitic or saprophytic to the host cell (Verma et

al., 1978). In nodules ineffective in fixing atmospheric nitrogen, there is an apparent variability in the rate of growth and stability of membrane envelopes, as well as in the average ratio of bacteroids per envelope (Werner et al., 1980). There are reports (Torrey, 1961; Phillips and Torrey, 1972) suggesting that the development of membrane envelope is paralleled by the production of cytokinins in Rhizobium. Although the mechanistic function of the membrane envelope remains unknown, it is clear that its development is a pre-requisite for both structural and functional establishment of the root nodule symbiosis.

(ii) Changes in Rhizobium During Nodule Development

Transformation of free-living Rhizobium into bacteroids is accompanied by a number of structural and morphological changes in the bacterium. Apart from differences in size and shape between vegetative cells and bacteroids, the latter appear to have altered nuclear material (Dart and Mercer, 1963b; Bergersen, 1955), ribosomes are seldom seen in electron micrographs (Bergersen, 1958), the deposits of poly-
-hydroxybutyric acid, glycogen and polyphosphate increase in size and number (Goodchild and Bergersen, 1966; Craig and Williamson, 1972), and mesosome-like structures of the cytoplasmic membrane become visible (Dart and Mercer, 1963c). Perhaps the most prominent structural differences between free-living Rhizobium and bacteroids are confined to the alterations of the cell wall (MacKenzie et al., 1973) and the consequent changes in osmotic sensitivity of bacteroids (Sutton et al., 1977). Recent biochemical studies indicate that the transformation of free-living Rhizobium into bacteroids is associated with significant alterations of gene expression, leading to the production of numerous

"bacteroid-specific" products. There is a 10-fold increase in cellular heme as well as protoporhyrin and coprotoporphynin contents, accompanied by a several-fold increase in the activities of two enzymes of heme biosynthesis (δ-aminolevulinic acid synthase and δ-aminolevulinic acid dehydrase) (Avissar and Nadler, 1978). Also, the Rhizobium bacteroid transformation is accompanied by the disappearance of cytochromes a, a_3, and the appearance of a CO-binding cytochrome 552 (Avissar and Nadler, 1978). Since this transformation is largely due to the anaerobic conditions in the host cytoplasm, which have been shown to induce nitrogenase in vitro (Tjepkema and Evans, 1975; Kurz and LaRue, 1975; McComb et al., 1975; Pagan et al., 1975), it is obvious that the development of bacteroids from vegetative cells is essential for nitrogen fixation. Comparison of enzyme activities of bacteroids from an effective (R. japonicum strain 61A101-induced) and ineffective (strain 61A24-induced) nodules of soybean, showed in the latter an 85% reduction in specific activity of alanine dehydrogenase, 50% reduction of 3-hydroxy-butyrate dehydrogenase, and an increase of glutamine synthetase to 400% (Werner et al., 1980). The observation that bacteroids deteriorate a few weeks before the nodule ceases to function (Bergersen, 1974) clearly indicates the relationship between the structure and function of root nodules, and also well documents the obligatory character of the Rhizobium-legume symbiosis.

Since nitrogen fixation can be measured as early as 3-5 days following detection of root nodules in soybean, and since at this stage the bacterial structure appears to be a little different from that of bacteria grown in culture, Bergersen and Goodchild (1973) conclude that

the bacteroid state may not be indispensable to nitrogen fixation by Rhizobium. The more recent biochemical evidence suggests, however, that the early "bacteroid-specific" changes in Rhizobium may not be closely followed by observable differences in the appearance and structure of the microsymbiont.

The breakdown of the root nodule symbiosis may take place not only due to aging of the tissue, but also due to incompatibility between host and bacteria. This may prevent nitrogen fixation (ineffective symbioses) or may reduce it. Incompatibility may occur at any stage of symbiosis; in the pre-infection phase, thus preventing infection (the so called "cross-inoculation group incompatibility"; Nutman, 1963), or at any point of nodule development, including the intracellular phase of rhizobial infection. It appears that the development as well as maintenance of the Rhizobium-legume symbiosis are controlled by both bacterial and host plant genes (see below under Genetics of Nitrogen Fixation). Verma et al. (1978) were able to simulate the initial steps of infection in pea using an incompatible (32H1) strain of R. japonicum by the treatment of etiolated pea seedings with IAA, but no symbiotic relationship was observed. Failure to develop symbiosis resulted in a saprophytic or parasitic association where nitrogen fixed under these conditions did not appear to be of any use to the plant.

2. BIOCHEMISTRY OF NITROGEN FIXATION

2.1. The Nitrogenase System

The term nitrogenase is used to denote the enzyme system capable of reducing dinitrogen. It is now well established that nitrogenase consists of two proteins: the iron protein (Fe protein), also called

azoferrodoxin, component II or azofer, and the molybdenum-iron protein (MoFe protein), also called molybdo-ferrodoxin, component I or azofermo (Ljones, 1974). The nitrogenase reaction, leading to the production of ammonia, requires the participation of at least six other components, including the two proteins, ATP, a divalent metal cation, a reductant, and an electron acceptor (Burns, 1977).

Component II of nitrogenase (Fe protein) occurs as a symmetric (α_2-type) dimer (Kennedy et al., 1976), with the molecular weight ranging from 56 kD to 67 kD. In C. pasteurianum its molecular weight is 56 kD (Dalton and Mortenson, 1972; Tso, 1974; Winter and Burns, 1976), in A. vinelandii (Shah and Brill, 1973; Kleiner and Chen, 1974; Winter and Burns, 1976) and A. chroococcum (Yates and Planqué, 1975) it is 64 kD, whereas in K. pneumoniae it was shown to be 66.8 kD (Eady et al., 1972; Smith et al., 1976). It appears that the Fe protein is rapidly inactivated by oxygen (Eady et al., 1972) particularly in the presence of ATP (Yates, 1972), and that this protein loses its activity upon prolonged storage at 0°C (Ljones, 1974). The Fe protein contains 4 iron atoms (Moustafa and Mortenson, 1969; Vandercasteele and Burns, 1970; Eady et al., 1972) and 4 acid-labile sulfide groups (Moustafa and Mortenson, 1969; Eady et al., 1972) per dimer. Whereas component II (Fe protein) of nitrogenase is involved in the reduction of nitrate, and thus is referred to as nitrate reductase (Kennedy et al., 1976), component I (MoFe protein) is believed to be responsible for binding the nitrogenase substrates (Burris et al., 1978).

There appears to be considerable variation in the structure and composition of the MoFe protein in different nitrogen-fixing organisms. The general consensus is that the MoFe protein occurs as an $\alpha_2\beta_2$-type

tetramer of molecular weight ranging from 200 kD to 227 kD, depending on the organism. In C. pasteurianum (Dalton and Mortenson, 1972), its molecular weight was found to be 220 kD (α, 59.5 kD, β, 50.7 kD), whereas in K. pneumoniae (Eady et al., 1972; Smith et al., 1976) it was 218 kD (α, 59.6 kD; β, 51.3 kD). The $\alpha_2\beta_2$-type subunit composition was not, however, observed in some other diazotrophs examined. The MoFe protein from R. japonicum, molecular weight of which is approximately 200 kD, appears to be composed of 4 identical 50 kD subunits (Israel et al., 1974; Winter and Burris, 1976). Similarly, the 227 kD MoFe protein of A. chroococcum was reported to be a tetramer of 60 kD subunits (Yates and Planqué, 1975). Despite the apparent variability and/or controversy in the subunit composition of the MoFe protein, it is generally defined as a tetramer of two 60 kD (α) and two 51 kD (β) subunits. This protein is also inactivated by oxygen, although not as rapidly as the Fe protein (Eady et al., 1972). Present knowledge suggests the presence of 24 to 32 iron atoms with an equivalent number of acid-labile sulfide groups and 2 molybdenum atoms per 200 kD molecule (Burris et al., 1978). The apparent difficulty in obtaining consistent values for the metal content may result from a variable loss of protein-bound metal during the isolation procedures (Ljones, 1974). Although the association of component I and component II in a complex is essential for nitrogenase activity, there is little known about the role and nature of this association. Since the two components elute separately from DEAE-cellulose and since the nitrogenase complex dissociates in very dilute solutions (Thorneley, 1975; Thorneley et al., 1975), Burris et al. (1978) postulated that the Fe and MoFe-containing proteins form a loose-binding complex. Moreover, it appears that the loose association between the two proteins

conditions nitrogenase activity. Emerich and Burris (1976) and Emerich (1977) showed that the MoFe protein from A. vinelandii and the Fe protein from C. pasteurianum form a tightly-binding complex which is not catalytically active. It has been postulated that this complex dissociates at each turn of the reduction cycle (Burris, 1978).

It has been well established that the Fe protein from C. pasteurianum (Biu and Mortenson, 1968; Tso and Burris, 1973) and K. pneumoniae (Thorneley and Eady, 1973) reacts specifically with MgATP to yield a stable complex. On complexation with MgATP, the Fe protein undergoes a dramatic negative shift of about 110 mV in its redox potential to a value of about -400 mV (Zumft et al., 1974). The complexation appears to proceed very slowly if only Fe protein, ATP and Mg^{++} are present, but the rate is greatly accelerated in the complete nitrogenase reaction mixture (Burns, 1977). The reaction with MgATP causes a transition from rhombic to axial conformation of the Fe protein, as well as it increases the sensitivity to inactivation by oxygen of this molecule (Burns, 1977). Burns (1977) also indicated that the failure of nucleoside triphosphates other than ATP to bind with Fe protein suggests involvement of the adenine moiety in binding. Since ADP and analogs of ATP, which are methylene-substituted for oxygen in the triphosphate moiety, form inactive complexes with the Fe protein, the nonfunctionality for these analogs emphasizes the essential role of specific phosphate bond hydrolysis for the catalytic function (Burns, 1977).

Numerous reaction mechanisms have been suggested to explain nitrogenase activity (Burns and Hardy, 1975: Eady et al., 1975; Newton et al., 1976; Burris and Orme-Johnson, 1976; Mortenson, 1976); however,

the exact sequence of events can not be postulated with certainty. According to Burns (1977) nitrogenase activity involves 6 major steps: 1. formation of a stable complex between MgATP and Fe protein; 2. reduction of MoFe protein by MgATP-Fe protein; 3. hydrolysis of ATP; 4. reduction of substrates by MoFe protein; 5. release of reduced product(s); and 6. reduction of Fe protein. It appears that the complex contains two ATP molecules per Fe protein, ie., one ATP per two iron atoms. The interaction between the reduced MgATP-Fe protein and MoFe protein, resulting in the hydrolysis of ATP, is also accompanied by evolution of hydrogen and/or reduction of molecular nitrogen or other substrates. Nitrogenase is capable of reducing in vitro a variety of substrates besides nitrogen (N_2), including N_2O (Hardy and Knight, 1966), N_3^- (Hardy and Knight, 1967; Schöllhorn and Burris, 1967), C_2H_2 (Dilworth, 1966; Schöllhorn and Burris, 1966), HCN (Kelly et al., 1967; Hardy and Knight, 1967), and CH_3CN (Hardy et al., 1971). The immediate donor of electrons to nitrogenase in Clostridium is ferredoxin (Mortenson, 1964; Buchanan and Arnon, 1970; Orme-Johnson, 1973), and in Azotobacter is azotoflavin (Shethna et al., 1966; Hinkson and Bulen, 1967; Benemann et al., 1969). Ferredoxin and azotoflavin act as reductants of the Fe protein, where their own redox state is regulated by hydrogenase (Kleiner and Burris, 1970; Nakos and Mortenson, 1971). Thus, hydrogen may also function as an electron source for nitrogenase by coupling through hydrogenase.

Although purified nitrogenase proteins have been obtained only from a limited number of diazotrophs, it appears that the basic structure, catalytic requirements and mechanism of this enzyme are similar and highly conserved. Combinations of the two nitrogenase components from

different organisms have in a number of cases shown nitrogen fixing activity, indicating a conserved character of this protein in evolutionary unrelated systems. Emerich (1977) demonstrated a very efficient action of such hybrid nitrogenases, comprising of proteins from A. vinelandii and R. japonicum, A. vinelandii and K. pneumoniae, R. japonicum and K. pneumoniae, R. rubrum and A. vinelandii, B. polymyxa and R. japonicum, and several other organisms. A highly conserved structure of the structural genes coding for nitrogenase has also been demonstrated (see Genetics of Nitrogen Fixation), suggesting a common origin of the nitrogenase system.

2.2. Symbiotic Nitrogen Fixation

Measurements of nitrogen fixation activity in Rhizobium spp. grown under anaerobic conditions (Kurz and LaRue, 1975; McCombe et al., 1975; Tjepkema and Evans, 1975) clearly indicated the presence of nitrogenase in the microsymbiont. The nitrogenase system of Rhizobium is believed to strongly resemble that of autonomous diazotrophs; it contains the Fe and MoFe-proteins, ferredoxin as a reductant, a variety of enzymes supplying ATP, as well as the hydrogenase system (Shanmugam et al., 1978). The levels of nitrogenase activity produced by free-living Rhizobium have been reported to approximate those from isolated bacteroids (Tjepkema and Evans, 1975; Bergersen et al., 1976; Keister and Evans, 1976). Using $^{15}N_2$, more than 94% of fixed nitrogen was found to be exported by free-living R. japonicum (O'Gara and Shanmugam, 1976a) as well as by isolated bacteroids (Bergersen and Turner, 1967). Both free-living rhizobia (O'Gara and Shanmugam, 1976a) and bacteroids (Bergersen and Turner, 1967) appear to excrete the fixed nitrogen as

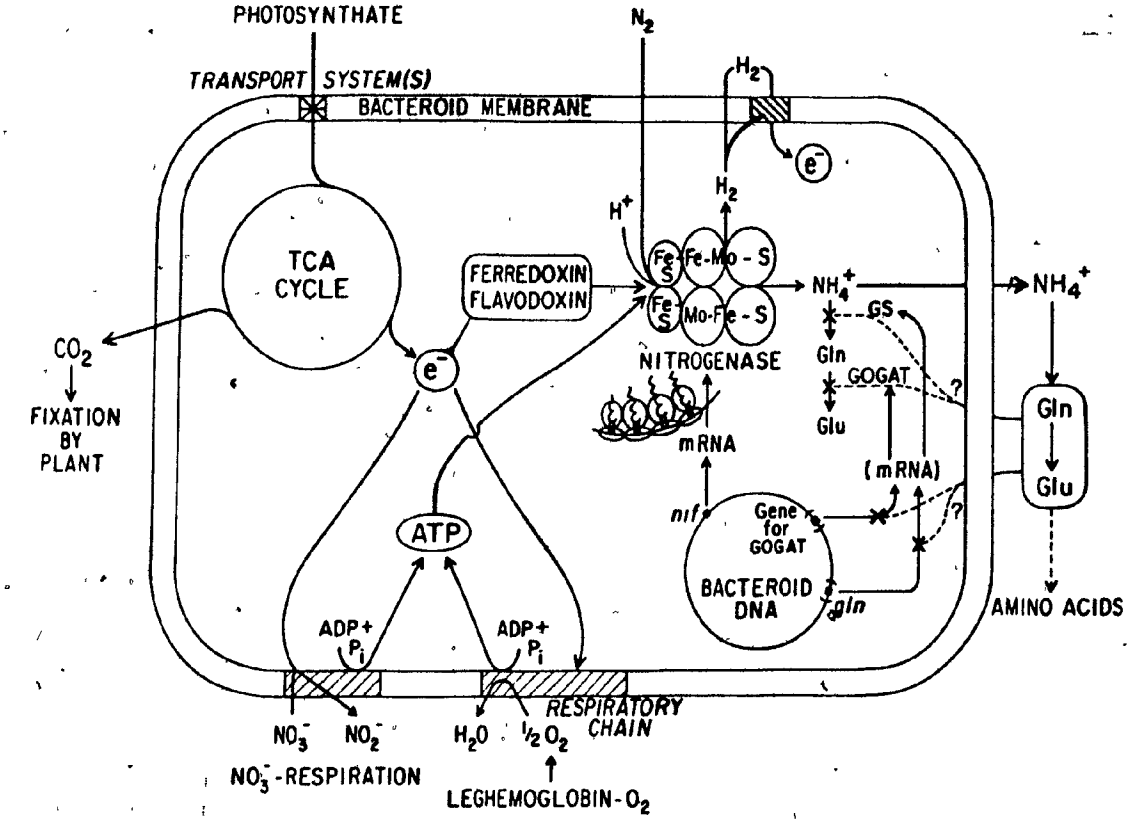
NH_4^+. There is indirect evidence that glutamate may play a role in the regulation of the nitrogenase system, leading to export of fixed nitrogen. For example, when R. trifolii (free-living) was treated simultaneously with glutamate and NH_4^+, it showed a marked preference for glutamate as nitrogen source (O'Gara and Shanmugan, 1976a), as well as it failed to utilize any detectable NH_4^+ as long as glutamate was present in the medium. In contrast, when glutamate in the medium was replaced by L-aspartate or L-leucine, R. trifolii utilized NH_4^+ for cellular growth (O'Gara and Shanmugam, 1976a and 1976b). The presence of either aspartate or leucine in the medium also caused induction of glutamate synthase activity, whereas addition of L-glutamate inhibited this enzyme. Since the presence or absence of glutamate synthetase corresponded well with the NH_4^+ utilisation pattern, and since NH_4^+ also repressed activity of glutamine synthetase, it is possible that the block in the conversion of NH_4^+ to glutamate is responsible for derepression of nitrogenase, even in the presence of NH_4^+ (O'Gara and Shanmugam, 1977). It thus appears that both glutamine synthetase and glutamate synthase may be involved in regulation of nitrogenase activity in root nodules (see Fig. 3).

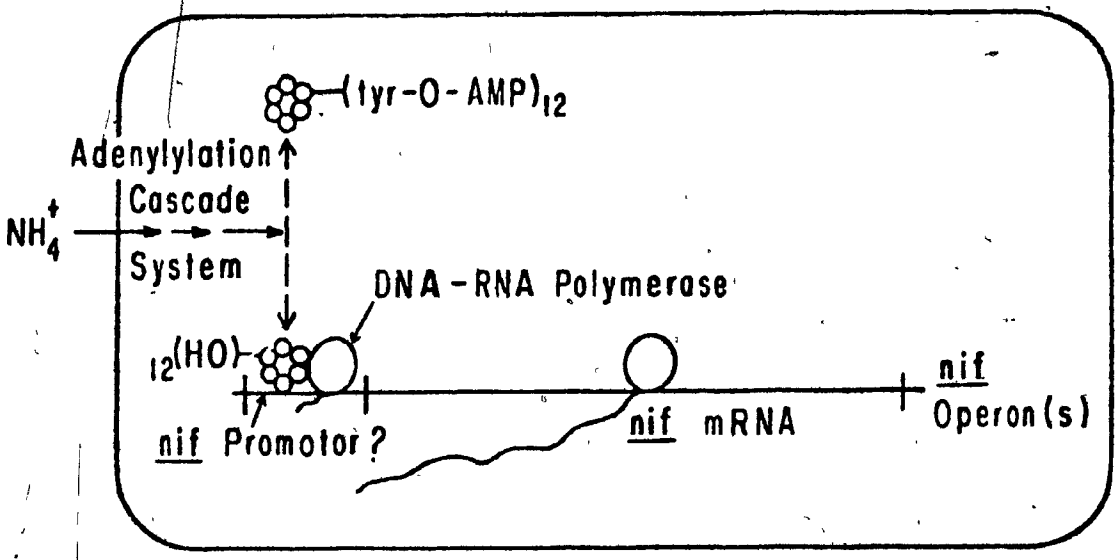
Bacteroids are known to metabolize various carbon compounds supplied by the plant cell (Bergersen, 1974) via an active TCA-cycle, coupled to an energy-yielding respiratory chain (Bergersen, 1971; Appleby et al., 1976). This system is known to function under low oxygen concentrations. The role of bacteroids in energy metabolism is well substantiated by two systems: the hydrogen uptake system (Dixon, 1972; Schubert and Evans, 1976) and energy-linked nitrate reductase (Rigaud et al., 1973). The involvement of hydrogen uptake system in the

FIGURE LEGENDS ON OTHER SIDE

Fig. 3. A model for symbiotic nitrogen fixation by bacteria (Shanmugam et al., 1978). GS, glutamine synthetase; GOGAT, glutamate synthase; nif and gln are genes for nitrogenase and glutamine synthetase, respectively. See Biochemistry of Nitrogen Fixation for details.

Fig. 4. The "adenylation cascade" model for regulation of the nitrogen fixation (nif) genes in Klebsiella pneumoniae (Shanmugam et al., 1976). See Genetics of Nitrogen Fixation for details.


PHOTOSYNTHATE
TRANSPORT SYSTEM(S)
BACTEROID MEMBRANE
N_2
H_2
e^-
H^+
TCA CYCLE
FERREDOXIN FLAVODOXIN
Fe-S
Fe-Mo-S
Mo-Fe-S
NH_4^+
GS
Gln
GOGAT
Glu
NITROGENASE
mRNA
(mRNA)
nif
Gene for GOGAT
BACTEROID DNA
gln
CO_2
FIXATION BY PLANT
ATP
ADP+ P_i
ADP+ P_i
NO_3^- NO_2^-
NO_3^--RESPIRATION
H_2O $\frac{1}{2}O_2$
RESPIRATORY CHAIN
LEGHEMOGLOBIN-O_2
Gln
Glu
AMINO ACIDS


(tyr-O-AMP)12
Adenylylation Cascade System
NH_4^+
DNA-RNA Polymerase
12(HO)
nif Promotor?
nif mRNA
nif Operon(s)

energy metabolism was shown by the use of isogenic strains of Rhizobium which lack the hydrogen utilization reactions by Schubert and Evans (1976), and Evans and Barber (1977). Since nitrogenase produces certain quantities of hydrogen during the reduction of nitrogen, the amounts of hydrogen and ethylene produced by nodules can be used to estimate the efficiency of energy used in the nodules. Apart from the hydrogenase uptake system, anaerobic reduction of nitrates was also shown to be an energy-yielding reaction in isolated bacteroids (Riguard et al., 1973). Interestingly, the hydrogenase system as well as the nitrate reductase are simultaneously induced in nitrogen-fixing cultures of free-living Rhizobium, thus further indicating their involvement in the process of nitrogen fixation.

It appears that one of the major limitations of nitrogen fixation by the bacteroids is the availability of photosynthate. Hardy and Hovelka (1975) demonstrated that increasing the rate of CO_2 fixation by the plant leads to higher rates of nitrogen fixation by the nodules. Although the mechanism of this phenomenon is unknown, it suggests the existence of a tight relationship between the physiology of the plant and the efficiency of nitrogen fixation by the microsymbiont.

2.3. The Role and Structure of Leghemoglobin

Leghemoglobin (Lb) was first reported by Kubo (1939) as a plant hemoglobin, able to reversibly bind oxygen in root nodules of legumes. Recent biochemical studies (Appleby, 1974), amino-acid sequence (Ellfolk, 1961; Ellfolk and Sievers, 1971; Ellfolk, 1972), and sequence alignment (Hunt, 1972), indicated that the structure of Lb is closely related to that of myoglobin and animal hemoglobins.

There have been a number of proposals for the involvement of leghemoglobin in symbiotic nitrogen fixation, including its function as electron acceptor in the transformation of N_2 to NH_2OH (Virtanen and Laine, 1946), as electron donor to nitrogenase bound to plant cell membranes (Bergersen, 1960), or as nitrogenase itself with ferrous LbN_2H_2 as intermediate species (Hanstein et al., 1967). It is now believed that leghemoglobin functions as an oxygen carrier maintaining a low and constant oxygen concentration in nodules, thus preventing inhibition of the oxygen-sensitive nitrogenase system (Appleby, 1974). The iron atom of ferrous heme of Lb was shown to strongly bind oxygen (Appleby, 1962), as well as carbon monoxide (Imamura et al., 1972), which acts as an effective competitive inhibitor of the oxygen-binding function of Lb. A clear indication of the importance of leghemoglobin for nitrogenase activity in bacteroids comes from the series of experiments performed by Bergersen et al. (1973). Measurements of oxygen uptake and nitrogenase activity of bacteroid suspensions in the presence and absence of oxygenated leghemoglobin (LbO_2), resulted in a 30% increase of oxygen consumption and a dramatic increase of acetylene reduction in the presence of LbO_2. The initial entry of oxygen from a gas phase into the cell cytoplasm appears to be a slow process (Scholander, 1965; Wittenberg, 1970). Leghemoglobin in root nodules appears to be only partially (20%) oxygenated (Appleby, 1969; Tjepkema, 1971), thus providing the concentration gradient essential for facilitated diffusion. Since the concentration of Lb-bound oxygen in the intact nodule exceeds that of free oxygen more than 1000-fold (Appleby et al., 1976), it is possible that the transport of oxygen in the host cell is entirely mediated by leghemoglobin. Appleby et al.

(1976) also postulated that the LbO_2/Lb system acts to buffer oxygen pressures within the host cell in the same way that acid-base systems act as pH buffers. This effect influences the diffusion of oxygen through such a protein solution and tends to stabilize its pressure at the point of delivery. Leghemoglobin-facilitated delivery was shown to be much more efficient than unfacilitated oxygen diffusion (Bergersen et al., 1973; Wittenberg et al., 1974). Moreover, the absence of Lb, with consequent loss of buffering and stabilizing effects on delivered oxygen tension, also causes the destruction of nitrogenase by free oxygen (Appleby et al., 1976). It remains possible, however, that increased oxygen concentrations stimulate a bacteroid oxidase, which might be responsible for maintenance of bacteroid respiration (Jones et al., 1972). A bacteroid oxidase involved in enzymic hydroxylation (Hollenberg and Hager, 1973), referred to as cytochrome P-450, is believed to act as a carrier or terminal oxidase in the pathway which receives its oxygen by facilitated diffusion via LbO_2 (Appleby et al., 1976). Whereas cyanide, a general inhibitor of oxidases including P-450, strongly inhibits both Lb-facilitated oxygen uptake and nitrogenase activity, N-phenylimidazole, which is a specific inhibitor of cytochrome P-450, inhibits Lb-facilitated nitrogenase activity only. These observations suggest that the transport of oxygen from LbO_2 into bacteroids may be mediated by cytochrome P-450 (Appleby et al., 1976).

The observed relationship between the Lb-facilitated diffusion of oxygen and nitrogenase activity appears to be influenced by the availability of ATP in the cell. Since it was well established that the Lb-facilitated diffusion of oxygen stimulates the oxidative phosphorylation system (Bergersen et al., 1973; Wittenberg et al., 1974), Appleby

et al., (1976) studied the relationship between the bacteroid ATP/ADP ratio and nitrogenase activity. A linear relationship was found, indicating that the stimulatory effect of LbO_2 on the nitrogenase system is mediated by increased ATP/ADP ratio in the bacteroid. Oxyleghemoglobin (LbO_2), like oxymyoglobin (George and Stratmann, 1952) and oxyhemoglobin (Misra and Fridovich, 1972) is sensitive to autoxidation in vitro, leading to the formation of metleghemoglobin (metLb), which is physiologically inactive (Appleby, 1974). Puppo et al. (1981) recently postulated that superoxide dismutase (SOD) from soybean nodules catalyzes the dismutation of LbO_2, and thus appears to play an important role in the Lb-mediated transport of oxygen. Superoxide dismutase may also prevent oxidation of nitrogenase (Henry et al., 1978). as well as ferredoxin (Misra and Fridovich, 1971) and some cytochromes (Cassell and Fridovich, 1975).

It has been established that at least the majority of heme present in leghemoglobin is synthesized in bacteroids (Godfrey, 1972; Appleby and Dilworth, 1974), whereas the globin apoprotein is produced by the host plant (Verma et al., 1974; Verma and Bal, 1976; Baulcombe and Verma, 1978). The synthesis of Lb apoprotein commences prior to that of nitrogenase in root nodules of soybean (Verma et al., 1979) and pea (Bisseling et al., 1979), and it occurs in a differential fashion (Verma et al., 1979) for the two electrophoretically distinguishable molecular species of leghemoglobin in soybean. Although the properties and structure of leghemoglobin, representing about 30% of the total soluble protein in soybean nodules, have been well determined, its localization within the plant cell is still in dispute.

Both X-ray microprobe analyses of fixed sections (Dart and Chandler, 1971) and biosynthetic studies (Verma et al., 1974; Verma and Bal, 1976) of nodules, indicated that leghemoglobin is localized entirely in the host plant cytoplasm. Bergersen and Goodchild (1973) postulated that leghemoglobin is localized within the membrane envelope surrounding bacteroids in both fresh and fixed soybean nodules. Due to the size and properties of membrane envelope, however, it is possible that this structure is broken during the preparation or extraction of the tissue and that the apparent intravesicular material is contaminated with cytosolic contents, including leghemoglobin. Although the structure and permeability of the membrane envelope remain largely unknown, it is unlikely that leghemoglobin can be transported across the membrane (Verma et al., 1979) by a vectorial discharge mechanism (Blöbel and Dobberstein, 1975), since it is a non-glycosylated protein (Ellfolk, 1972).

Despite its host origin, the levels of leghemoglobin in the plant cell appear to be strongly influenced by mutations in Rhizobium spp. Verma et al. (1981) reported that ineffective nodules, developed by Rhizobium strains SM4, SM5 and 61A24, contain reduced amounts of leghemoglobin. Whereas the mutation in strain SM4 and strain incompatibility in 61A24 are poorly understood, it is believed that the mutation in SM5 affects the function of nitrogenase only (Maier and Brill, 1976). The observed amount of leghemoglobin in the SM5-induced nodules represents only 30-40% of that found in a wild type (strain 61A76-induced) nodules (Verma et al., 1981), providing additional evidence for the apparent lack between the presence or activity of nitrogenase and leghemoglobin.

Purification of Lb-mRNA from soybean root nodules (Baulcombe and Verma, 1978) has led to the construction Lb-cDNA clones (Truelsen et al., 1979; Sullivan et al., 1981). Subsequent isolation of several genomic clones of leghemoglobin from soybean nodules (Sullivan et al., 1981; Jensen et al., 1981) revealed the presence of at least seven EcoR1 fragments containing leghemoglobin genes in soybean. One of these genes has been shown to be interrupted with three intervening sequences (Jensen et al., 1981). Further investigations of the structure and complexity of leghemoglobin genes may elucidate the evolutionary origin of this protein. Since the globin apoprotein of Lb shares an extensive structural homology with common animal globins (Hunt, 1972), the origin of this class of genes must be sought in an early phylogenesis of the animal and plant kingdoms.

3. GENETICS OF NITROGEN FIXATION

3.1. Structure and Regulation of the Nitrogen Fixation (nif) Genes

Klebsiella pneumoniae is the most widely used organism for studying the genetics of nitrogen fixation because it can fix nitrogen in a free-living state and is easy to manipulate in cultures. The genes for nitrogen fixation (nif genes) were shown to be located near the operon for histidine biosynthesis in K. pneumoniae by cotransduction of his and nif using the generalized transducing bacteriophage P1 (Streicher et al., 1971). At the same time, Dixon and Postgate (1971) performed R144-mediated conjugation experiments between His^+ nif^+ donors and His^-Nif^- recipients in which His^+ exconjugants had regained the ability to fix nitrogen, thus confirming linkage between his and nif genes. A short time later, they showed that the nif genes could be transferred by

conjugation from K. pneumoniae to E. coli C, a strain which normally does not fix nitrogen (Dixon and Postgate, 1972). Shanmugan et al. (1974) analyzed deletion mutants of the his region in K. pneumoniae and found that the nif genes are located between his and shi A on the bacterial chromosome. On the basis of analogy with the chromosome map of E. coli, nif genes were placed in a counter-clockwise position from the his operon. Some of the individual genes involved in nitrogen fixation were identified and ordered by St. John et al. (1975). Their results suggested the following order and gene products: his D (histidinol dehydrogenase), nif B (Mo-cofactor), nif F (electron transfer protein), nif D (nitrogenase MoFe protein), nif H (nitrogenase Fe protein), and nif G (regulatory component). Fine-structure mapping and complementation analysis of nif genes have led to identification of total of 14 nitrogen fixation genes in K. pneumoniae (MacNeil et al., 1978). In this study, several hundred Nif$^-$ strains containing point mutations (Mu insertions and Mu-induced deletions) were used in complementation analysis with plasmids containing nif mutations. The nif mutations, mapped by deletion analyses, were ordered into almost 50 deletion groups with a gene order of his ... nif QBALFMVSNEKDHJ. MacNeil et al. (1978) postulated the presence of seven operons; five polycistronic and two monocystronic. Recent studies by Dixon et al. (1977), Kennedy (1977), and Roberts et al. (1978), resulted in identification of a number of nif gene products other than nitrogenase (Table 2). Many of the nif-coded polypeptides were identified by means of two-dimensional polyacrylamide gel electrophoresis (Roberts et al., 1978) and assigned to specific nif genes. It is now believed that there are 17 nif genes organized in seven operons on the chromosome of K.

TABLE 2

IDENTIFICATION OF *nif*-ASSOCIATED PROTEINS IN *K. PNEUMONIAE*

Gene assignment[a]	MW/pI	Putative function	Reference
nif A	60 kD/?	Derepression of *nif*; regulatory	Dixon et al., 1977; Kennedy, 1977
nif B	Unknown	Essential in FeMo-Co[b] synthesis	St. John et al., 1975; Roberts et al., 1978
nif D	56 kD/6.1	Encoding nitrogenase (component I[c])[d]	St. John et al., 1975; Roberts et al., 1978
nif E	46 kD/6.8	Involvement in FeMo-Co[b] synthesis	Dixon et al., 1977; Roberts et al., 1978
nif F	17 kD/5.0	Electron transport factor	St. John et al., 1975; Roberts et al., 1978,
nif H	35 kD/4.9	Encoding component II[e]	St. John et al., 1975; Roberts et al., [illegible]
nif J	120 kD/6.0	Regulatory, unrelated to components I, II	Kennedy, 1977; Roberts et al., 1978
nif K	60 kD/5.7	Encoding nitrogenase (component I)[f]	MacNeil et al., 1978; Roberts et al., [illegible]
nif L	50 kD/?	Repression of *nif*; regulatory	Kennedy, 1977; Kennedy, 1980
nif M	28 kD/?	Involvement in synthesis of component II	MacNeil et al., 1978; Klipp and Pühler, 1980
nif N	50 kD/6.7	Essential for FeMo-Co synthesis	St. John et al., 1975; Roberts et al., 1978
nif Q	Unknown	Unrelated to components I, II	MacNeil et al., 1978; Roberts et al., 1978
nif S	18 kD/51	Involvement in synthesis of component II	MacNeil et al., 1978; Roberts et al., 1978
nif U	28 kD/?	Unknown	Klipp and Pühler, 1980
nif V	42 kD/?	Unrelated to component I, II	MacNeil et al., 1978; Klipp and Pühler, 1980
nif Y	24 kD/?	Unknown	Klipp and Pühler, 1980
nif X	18 kD/?	Unknown	Klipp and Pühler, 1980

[a] Genes are listed according to alphabetical order. The actual sequence of the *nif* genes on *K. pneumoniae* chromosome is: *his* ... *nif* QBALFMVSUXNEYKDHJ (Kennedy, 1980)

[b] FeMo-Co is a cofactor of nitrogenase (component I) at its active site (Shah and Brill, 1977)

[c] Component I is nitrogenase, a tetramer $\alpha_2\beta_2$ of two polypeptides of 54 kD (β subunit) and 57 kD (α subunit) (Kennedy et al., 1976).

[d] The α subunit of nitrogenase

[e] Component II is nitrate reductase, a dimer of identical polypeptides of 34 kD (Kennedy et al., 1976)

[f] The β subunit of nitrogenase

pneumoniae. Three genes code for nitrogenase proteins; nif H for the iron protein of nitrate reductase (component II) and D and K for the α and β subunits of the molybdenum-iron protein (component I) respectively (Kennedy et al., 1976). Identification of the remaining nif gene products (Table 2) is at its preliminary stage and detailed functions have yet to be assigned to most of these proteins. A number of other nif loci have been reported, including nif G (St. John, 1975) and nif T (Ausubel et al., 1977), but in each case the report has been based on only a single mutant strain which was not well characterized biochemically.

As suggested above, the expression and regulation of nif is under a complex control mechanism of glutamine synthetase. Ginsburg and Stadtman (1973), and Wohlhueter et al. (1973) postulated the presence of a regulatory model for nif expression, referred to as "adenylation cascade" (see Fig. 4). According to this model, addition of NH_4^+ or any other source of nitrogen, known to repress nitrogenase (Dixon and Postgate, 1972), triggers a cascade of enzymatic reactions modifying glutamine synthetase. The specific attachment or removal of adenyl moieties to or from tyrosine residues changes dramatically the catalytic properties of glutamine synthetase. Adenylation of the enzyme (one adenyl residue per each of 12 identical subunits) blocks the binding of glutamine synthetase to the nif promoter or, conversely, deadenylation leads to binding of the enzyme and subsequent activation of transcription of nif operon. More recent studies with three glutamine-requiring auxotrophs (glu A^-) (Shanmugam et al., 1976) further supported the "adenylation cascade" model. The glu A^- mutants, lacking catalytic activity of glutamine synthetase, failed to synthesize nitrogenase under

a variety of conditions. Shanmugam et al. (1976) postulated that the loss of catalytic activity by glutamine synthetase simultaneously destroys the regulatory properties of the protein. On the other hand, transfer of an E. coli episome F133, carrying an effective glutamine synthetase gene, to a glu A^- strain (5060) of K. pneumoniae restores the production of nitrogenase (Low, 1972; Shanmugam et al., 1976). The original reports, postulating that glutamine synthetase is a positive controlling element for nitrogenase derepression (Streicher et al., 1974; Tubb, 1974), were also evidenced by the isolation of mutants mapping within the glutamine synthetase structural gene, which led to no loss of biosynthetic activity of this enzyme but gave rise to marked irregularities in nif and hut expression (Ausubel et al., 1977). In addition, an apparent sensitivity to ammonia in K. pneumoniae mutants glu A^+R^-nif suggested that glutamine synthetase is not the only regulatory element controlling nitrogenase derepression (Streicher et al., 1974), but that there may be a second controlling element, a nif$^-$-specific repressor (Ausubel et al., 1977). It is believed that within a narrow range of ammonia concentrations, the activator (derepressor) and the postulated repressor compete for the control of nif transcription.

Since the nif genes map as a cluster between the his operon and shi A (shikimate permease) on the K. pneumoniae chromosome (Streicher et al., 1971; Dixon and Postgate, 1971; Shanmugam et al., 1974; St. John et al., 1975), the possibility of a coordinate expression of the nitrogen fixation genes was examined by Shah et al. (1972). The results indicated that both component I and component II of nitrogenase in Azotobacter vinelandii are cordinately synthesized during derepression

and coordinately lost during repression of the enzyme. Moreover, Gordon and Brill (1972) isolated revertants of nif⁻ mutants which lacked both component I and component II activities; several revertants produced nitrogenase constitutively and were insensitive to ammonia repression. These observations suggest that the structural genes for nitrogenase are controlled by a common regulatory gene.

3.2. Nodulation and Nitrogen Fixation Genes of Rhizobium

A major difficulty associated with studies of Rhizobium genetics is the fact that the genes of Rhizobium involved in root nodule symbiosis do not generally express in vitro, but are revealed in the presence of the host plant.

Schwinghamer (1964) studied the association between the antibiotic resistance and modification of effectiveness in spontaneous mutants of R. leguminosarum, R. meliloti and R. trifolii. He distinguished three groups of antibiotics according to the modification of symbiotic properties. In group I, resistant to chloramphenicol, spectinomycin, spiramycin and streptomycin, most of the mutants did not lose effectiveness. In group II, resistant to novobiocin, penicillin and vancomycin, there was a partial or total loss of effectiveness in about 50% of mutants, whereas in group III, resistant to neomycin and viomycin, most of the mutants were completely ineffective in fixing nitrogen. Subsequent studies of viomycin resistance (Vio^r) in R. meliloti showed that the Vio^r mutants accumulate phospholipids in the cell wall (McKenzie and Jordan, 1970; McKenzie and Jordan, 1972) and have fewer negatively charged sites on the envelope (Yu and Jordan, 1971). Since no bacteroids were detected in the nodules, it is believed

that these vegetative bacteria are not converted into bacteroids and degenerate (Hendry and Jordan, 1969). Surprisingly, almost all the antibiotic-resistant mutants of Rhizobium studied are infective, thus providing a suitable system for identification of the genes related to the effectiveness of symbiotic nitrogen fixation.

Apart from the studies of effectiveness in spontaneous mutants of Rhizobium, selected according to the antibiotic resistance (Schwinghamer, 1964) and the resistance to metabolic inhibitors (Schwinghamer, 1968), a variety of genetic techniques have been used in the studies of the Rhizobium genetics, including transformation, transduction, transfection, conjugation and mutagenic treatments. As early as in 1953, the genetic transformation of host specificity by bacterial lysates of R. lupini was reported (Balassa, 1953). When exposed to DNA from R. meliloti strain M, some cells of R. lupini strain H-13 became infective on alfalfa (Medicago sativa). When the donor strain was Str^r and the recipient Str^s, bacteria isolated from the resulting nodules retained the Str^s character. When strain H was transformed to Str^r with DNA of Str^r strain M, the transformants did not form nodules on alfalfa. A Cys^- mutant of R. lupini strain H-13, when transformed with DNA from strain M, gave cultures forming ineffective nodules on alfalfa. Bacteria isolated from these nodules were either Cys^- or Cys^+, the latter being spontaneous revertants (Balassa, 1953), and continued to induce the same type of ineffective nodules on alfalfa (Imshenetski and Puriiskaya, 1973). If transformed a second time by DNA of strain M, the resulting cultures formed several effective nodules. Since similar results were obtained in R. trifolii mutagenized by UV or X-ray irradiations, where the bacteria formed ineffective nodules on pea

(Schwinghamer, 1962), it appears that host specificity of Rhizobium can be modified by transformation or a single-step mutation (Dénarié and Truchet, 1976). The ineffectiveness of nodules formed on a new host and the observation that the nitrogen fixation ability restored only after further transformations, suggest that the nodulation and effectiveness genes may not be closely related. Further evidence for this hypothesis was provided by Maier and Brill (1976), who recently isolated five symbiotically defective mutants of R. japonicum among 2,500 survivors of a mutagenic treatment. Some of these mutants (strains SM3, SM4 and SM5) produced nodules, but they did not fix nitrogen. R. japonicum strain SM5 was shown to be defective in the production of nitrogenase component II. Some additional evidence for the apparent lack of a direct relationship between nodulation and symbiotic effectiveness in Rhizobium has been also obtained using auxotrophs. A riboflavin-dependent mutant of R. trifolii showed full restoration of the effectiveness in prototrophic revertants following addition of riboflavin or its coenzymes (Schwinghamer, 1969; Schwinghamer, 1970). Similarly, in a leucine auxotroph of R. meliloti, the effectiveness could be restored by the addition of leucine (Kowalski and Dénarié, 1972).

The observation that about 70% of all R. meliloti strains examined are phage carriers (Kowalski, 1965) was encouraging for the prospect of obtaining transduction among different species of Rhizobium. Phage L5, whose prophage resides in the lysogenic R. meliloti strain L5 (L5), was shown to be a generalized transducing phage (Dénarié and Truchet, 1976). The use of phage L5 indicated that an ineffective lysine-requiring mutant of R. meliloti carries at least two mutations: one in a gene controlling lysine biosynthesis (lys) and the other in a gene for

effectiveness (eff-2) (Kowalski, 1970). Analysis of a number of other ineffective leucine-requiring mutants (Kowalski and Dénarié, 1972; Kowalski, 1974) suggested that all the leu genes are clustered in the same region of the chromosome. Two of these mutants, the prototrophic transductants and revertants, were fully effective (strains B11 and E66) (Kowalski, 1974). Additional experiments also suggested that the B11 strain of R. meliloti carries an eff-1 mutation which is situated in the leu region of the chromosome (Kowalski, 1974; Dénarié et al., 1974). Whereas Kowalski's results involved generalized transduction, a specialized transduction was also reported in literature. Staniewski et al. (1971) demonstrated transfection (infection of bacteria with phage DNA) in R. meliloti. A low frequency was observed for transfection of intact cells of Rhizobium by a temperate phage, but use of spheroplast cells and helper phages gave a 300-fold increase in frequency. Although transfection in Rhizobium remains at its preliminary stage and no conclusive data are as yet available, this approach may be a powerful tool for genetic mapping of the closely-related rhizobia.

Since generalized transformation and transduction allow the transfer of only a small part (about 1%) of the bacterial chromosome (Dénarié and Truchet, 1976), a system allowing genetic transfer of large chromosomal segments would be of major interest in the investigation of the symbiotic role of Rhizobium. According to Dénarié and Truchet (1976), two approaches to such a system can be distinguished: the search for natural conjugation systems in root nodule bacteria, and the introduction of sex factors from other bacteria (eg. E. coli or Pseudomonas) into Rhizobium spp. Higashi (1967) reported the transfer of host specificity from R. trifolii to R. phaseoli by conjugation; however, no

further characterisation and genetic identification followed this work. A successful transfer of the resistance factor RP_4, conferring resistance to ampicillin, kanamycin and tetracycline, from Pseudomonas aeruginosa to R. lupini was reported by Pühler et al. (1972). In another report on conjugation among five strains of R. trifolii (Lorkiewicz et al., 1971), crosses between singly auxotrophic mutants or between auxotroph and wild type yielded a number of prototrophic back mutants from ineffective auxotrophs which remained ineffective. Finally, a successful transfer of the R-factor, responsible for a multiple drug resistance to chloramphenicol, neomycin and penicillin G, was reported from R. japonicum to A. tumefaciens (Cole and Elkan, 1973). Although a number of reports describing transfer of sex factors from Pseudomonas and Enterobacteriaceae to Rhizobium have appeared in literature (Datta et al., 1971; Datta and Hedges, 1972; Pühler et al., 1972), including that reporting the first complementation test used in Rhizobium (Datta and Hedges, 1972), all of them failed to demonstrate chromosome transfer in rhizobia. Considerable progress in this area, however, was reported by Dunican and Tierney (1974) who showed that the F-like R plasmid (Rl-drd) acts as a sex factor in R. trifolii and promotes the conjugative transfer of genes controlling nitrogen fixation into Klebsiella aerogenes.

During the past few years there have been numerous reports suggesting an extrachromosomal inheritance of factors involved in symbiotic properties of Rhizobium. Convincing evidence for plasmid inheritance in R. trifolii comes from the report by Dunican and co-workers (Cannon et al., 1971). Viomycin resistance, known to be closely associated with loss of effectiveness (Schwinghamer, 1964; Hendry and Jordan, 1969; also

see above) was used as a marker for detecting loss of effectiveness following treatment of effective, viomycin-sensitive bacteria with plasmid-eliminating agents. Treatment with acridine orange or ethidium bromide significantly increased the frequency of viomycin resistance, whereas a prophage-inducing agent and a mutagen did not. Thus it was presumed that factors for both viomycin sensitivity and symbiotic efficiency could be located on plasmids, possibly on the same one. However, according to Schwinghamer (1977), the validity of this conclusion depends on the absence of selective enrichment of spontaneous mutants by plasmid-curing agents. Most recent studies indicate the presence of 2 (Kondorosi et al., 1980) to 4 (Pühler et al., 1980) plasmids in R. melilotii, ranging from 3 to 260 Md in size. According to Badenoch-Jones et al. (1980), at least four classes of Rhizobium plasmids can be distinguished: class I, greater than 200 Md; class II, 95-125 Md, class III, 40-65 Md; class IV, 3-7 Md. It is believed that the Rhizobium plasmids exist in the covalently closed circular (CCC) form, representing about 10-20% of total cellular DNA, and that they are stringently replicated and non-amplifiable (Badenoch-Jones et al., 1980). In 1979 Nuti et al. reported that some of the nitrogen fixation (nif) genes of K. pneumoniae, cloned in two recombinant plasmids (pSA30, containing nif KDH and a part of nif E, and pCM1, containing nif Q-E and part of nif K), hybridized with plasmid DNA from R. leguminosarum. The hybridization data clearly indicated that the plasmid DNA contains genes encoding both component I and component II of nitrogenase (nif KDH), but no other gene of the nif cluster could be detected in Rhizobium. Similarly, the presence of at least two structural nif genes encoding nitrogenase (nif DH) on rhizobial plasmid(s) was recently reported in

R. japonicum (Hennecke, 1980). However, Ausubel and co-workers (Szeto et al., 1980) failed to localize any of the nif genes on the 90-200 Md plasmids examined in R. meliloti. Both Ausubel (Szeto et al., 1980) and Fühler et al. (1980) raised the possibility that at least some of the nif genes of R. meliloti may be harbored on very large plasmids which co-purify with the chromosomal DNA; and hence, it is difficult to define their location at this stage.

A great deal of information regarding the apparent conserved character of nif genes, cleary exceeding the Rhizobium genus, was reported by Ruvkun and Ausubel (1980) and Mazur et al. (1980). All of the 19 strains of different nitrogen-fixing procaryotes, including Gram-positive and Gram-negative bacteria, cyanobacteria and the actinomycete Frankia, showed the presence of nif KDH genes (Ruvkun and Ausubel, 1980). Similar results were observed in cyanobacteria (Mazur et al., 1980). Interestingly, none of the remaining 14 structural genes of the nif cluster (see chapter 3.1) were detected in all the examined species (Ruvkun and Ausubel, 1980).

It is believed that also the nodule-forming ability (Inf^+) in some species of Rhizobium is under a plasmid control. Higashi (1967) reported a loss of this character after an acridine orange treatment. Due to the high concentration of acridine orange; however, the effect of this agent as a mutagen could not be ruled out. In R. meliloti strain L-1, subbacteriostatic doses of acridine orange increase the frequency of Inf^- mutations with up to 30% loss of Inf^+ (Pariiskaya, 1973). Similar doses of acriflavine or sodium dodecyl sulphate (SDS) were also reported to induce a loss of Inf^+ for R. trifolii strain T37 with a very

high frequency (90 to 100 percent) (Zurkowski et al., 1973). On the other hand, treatments with acridine orange, SDS or with short-wave UV light irradiation failed to abort infectivity in R. trifolii, R. meliloti and R. japonicum (Dénarié and Truchet, 1976). It was recently observed that incubations of the rhizobia at elevated temperatures frequently result in the loss of nodulating ability (Zurkowski and Lorkiewicz, 1978; Zurkowski and Lorkiewicz, 1979; Kondorosi et al., 1980). Using R. trifolii, Zurkowski (1980) demonstrated that there is a correlation between the loss of the nodulating ability and the loss of a large (95-125 Md) plasmid, whose size varies depending on the strain used. Interestingly, the absence of the plasmid had also an effect on cell wall synthesis and cell division (Zurkowski, 1980), although it could not be concluded if these characters are confined to the symbiotic process. Very similar results were reported in R. meliloti (Kondorosi et al., 1980). Using a heat-treatment procedure developed for the elimination of R-plasmids from Rhizobium, about 5-30% of the cells became defective in symbiotic functions (Nod$^-$ or Inf$^-$, and Fix$^-$ or Eff$^-$). In some Nod$^-$ mutants, a deletion of a 25 kb DNA segment of the larger (> 200 Md) plasmid was observed. Since this DNA fragment also contained the nif region (Kondorosi et al., 1980), it was concluded that both nod and nif genes are harbored on the larger plasmid of R. meliloti, and that they are located relatively close to each other. Further evidence for the apparent presence of nodulation genes on plasmids was reported by Scott (1980). A long-term storage of R. trifolii on agar slants at 4°C resulted in the formation of non-nodulating (Nod$^-$) mutants within cultures. Plasmid analysis of these strains showed that the wild type strain had two plasmids of molecular

weights 120 Md and 160 Md, whereas the non-nodulating mutants had lost the 120 Md plasmid. The above observations thus strongly suggested that the genes involved in nodulation (nod) and at least some structural genes for nitrogen fixation (nif) in Rhizobium are present on large plasmids rather than the chromosome.

Plasmid DNA of Rhizobium spp. is also believed to harbor genetic factors controlling the production of bacteriocins (Novick, 1969). Bacteriocins are considered temperate phages of bacterial origin, capable of killing other bacteria within a particular strain-specificity. Bacteriocin 37 of R. trifolii (Schwinghamer et al., 1973) was shown to be a DNA-containing nucleoprotein, hexagonal in outline, and resembling other colicins from Rhizobium spp. (Schwinghamer, 1971; Roslycky, 1967), perhaps with the exception of a bacteriocin from R. lupinus (Lotz and Mayer, 1972), which is apparently devoid of DNA. Since the structure and biology of bacteriocins, occurring widely in all species of Rhizobium, remain largely unknown, it is difficult to speculate on their function. To date there have been no reports suggesting the involvement of bacteriocins prior to or during the Rhizobium-legume symbiosis.

3.3. Plant Genes Involved in the Rhizobium-Legume Symbiosis

Studies of the genetic role of the host plant in root nodule symbiosis have been carried out in two major aspects: the ability of nodulation and the effectiveness of the nitrogen fixation. The most easily recognized host-specific interaction in legumes is the inability to develop nodules (non-nodulation) by the plant infected with a genetically compatible species of Rhizobium. Nutman (1949) reported the

presence of a recessive gene r r in red clover (Trifolium pratense) which controls nodulation of the plant by R. trifolii, possibly at its very early stage. Williams and Lynds (1954) reported that a single recessive plant gene no no controls nodulation in soybean (Glycine max). This gene, later designated rj_1 rj_1 (Caldwell, 1966), was believed to control the production of a specific factor localized in the root which inhibits nodulation (Elkan, 1961). Subsequent studies of Hubbell and Elkan (1967a) demonstrated some physiological changes in the non-nodulating strains of soybean, characteristic of the rj_1 rj_1 mutation. Comparison of the wild type Rj_1 Rj_1 and mutant rj_1 rj_1 prior to and after rhizobial infection, also revealed significant differences in the amount of protein, reducing sugars and free amino-acids (Hubbel and Elkan, 1967b). Whereas the presence of the recessive genes r r in red clover (Nutman, 1949) and rj_1 rj_1 in soybean (Williams and Lynch, 1954; Caldwell, 1966) was shown to condition nodulation in the host plant regardless of the environmental conditions, a dominant gene Sym 1 was reported to condition nodulation of field pea (Pisum sp.), cultivar "Iran", in a temperature-sensitive fashion (Lie, 1971; Holl, 1973). Another plant gene of field pea, sym 2 sym 2, occuring in cultivar "Aphganistan", was identified as a recessive gene conditioning all strains of R. leguminosarum, regardless of the environmental conditions (Lie, 1971; Holl, 1973).

A gene of red clover, ie ie, conditions ineffective response with a number of bacterial (R. trifolii) strains, causing abnormal host cell divisions and no bacteroid formation (Nutman, 1957; Bergersen and Nutman, 1957). Nutman (1968) also reported the presence in red clover of two other genes, designated n n and d d, which condition

ineffectiveness of nitrogen fixation in the small nodules produced. In soybean there are at least 3 dominant genes involved in effectiveness of nitrogen fixation: $\underline{Rj_2}$ (Caldwell, 1966; Caldwell et al., 1966), $\underline{Rj_3}$ (Vest, 1970), and $\underline{Rj_4}$ (Vest and Caldwell, 1972). Phenotypically, the presence of the $\underline{Rj}$ genes leads to the formation of small, white nodules, unable to fix nitrogen. In field pea there are a minimum of three recessive genes influencing the effectiveness of nitrogen fixation: two unidentified genes conditioning the number of nodules (Gelin and Blixt, 1964), and a $\underline{sym}$ 3 $\underline{sym}$ 3 gene affecting the function of nitrogenase and causing accumulation of polysaccharides in periphery of infected cells (Holl, 1973).

Since all of the plant genes involved in the nodule formation and function were found by means of classical Mendelian genetics, our knowledge of their location, regulation and expression is very incomplete. From the data available, it appears that the gene $\underline{Rj_2}$, involved in the effectiveness of nitrogen fixation in soybean, is independent of the non-nodulating gene $\underline{rj_1}$ $\underline{rj_1}$ (Caldwell, unpublished), and that the genes $\underline{Rj_2}$ and $\underline{Rj_3}$ may be linked (Vest, unpublished). While the control of the $\underline{Rj_2}$ (Caldwell et al., 1966) and $\underline{Rj_3}$ (Caldwell and Vest, 1977) types of ineffectiveness was shown to occur within the root system, Fischer et al. (1972) reported that the efficiency of nitrogen fixation involves both the root and the scion of the plant. A series of interesting studies regarding the role of plant genes in the symbiotic process was initiated by the observation that the association of plant with Rhizobium may often lead to chlorosis (Erdman et al., 1956). Johnson et al. (1958), and Johnson and Clark (1958) showed that the Rhizobium-induced chlorosis was observed when certain soybean genotypes

were inoculated with certain strains of R. japonicum. Although the root nodules formed on severely chlorotic plants were of normal size, shape and distribution, Johnson and Clark (1958) demonstrated that control of the expression of chlorosis was confined to the roots. Later studies showed that the chlorosis is caused by a toxin (Owens and Wright, 1965) which is indeed synthesized in the nodules. Due to its translocation to young developing leaves (Johnson and Clark, 1958), it could be easily isolated from chlorotic leaves. The biochemical action of this toxin, referred to as rhizobitoxine, was well elucidated by Owens et al. (1968). Susceptibility of soybean genotypes to rhizobitoxine appears to vary in different cultivars (Johnson and Means, 1960), nevertheless the involvement of a single plant gene with modifiers was postulated (Caldwell and Vest, 1977) in this phenomenon.

Nodule distribution also appears to be controlled genetically by the plant. Bhaduri and Sen (1966) reported several nodulation patterns for Phaseolus spp. When P. aureus var. N.P.28, which had a localized nodulation pattern, was crossed with P. trilobus of a diffuse nodulation pattern, the diffuse pattern was dominant (Bhaduri and Sen, 1968). There are also a few reports on increasing nodule mass by genotypic selection in the host. Jones and Burrows (1968) observed that certain crosses between different cultivars of white clover (Trifolium repens) and a selected, effective strain of R. trifolii, resulted in the formation of especially large root nodules. They also observed, however, that the increasing size of nodules was accompanied by a decreased efficiency of nitrogen fixation. These results are in agreement with those of Chen and Thornton (1940) with red clover and with the results of Jones (1962) with white clover.

That the plant genome is directly involved in both structural and functional establishment of the Rhizobium-legume symbiosis has been suggested in a number of reports, in which different species or cultivars of the plant were shown to form different nodules with the same Rhizobium strain. Although most of these studies failed to indicate or identify the plant gene(s) involved, some information related to the development of the symbiosis was obtained. Studies of the root nodules formed by R. lupini strain D25 on Lupinus luteus and Ornithopus sativus (Kidby and Goodchild, 1966) demonstrated that the plant genes influence the shape of bacteroids and infection thread, as well as they determine the number of bacteroids per membrane envelope. Similar observations were made in root nodules of the same rhizobial origin on Lotus and Astragalus, where both the morphology of nodules and number of bacteroids per membrane envelope appeared to be under genetic control of the host (see Dart, 1977). The conclusion that nodule senescence of Astragalus begins at the nodule base and for Lotus (infected with the same Rhizobium strain) it begins throughout the bacteroid zone (Dart, 1977), suggests that the involvement of the plant in symbiosis also occurs at late stages.

An increased ploidy of the root cells associated with the initiation of nodules (Wipf and Cooper, 1940) has been a controversial issue. Although the infected plant cells were shown to contain increased levels of DNA (4c, 8c and even 16c) in P. sativum (Mitchell, 1965) and V. unguiculata (Kodama, 1970), the actual cell divisions in the development of nodules are diploid (Kodama, 1970). It is believed that the polyploidy in nodules is a consequence of the infection process rather than a pre-requisite for nodule formation (see Dart, 1977).

Libbenga et al. (1973) and Libbenga and Torrey (1973) proposed that cytokinins and auxins produced by Rhizobium (Thimann, 1936) and/or by the host plant (Dullaart, 1970) induce endoreduplication, followed by an additional round of DNA synthesis and the subsequent tetraploid cell division in the nodules. Although the natural occurrence of tetraploid cells in nodules has been known for long (Wipf and Cooper, 1938, 1940), it should be noted that uninvaded cells in the outer cortex and meristematic zones of nodules are also polyploid (Dart, 1977).

Despite the numerous studies regarding the role of plant genes in the development of Rhizobium-legume symbiosis, which have been carried out mostly by means of classical genetics and physiological techniques, our knowledge of specific plant gene products involved in this process remains very incomplete. The only product of plant origin known to this date, whose expression is restricted to root nodules, has been leghemoglobin (see under Biochemistry of Nitrogen Fixation). This thesis reports the presence of several other "nodule-specific" plant proteins in soybean, referred to as nodulins, including nodulin-35 (Legocki and Verma, 1979) and about 20 other nodulins (Legocki and Verma, 1980). Recent advances in molecular biology with recombinant DNA techniques in particular, will certainly contribute to our understanding of the genetic role of the plant in association with Rhizobium. Due to the possibility of bacteroid secretions into the host cell cytoplasm, as well as due to the possible disruption of bacteroids upon homogenisation procedures, a mere localization of putative plant products in the host cytoplasm ought to be substantiated by sufficient biosynthetic studies.

The concept of "nodule-specific" plant proteins in the root nodule symbiosis, postulated in this thesis, involves studies of host proteins which are absent from uninfected roots of soybean and appear only after rhizobial infection. These proteins are synthesized on the 80S-type polysomes in the host cytoplasm, and their composition and appearance are differentially affected by mutations in Rhizobium. Identification and a preliminary characterization of nodulins presented here, may contribute to our better understanding of the role of leguminous plants in the symbiotic association with Rhizobium spp.

MATERIALS AND METHODS

BIOLOGICAL MATERIALS

Soybean (Glycine max L. var. Prize) seeds (Strayer Seed Farm, Hudson, Iowa).

Rhizobium japonicum, strains 61A76 and 61A24 (Nitrogen Co., Milwaukee); strains SM3, SM4, and SM5 (from Dr. W. Brill, University of Wisconsin).

Uninfected Roots of Soybean

Uninfected roots of soybean from two developmental stages were used: 3 day-old hypocotyls and 21-day mature roots. Seeds were germinated in the dark on large trays containing vermiculite at 28°C for three days, and 1.5 - 2.0 cm long root tips were harvested using a razor blade.

To obtain mature uninfected roots, the seeds were sterilized in 5% sodium hypochlorite (Hypo 12), thoroughly washed in water, and grown on vermiculite in a controlled environment as described by Verma et al. (1974). Mature uninfected roots of soybean were also obtained by excising primary and secondary root sections free of nodules using a razor blade.

Root Nodules

Three day-old seedlings of soybean were inoculated with Rhizobium japonicum (strain 61A76), and grown on vermiculite in a controlled environment (Verma et al., 1974). Root nodules were harvested usually 3

weeks following infection, or earlier for developmental studies. The effectiveness of the nodules in nitrogen fixation was tested by the acetylene reduction assay (Hardy et al., 1968).

Storage of Tissue

Both uninfected roots and root nodules were stored in liquid nitrogen immediately after harvesting.

Rhizobium Cultures

Both effective (61A76) and ineffective (61A24, SM3, SM4 and SM5) strains of Rhizobium japonicum were grown on liquid media as described by Sutton (1974). The cultures were incubated in the dark with a gentle shaking at 28°C and cells, grown to a late log phase, were collected by centrifugation at 6,000 x g. Prior to inoculating soybean hypocotyls, the cells were suspended in a small volume of 10% (v/v) glycerol or 5% (w/v) sucrose.

Wheat Germ

Cell-free in vitro translation was carried out using wheat germ extracts (S23). Wheat germ (General Mills Inc., Vallejo, California) was stored at 4°C in a sealed container.

METHODS

(i) Preparation of Soluble Cytoplasmic Proteins from Nodules

Tissue was pulverized with liquid nitrogen in a mortar and pestle, transferred into buffer A (10 ml/g tissue) containing 50 mM Tris-HCl (pH 8.7), 20 mM KCl, 10 mM $MgCl_2$ and protease inhibitors[1], and homogenized at 4°C. Following removal of cell debris and bacteroids at 20,000 x g, the supernatant was recentrifuged at 105,000 x g for 2 hr. For storing, the postribosomal supernatant (soluble cytoplasmic proteins) was lyophilized or frozen at -20°C. Protein samples were dissolved in H_2O for immunoelectrophoresis, or in sample buffers according to Laemmli (1970) and O'Farrell (1975) for polyacrylamide gel electrophoresis.

(ii) Isolation of Bacteroids

Three week-old fresh nodules (4 g) were ground in a mortar and pestle with 5 ml of buffer A for 15 sec and, following addition of another 10 ml of the buffer, homogenisation was continued for 30 more sec. Cell debris was removed at 750 x g and the supernatant was recentrifuged at 6,000 x g to sediment bacteroids (Sutton et al., 1977). The pellet was gently resuspended in 4 ml of deionized water and centrifuged through a 20% (w/v) sucrose cushion at 30,000 x g for 10 min. To obtain cellular extracts of bacteroids, the membrane envelope contaminations were removed by resuspending the pellet in 0.5% (v/v)

[1] 1 mM p-aminobenzamidine (dihydrochloride);
1 mM N-α-p-tosyl-L-lysine;
1 mM phenylmethyl-sulfonylfluoride;
1 mM L-1-tosylamide-2-phenyl-ethylchloromethyl ketone (TPCK), all from Sigma.

Nonidet P40 (Bethesda Research Laboratories, Rockville, Maryland) (Verma et al., 1978). The bacteroids were suspended in 0.5 ml of 20 mM Barbital Buffer III (pH 8.6) (Bio-Rad, Richmond, California) or in water, and sonicated with a microprobe (Sonifier Cell Disrupter, model W140D; Ultrasonics) twice for 30 sec at 4°C. Following removal of cell debris by centrifugation, the supernatant was used for analysis.

(iii) Labeling of Root Nodules, Free-living Rhizobia and Bacteroids

Fourteen day-old freshly harvested nodules (0.3 g tissue) were incubated in 0.5 ml of deionized water containing 75 μCi of ^{35}S-methionine (880 Ci/mmole, Amersham Corp., Illinois) or 100 μCi of ^{3}H-leucine (137 Ci/mmole, Amersham) for 4 hr at 28°C, under vigorous shaking (220 rpm) in a Gyrotory shaker (New Brunswick Scientific Co., New Jersey). Following incubation, the nodules were washed with deionized water at room temperature and homogenized in 50 mM Tris-HCl (pH 8.7) with a mortar and pestle at 4°C. Cytoplasmic proteins were prepared as outlined above.

Free-living rhizobia (strain 61A76) were grown in liquid media as described by Sutton (1974), and cells (50 ml) in late log phase were harvested by centrifugation at 6,000 x g for 10 min. The bacterial pellet was resuspended in 0.5 ml of deionized water and incubated with 100 μCi of ^{3}H-leucine or 75 μCi of ^{35}S-methionine for 3-4 hr as described above. The cells were washed thoroughly with H_2O at room temperature and sonicated in 0.5 ml of 20 mM Barbital Buffer III (pH 8.6). Following removal of cell debris by centrifugation, the supernatant was used for immunological and electrophoretic assays.

Isolation of bacteroids for *in vivo* labeling was carried out as described above and, following centrifugation through a sucrose cushion, the cells (0.2 g wet weight) were resuspended in 0.5 ml of H_2O and incubated with 250 μCi of ^{35}S-methionine. Cells were removed by centrifugation and the incubation medium was passed through a millipore filter (0.8 μm; Gelman Instrument Co., Michigan), lyophilized, and analyzed for the presence of secretory products of bacteroids. The labeled bacteroids were processed for cellular proteins as described above.

(iv) Rhizobium Cultures Induced for Nitrogenase

Rhizobium japonicum strain 61A76 was grown to an early log phase as outlined above, and the growth was continued under unaerobic conditions (Avissar and Nadler, 1978). In order to create a low O_2 tension, the cultures were thoroughly flushed with sterile nitrogen and grown at 28°C in sealed flasks under a slight N_2 pressure. Rhizobia were examined for induction of nitrogenase by the acetylene reduction assay (Hardy et al., 1968).

(v) Polyacrylamide Gel Electrophoresis Under Denaturing Conditions (SDS-PAGE)

Cytoplasmic proteins were examined by means of discontinuous sodium dodecyl sulphate (SDS) slab gel electrophoresis [16.5% acrylamide, 0.07% (w/v) bisacrylamide (Laemmli, 1970)], as well as by two-dimensional polyacrylamide gel electrophoresis (O'Farrell, 1975). The pH range of the isoelectric focusing dimension was from 5.2 - 7.2. The gradient was formulated with the following final concentrations of carrier Ampholines

(Bio-Lytes, Bio-Rad): 0.8%, pH 5-7, 0.8%, pH 6-8, and 0.4% (w/v), pH 2-11. Electrophoresis in the second dimension was performed in slab (1.5 mm thick) gels containing 17.5% acrylamide, 0.07% bisacrylamide and 0.1% (w/v) SDS. To improve electrophoretic resolution of small molecular weight proteins, SDS-PAGE was also carried out in gels containing a higher concentration of bisacrylamide. These gels, referred to as "high bisacrylamide gels", were identical to the "low bisacrylamide gels" described by Laemmli (1970), except that the concentrations of acrylamide and bisacrylamide were 12.5% and 0.33% (w/v), respectively. The gels were stained in 0.3% (w/v) Coomassie brilliant blue R (Sigma) with 50% methanol, 10% acetic acid and destained in 30% methanol, 7% (v/v) acetic acid.

(vi) Gel Fluorography

Following treatment with dimethyl sulfoxide (Me_2SO; Fisher Scientific) and 20% (w/v) 2,5-diphenyloxazole (New England Nuclear) in Me_2SO for 2 hr, the gels were thoroughly washed in H_2O, dried, and exposed to prefogged X-ray films (Kodak RP-X-omat) for quantitative autoradiofluorography (Laskey and Mills, 1975).

(vii) Non-Denaturing Polyacrylamide Gel Electrophoresis (ND-PAGE)

Non-denaturing PAGE was carried out according to the procedure of Davis (1964). The separating gel, containing 7% acrylamide and 0.18% (w/v) bisacrylamide was polymerized chemically with ammonium persulphate and TEMED (Eastman Kodak Co.), whereas the stacking gel, containing 2.5% acrylamide and 0.62% (w/v) bisacrylamide was photopolymerized using riboflavin and TEMED. The running buffer was identical to that

described by Laemmli (1970) except that no SDS was used. The non-denaturing PAGE was carried out using both slab and cylindrical gels, where approximately 30 mA of constant current was applied per 2 cm^2 surface of the gel. Electrophoresis in non-denaturing gels was usually carried out at 4°C. Staining and destaining procedures were identical to those described above.

(viii) Electrophoresis of Small Molecular Weight Proteins

In order to resolve polypeptides of small molecular weights, high Tris/urea polyacrylamide gel electrophoresis was used (Goldsmith et al., 1979). To obtain a good resolution, it is desirable to use specially purified acrylamide (BDH Chemicals Ltd., electrophoresis grade) and urea (BDH). The resolving gel contained 9.4% acrylamide, 0.6% bisacrylamide, 0.45% (w/v) SDS, 6.4 M urea and 0.675 M Tris-HCl (pH 8.9). The gel was made from stock solutions of 3 M Tris-HCl (pH 8.9), 20% (w/v) SDS, 10 M urea, and crystalline acrylamide and bisacrylamide. Polymerization of gels was carried out with 0.0035% (w/v) of freshly prepared ammonium persulfate and 0.005% (v/v) TEMED. The stacking gel contained 3% acrylamide, 0.08% bisacrylamide, 0.5% (w/v) SDS, and 0.125 M Tris-HCl (pH 6.8), and it was polymerized with 0.008% (w/v) ammonium persulfate and 0.04% (v/v) TEMED. Electrophoresis was carried out using standard (Bio-Rad) glass plates at 5-7°C at 20 mA constant current in the stacking gel, and at 40 mA in the resolving gel. Gels were fixed in 20% (w/v) TCA for at least 1 hr and stained in 0.3% (w/v) Coomassie brilliant blue R, as above.

(ix) Preparative Gel Electrophoresis

Nodulin-35 was purified by means of preparative SDS gel electrophoresis. Electrophoresis was carried out in a 17.5% acrylamide, 0.07% (w/v) bisacrylamide cylindrical (1.5 x 10 cm) gel (Savant Instruments Inc.) in the buffer system described by Laemmli (1970). To ascertain a flat surface of the gel in the elution chamber, 1% (w/v) agarose was polymerized at the bottom of gel. Elution buffer [electrophoresis buffer (Laemmli, 1970) without SDS] was run across the chamber at 200 µl/min, and absorbance was monitored at 280 nm (ISCO Instrumentation Specialties Co.). Fractions (2 ml each) were collected and dialized overnight, lyophilized and examined by SDS-PAGE.

(x) Preparation of R-type Antisera

New Zealand White rabbits were immunized subcutaneously with 5-20 mg of protein extracts in complete Freund's adjuvant (Difco Laboratories, Detroit) every week for one month, and bled 5 days after the fourth injection. Monospecific antibodies to nodulin-35 required approximately 150 µg of pure protein per injection. Beginning two weeks after the initial injection, the appearance and titer of antibodies was monitored in the serum by double immunodiffusion tests (Byrne et al., 1975). Rabbits were bled from ear (up to 40 ml of blood) and by cardiac puncture (up to 150 ml). The blood was allowed to stand at room temperature for 30 min and centrifuged at low speed to isolate serum. Antisera were stored at -70°C in small aliquots or further processed for isolation of IgG (see below).

(xi) Isolation of IgG from Antisera

To isolate the immunoglobulin fraction from crude antiserum, a protein A-Sepharose CL-4B column (Sigma, St. Louis) was used. A 2 ml column was equilibrated with buffer A containing 150 mM NaCl, 10 mM Tris-HCl pH 8.3, 10 mM Na-EDTA and 100/ml Kallikrein Inhibitor Units of Aprotinin (Sigma) (Lingappa et al., 1978). Four to five ml of antiserum were passed through the column twice and the gel was thoroughly washed with buffer A. Elution of IgG was carried out with buffer B containing 0.1 M glycine-HCl pH 2.2, 20 mM $MgAc_2$ and 50 mM KCl, and the eluate was immediately neutralized with Tris-base to prevent possible damage to IgG. Following dialysis against phosphate-buffered saline [PBS: 0.8 g NaCl, 0.02 g KCl, 0.115 g Na_2HPO_4, 0.2 g KH_2PO_4, 0.01 g $MgCl_2 \cdot H_2O$ (pH 7.2)], the immunoglobulin fraction was concentrated on CM-cellulose (Sigma) to about 1.3 mg/10 µl and stored at -70°C in small aliquots.

(xii) Double Immunodiffusion (Ouchterlony's) Test

The test was carried out for preliminary analyses of antisera as well as to determine reaction of identity (Crowle, 1973a) using various sources of antigens. One percent (w/v) agarose (Bio-Rad) gels containing 20 mM Barbital Buffer III (pH 8.6) (Bio-Rad) were polymerized on small glass plates (7.5 x 2.5 cm). Depending upon the number of antigens applied, 3-6 wells were made in a circle, each of them at about 7 mm distance from the central application well. Up to 5 µl of antigen samples (10 - 250 µg protein) were applied into the sample wells, whereas the antiserum was usually placed in the center. Immunodiffusion was carried out at room temperature overnight (Byrne et al., 1975) in a closed chamber at high humidity. In case of no apparent precipitation

arcs, the gels were processed and stained as described under Rocket Immunoelectrophoresis.

(xiii) Rocket Immunoelectrophoresis

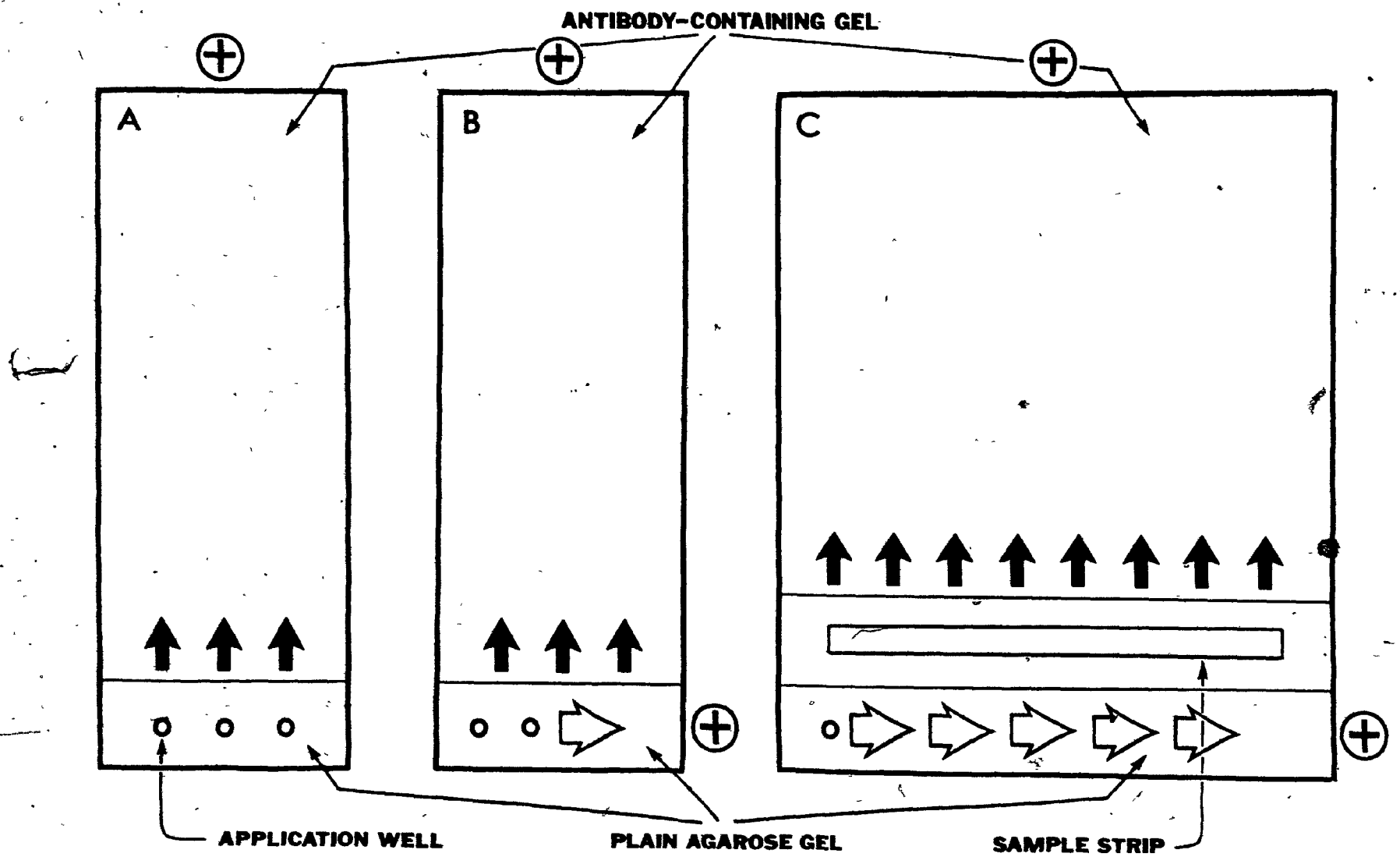
Rocket immunoelectrophoresis was carried out in 1% (w/v) agarose gels containing 20 mM Barbital Buffer III, pH 8.6, as described by Weeke (1973). Gels with 10% (v/v) antiserum were polymerized on glass plates (10 x 5 cm or 7.5 x 2.5 cm), where the agarose solution was cooled to 55°C prior to the addition of antiserum. The section of the gel (1 cm wide) containing sample wells was polymerized without antiserum (Fig. 5). Gels were placed in an electrophoresis chamber, connected to the buffer by paper (Whatman No. 3) wicks, and approximately 4 µl of antigen samples (30-120 µg protein) were placed into application wells (see Fig. 5 A). Electrophoresis was carried out at 4°C for 4-12 hr at 2 V/cm until the bromophenol blue marker reached the anodic end of the gel. After immunoelectrophoresis, the gels were pressed dry with Whatman No. 3 paper and washed in 0.1 M NaCl at least 5 times to remove unprecipitated proteins (Weeke, 1973). Gels were stained in 0.2% (w/v) Kenacid blue R (BDH Biochemicals) in 50% methanol, 10% (v/v) acetic acid for 15 min, and destained in 30% methanol, 7% (v/v) acetic acid.

(xiv) Tandem-Crossed Immunoelectrophoresis

Gels containing 1% (w/v) agarose and 10% (v/v) antiserum were prepared on small glass plates as outlined above. Two sample wells were made in the plain agarose gel as depicted in Fig. 5B. Following application of two antigen samples containing bromophenol blue as a

FIGURE LEGEND ON OTHER SIDE

Fig. 5. Schematic illustration of rocket immunoelectrophoresis (A), tandem-crossed immunoelectrophoresis (B), and crossed-line immunoelectrophoresis (C). Tandem-crossed and crossed-line immunoelectrophoreses are carried out in two dimensions, where antigens are first resolved in a plain agarose gel (open arrows), and then electrophoresed into the antibody-containing gel (black arrows). See also Results and Discussion.


ANTIBODY-CONTAINING GEL
A
B
C
APPLICATION WELL
PLAIN AGAROSE GEL
SAMPLE STRIP

marker, proteins were resolved electrophoretically in the first dimension to the distance equivalent to that separating the application wells. The electrophoresis in the second dimension was carried out perpendicular to that into the antibody-containing gel (Fig. 5 B), until the bromophenol blue reached the upper end of the gel (Kroll, 1973a). Electrophoretic conditions and processing of gels after electrophoresis were identical to those described under Rocket Immunoelectrophoresis.

(xv) Crossed-Line Immunoelectrophoresis

The experimental set-up for crossed-line immunoelectrophoresis (Kroll, 1973b) was essentially the same as for rocket immunoelectrophoresis, except that a larger (6 x 7 cm) gel was used. The section of the gel polymerized without antiserum was divided in two parts (each 1 cm wide), where the lower part contained the application well, and the upper one the sample strip (Fig. 5 C). The sample strip (5 x 0.4 cm) was prepared from 1% (w/v) agarose polymerized with or soaked in concentrated extracts of cytoplasmic proteins from uninfected roots (8 mg/ml), but it was placed in the gel only after electrophoresis of the main (nodule) antigen in the first dimension. This electrophoresis was carried out at 2 V/cm in the cold, as described above, to a distance of approximately 5 cm. The sample strip was then moulded into a 1.5 mm thick contact gel by pouring agarose around the gel strip between the barriers formed by the lower part of the plain agarose gel and the antibody-containing gel (Fig. 5 C). Immunoelectrophoresis was carried out perpendicular to the first dimension until the dye (bromophenol blue) front reached the top of the gel. Electrophoretic conditions, removal of non-precipitated proteins, drying and staining procedures, were carried out as described under Rocket Immunoelectrophoresis.

(xvi) Electroimmunodiffusion*

To examine monospecificity of antiserum against nodulin-35, one-dimensional electroimmunodiffusion (Crowle, 1973b) was used. One percent (w/v) agarose gels were polymerized on small glass plates as described under Double Immunodiffusion Test.

Following electrophoresis of antigens parallel to the long axis of gel, the antiserum was placed longitudinally in the application slot (45 x 2 mm) and incubated at room temperature for 12 hr. Lateral diffusion of antiserum and electrophoretically resolved antigen(s) resulted in formation of precipitation arcs within 12 - 18 hrs. To visualize possibly all precipitation arcs, the gels were processed and stained as described under Rocket Immunoelectrophoresis.

(xvii) Preparation of "Nodule-Specific" Antiserum by Adsorption

"Nodule-specific" antiserum was prepared by adsorption of the anti-nodule serum (raised against total cytoplasmic proteins of nodules) with the cytoplasmic proteins from uninfected roots. The reaction was carried out in 200 µl aliquots of the anti-nodule serum by addition of increasing amounts of the root cytoplasmic proteins (80 µg/µl) in the antiserum/antigen range of 50:1, 25:1, 15:1, 7:1 and 3:1 (v/v) (total antigen added = 6.2 mg). Addition of the root proteins was done every 24 hr for 5 days. Each time, following incubation at 28°C for 30 min, the anti-nodule serum was kept at 4°C overnight, centrifuged to remove immunoprecipitates, and treated with the consecutive aliquot of the root

* This technique is also referred to as immunoelectrophoresis (Crowle, 1973b).

proteins. After five treatments, adsorption appeared to be complete and no further precipitation was observed. The resulting "nodule-specific" antiserum was stored at -70°C in small aliquots (see also Results and Discussion).

(xviii) Isolation of Polysomes and In Vitro Translation

Total (free and membrane-bound polysomes) from uninfected roots and root nodules were isolated according to procedure by Verma et al., 1974). Tissue was pulverized with liquid nitrogen in a mortar and pestle. Homogenisation was carried out in buffer A containing 150 mM Tris-acetate (pH 8.5), 20 mM KCl, 5 mM $MgAc_2$, 200 mM sucrose (ribonuclease-free, Sigma), 5 mM β-mercaptoethanol and 0.4% (v/v) Nonidet P40 at 2°C. Following removal of cell debris at 20,000 x g, the polysomes were centrifuged through a 2 ml sucrose cushion (1.5 M sucrose, 50 mM Tris-acetate pH 8.5, 20 mM KCl, 5 mM $MgAc_2$) for 2 hr at 105,000 x g. The polysomal pellet was resuspended in 20 mM KCl and 1 mM $MgAc_2$. Polysomes could be stored in liquid nitrogen either in the same buffer containing 20% (v/v) glycerol or as a pellet.

Polysomes were translated in a wheat germ cell-free system prepared according to Marcu and Dudock (1974). Prior to translation, polysomes were treated with micrococcal nuclease (150 units/ml) at 20°C for 10 min (Pelham and Jackson, 1976) to remove endogenous mRNA activity (Verma and Ball, 1977). In vitro translation was carried out in 100 µl aliquots containing 1-2 A_{260} units of polysomes, 15 µl of micrococcal nuclease-treated wheat germ S23, 1 mM ATP, 8 mM creatinine phosphate, 4 µg of creatinine phosphokinase, 25 µM GTP, 2 mM dithiothreitol, 90 mM potassium acetate, 2.5 mM $MgAc_2$, 2 µg of wheat germ tRNA, 40 µM

spermidine tetrahydrochloride (neutralized), 10 μCi of ^{3}H-leucine (Amersham Corp.) or 15 μCi of ^{35}S-methionine (Amersham) (Verma et al., 1980). Following incubation at 25°C for 90 min., the mixtures were ultracentrifuged (105,000 x g for 45 min) to remove nascent polypeptide-ribosome complexes, and the released product was used for analyses.

(xix) Immunoprecipitations

Total nodule polysomes (including free and membrane-bound) were prepared as described previously (Verma et al., 1974), and translated *in vitro* (Verma et al., 1979) in a wheat germ cell-free system (see Preparation of Polysomes and *In Vitro* Translation). The released *in vitro* translation products, as well as *in vivo* labeled cytoplasmic proteins, were precipitated with cold 5% (w/v) TCA, dissolved in 1% (w/v) SDS, and diluted 10-fold with buffer B [150 mM NaCl, 10 mM Tris-HCl (pH 8.3), 10 mM Na-EDTA, 1% (v/v) Triton X-100 and 100/ml Kallikrein Inhibitor Units of Aprotinin (Sigma)], as described by Lingappa et al. (1978). The samples were pre-adsorbed with a nonimmune rabbit serum for 3 hr at 25°C and 3 hr at 4°C, and treated with staphylococci-bound protein A [0.6g cells per 10 ml, The Enzyme Center Inc., Boston, prepared as described by Kessler (1975)](Lingappa et al., 1978). Following the removal of nonspecific antibody-antigen complexes, the samples were incubated with the immune sera (3 μl for *in vitro* and 7 μl for *in vivo* labeled material) for 12 hr at 25°C and 12 hr at 4°C. Antibody-antigen complexes were sedimented with staphylococci-bound protein A (20 μl suspension of cells per μl of antiserum) for 3 hr at 25°C. The suspension was centrifuged at 12,000 x g for 3 min and the pellet was washed extensively (3-5 times) in buffer B. The products to

be analyzed by two-dimensional PAGE were released by treatment of the pellet with 9.5 M urea, 2% (v/v) Nonidet P40, 2% (w/v) Ampholines, 5% (v/v) β-mercaptoethanol (O'Farrell, 1975) for 15 min at 35°C, whereas those subjected to one-dimensional SDS-PAGE were dissolved directly into the sample buffer and boiled for 2 min. The staphylococci cells were removed by centrifugation at 12,000 x g for 3 min and the supernatant was used for analyses.

(xx) Isolation of Specific mRNAs by Immunoprecipitation of Polysomes

Total (free and membrane-bound) polysomes were isolated from 1g of 8 day-old nodules as described under Isolation of Polysomes and In Vitro Translation, except that Na-heparine (100 µg/ml) and cycloheximide (10 µg/ml) were added in homogenisation buffer. The polysome pellet was resuspended in 1 ml of solution containing 20 mM KCl and 1 mM $MgAc_2$, and centrifuged at 10,000 x g for 10 min to remove aggregated polysomes. Approximately 20 A_{260} optical units of polysomes were subsequently incubated for 45 min on ice with 1.3 mg of monospecific IgG. After incubation with antibody, the mixture was slowly passed through a 1 ml protein A-Sepharose CL-4B column, prepared as outlined under Isolation of IgG from Antisera, at 2°C. The column was washed thoroughly with buffer A and mRNAs were eluted with 10 ml of buffer B containing 10 mM Hepes (pH 7.6) and 25 mM EDTA. The eluate was made 0.4 M NaCl, passed through oligo (dT)-cellulose (Collaborative Research) and the obtained poly A(+) mRNA was examined by in vitro translation. Protein A-Sepharose Cl-4B was regenerated in buffer C containing 0.1 M glycine-HCl (pH 2.2), 20 mM $MgAc_2$, 50 mM KCl, and stored in buffer A for further use.

(xxi) Multiple Immunoreplica Technique

Following electrophoresis as described under Polyacrylamide Gel Electrophoresis Under Denaturing Conditions, the gels were washed in the electrode buffer containing 25 mM Tris-base, 192 mM glycine and 20% (v/v) methanol for 20 min, and the proteins were transferred to nitrocellulose paper (Schleicher and Schuell, Inc.) according to the method of Towbin et al. (1979). A series of partial electrophoretic transfers from one gel containing approximately 200 µg protein were carried out at 300 mA for 1 hr each. Two to three replicas containing sufficient amounts of protein to react with antibodies or to stain were routinely obtained from one gel. After the transfer, nitrocellulose paper (replica) was equilibrated with buffer A [saline (0.9% (w/v) NaCl, 10 mM Tris-HCl pH 7.4) containing 3% (w/v) BSA (Sigma)] (Towbin et al., 1979) for 1 hr at room temperature and incubated with antiserum (20 µl per ml of the above buffer) overnight at 30°C. The reaction vessel was gently shaken on a gyratory shaker. Following a thorough washing of the nitrocellulose paper with saline (at least 5 changes of buffer) the paper was treated with ^{125}I-protein A in buffer A [1.5×10^6 cpm per ml; prepared by a modified version of the chloramine T method (Granger and Lazarides, 1979), see also Iodination of Proteins] for 1 hr at room temperature. The nitrocellulose paper was washed to remove unbound ^{125}I-labelled protein A with saline, blotted dry on paper towels, and exposed to unprefogged X-ray films (Kodak RP-X-omat or Dupont Cronex). In order to react the same replica with a second antibody, IgG-[^{125}I-protein A] complex was removed from the nitrocellulose paper with buffer B (0.1 M glycine - HCl pH 2.2, 20 mM $MgAc_2$, 50 mM KCl) for 1.5 hr at room temperature. The regenerated paper was neutralized by a

brief wash in saline and, following incubation in buffer A, reacted with the second antiserum as described above, or stored at 4°C in a sealed bag for future use. Staining and destaining of polyacrylamide gels and nitrocellulose paper was carried out according to standard procedures described above.

(xxii) Preparation of Nodules for Light Microscopy

Root nodules developed by Rhizobium strains 61A76, 61A24 and SM5 were prepared for light microscopy according to standard procedures (Drury and Wallington, 1967). The tissue was fixed in formalin-ethanol-acetic acid (FAA) overnight, subjected to the dehydration series of ethanol and, following incubation in 50% and 100% (v/v) xylene, molded in paraffin wax. Fifteen μm sections were prepared by means of a rotary microtome (American Optical 820), and tissue was stained in Delafeld's hematoxylin (Drury and Wallington, 1967). A series of sections were mounted in xylene on a microscope slide, and examined by light microscopy at 100x magnification.

(xxiii) Peptide Mapping of Purified Polypeptides

In order to compare identity of nodulin-35 from various effective and ineffective root nodules, the purified polypeptide was digested with α-chymotrypsin (Sigma) during electrophoresis in the stacking gel (Cleveland et al., 1977). The gel was prepared as outlined in Polyacrylamide Gel Electrophoresis Under Denaturing Conditions, except that a longer (4 cm) stacking gel was polymerized. Bands from SDS gels stained with Coomassie blue R were placed in the sample wells of a second SDS gel, and overlayed with protease (4 μg α-chymotrypsin). The

individual bands were soaked for 30 min in 10 ml of buffer A containing 0.125 M Tris-HCl (pH 6.8), 0.1% (w/v) SDS, 1 mM EDTA, and placed over the second gel in the same buffer containing 20% (v/v) glycerol. Prior to electrophoresis, 10 ul of buffer A containing 10% (v/v) glycerol and 4 µg of protease was overlayed into each slot. Proteolytic digestion was allowed to take place in the stacking gel by turning off the current for a total of 90 min (2 times 45 min with a short interval). The gel was stained with Coomassie blue as described above.

(xxiv) Iodination of Proteins

Soluble protein A (Pharmacia) was iodinated by the chloramine T method of Greenwood et al. (1963), except that the reaction was terminated by adding an excess of tyrosine (Granger and Lazarides, 1979). 100 µl of 0.5 M potassium phosphate (pH 7.5) were added to 1 mCi of Na ^{125}I [New England Nuclear; in 2 µl of NaOH (pH 8.8)]; 20 µl of protein A (5 mg/ml) and 20 µl of chloramine T (Sigma; 2.5 mg/ml) were added next. After 2 min, 150 µl of tyrosine (0.4 mg/ml; Pfanstiehl Laboratories, Inc., Waukegan) were added. The mixture was passed through a 3 ml bed of Sephadex G-25 and the void fraction was diluted to 100 ml with saline [0.9% (w/v) NaCl, 10 mM Tris-HCl pH 7.4] containing 3% (w/v) BSA. Following the measurement of radioactivity associated with protein A, the solution was stored in a sealed container at 4°C.

(xxv) Bio-Gel P-200 Filtration

In order to isolate proteins of molecular weights below 20,000, a Bio-Gel P-200 filtration was carried out under denaturing conditions (8 M urea). A long (118 x 1.2 cm) column was packed at room temperature

with Bio-Gel P-200 (50-100 mesh, Bio-Rad Laboratories) with a minimum hydrostatic pressure applied. The gel was equilibrated with 8 M urea (Sigma) and de-gassed prior to packing. The column was washed with 8M urea overnight. Protein sample (14 mg) was dissolved in 1 ml of 8.5 M urea and any undissolved material was removed by centrifugation prior to applying it to the column. Fractions (4 ml each) were collected, dialyzed against water at 4°C, lyophilized and dissolved in SDS sample buffer (Laemmli, 1970) or urea lysis buffer (O'Farrel, 1975) for electrophoretic analyses.

(xxvi) Protein Fractionation by Sevag's Method

Sevag's method has been originally described (Sevag, 1934; Staub, 1965) as a procedure for the gradual removal of proteins and isolation of glycoproteins and saccharides from protein extracts. Soluble cytoplasmic proteins from root nodules were treated with 0.2 volume of chloroform and 0.04 volume of n-butanol, and the mixture was shaken at 270 rpm for 10 min (Gyrotory shaker; New Brunswick Scientific Co., New Jersey) at room temperature. Following centrifugation at 6,000 x g, the aqueous phase was collected and re-extracted 3 more times with chloroform-butanol as above. Precipitated proteins, sedimenting at the chloroform-aqueous interface, were also retained for electrophoretic analyses after each extraction. Following 4 extractions, the aqueous phase was saturated with ammonium sulphate and the precipitated hydrophilic proteins were dissolved in a small volume of H_2O. The protein suspension was subsequently precipitated with TCA, and pellet washed at least 2 times with absolute ethanol. Following the removal of ethanol in a vacuum centrifuge, the protein was stored as a dry powder at 4°C.

(xxvii) Chromatography on DEAE-Cellulose

DEAE-cellulose (DE52, Whatman) was treated with 0.5 N HCl for 30 min, washed with water, and treated with 0.5 N NaOH for another 30 min. Following de-gassing, the cellulose was placed in a small column and thoroughly equilibrated with 5 mM K-acetate buffer (pH 5.2). Approximately 12 mg of nodule cytoplasmic proteins were applied to a 4 cm column in the same buffer, and the column was washed with 5 mM K-acetate (pH 5.2) to elute unbound material. Following application of a 5 mM - 50 mM gradient of the same buffer, the column was eluted stepwise with 100 mM, 200 mM, and 400 mM K-acetate (pH 5.2). Fractions (about 1.2 ml each) collected during the chromatography were dialyzed, lyophilized, and, following measurements of protein contents, analyzed electrophoretically.

(xxviii) Hydrophobic Chromatography on Phenyl-Sepharose

Phenyl-Sepharose CL-4B (Pharmacia) was washed in a 10 mM sodium phosphate (pH 7.6) buffer, de-gassed, and placed in a small (1 x 5 cm) column. The column was equilibrated with the same buffer containing 1 M ammonium sulphate. Protein sample, obtained as described under Preparation of Soluble Cytoplasmic Proteins from Nodules, was made 1 M with ammonium sulphate and centrifuged at 12,000 x g for 10 min prior to applying to the column. Upon application of samples, unbound material was collected for analyses. Column was eluted stepwise with decreasing ionic strengths of the salt (0.5 M, 0.1 M and no ammonium sulphate in 10 mM sodium phosphate buffer), until zero absorbance at A_{280} (ISCO Instrumentation Specialties Co.) of the eluate. Elution of proteins tightly bound to Phenyl-Sepharose was carried out in 10 M urea

(de-ionized with a Mixed Bed Resin AG501-X8). Collected fractions (0.5 ml each) were thoroughly dialyzed against 10 mM Tris-HCl (pH 8.7), lyophilized, and analyzed electrophoretically.

(xxix) Silver Stain for Proteins

A simplified procedure for staining proteins with silver (Oakley et al., 1980) was found advantageous to the original technique reported by Switzer, III et al. (1979) and Merril et al. (1979). To minimize background, the polyacrylamide gels were routinely pre-fixed in 50% methanol, 10% (v/v) acetic acid for 30 min or longer, and washed in 5% methanol, 7% (v/v) acetic acid overnight (Oakley et al., 1980). The gels were soaked in 10% (v/v) unbuffered gluteraldehyde (biological grade; E.M. Sciences) and washed in glass-distilled water for 12-16 hr. Staining of proteins was carried out in the silver solution prepared as described by Oakley et al. (1980) for 8-9 minutes. [To prepare 100 ml of the silver stain, 1.4 ml of fresh NH_4OH were added to 21 ml of 0.36% (w/v) NaOH. With vigorous agitation, 4 ml of 19.4% (w/v) $AgNO_3$ were added, and glass-distilled water was added to 100 ml]. The gels were vigorously shaken to prevent silver precipitation during staining. Immediately after staining, gels were washed with water for 2 min and proteins visualized in a freshly prepared solution containing 0.005% (w/v) citric acid and 0.019% (v/v) formaldehyde (Fisher Scientific). Reaction was stopped by removing the reducer and washing gels in water. Overstained gels can be destained in Kodak rapid fixer. It is important that acid-washed glassware and glass-distilled water be used throughout this staining procedure.

Gels previously stained with Coomassie blue R can also be stained with silver. Prior to fixing in gluteraldehyde (see above), Coomassie blue-stained gels were thoroughly destained to a clear background and incubated overnight in water upon shaking. Following treatment with gluteraldehyde, the gels were processed and stained with silver as described above.

(xxx) Measurement of Radioactive Proteins (TCA-Precipitation)

To estimate the amount of radioactive precursor(s) incorporated into protein, samples were precipitated with hot TCA and the radioactivity of TCA-insoluble material was measured on glass fiber filters in a scintillant. The radioactive sample (usually 10 μl aliquot) was first treated with equal volume of 20% (w/v) TCA, containing 0.1 M of unlabelled amino-acid, and diluted to about 4 ml with cold 5% TCA. Following incubation for 5 min on ice, the sample was boiled for 10 min, kept on ice for about 10 min, and filtered through a GF/A glass fiber filter (Whatman). The filter was washed with at least 15 ml of cold 5% TCA, dried under a heat lamp, and placed in 5 ml of toluene scintillant for counting.

(xxxi) Protein Estimation

Protein contents were measured according to the method of Lowry et al. (1951). Quantitation was based upon standard curves prepared with the use of bovine serum albumin.

RESULTS AND DISCUSSION

I. ANALYSIS OF SOLUBLE CYTOPLASMIC PROTEINS FROM ROOT NODULES

To preliminarily examine total cytoplasmic proteins from soybean roots and Rhizobium prior to and after infection, protein extracts were prepared as described in Methods and analyzed by one-dimensional SDS-PAGE (Fig. 6). Direct comparison of soluble proteins from uninfected and infected roots (lanes a and b, respectively) showed that the majority of polypeptides are common to both tissues. However, in addition to leghemoglobin, a polypeptide having a molecular weight of approximately 35,000 was found in root nodules (lanes b and d), and it was not detectable in uninfected roots (lane a), bacteroids (lane c), or free-living Rhizobium (lane k); nor was it detectable in Rhizobium induced for nitrogenase (lane l). These data suggested that the 35,000 molecular weight polypeptide could represent a first "nodule-specific" protein other than leghemoglobin of host origin. This protein is referred to as nodulin-35 (Legocki and Verma, 1979).

II. CHARACTERIZATION OF NODULIN-35

(i) Purification

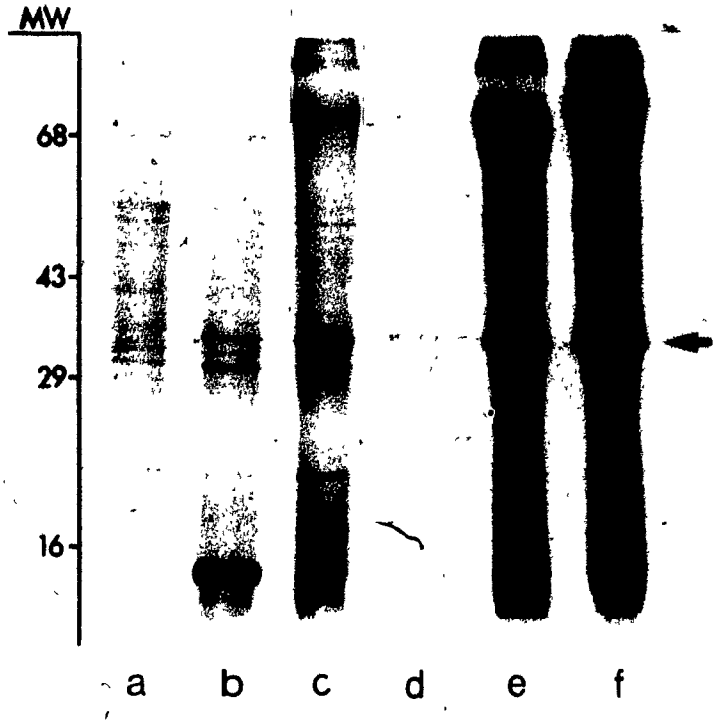
Fractionation of protein extracts from nodules with ammonium sulphate indicated that precipitation of nodulin-35 is virtually complete at 30% saturation and hence it facilitated purification of this polypeptide. Figure 7 shows that the 35,000 molecular weight protein

FIGURE LEGENDS ON OTHER SIDE

Fig. 6. SDS gel electrophoresis of cytoplasmic proteins extracted from soybean roots, nodules, bacteroids, and free-living Rhizobium. Total cytoplasmic proteins from (a) uninfected roots; (b) nodules induced by an effective strain of Rhizobium japonicum (strain 61A76, wild type); (c) wild-type bacteroids; (d to h) ammonium sulphate-precipitated fraction (30% saturation) of cytoplasmic proteins from nodules induced by Rhizobium strains 61A76, SM3, SM4, SM5, and 61A24, respectively; (i) wild-type bacteroids; (j) bacteroids from Rhizobium strain SM4; (k) free-living wild-type Rhizobium; (l) Rhizobium induced for nitrogenase; and (m) molecular weight markers: N35, nodulin-35; and Lb, leghemoglobin. Electrophoresis was carried out on discontinuous SDS slab gels (low bisacrylamide), as described in Polyacrylamide Gel Electrophoresis Under Denaturing Conditions.

Fig. 7. Isolation of nodulin-35 by ammonium sulphate fractionation. Total soluble cytoplasmic proteins from uninfected roots (a) and root nodules (b) of soybean; nodule cytoplasmic proteins fractionated with ammonium sulphate at 0-30% (c); 0-10% (d); 10-20% (e), and 20-30% saturation (f). Arrow indicates position of nodulin-35.


MW
68kD
43kD
29kD
18kD
N35
Lb
a b c d e f g h i j k l m


MW
68
43
29
16
a b c d e f

can be highly enriched by ammonium sulphate fractionation from 10% to 30% saturation (lanes d to f). A similar fractionation of cytoplasmic proteins from uninfected roots did not result in any enrichment of protein(s) in the 35,000 molecular weight region (data not shown). Electrophoretic analyses of cytoplasmic proteins precipitated with ammonium sulphate at concentrations higher than 30% indicated only trace amounts of nodulin-35 (data not shown).

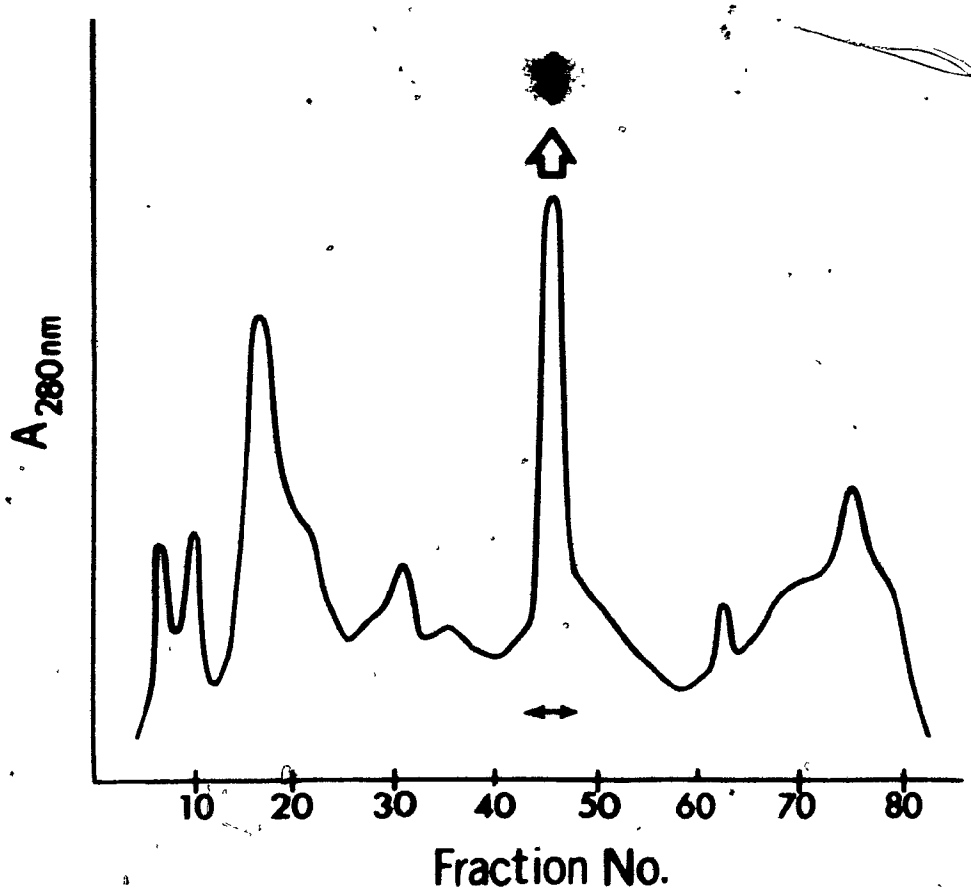
Nodulin-35 was purified to homogeneity by means of preparative PAGE in the presence of SDS. Electrophoresis of the 30% ammonium sulphate-precipitated fraction was carried out in a discontinuous buffer system (Laemmli, 1970) as described in Methods, and eluted proteins were monitored at A_{280} (Fig. 8). Collected fractions (about 90) were dialyzed, lyophilized, and analyzed by SDS-PAGE to localize nodulin-35. Figure 9 represents one of the analytical electrophoregrams showing fractions containing nodulin-35. The peak fractions (Fig. 8, arrow) were combined and used for raising R-type monospecific antibodies (see below).

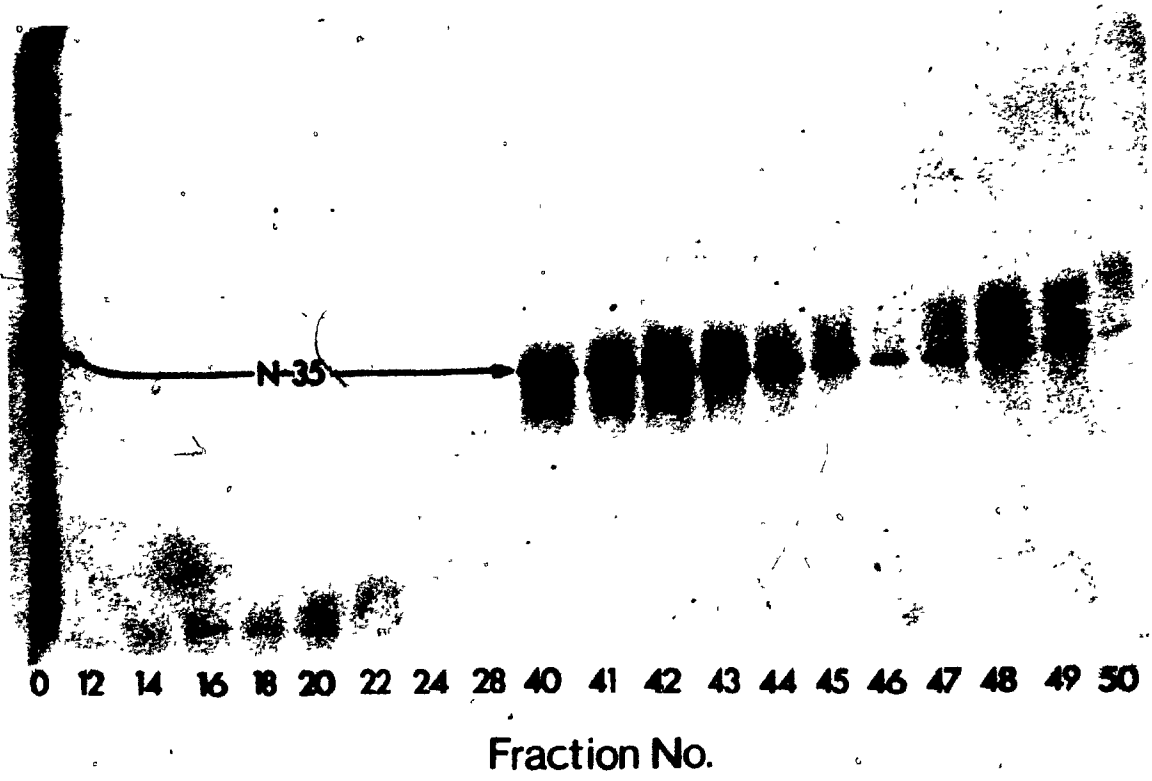
Since nodulin-35 precipitates at low concentrations of ammonium sulphate, it appears that it is a relatively hydrophobic protein. This characteristic was further documented by means of another method for protein fractionation, referred to as Sevag's method (Sevag, 1934). This technique is based upon repeated treatments of protein extracts with a chloroform-butanol mixture, leading to a gradual removal of hydrophobic proteins from solution (see Methods for details). Nodulin-35 was found among the most hydrophobic proteins and precipitated upon initial extractions with the chloroform-butanol mixture.

FIGURE LEGENDS ON OTHER SIDE

Fig. 8. Purification of nodulin-35 by preparative electrophoresis. A 0–30% ammonium sulphate-precipitated fraction of nodule cytoplasmic proteins was subjected to a preparative electrophoresis (Methods), and elution of proteins was monitored photometrically at 280 nm. Fractions corresponding to nodulin-35 were collected (arrow), and examined electrophoretically (see also Fig. 9).

Fig. 9. An electrophoregram of selected protein fractions after preparative gel electrophoresis. Arrows indicate position of nodulin–35 (N35) in the sample prior to (fraction 0) and after (fractions 40 to 50) the electrophoresis.


A280nm
10
20
30
40
50
60
70
80
Fraction No.


N-35
0
12
14
16
18
20
22
24
28
40
41
42
43
44
45
46
47
48
49
50
Fraction No.

Glycoprotein staining (Zacharius et al., 1969) indicated that nodulin-35 is a non-glycosylated polypeptide.

(ii) Monospecific Antibodies to Nodulin-35

To raise antiserum against nodulin-35, New Zealand White rabbits were injected weekly with approximately 150 µg of purified protein in complete Freund's adjuvant, as described in Methods. Electro-immunodiffusion tests of pure nodulin-35 as well as of total cytoplasmic proteins from nodules, resulted in single immunoprecipitation arcs, suggesting monospecific nature of this antiserum. A 30% ammonium sulphate-precipitated fraction prepared from both effective and in-effective nodules, along with non-nodulated roots (used as a control), was reacted with the antiserum to nodulin-35. Figure 10, representing Ouchterlony's double immunodiffusion test, shows a common precipitation line between cytoplasmic proteins obtained from nodules developed by different strains of Rhizobium (wells b to e), whereas nodulin-35 is not detectable in uninfected roots (well f). Furthermore, extracts from free-living Rhizobium (including cultures induced for nitrogenase) and from bacteroids do not cross-react with the antiserum, suggesting host origin of nodulin-35.

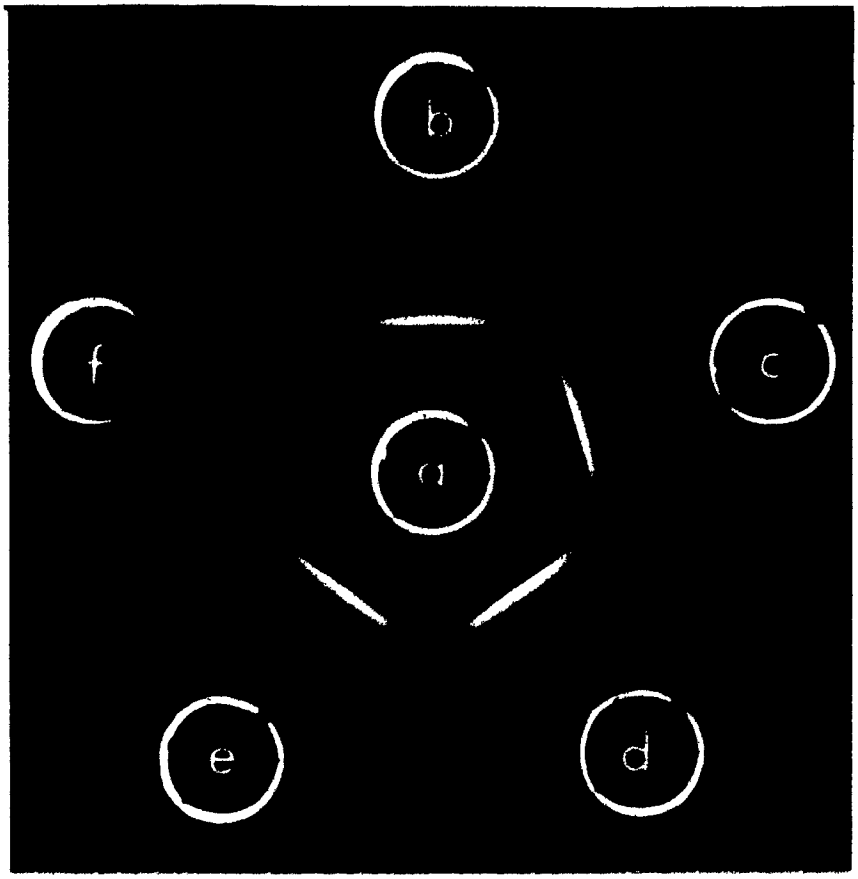
(iii) Abundance of Nodulin-35 in Root Nodules

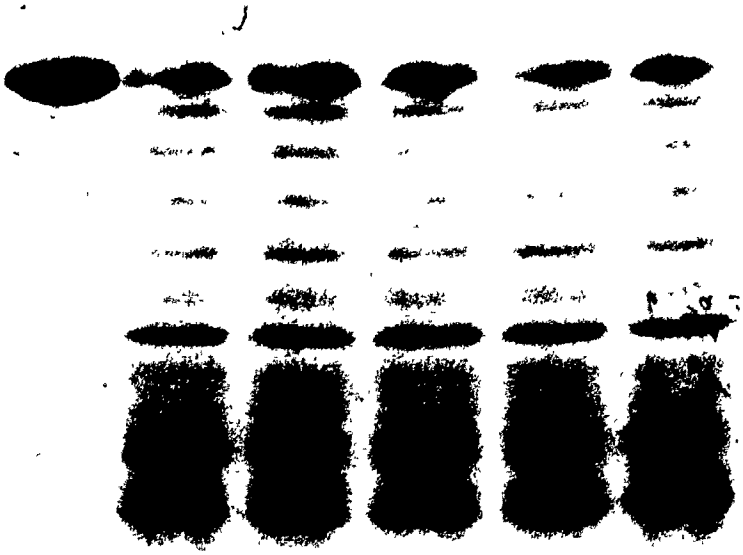
To measure the relative amount of nodulin-35 in root nodule cytoplasm, approximately 100 µg of total cytoplasmic proteins were resolved electrophoretically, stained with Coomassie blue, and scanned with a spectrophotometer in parallel with the purified polypeptide. Assuming that all protein bands stained quantitatively, nodulin-35 represents about 4% of the total cytoplasmic fraction in root nodules. In spite of its abundance in the tissue, the rate of biosynthesis of nodulin-35 appears to be low. Electrophoretic analysis of cytoplasmic

FIGURE LEGENDS ON OTHER SIDE

Fig. 10. Ouchterlony immunodiffusion test of (a) antiserum to nodulin-35 with the 30% ammonium sulphate-precipitated fraction of cytoplasmic proteins from root nodules formed by Rhizobium strains (b) 61A76, (c) SM4, (d) SM5, (e) 61A24, and (f) from non-nodulated roots. Reaction was performed on Hyland agarose immunodiffusion plates at room temperature (Methods).

Fig. 11. Peptide maps of nodulin-35 isolated from effective and ineffective root nodules. The pure protein was digested with α-chymotrypsin during SDS slab gel electrophoresis (Cleveland et al.(1977); see also Methods). (a) α-chymotrypsin, (b) undigested nodulin-35, (c to g) digestion products of nodulin-35 from nodules induced by Rhizobium strains 61A76, SM3, SM4, SM5, and 61A24, respectively.





a b c d e f g

proteins from young (7 day-old) and mature (14 to 16 day) nodules indicated similar amounts of nodulin-35 at these stages of development, suggesting that this protein may be synthesized at very early stages of the symbiosis. This was further substantiated by the following two observations: in vivo labeling of both young and mature nodules with ^{3}H-leucine and/or ^{35}S-methionine results in a very poor labeling of nodulin-35; rate of synthesis of nodulin-35, as measured by immunoprecipitation of in vitro translation products of 80S-type host polysomes is low, even in very young nodules. It is thus possible that this "nodule-specific" protein is synthesized soon after infection of root by Rhizobium.

(iv) Appearance of Nodulin-35 in Effective and Ineffective Nodules

In order to determine whether this protein is present in nodules that do not fix nitrogen, cytoplasmic proteins were prepared from ineffective root nodules developed by several mutant strains of R. japonicum (strains SM3, SM4, SM5, and 61A24) and analyzed on SDS-polyacrylamide gels (Fig. 6, lanes e to h). The presence of a common protein band at molecular weight 35,000 in all nodules demonstrated that the appearance of nodulin-35 is not related to the effectiveness of the nodules in nitrogen fixation. The identity of this 35,000 molecular weight protein was further determined in nodules induced by different strains of R. japonicum by analyzing peptide maps of nodulin-35 on SDS gels (Methods). All profiles of proteolytic digestion of this protein are similar (Fig. 11), suggesting that it is the same protein and its presence in different nodules is not simply a result of electrophoretic co-migration or cross-reactivity with antibody. The apparent structural identity of the 35,000 molecular weight protein in nodules developed by various strains of Rhizobium also suggested plant rather than bacterial origin.

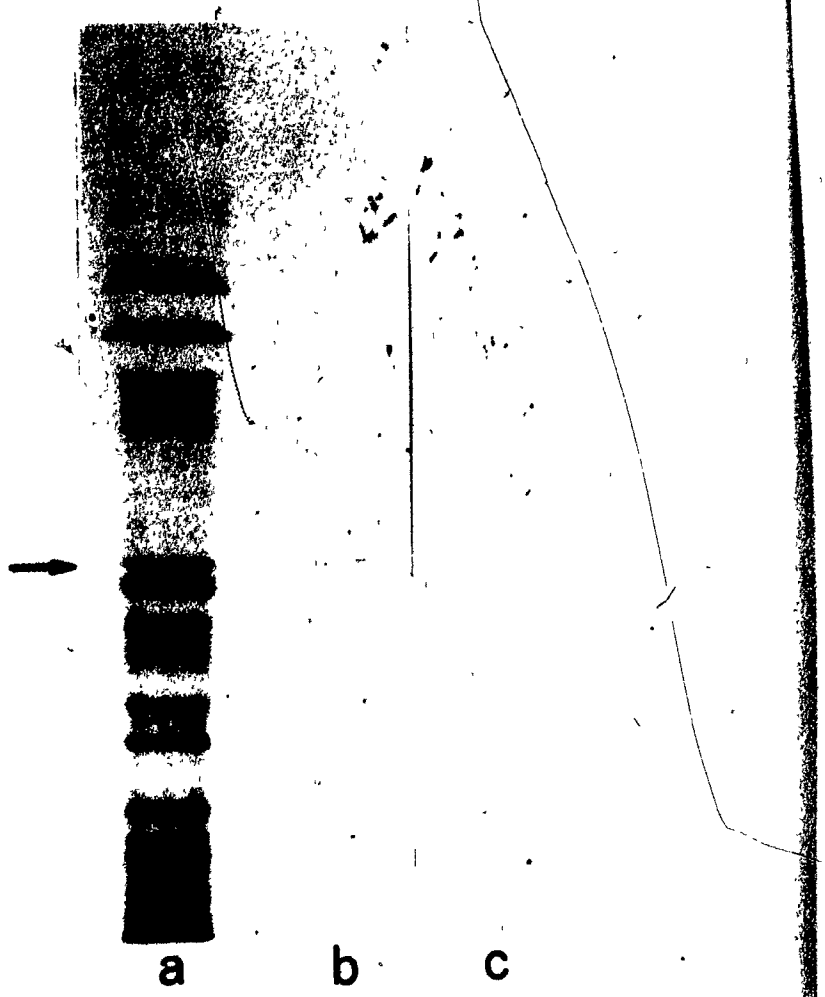
(v) Origin of Nodulin-35

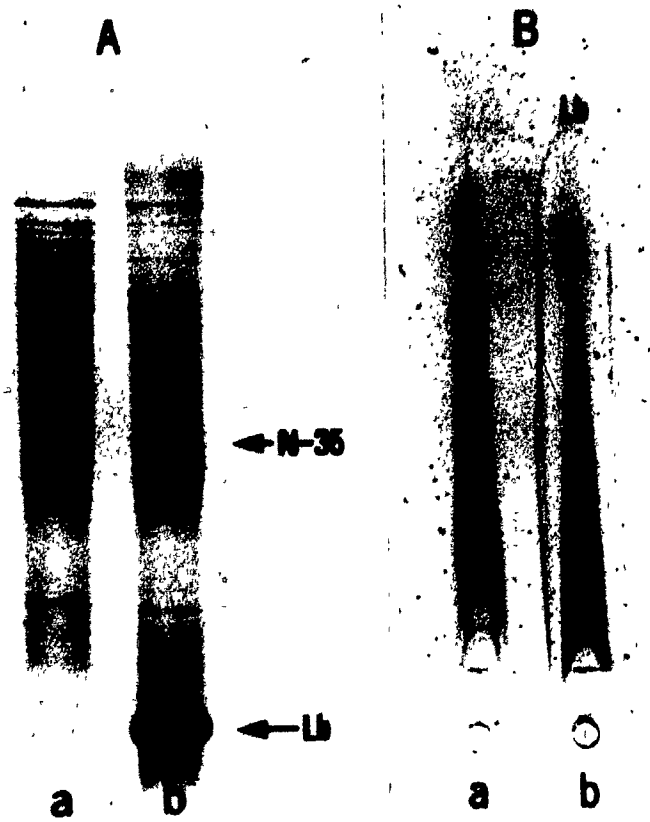
That nodulin-35 is synthesized by the host plant was shown by immunoprecipitation of the translation product of the nodule 80S-type polysomes and its inhibition of synthesis with cycloheximide (Fig. 12). The polysomes used have previously been shown to be free of bacteroid ribosome contaminations (Verma and Bal, 1976), providing a suitable system to study the biosynthesis of host proteins. In vitro translation of polysomes was carried out in a micrococcal nuclease-treated wheat germ translation system as described in Methods, in the presence of ^{3}H-leucine. Lane a in Fig. 12 represents total translation product, lanes b and c show immunoprecipitation of the translation product with antibody against nodulin-35. Translation in the presence of cycloheximide (1 µg/ml) (lane c) resulted in 89% inhibition of total TCA-precipitable counts. After translation, the ribosomes were removed by ultracentrifugation (105,000 x g for 2 hr) and the supernatant containing released polypeptides was prepared for electrophoresis as outlined in Methods. Immunoprecipitation of TCA-precipitated product was carried out as described by Lingappa et al. (1978), using staphylococci-bound protein A to precipitate antigen-antibody complexes (Methods). Since no immunoreactive material was obtained with the translation product of control root polysomes and since extracts from free-living Rhizobium and bacteroids did not cross-react with the antiserum, nodulin-35 appeared to be a "nodule-specific" protein of host origin.

FIGURE LEGENDS ON OTHER SIDE

Fig. 12. SDS slab gel electrophoresis of *in vitro* translation products from soybean polysomes (autoradiofluorogram). (a) total translation product of 80S-type nodule polysomes; (b and c) immunoprecipitation of the translation product with antibodies against nodulin-35, and (c) translation in presence of 1 μg of cycloheximide per milliliter, which gave 89% inhibition of total TCA-precipitable counts. Arrow indicates the position of nodulin-35 run as a marker in parallel.

Fig. 13. Analysis of soluble cytoplasmic proteins from uninfected roots (a) and root nodules (b) of soybean by SDS-PAGE (A) and rocket immunoelectrophoresis (B) using anti-nodule serum (Methods). SDS-PAGE was carried out with 100 μg of root and nodule proteins, while 30 μg from each were used for rocket immunoelectrophoresis. The gels were processed and stained as described in Methods. N-35, nodulin-35; Lb, leghemoglobin.


a
b
c


A
B
N-35
Lb
a
b
a
b

III. IDENTIFICATION OF OTHER "NODULE-SPECIFIC" HOST PROTEINS (NODULINS)

(i) Justification of an Immunological Approach

Despite a rapid development of techniques for identification of proteins in the recent years, a direct comparison and quantitation of rare polypeptides encounters many problems. Within the limited number of physical and chemical parameters of proteins (molecular size, solubility, isoelectric point, etc.), their detectability, localization or identity, may be difficult to assess by conventional methods. Since immunological reactions are highly specific, and since no "nodule-specific" proteins other than leghemoglobin and nodulin-35 could be detected by classical methods (see above), an attempt was made to develop and use a "nodule-specific" antibody probe.

R-type antisera against soluble cytoplasmic proteins from uninfected roots and root nodules, referred to as anti-root and anti-nodule sera respectively, were prepared as described in Methods. Following determination of the titer for each antiserum (10 µl of the anti-root serum precipitated 28 µg of its antigen, while 10 µl of the anti-nodule serum precipitated 36 µg of the root nodule antigen), the antisera were thoroughly tested by a variety of immunoelectrophoretic techniques (see below).

(ii) Evidence for the Presence of "Nodule-Specific" Proteins Other than Nodulin-35

Analysis of the soluble cytoplasmic proteins from uninfected roots and 3 week-old effective (*Rhizobium japonicum* strain 61A76-induced) nodules of soybean by SDS-PAGE, showed that the majority of the

polypeptides are common to both tissues (Fig. 13A). This was also evidenced by an extensive cross-reactivity of proteins from uninfected roots with the anti-nodule serum (Fig. 13B). In addition to the precipitation arcs formed by the root proteins (Fig. 13B,a), several other arcs were observed in the reaction of the anti-nodule serum with its homologous antigen (Fig. 13B,b). The similarity in the appearance of the precipitation arcs in rocket immunoelectrophoresis could not, however, demonstrate that they are formed by identical antigens.

Tandem-crossed immunoelectrophoresis (Kroll, 1973a) is an especially applicable technique for visualization of both common and tissue-specific proteins. This method is a combination of electrophoretic separation in a plain agarose gel of two samples containing antigens, followed by electrophoresis perpendicular to that in an antibody-containing gel (Methods). The related precipitin peaks formed from respective samples fuse into double precipitation arcs in the final pattern, indicating their common antigenicity. In addition, proteins that do not have their antigenic counterparts present in the other sample form single precipitation peaks with their respective antisera. Since the area enclosed by an individual precipitation arc is proportional to the antigen/antibody ratio, it is possible not only to quantitate proteins common to the two samples, but also to specifically identify the number and relative amounts of the unique antigens. Such analysis of the root and nodule proteins using the anti-nodule serum showed several (at least nine) proteins present in root nodules which do not have their counterparts in the uninfected root tissue, as evidenced by the lack of common precipitation arcs (Fig. 14). Tandem-crossed immunoelectrophoresis has also indicated that there may be some

FIGURE LEGENDS ON OTHER SIDE

Fig. 14. Tandem-crossed immunoelectrophoresis of soluble cytoplasmic proteins from uninfected roots (R) and root nodules (N) of soybean against the anti-nodule serum. (B) is an enlargement of (A); (C) outlines the precipitation arcs which do not have their counterparts in roots and thus are unique to nodules. The antigens (30 μg protein each), containing bromophenol blue as a marker, were electrophoresed horizontally in an agarose gel containing no antibody to a distance of about 1 cm. This was followed by electrophoresis in the antibody-containing gel, perpendicular to the first dimension. Arrows in (B) indicate the immunoprecipitation arcs formed by root proteins which are greatly reduced in nodules. See also Methods.

Fig. 15. Crossed-line immunoelectrophoresis of soluble cytoplasmic proteins from uninfected roots (R) and root nodules (N) of soybean against the anti-nodule serum. Following electrophoresis of about 75 μg of the nodule antigen in a plain agarose gel, a sample strip containing proteins from uninfected roots was moulded into the gel, and the two antigens were electrophoresed into the antibody-containing gel (see Methods for details). A common precipitation line of the slow (LbS) and fast-moving (LbF) components of leghemoglobin suggests their common antigenicity.


A
B
C
1
2
3
4
5
6
7
8
9
R
N


R
N

proteins present in soybean roots which are greatly reduced upon infection with Rhizobium (two such proteins are indicated by arrows in Fig. 14B). This conclusion was further substantiated by both one and two-dimensional electrophoretic analyses of proteins from uninfected and infected roots (see Fig. 13A and Figures 18A and 18B).

Concentrations of the common proteins vary in the two tissues, supporting the observation made with rocket immunoelectrophoresis (Fig. 13B). Among the apparent "nodule-specific" antigens, leghemoglobin is the predominant component, as indicated by the size of its precipitation arc. A variety of techniques used showed that the electrophoretically slow and fast moving components of leghemoglobin (Ellfolk, 1972) exhibit virtually identical antigenicity (Figures 13B and 14A). Results obtained from crossed-line immunoelectrophoresis (Kroll, 1973b) indicated the presence of several "nodule-specific" proteins as well as directly illustraed the common antigenicity of leghemoglobins (Fig. 15; see also Crossed-Line Immunoelectrophoresis under Methods). In this technique, proteins common to uninfected roots and nodules, migrating from the sample strip saturated with the uninfected root proteins into the antibody-containing gel, form parallel precipitin lines fused with rocket-type arcs (Fig. 15). The rocket-type arcs are generated from identical antigens (proteins) present in root nodules and resolved electrophoretically in the first dimension (see Fig. 5), thus leading to the formation of common immunoprecipitins with the lines. Antigens present in uninfected root extracts at higher concentration than in root nodules form parallel lines only, whereas those specific to nodules form the rocket-type arcs.

Although the existence of several proteins unique to nodules was apparent, due to the possibility of bacteroidal contamination in the

host cell cytoplasm, it could not be determined at this stage whether all these "nodule-specific" proteins were of host origin.

(iii) Development of a "Nodule-Specific" Antiserum

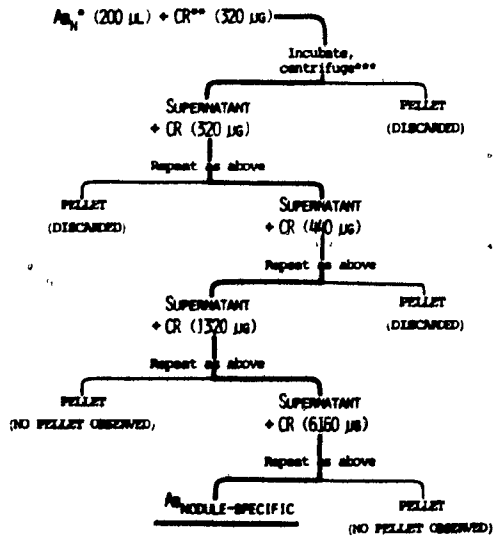
Since the majority of cytoplasmic proteins from root nodules are also present in uninfected roots, the anti-nodule serum contained a large quantity of antibodies strongly reactive with the cytoplasm of uninfected roots. To selectively remove the antibodies against proteins common to the uninfected and infected roots, and hence obtain a "nodule-specific" antiserum, the anti-nodule serum was adsorbed with increasing amounts of proteins from the uninfected roots. The adsorption was carried out step-wise from a high (50:1) to low (3:1) antiserum/antigen ratio (v/v) every 24 hr for 5 days, as shown in Fig. 16 (for details see Methods). This procedure, carried out entirely in liquid phase, allowed both antigens and antibodies to maintain their native conformations and thus ascertain a high fidelity of the antibody-antigen binding. Alternative techniques of adsorption involve chemical coupling of either antibodies or antigens to a stationary phase (e.g. Sepharose or Bio-Gel polyacrylamide; Bernfeld and Wan, 1963) and may lead to a non-specific binding to the matrix and/or to a decreased reactivity of antibodies with antigens due to their modification upon coupling. Since a selective removal of the common antibodies could not be carried out by means of protein A, which is known to react with all IgG's in a generalized fashion (Forsgren and Sjöquist, 1966; Kronvall and Frommel, 1970; Goding, 1978), it was important to use an equimolar excess of antibodies and thus allow formation of the secondary antibody-antigen complexes during the adsorption (Crowle, 1973c). Such complexes are precipitable and were removed by centrifugation (Methods). On the contrary, even a slight equimolar excess of antigens could prevent the

FIGURE LEGENDS ON OTHER SIDE

Fig. 16. A scheme illustrating preparation of the "nodule-specific" antiserum by adsorption. Following a series of treatments of the anti-nodule serum with concentrated extracts of protein from uninfected roots (see Methods), the resulting antiserum remains reactive with "nodule-specific" proteins, while its reactivity with proteins common to roots and nodules is negligible (see also Table 3).

Fig. 17. SDS-PAGE (fluorogram) of <u>in vitro</u> translation products of nodule total polysomes (a), immunoprecipitated with the "nodule-specific" antiserum (b) and the anti-root serum (c). While approximately $1x10^5$ cpm of ^{35}S-methionine-labeled translation products were analyzed in lane (a), the immunoprecipitation was carried out using $2.5x10^6$ cpm of material and resulting products were analyzed in lanes (b) and (c). Due to an excess of the antibodies in the reaction mixture, all corresponding antigens should be precipitated. As a result, each of the immunoprecipitated bands would be enriched approximately 25 fold in relation to those in the total translation product. Molecular weight (MW) markers were visualized by staining.

Preparation of Nodule-Specific Antiserum by Adsorption



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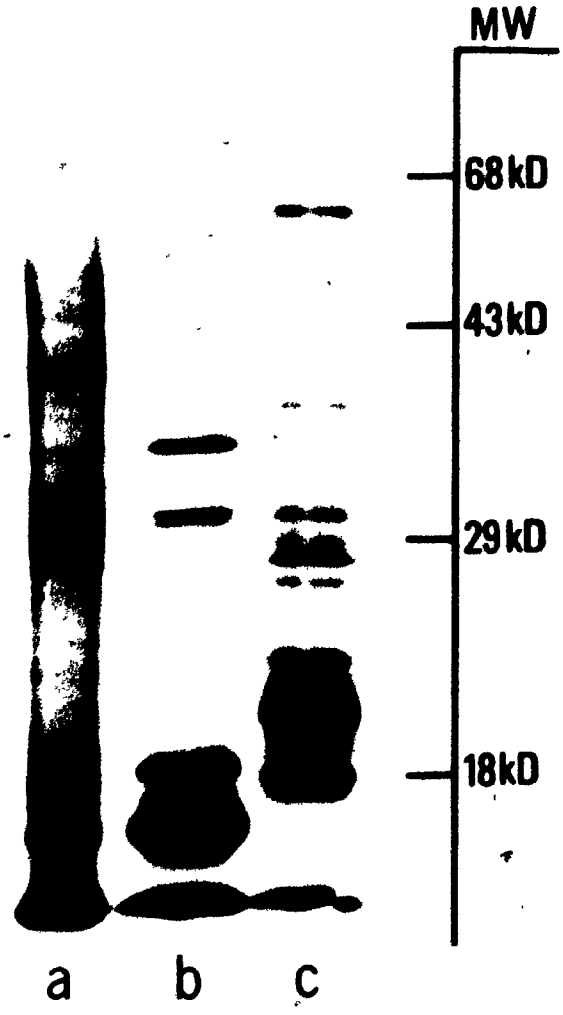
* Antiserum against host cytoplasmic proteins from root nodules (85 mg/ml). 10 ul of Ab_N precipitates 36 µg of homologous antigen.

** Extract of cytoplasmic proteins from uninfected roots (80 mg/ml).

*** Incubation at 28°C (12 hr) and 4°C (12 hr), followed by centrifugation (12,000 g for 5 min).



transition from the primary (soluble) to the secondary (insoluble) antibody-antigen complex (Crowle, 1973c), leading to an incomplete adsorption, where they could not be removed by centrifugation. Since the polyspecific anti-nodule serum contained a number of different antibodies at varying concentrations, it was desirable to use the broad range of the antiserum/antigen ratio during the adsorption.

Examination of the "nodule-specific" antiserum by rocket immunoelectrophoresis showed a significant reactivity of this serum with the cytoplasmic proteins from nodules and a negligible cross-reactivity with the proteins from uninfected roots. The degree of cross-reactivity was estimated by using equal amounts of the root and nodule extracts (30 or 120 μg of protein from each). In addition, no detectable cross-reactivity of the "nodule-specific" antiserum was observed with cellular extracts of bacteroids. The possibility that some bacteroidal proteins may be secreted into the host cell cytoplasm was examined by *in vivo* labeling of bacteroids and analysis of the secreted products, following immunoprecipitation with the "nodule-specific" antiserum (see below).

(iv) *Cross-Reactivity of the "Nodule-Specific" Antiserum*

To measure the percentage of protein represented by "nodule-specific" proteins in mature nodules, *in vitro* translation products of root nodule polysomes were reacted with the "nodule-specific" antiserum. Table 3 shows that 66% of the ^{3}H-leucine-labeled *in vitro* translation product is immunoreactive, while only 12% of the ^{35}S-methionine-labeled product reacts with this antiserum. A negligible cross-reactivity of the antiserum was observed with the *in vitro* labeled proteins from uninfected roots, as well as with *in vivo* labeled proteins of free-living *Rhizobium* and bacteroids. Thus, taking

TABLE 3

CROSS-REACTIVITY OF THE "NODULE-SPECIFIC" ANTISERUM

Source of antigen*	Radioisotope	Radioactivity (cpm) Total	Immunoprecipitated	% Protein precipitated
TP(WT)	^{3}H-Leu	149,000	98,896	66.4
TP(WT)	^{35}S-Met	115,000	13,907	12.1
TP(SM5)	^{35}S-Met	123,769	13,615	11.0
TP(61A24)	^{35}S-Met	126,400	11,123	8.8
TP(R)	^{35}S-Met	120,000	3,238	2.7
B	^{3}H-Leu	100,000	530	0.5
Bd(c)	^{3}H-Leu	100,000	503	0.5
Bd(c)	^{35}S-Met	116,796	798	0.7
Bd(s)	^{35}S-Met	138,664	1,680	1.2

*TP(WT), TP(SM5), TP(61A24): *in vitro* translation products of the 80S-type polysomes from root nodules developed by wild-type (61A76), SM5 and 61A24 strains of *Rhizobium*, respectively; TP(R), *in vitro* translation products of the 80S-type polysomes from uninfected roots; B, *in vivo* labeled total cellular proteins of free-living *Rhizobium* (wild type); Bd(c), *in vivo* labeled total cellular proteins of wild-type bacteroids; Bd(s), *in vivo* labeled total secretory proteins of wild-type bacteroids.

into account the cross-reactivity of the "nodule-specific" antiserum with extracts from uninfected roots as well as both cellular and secretory products of bacteroids, approximately 7-11% of the ^{35}S-methionine-labeled protein synthesized in nodules may be considered "nodule-specific" (see Table 3). Since soybean leghemoglobins are free of methionine residues (Ellfolk and Sievers, 1971; Nicola, 1975; Hurrell and Leach, 1977), provided that other nodule proteins possess an average methionine content, the observed difference between the immunoprecipitation of leucine and methionine-labeled products should be due to leghemoglobin. About 40% of ^{3}H-leucine-labeled in vitro translation product is immunoreactive with antibodies against purified leghemoglobin (Verma et al., 1981). The synthesis of nodulins appears to decrease in ineffective (unable to fix nitrogen) nodules induced by the SM5 and 61A24 strains of Rhizobium (see below).

(v) Identification of Nodulins

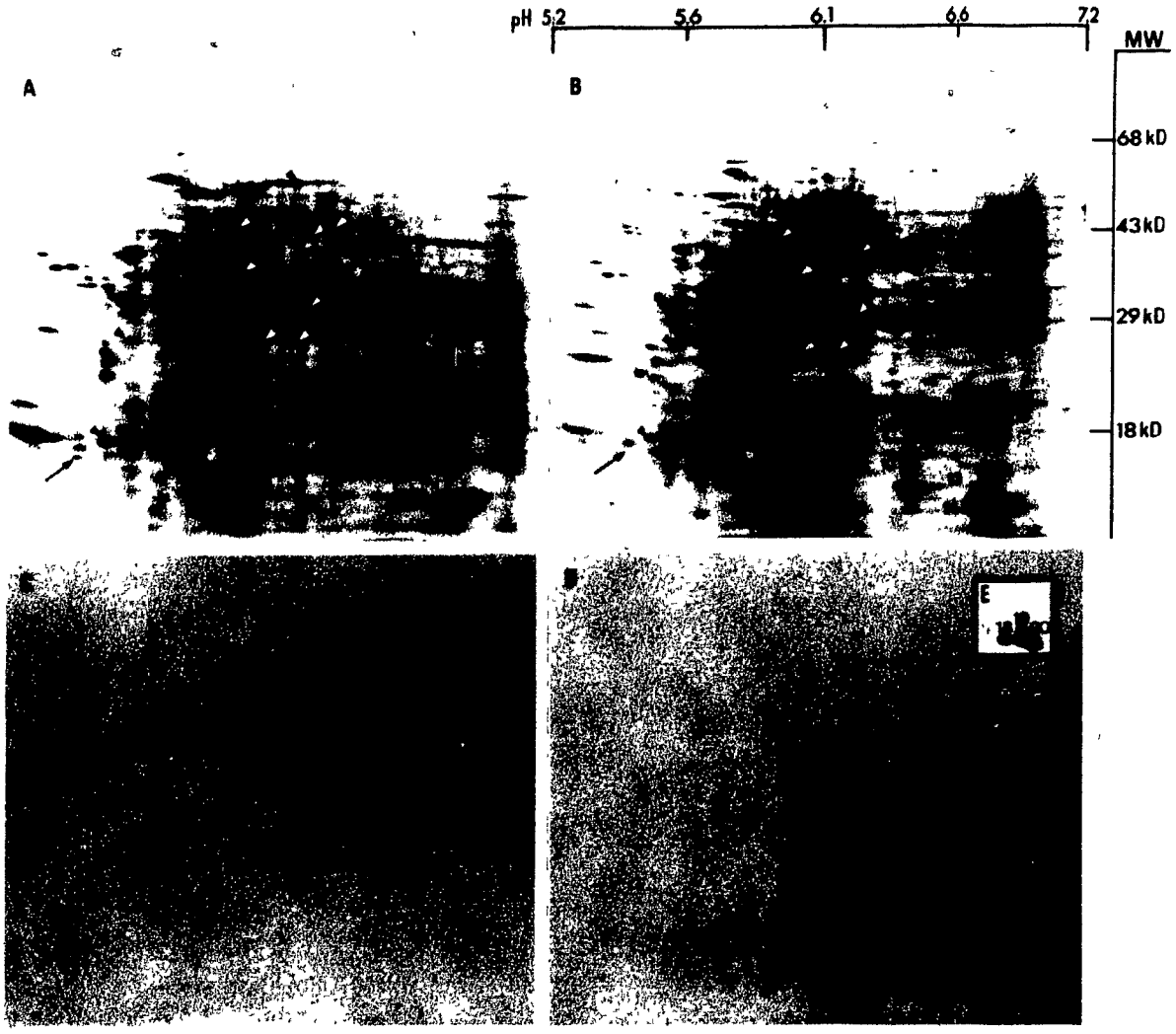
Identification of nodulins in the in vivo labeled tissue is difficult due to their low abundance and a low specific activity of the labeled products, especially in mature nodules. In vitro translation of the 80S-type nodule polysomes in the presence of ^{35}S-methionine, followed by immunoprecipitation with the "nodule-specific" antiserum, allowed the visualization of nodulins. The polysomes used have previously been shown to be free of 70S-type polyribosomes of bacteroidal or organellar origin (Verma and Bal, 1976). Analysis of the total translation products and immunoprecipitates on discontinuous SDS-PAGE is shown in Figure 17. Antibodies against proteins from uninfected roots (anti-root serum) immunoprecipitated a wide spectrum of polypeptides from the translation product of nodule polysomes (Fig. 17, lane c). A parallel experiment with the "nodule-specific" antibodies

yielded most of those peptides which were not precipitated by the anti-root serum (Fig. 17, lane b). A discrimination between nodule peptides by the two antisera also demonstrated the validity of "nodule-specific" antibodies. Due to the differential antigenicity of proteins giving rise to polyspecific antisera (Crowle, 1973d), the efficiency of immunoprecipitation of some polypeptides does not reflect their relative amounts in the total translation product (see also legend to Fig. 17).

To further establish the identity and an approximate number of nodulins in soybean, the total ^{35}S-methionine-labeled *in vitro* translation product of root and nodule polysomes was analyzed on two-dimensional polyacrylamide gels. Figures 18A and 18B show that there are a number of polypeptides present specifically in nodules. On the other hand, several peptides abundant in the root are greatly reduced or disappear in nodules (Fig. 18, black arrowheads). Analysis of immunoprecipitates formed by the "nodule-specific" antiserum with the translation products of nodule polysomes (Fig. 18D) demonstrated that a majority of the peptides unique to nodules can be selected with this antiserum. In addition to the clearly visible nodulins, designated by numbers, there are several other peptides in these (effective) nodules, which become more prominent in the ineffective (unable to fix nitrogen) nodules (see below). The "nodule-specific" antibodies reacted with only one peptide from the control root (Fig. 18C). This peptide does not appear to be synthesized in nodules at this stage of development (see Figures 18A and 18B, arrow), and represents one of the polypeptides whose synthesis is greatly reduced following infection of the root with bacteria. However, it must have been present in the nodule tissue to allow the production of its antibody, which was not

FIGURE LEGEND ON OTHER SIDE

Fig. 18. Two-dimensional PAGE (fluorogram) of ^{35}S-methionine-labeled *in vitro* translation products of total polysomes from uninfected roots (A) and nodules (B) of soybean. After pre-adsorption with a non-immune serum (see Methods), equal amounts (2.5×10^6 cpm) of the translation products were reacted with the "nodule-specific" antiserum and the resulting immunoprecipitates were washed thoroughly with a buffer containing non-ionic detergent (Lingappa et al., 1978; see also Methods). Following dissociation from staphylococci as outlined in Methods, the antigens were analyzed on two-dimensional gels. (C) Immunoprecipitation of proteins from uninfected roots, (D) immunoprecipitation of proteins from root nodules. Isoelectric focusing carried out in a narrower pH range results in a better resolution of polypeptides numbers 18, 19 and 20 (E, two fold magnification). Black arrowheads indicate several root polypeptides which disappear in nodules; white arrowheads show the presence of other "nodule-specific" proteins which are not immunoprecipitated by this antiserum. Among the nodulins (D) there are two peptides (shown in paranthesis) which appear to be in common with the uninfected root (A), but are not immunoprecipitated from the latter (C). Arrow indicates a polypeptide common to uninfected roots and nodules which, however, is not synthesized at this stage of root nodule development (see text).


pH 5.2
5.6
6.1
6.6
7.2
MW
A
B
68kD
43kD
29kD
18kD
E

completely removed during preparation of the "nodule-specific" antiserum.

Isolation of polysomes from uninfected roots homogenized in the absence and presence of a large amount of bacteroids (prepared from equal quantities of nodule tissue), followed by analysis of the *in vitro* translation product by two-dimensional gel electrophoresis, resulted in an identical profile (data not shown). This suggests that the presence of bacteroids does not have any effect on the isolation of plant polysomes or their translatability *in vitro*.

To rule out the possibility that some of the apparent nodulins are related to the developmental changes in the root and not symbiosis with *Rhizobium*, *in vitro* translation products of the nodule polysomes were immunoprecipitated with the "nodule-specific" antibodies in the presence of a vast excess (5 mg) of cytoplasmic proteins from uninfected mature roots. Two-dimensional analysis of this immunoprecipitate resulted in a profile (data not shown) identical to that in Figure 18D, suggesting that the appearance of nodulins is restricted to the development of the root nodule symbiosis.

The above observations indicate the presence of at least 18-20 "nodule-specific" host polypeptides other than leghemoglobin (nodulins), the molecular weights of which range from 12,000 - 20,000 (Fig. 18D). These proteins should be encoded by mRNAs of 8-10S size. Previously observed heterogeneity in the 9S mRNAs of nodules (Baulcombe and Verma, 1978), resulting in a biphasic kinetics of hybridization of its cDNA, could in part be due to the presence of mRNAs coding for nodulins. Since antibodies raised against purified leghemoglobin (Verma and Bal, 1976; Verma et al, 1979) do not precipitate nodulins (not shown),

and since all leghemoglobins are free of methionine (Ellfolk and Sievers, 1971; Nicola, 1975; Hurrell and Leach, 1977), these two groups of "nodule-specific" proteins may be unrelated.

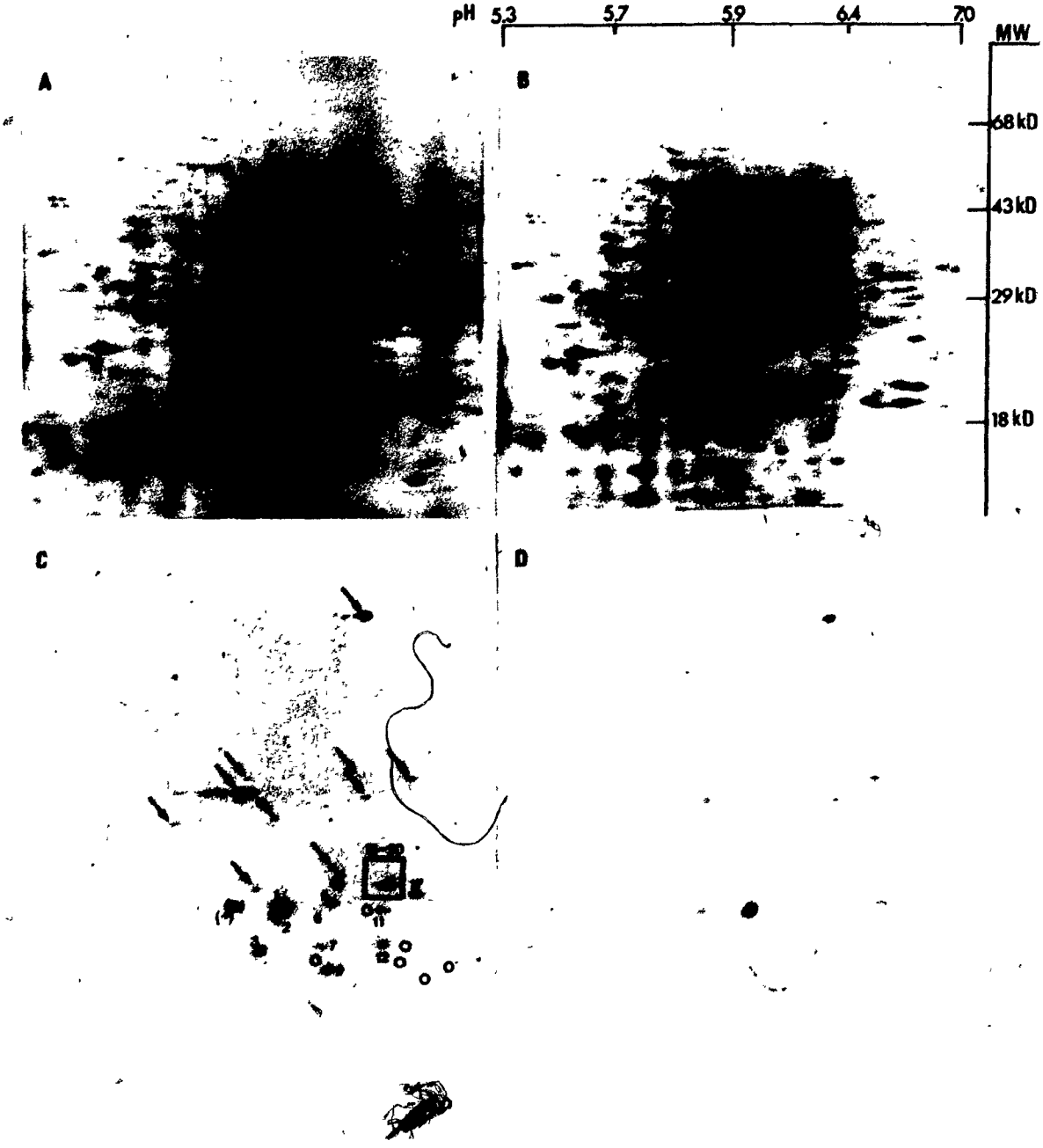
(vi) Synthesis of Nodulins in Ineffective Nodules

The existence of "nodule-specific" proteins in effective root nodules suggests either that they are involved in the development of nodules and, like nodulin-35 (Legocki and Verma, 1979), would be present in both effective and ineffective nodules, or that they are involved in processes related to nitrogen fixation. In vitro translation of host polysomes from effective and ineffective root nodules in the presence of ^{35}S-methionine indicated that the overall rate of protein synthesis is similar in the two types of nodules (Verma et al., 1981). A reduced level of synthesis of nodulins was observed, however, particularly in the 61A24-induced tissue (Table 3). This is accompanied by the disappearance of some nodulins (numbers 8, 10, 13-16; Figures 19C and 19D; compare Fig. 18D) in both SM5 and 61A24-induced nodules. On the other hand, several other "nodule-specific" polypeptides of very low abundance in the effective nodules become well detectable in the ineffective nodules (Fig. 19, arrows)[1].

The observed differences among nodulins in effective and ineffective nodules suggest that mutations in Rhizobium influence the expression of specific host genes, either directly or through a change in the physiology of the host tissue. Rhizobium does not appear to fully differentiate into bacteroids in 61A24-induced nodules (Werner et al., 1980), and the presence of these undifferentiated bacteria may influence the expression of host genes during the root nodule development (Verma et al., 1981) in a manner different from that of the effective (61A76) strain. It thus appears that some of the "nodule-specific" proteins may

FIGURE LEGEND ON OTHER SIDE

Fig. 19. Two-dimensional PAGE (fluorogram) of ^{35}S-methionine-labeled *in vitro* translation products from ineffective nodules formed by *Rhizobium japonicum* strains SM5 (A) and 61A24 (B), and immunoprecipitates formed by the "nodule-specific" antiserum (C) and (D) from (A) and (B), respectively. Numbers designate nodulins; circles indicate positions of nodulins number 8, 10 and 13-16, which are present in wild-type nodules (see Fig. 18D) and disappear from the ineffective nodules. Although immunoprecipitable with the "nodule-specific" antiserum, the two peptides shown in paranthesis are not considered as nodulins (see legend to Fig. 18).


pH 5.3
5.7
5.9
6.4
7.0
MW
A
B
68kD
43kD
29kD
18kD
C
D

be involved in the development of the nodules, while others are involved in their effectiveness.

IV. PROTEIN SYNTHESIS AND ACCUMULATION IN THE HOST CYTOPLASM DURING DEVELOPMENT OF THE NODULES

(i) Morphology of Effective and Ineffective Nodules

There are a number of mutant strains of Rhizobium japonicum in which the ability to form root nodules is maintained, however no nitrogen fixation takes place (Maier and Brill, 1978). Nodules formed by mutant strain SM5 and ineffective wild-type strain 61A24 were of particular interest to this study, as both are ineffective (do not fix nitrogen) and their morphology and structure significantly differ. Root nodules induced by R. japonicum SM5 are relatively large and brown inside, whereas those induced by R. japonicum 61A24 are small and green inside (Maier and Brill, 1976). To directly compare the cellular structure of the SM5 and 61A24-induced nodules with that of the effective (strain 61A76-induced) nodules, 15 µm sections were prepared from the three tissues and examined by light microscopy. Figure 20

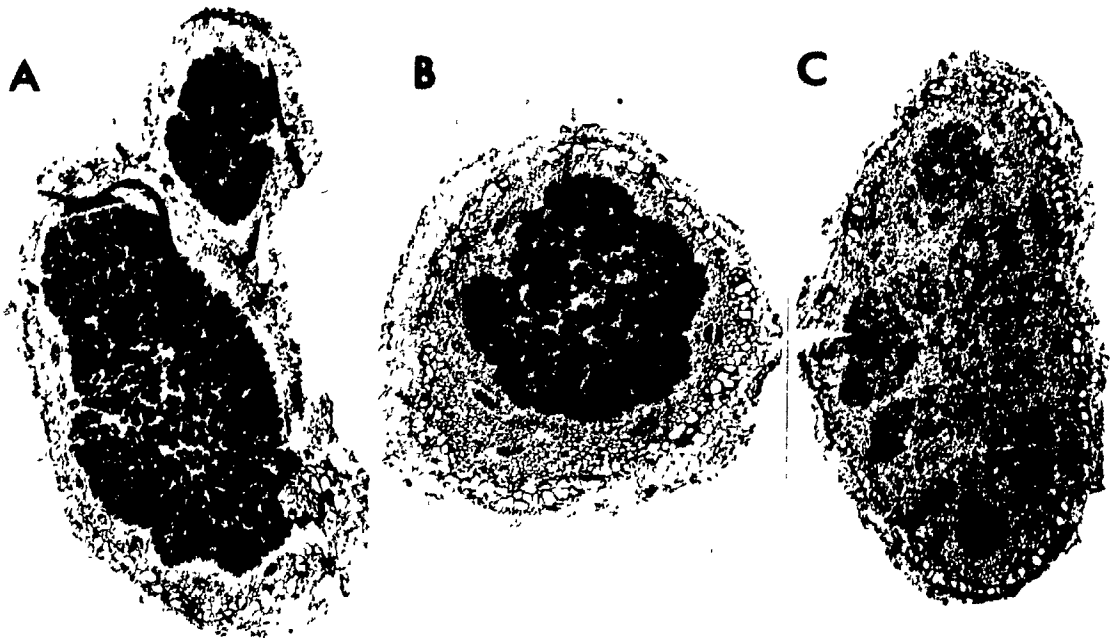
---

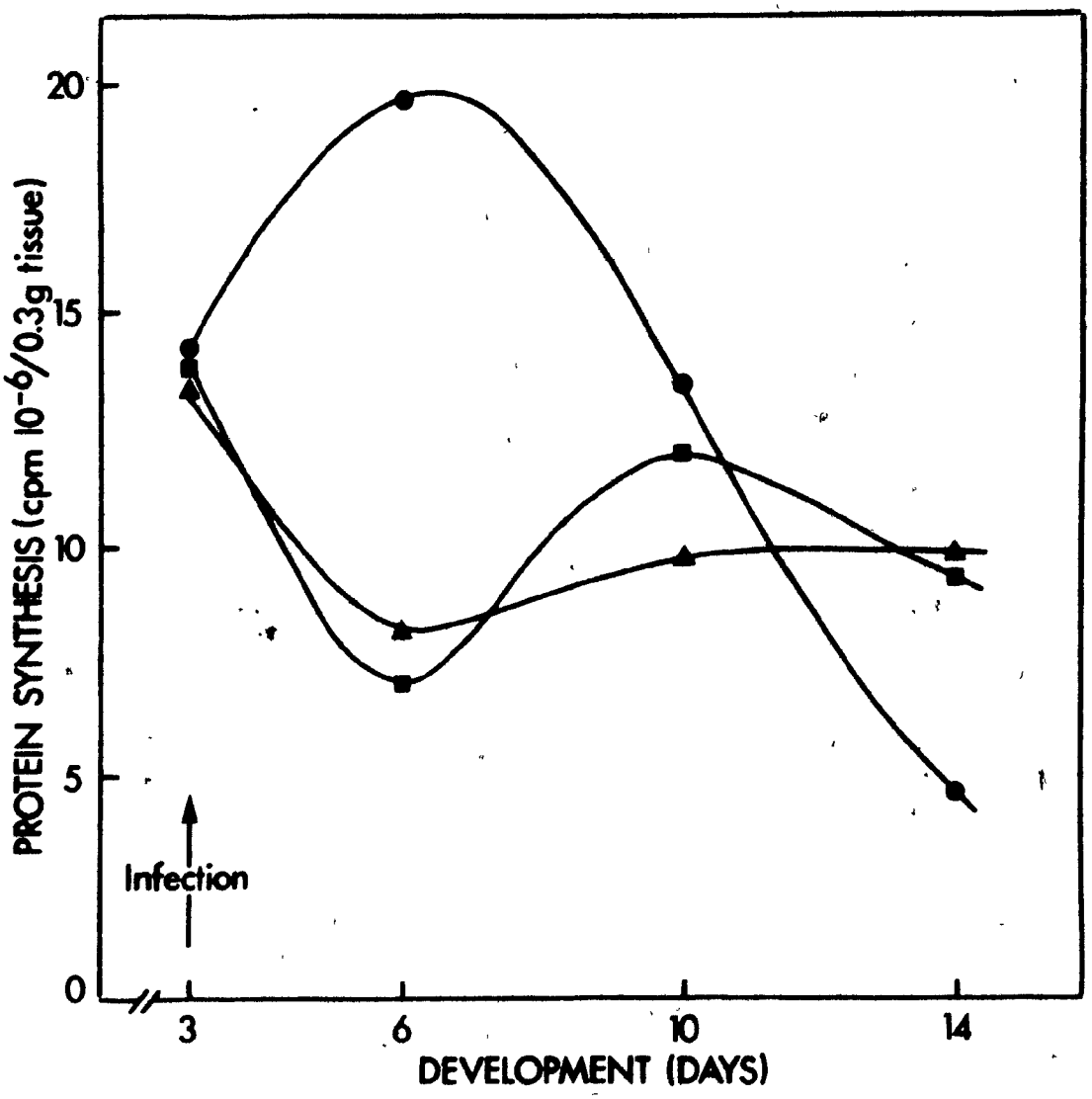
[1] Since the "nodule-specific" antiserum was raised against proteins that accumulated in the cytoplasm of mature nodules during the entire period of their development, whereas the analysis of nodulins was carried out at a particular stage of nodules (3 weeks), identification of the "nodule-specific" proteins may be restricted to the time of their biosynthesis. This may affect the apparent abundance of nodulins in the immunoprecipitates.

FIGURE LEGENDS ON OTHER SIDE

Fig. 20. Cross-sections of soybean root nodules formed by an effective (61A76; panel A) and ineffective (SM5 and 61A24; panels B and C, respectively) strains of Rhizobium japonicum. Fifteen μm sections of nodules were stained in Delafeld's hematoxylin (Methods) and examined by light microscopy at 100x magnification.

Fig. 21. Kinetics of protein biosynthesis in uninfected roots (circles) and root nodules of soybean formed by strains 61A76 (effective; squares) and 61A24 (ineffective; triangles) of Rhizobium japonicum. Tissue (0.3 g each) was labeled in vivo with 250 μCi of ^{35}S-methionine as outlined in Methods and, following isolation of soluble cytoplasmic proteins, total TCA-precipitable counts were determined.


A
B
C


PROTEIN SYNTHESIS (cpm 10⁻⁶/0.3g tissue)
20
15
10
5
0
Infection
3
6
10
14
DEVELOPMENT (DAYS)

shows that while the SM5 and 61A76-induced nodules appear to have similar structures and percentages of infected cells (45.4% and 45.9%, respectively), the 61A24-induced tissue is different in its organization and number of infected cells (approximately 16.2%). To establish an accurate number of host cells infected with Rhizobium, each nodule was sectioned at its largest diameter and 4-6 measurements were carried out, each based on analysis of 80-140 cells across the section. Figure 20 shows that cells infected with bacteroids are at least 2 times larger than those uninfected, and that their nuclei are enlarged and well visible. The intracellular structure of R. japonicum 61A24-induced nodules appears to be somewhat disorganized in an uneven distribution of the infected cells as well as in their size and shape. Studies of protein synthesis in R. japonicum 61A24 indicated that it is also very different from that in strains SM5 and 61A76 (see below).

(ii) Protein Synthesis in Root Before and After Infection

To measure the effect of rhizobial infection on the rate of protein synthesis in the host cell, uninfected roots and root nodules were in vivo labeled with ^{35}S-methionine at various stages of development. To obtain a high specific activity of labeled products, 0.3 g of tissue was incubated with 250 μCi of ^{35}S-methionine for 2.5 hr. Isolation of the soluble cytoplasmic proteins was carried out as described in Methods, except that the bacteroid fraction was also isolated for analyses at each developmental stage. The amount of protein synthesized was measured by TCA precipitation of small aliquots from each sample.

Figure 21 shows that a high rate of protein synthesis occurs in young uninfected roots, however it decreases rapidly in development. The level of protein synthesis in root nodules appears to be much lower

than that in uninfected roots particularly in a very young (6 day-old) nodule tissue. It is also apparent that synthesis of the cytoplasmic proteins in very young nodules, whether effective (R. japonicum 61A76-induced) or ineffective (61A24-induced), occurs at about the same level in the later stages of development (Fig. 21). Since the use of methionine as a radioactive precursor eliminated detection of leghemoglobin in this study (Ellfolk and Sievers, 1971; Nicola, 1975; Hurrell and Leach, 1977), it can be concluded that the marked (up to 35%) differences between the level of protein synthesis in the development of nodules are primarily due to leghemoblogin (see also Verma et al., 1981). This was further substantiated by electrophoretic analyses of the cytoplasmic proteins from several developmental stages (7,9,12 and 14 day-old nodules) which did not indicate any abundant polypeptides developmentally regulated other than leghemoglobins (see below). It is possible that the expression of "nodule-specific" proteins is accompanied by rapid developmental changes in the root cytoplasm prior to the appearance of nodule structure. It is interesting to note that the level of protein synthesis in the root cytoplasm is significantly reduced soon after initiation of infection (Fig. 21). Whether this is due to the presence of bacteroids and the alterations of the intracellular structure of the host cell, or whether there is a specific suppression mechanism affecting the synthesis of plant proteins following rhizobial infection, remains unknown.

(iii) Correlation Between Biosynthesis of Leghemoglobin, Nodulin-35 and Other Nodulins

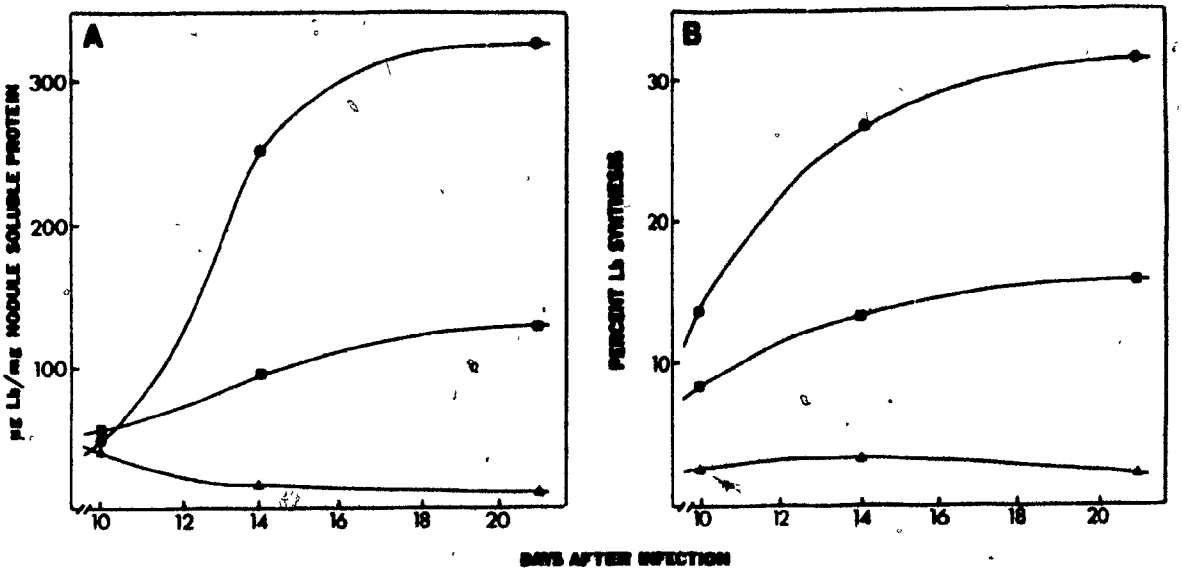
In effective soybean nodules, leghemoglobin represents up to 35% of the total soluble protein (Verma and Bal, 1976). In order to measure the amount of leghemoglobin in both effective (strain 61A76-induced or

wild-type) and ineffective (SM5 and 61A24-induced) nodules, total cytoplasmic protein from nodules (see Methods) was reacted with an anti-leghemoglobin serum (Verma and Bal, 1976; Verma et al., 1979) by means of rocket immunoelectrophoresis (Weeke, 1973). Quantitation was based upon the reactivity of anti-leghemoglobin serum with a known amount of purified leghemoglobin in the same gel, and direct measurements of the area enclosed by precipition arcs. Figure 22A shows that there is a significant amount of leghemoglobin in SM5-induced nodules, representing approximately 40% of that in wild-type, whereas the 61A24-induced tissue contains a very low level of leghemoglobin (about 4% of that from wild-type; data from R. Haugland in Verma et al., 1981).

To determine if the rate of leghemoglobin accumulation parallels its biosynthesis, total (free and membrane-bound) polysomes were isolated from the effective and ineffective nodules at various stages of development and translated in vitro in the wheat germ cell-free system (see Methods). Each of the ^{3}H-leucine-labeled translation products was reacted with the anti-leghemoglobin serum and radioactivity of the resulting immunoprecipitates was determined following TCA precipitation. Figure 22B illustrates the synthesis of apoleghemoglobin in 61A76, SM5 and 61A24-induced nodules. Comparison of the accumulation (Fig. 22A) and synthesis (Fig. 22B) of leghemoglobin throughout the development indicates that there is a good correlation between the two events in both effective and ineffective nodules. The comparable levels of leghemoglobin content (34% of total cytoplasmic protein) and its synthesis (32% of total translation product) in mature nodules suggest that the biosynthesis and accumulation of this protein occur in a highly coordinate fashion. While the mutations in R. japonicum have an obvious

FIGURE LEGEND ON OTHER SIDE

Fig. 22. Accumulation (A) and synthesis (B) of leghemoglobin during the development of root nodules formed by Rhizobium strains 61A76 (effective; circles), SM5 (ineffective; squares), and 61A24 (ineffective; triangles). Accumulated leghemoglobin was measured by quantitative rocket immunoelectrophoresis, whereas its synthesis was estimated from immunoprecipitations of in vitro translation products with the leghemoglobin antibodies (see text). Lb, leghemoglobin.


A
B
300
200
100
30
20
10
10
12
14
16
18
20
DAYS AFTER INFECTION

effect on the level of expression of leghemoglobin genes (Fig. 22; see also Verma et al., 1981), they do not appear to alter the observed correlation between the rate of synthesis and accumulation of leghemoglobin in the host cytoplasm.

In contrast to leghemoglobin, the synthesis and accumulation of nodulin-35 do not seem to occur coordinately during the development of root nodules. Moreover, the presence of nodulin-35 in the host cytoplasm is not affected by mutations in R. japonicum strains SM3, SM4, SM5, and 61A24 (see Fig. 6). Whereas the level of nodulin-35 remains unchanged in very young and mature nodules, its synthesis in vivo could only be detected in a very young (7 day-old) nodule tissue. On the other hand, immunoprecipitation of ^{35}S-methionine-labeled in vitro translation product of total poly A(+) RNA from 80S-type polysomes (8 day nodules) with purified IgG to nodulin-35 indicated that the synthesis of this protein represents only about 0.6 - 1.4% of the total translation product. Assuming that there is a direct relationship between the amount of mRNAs and their specific products in the cell-free translation system, and that no mRNA is translated preferentially, the mRNA encoding nodulin-35 may also represent about 0.6 - 1.4% of the total poly A(+) RNA in the host cytoplasm at this stage of development. (see Appendix II for isolation of nodulin-35 mRNA). Since nodulin-35 represents approximately 4% of the total cytoplasmic protein in both young and mature nodules (see Identification of Nodulin-35), it appears that its synthesis takes place in very early stages of the development.

Developmental studies of effective (Rhizobium 61A76-induced) and ineffective (Rhizobium 61A24-induced) nodules indicated that most of the low molecular weight nodulins are already present in very young (7 day-old) tissue and, similarly to nodulin-35, their synthesis in vivo

does not increase significantly later in development. In contrast to nodulin-35, however, these proteins do not accumulate in the tissue at a high rate, and their visualization by staining is difficult. Thus, electrophoretic analyses of ^{35}S-methionine-labeled soluble cytoplasmic proteins from nodules at 7, 9, 12 and 14 days following infection, failed to show any appreciable developmental changes in the synthesis and accumulation of proteins, with the exception of leghemoglobin (data not shown). Comparison of cytoplasmic proteins synthesized (fluorography) and accumulated (stain) during the development of the 61A76, SM5, and 61A24-induced nodules indicated almost identical profiles on two-dimensional gels, suggesting a very similar protein composition of the host cell cytoplasm, regardless of the Rhizobium strain used. This observation was further substantiated by almost identical profiles of in vitro translation products of polysomes from both effective (61A76-induced) and ineffective (SM5 and 61A24-induced) nodules (Fig. 18B and Figures 19A and 19B). Due to the low abundance of "nodule-specific" proteins other than leghemoglobin, their different appearance in ineffective nodules could only be detected by means of the "nodule-specific" antiserum and electrophoretic analysis of immunoprecipitated products (Figures 18 and 19).

V. PROTEIN SYNTHESIS AND ACCUMULATION IN RHIZOBIUM JAPONICUM BEFORE AND DURING SYMBIOSIS

(i) Antibodies Against Proteins from Nitrogenase-Induced Rhizobia

To obtain an antibody probe against rhizobial proteins which are free of any host contaminations, but at the same time which strongly resemble bacteroids, free-living rhizobia were induced for nitrogenase in vitro (Avissar and Nadler, 1978) and their total protein extracts

were used as antigens. Rhizobium japonicum strains 61A76 (effective) and 61A24 (ineffective) were grown in liquid media to an early log phase and the growth was continued under anaerobic conditions (Methods). Following detection of nitrogenase activity in the cultures by the acetylene reduction assay, the cells were lysed in the presence of SDS and used for raising R-type antisera (Methods). The denaturing conditions used (SDS, boiling) are believed to enhance the availability of all polypeptides to the immunogenic system of the animal (Schild and Pereira, 1965; Crowle, 1973), thus resulting in a more complete population of antibodies in polyspecific antisera.

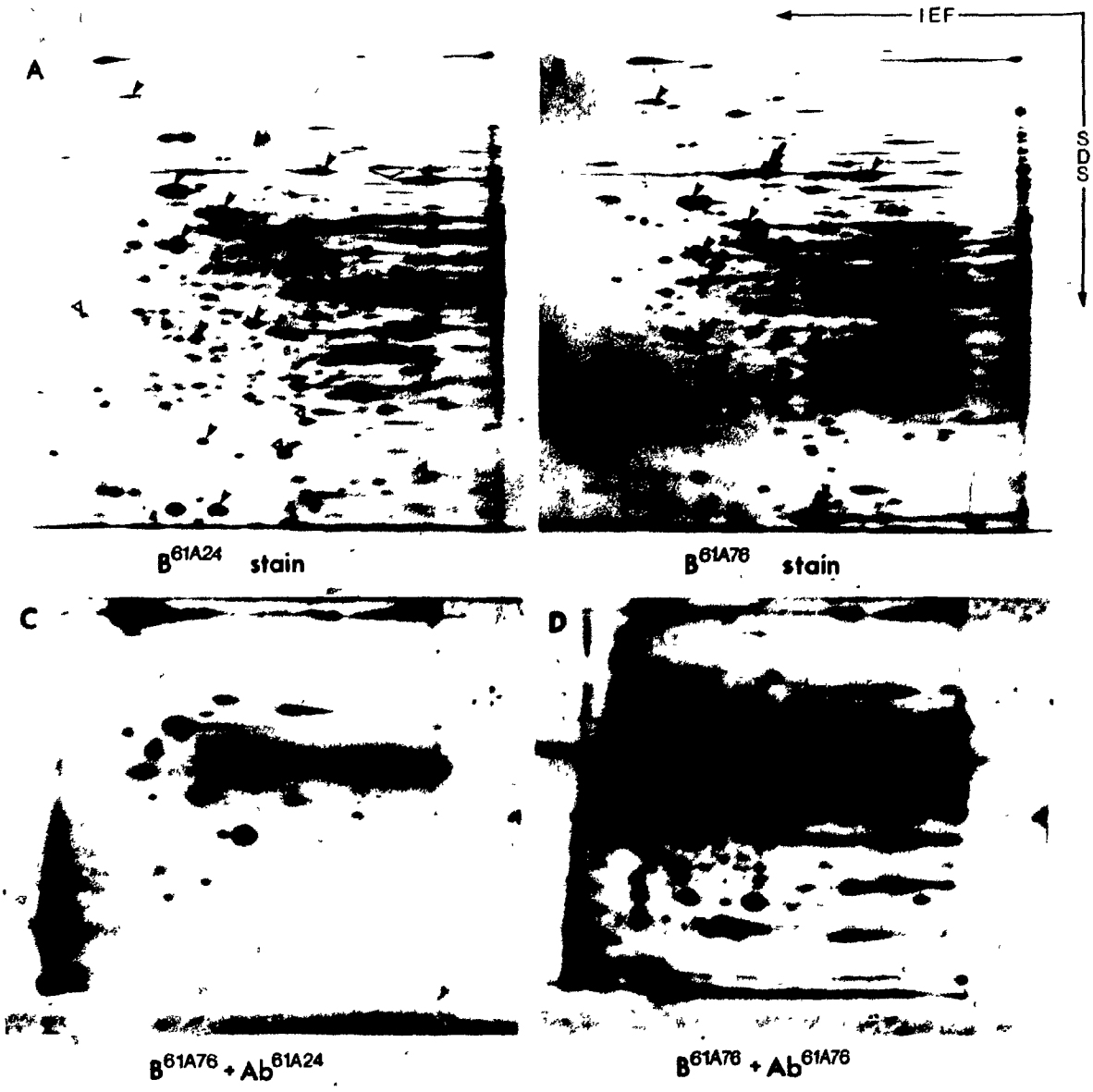
Although the use of bacteroid extracts as a source of antigen may be considered advantageous to the nitrogenase-induced cultures of Rhizobium, the isolation of bacteroids involves disruption of the host cell membranes and the bacteroidal pellet could contain at least trace amounts of the plant material, e.g. mitochondrial contaminations. Moreover, since the number, appearance and ultrastructure of bacteroids and the infected cells are very different in nodules formed by the 61A76 and 61A24 strains of R. japonicum (Werner et al. 1980; see also Morphology of Effective and Ineffective Nodules), the bacteroids from the two tissues may contain different contaminations which, consequently, could lead to an inaccurate comparison of proteins from the two bacteroid strains.

(ii) Comparison Between Free-living R. japonicum Strains 61A76 and 61A24

To directly compare proteins from the effective (strain 61A76) and ineffective (strain 61A24) rhizobia in their free-living state, total protein extracts were prepared as described in Methods and analyzed by two-dimensional SDS-PAGE. Figures 23A and 23B show that there is some

FIGURE LEGEND ON OTHER SIDE

Fig. 23. Two-dimensional PAGE / multiple immunoreplica technique of total cytoplasmic proteins from free-living Rhizobium japonicum strains 61A76 (B^{61A76}) and 61A24 (B^{61A24}). A nitrocellulose replica of B^{61A76} was reacted with antibodies to total protein extracts from Rhizobium 61A24 (Ab^{61A24}) and, following the removal of IgG and ^{125}I-protein A at pH 2.2, it was reacted with its homologous antibodies (Ab^{61A76}). Black arrowheads indicate some of the peptides common to the two strains of Rhizobium; open arrows in panel (B) indicate some of the polypeptides unique to strain 61A76; open arrowheads in panel (A) indicate the presence of polypeptides unique to strain 61A24. See Methods and Appendix for a detailed description of the multiple immunoreplica technique.


IEF
SDS
A
B
C
D
B61A24 stain
B61A76 stain
B61A76 + Ab61A24
B61A76 + Ab61A76

homology between the protein populations in the two strains (arrowheads) but the ineffective strain seems to lack a number of polypeptides (arrows). Since there are also several proteins unique to the ineffective strain of R. japonicum (Fig. 23A, open triangles), the two strains of rhizobia appear to significantly differ in their total protein populations. This observation was further substantiated by a poor cross-reactivity of protein extracts from the 61A76 strain with antiserum to the Rhizobium strain 61A24 (Fig. 23C; see Appendix I for detailed procedure). Since the homologous reactions between proteins and their respective antisera in Rhizobium 61A76 (Fig. 23D) and Rhizobium 61A24 (not shown) resulted in detection of about 4-5 times more polypeptides than those which are believed to be common (or immunologically cross-reactive) to the two strains of rhizobia, it is apparent that free-living Rhizobium 61A76 and 61A24 are very different in their total protein populations. Despite the similar results obtained using heterologous antisera to Rhizobium 61A76 and 61A24 (i.e. antisera against 61A24 and 61A76, respectively), it should be noted that not all polypeptides contain their antibodies and thus this study cannot be quantitative. Moreover, since the antigenicity rather than concentration of a particular protein is critical to its antibody titre, reactivity of a polyspecific antiserum with a complex population of proteins usually does not follow their abundance. Whether or not the substantial differences in total protein composition between the effective (61A76) and ineffective (61A24) strains of R. japonicum account for the incompatibility of the latter, leading to the formation of ineffective nodules on soybean roots, remains subject to further investigations. The mutant strain SM5 of R. japonicum, which is

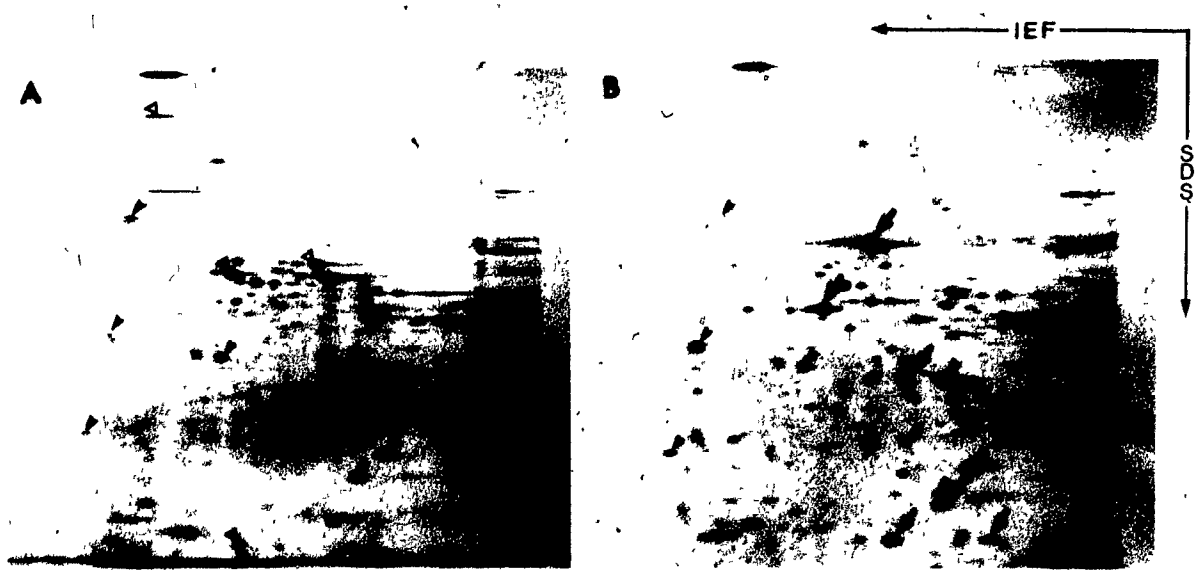
believed to contain a lesion in the gene specifying nitrogenase component II (Maier and Brill, 1976), shares almost an identical population of proteins with the effective strain 61A76, as examined by two-dimensional PAGE of ^{35}S-methionine-labeled products (data not shown). These observations, along with the studies of protein synthesis and accumulation, indicate a large homology between R. japonicum 61A76 and SM5, and some basic differences in the strain 61A24. This conclusion appears to be supported by a recent study of Haugland and Verma (1981), who showed only limited (about 25%) DNA sequence homology between the 61A76 and 61A24 strains of R. japonicum. Since the processes of infection and nodulation are not affected by any of these rhizobia, but the morphology and number of bacteroids are very different, particularly in Rhizobium 61A24 (see Morphology of Effective and Ineffective Nodules), it is possible that the observed differences in protein composition of the 61A24 strain are responsible for its incompatability in forming effective nodules. This strain does have complete nitrogenase genes and it resembles the effective strain 110 (see Haugland and Verma, 1981).

(iii) Comparison Between Bacteroids of R. japonicum Strains 61A76 and 61A24

A comparison of the total intracellular protein from 2 week-old bacteroids of Rhizobium 61A76 and 61A24 was carried out by two-dimensional SDS-PAGE, using homologous and heterologous antisera against protein extracts from the two bacterial strains. Figures 24A and 24B show that in addition to some polypeptides common to Rhizobium 61A76 and 61A24 bacteroids (arrowheads), there are a large number of proteins unique to the strain 61A76 (arrows) and strain 61A24 (open triangles). It is thus believed that the intracellular protein composition of the

FIGURE LEGEND ON OTHER SIDE

Fig. 24. Two-dimensional PAGE / multiple immunoreplica technique of total cytoplasmic proteins from bacteroids of Rhizobium japonicum strains 61A76 (Bd^{61A76}) and 61A24 (Bd^{61A24}). A nitrocellulose replica of Bd^{61A76} was reacted with antibodies to total protein extracts from Rhizobium 61A24 (Ab^{61A24}) and, following the removal of IgG and ^{125}I-protein A at pH 2.2, it was reacted with its homologous antibodies (Ab^{61A76}). Black arrowheads indicate some of the polypeptides common to the two strains of Rhizobium; arrows in panel (B) indicate some of the polypeptides unique to strain 61A76; open arrowheads in panel (A) indicate the presence of polypeptides unique to strain 61A24.



Bd^{61A24} stain

Bd^{61A76} stain

$Bd^{61A76} + Ab^{61A24}$

$Bd^{61A76} + Ab^{61A76}$

two strains of rhizobia is very different in both the free-living state (Fig. 23) and bacteroids. Moreover, there appears to be a dramatic change in protein composition upon the transformation from the free-living to bacteroid state in both effective (61A76) and ineffective (61A24) strains of R. japonicum. This conclusion is justified by the fact that, although all protein extracts were prepared according to the same procedure (Methods) and subsequently examined on gels which were run in parallel, the postulated differences were observed by both protein staining and reactivity with antibodies. Similarly to the results obtained using free-living rhizobia, the cross-reactivity of antisera with the heterologous strain (e.g. proteins from strain 61A76 reacted with antiserum to 61A24, Fig. 24C) was about 4 times lower than the homologous reaction (proteins from strain 61A76 reacted with its own antibody, Fig. 24D).

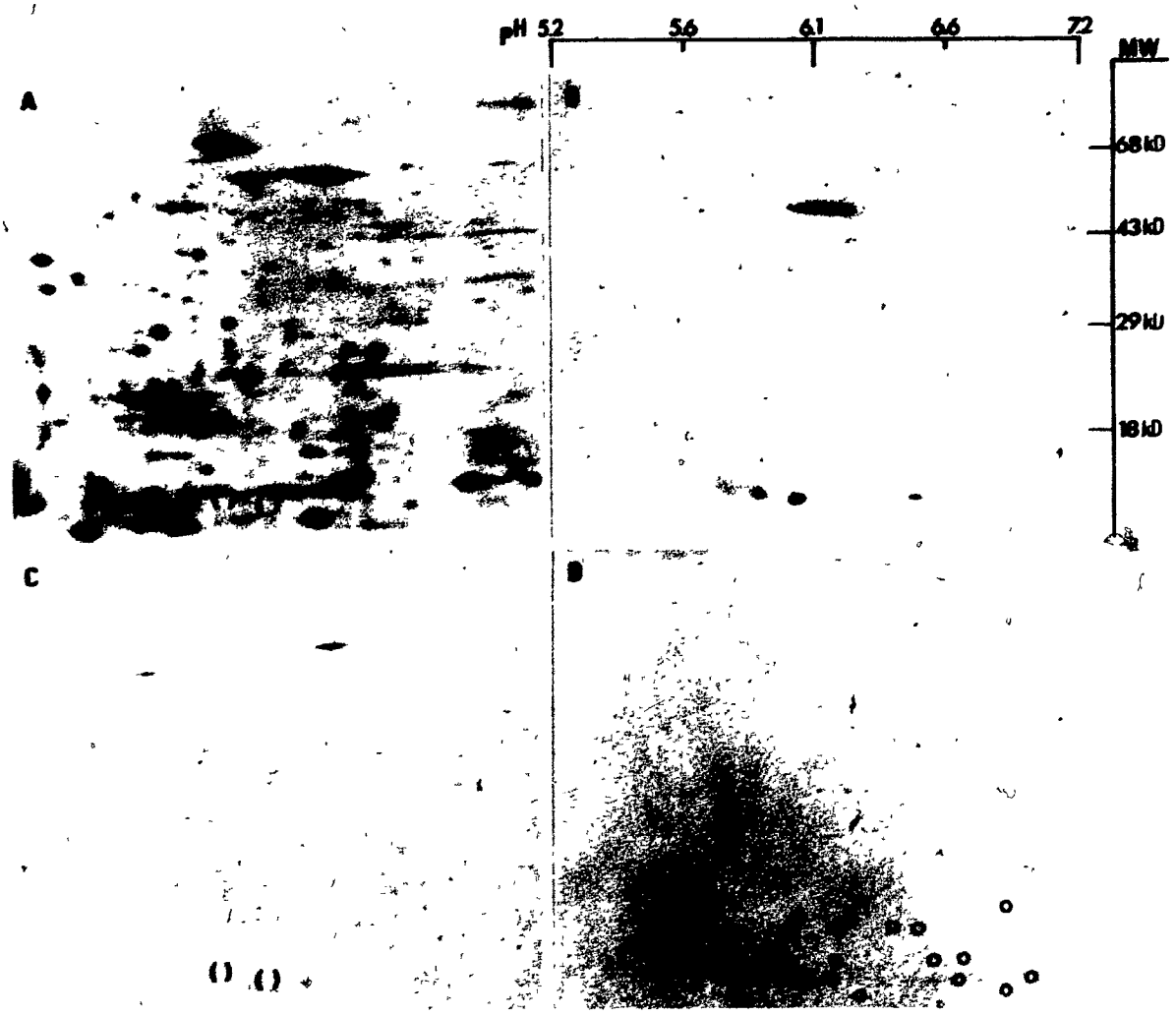
(iv) Presence of Bacteroid Secretory Proteins in Nodules

In spite of biosynthetic evidence for the host and not bacterial origin of nodulin-35 and the other (20) nodulins, it was desirable to directly examine the host cell cytoplasm for the presence of any bacteroidal secretory proteins. Two week-old bacteroids were isolated from the effective (R. japonicum strain 61A76-induced) nodules as described in Methods, and labeled in vivo with ^{35}S-methionine. Following removal of the cells by centrifugation, the incubation medium was passed through a 0.8 μm millipore filter and TCA-precipitable counts were determined. Table 3 shows that the "nodule-specific" antiserum immunoprecipitated approximately 1.2% of the secretory and 0.7% of the intracellular material associated with the bacteroid fraction. Although the observed cross-reactivity seemed negligible, the resulting immunoprecipitates were examined electrophoretically. First,

it was essential to determine whether the extracellular material found in the incubation medium was due to a genuine secretion and not merely a result of cell lysis. Analysis of the bacteroid cellular proteins as well as the extracellular material (Figures 25A and 25B) showed that the two populations of proteins are different, indicating that no appreciable cell lysis occurred during incubation. If the observed secretion takes place in the intact nodules, these proteins may be transferred into the host cell cytoplasm. Alternatively, they may remain within the membrane envelopes enclosing bacteroids and may be released during the preparation of soluble proteins (Verma et al., 1978). In either case, the "nodule-specific" antiserum should contain antibodies against these proteins. Examination of the immunoprecipitates formed by this antiserum with the extracellular products of bacteroids showed two secretory polypeptides that appear to be secreted into the host cell cytoplasm (Fig. 25D). These peptides are not detected inside the bacteroids, which suggests that they do not accumulate in the cells (Fig. 25C). The molecular weight of the secretory proteins is approximately 11,000, and their size as well as isoelectric points are different from those of nodulins and leghemoglobins. A negligible cross-reactivity of the "nodule-specific" antiserum with cellular extracts of bacteroids (Fig. 25C; see also Table 3) suggested that the procedure used for isolation of the host cytoplasmic proteins from nodules did not result in a significant breakage of bacteroids. The possibility remains that isolated bacteroids do not synthesize proteins identical to those made inside the nodules, and thus they would not be detected in this study. Since the development of symbiosis between the rhizobia and host plant may be

FIGURE LEGEND ON OTHER SIDE

Fig. 25. Analysis of cellular (A) and secretory (B) ^{35}S-methionine-labeled *in vivo* products of wild-type bacteroids by two-dimensional PAGE (fluorogram). Approximately 2×10^6 cpm of the cellular and secretory products were pre-adsorbed with a non-immune serum and reacted with the "nodule-specific" antiserum, as described in Methods. The resulting immunoprecipitates are shown in (C) and (D), respectively. A positive cross-reactivity of the "nodule-specific" antiserum with at least two secretory peptides of bacteroids suggests their presence in the cytoplasm of infected cells (see text), whereas these proteins are not accumulated inside bacteroids (paranthesis in panels A and C). The "nodule-specific" proteins were run in a gel parallel to that containing the secretory polypeptides, and the resulting profiles are superimposed for direct comparison (positions of nodulins numbers 1 to 17 are indicated by circles in panel D; see Fig. 18D).


pH 5.2
5.6
6.1
6.6
7.2
MW
68kD
43kD
29kD
18kD
A
B
C

strongly affected by the genetic compatibility of the microsymbiont (Maier and Brill, 1976; see also Introduction and Literature Review), a similar study on bacteroidal secretions in ineffective strains of Rhizobium, including R. japonicum SM3, SM4, SM5 and 61A24, could elucidate the important question of communication between the micro and macrosymbionts of root nodules.

VI. ATTEMPTS TO ISOLATE "NODULE-SPECIFIC" PROTEINS OTHER THAN LEGHEMOGLOBIN

Immunological identification of nodulins presented in this study has indicated that, except for nodulin-35, all of these polypeptides are of small molecular weight. Procedures for isolation or enrichment of these proteins, therefore, were primarily based upon their low (less than 20,000) molecular weight. While the use of ^{35}S-methionine-labeled products easily eliminated leghemoglobin during identification of nodulins (see Identification of Nodulins), leghemoglobin represents about 30% by weight of cytoplasmic protein in nodule cytoplasm. Considering the low molecular weight of this protein, it was essential to remove leghemoglobin prior to screening for the "nodule-specific" polypeptides.

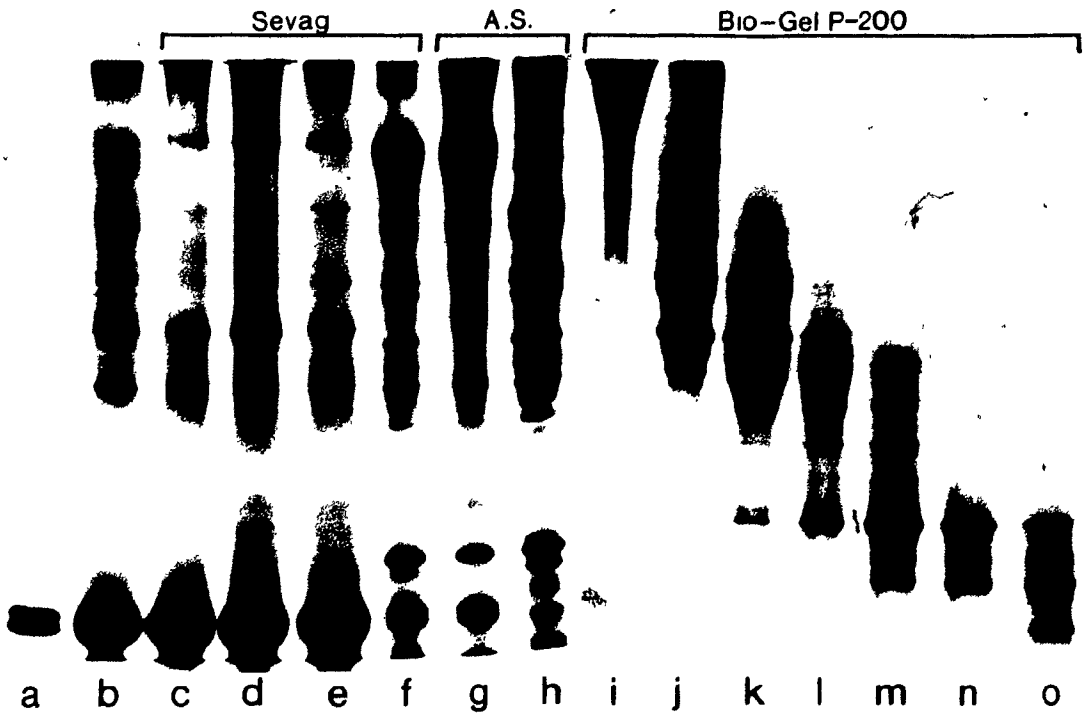
A number of procedures were used in an attempt to isolate or enrich the low molecular weight nodulins, including ammonium sulphate-fractionation, chromatography on DEAE-cellulose, and hydrophobic chromatography on Phenyl-Sepharose. These techniques, described in Appendix III, did not lead to a satisfactory enrichment of "nodule-specific" proteins and/or sufficient removal of leghemoglobin, nevertheless their use suggested that at least some "nodule-specific" proteins are of hydrophilic nature.

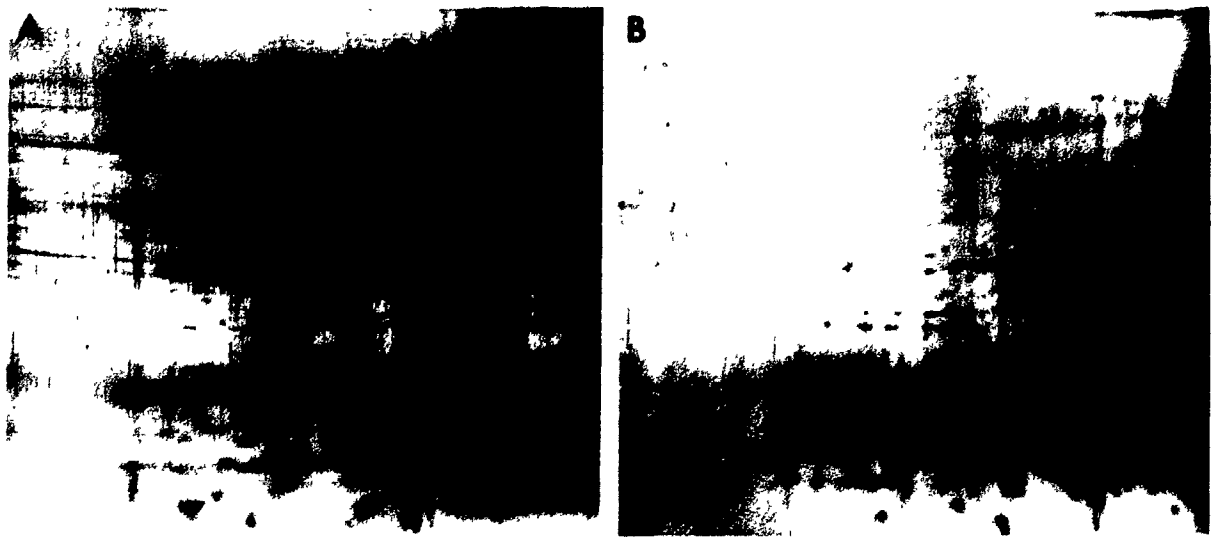
Application of Sevag's method, in conjunction with ammonium sulphate-fractionation and Bio-Gel P-200 filtration, has led to a high enrichment of at least three "nodule-specific" proteins of plant origin, of which two were shown to be actively synthesized in mature nodules. Sevag's method gradually removes proteins according to their hydrophobicity (Sevag, 1934; Staub, 1965). Repeated extractions of proteins with chloroform and n-butanol (0.2 and 0.04 volume of the protein solution, respectively) precipitate hydrophobic proteins, whereas the other cytoplasmic components, including hydrophilic polypeptides and polysaccharides, remain in the aqueous phase. Although the concentration of chloroform does not change during consecutive extractions (following each extraction the chloroform phase is removed by centrifugation), the amount of butanol increases in the aqueous phase, thus causing a gradual removal of proteins. To evaluate this procedure in more detail, the same conditions of extraction were maintained in a series of experiments, and protein samples were routinely saved at all stages of extraction for electrophoretic analyses. Figure 26 illustrates some purification steps of Sevag's method, leading to a considerable enrichment of hydrophilic proteins from nodules. Consecutive extraction of the nodule cytoplasmic proteins (lane b) with the chloroform-butanol mixture, resulted in the removal of hydrophobic proteins, including nodulin-35 and leghemoglobin (lanes c to e). After 4 extraction, the aqueous phase (lane f), representing hydrophilic proteins, appears to be free of nodulin-35 and contains much lower quantities of leghemoglobin. On the other hand, several polypeptides are strongly enriched in this fraction, particularly in the small molecular weight region. Subsequent fractionation of the hydrophilic proteins with ammonium sulphate at 0-60% and 60-100% AS

FIGURE LEGENDS ON OTHER SIDE

Fig. 26. Isolation of small molecular weight hydrophilic proteins from nodules by Sevag's method / AS-fractionation / Bio-Gel filtration: SDS-PAGE of selected fractions. (a) Purified leghemoglobin as a marker; (b) total cytoplasmic proteins prior to fractionation; (c to e) three consecutive pellets after Sevag's extraction with chloroform-butanol; (f) hydrophilic proteins after four Sevag's extractions; (g) 0-60% AS-precipitated fraction of (f); (h) 60-100% AS-precipitated fraction of (f); (i to o) Bio-Gel filtration of (h) in the presence of 8 M urea. The gel was stained with Coomasie blue (see also next figure).

Fig. 27. Two-dimensional PAGE of cytoplasmic proteins from nodules prior to (A) and after (B) the purification procedure presented in Fig. 26, showing the enrichment of hydrophilic proteins of low molecular weights. Proteins in (B) are identical to those shown in lanes (n) and (o) of Fig. 26, except that they were stained with silver.


Sevag
A.S.
Bio-Gel P-200
a b c d e f g h i j k l m n o


B

(lanes g and h, respectively), led to a further enrichment of small proteins, and to an almost complete removal of leghemoglobin from this fraction (see also Fig. 27B). The protein fraction enriched in low molecular weight proteins was subjected to a Bio-Gel P-200 filtration in the presence of 8 M urea (Fig. 26, lanes i to o; see also Methods). Analysis of the small molecular weight proteins resulting from the Bio-Gel P-200 filtration (lane o) by two-dimensional PAGE, shown in Fig. 27B, illustrates the high degree of enrichment of polypeptides in a 11,000 - 20,000 molecular weight region obtained by this procedure. It should be noted at the same time, that the conventional staining of proteins with Coomassie blue (Fig. 26, lanes n and o) is no longer satisfactory for an assessment of protein purity (Fig. 27B displays the same proteins stained with silver).

To further evaluate the purification procedure illustrated in Fig. 26, relative contents of the small (13,000 - 20,000 molecular weight) proteins, as well as those of nodulin-35 and leghemoglobin used as internal markers, were determined at each stage of purification. This was accomplished by a quantitative scanning of the original gels (ORTEC Scan, 0.1 OD set-up, 12.5 cm/min), and by calculating the percentage of a particular protein present in the total sample subjected to electrophoresis. Results obtained indicated that the total fraction of cytoplasmic proteins from root nodules (Fig. 26, lane b) consists of only about 6.3% of polypeptides ranging from 13,000 to 20,000 MW, 36.9% of leghemoglobin, and 4.7% of nodulin-35. After four Sevag's extractions, the level of small molecular weight proteins in the sample increased to 17.0%, whereas the contents of leghemoglobin and nodulin-35 were reduced to 11.2% and 1.2%, respectively (Fig. 26, lane f). Following fractionation with ammonium sulphate, the 60-100% AS

fraction was found to contain about 26.4% of the small molecular weight proteins, 3.9% leghemoglobin, and 1.0% nodulin-35 (Fig. 26, lane h). Finally, the sample representing the small molecular weight proteins obtained from a Bio-Gel P-200 filtration (Fig. 26, lanes n and o) was composed of approximately 85.1% of the 13,000 - 20,000 MW polypeptides, and it contained only 3.3% leghemoglobin and 0.5% nodulin-35. Estimation of protein at each purification stage indicated that, following Sevag's extractions, the 60-100% AS fraction of the hydrophilic proteins represents approximately 5.3% of the total cytoplasmic protein subjected to this purification procedure. Considering the fact that the 60-100% AS fraction contains about 26.4% of the small molecular proteins other than leghemoglobin, the overall yield of these polypeptides is 1.4% in the original sample used for this procedure. Since the level of the small molecular weight proteins in an untreated cytoplasm was estimated at 6.3%, the observed difference must account for a partial loss of these proteins throughout the procedure, particlarly in the pellet fractions of the Sevag's method (Fig. 26, lanes c,d, and e). Despite its relatively low efficiency, this purification procedure proved a successful method for isolation of small molecular weight proteins other than leghemoglobin, including at least three "nodule-specific" proteins (see below). First, this technique resulted in a nearly 14-fold enrichment of the 13,000 - 20,000 MW polypeptides in the sample. Secondly, it resulted in the removal of about 91% of leghemoglobin, thus facilitating studies of the "nodule-specific" protein other than the abundant leghemoglobins. Finally, since the whole procedure, except for the Bio-Gel filtration, can be completed within a two-hour time, it may represent a convenient method for isolation of small molecular weight proteins form the root nodule cytoplasm on a preparative scale.

To examine the low molecular weight proteins for the presence of nodulins, the purified sample, shown in lanes n and o of Fig. 26, was resolved electrophoretically by one-dimensional SDS-PAGE, and transferred to nitrocellulose for immunological analyses (Methods). The nitrocellulose paper was reacted with three different antibodies, according to the multiple immunoreplica technique (for a detailed description of the technique see Appendix I). Figure 28 shows reactivity of the purified proteins with antisera against soluble cytoplasmic proteins from root nodules (lane a), uninfected roots (lane b), and R. japonicum strain 61A76 (lane c). Whereas antbodies to uninfected roots signal the presence of several polypeptides higher than 20,000 MW in the sample (lane b), their reactivity with the low molecular weight proteins is very low. Although the conventional staining with Coomassie blue of the purified sample did not show the trace amounts of larger polypeptides (Fig. 26, lanes n and o), their presence was detected by staining with silver (Fig. 27B). On the other hand, antibodies to root nodule cytoplasm, which have a titer comparable to antibodies against uninfected root cytoplasm (Methods), strongly reacted with several proteins (Fig. 28, lane a).

To determine if these apparent "nodule-specific" proteins are indeed of host and not bacterial origin, the same nitrocellulose replica was reacted with antibodies to strain 61A76 of R. japonicum. As shown in Fig. 28, lane c, a single polypeptide reacted, however it does not correspond to any of the apparent nodulins. It thus appeared that at least some of the "nodule-specific" proteins, originally identified by immunoprecipitation of in vitro translation products, can be isolated from the cytoplasm of root nodules on a preparative scale.

FIGURE LEGEND ON OTHER SIDE

Fig. 28. Screening of the low molecular weight polypeptides, isolated as shown in Fig. 26, for the presence of "nodule-specific" polypeptides by means of multiple immunoreplica technique. Proteins identical to those shown in lanes (n) and (o) of Fig. 26 were transferred to nitrocellulose and reacted with antibodies against cytoplasmic proteins from nodules (a), uninfected roots (b), and R. japonicum strain 61A76 (c). Whereas the presence of polypeptides larger than 20,000 MW in this preparation was confirmed only by staining with silver (see Fig. 27 B), they were easily visualized by the use of antibodies (lane b).

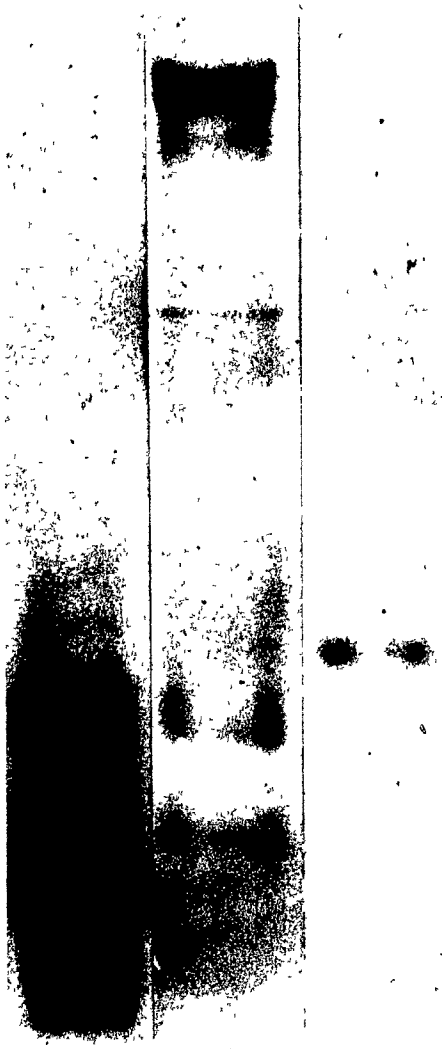
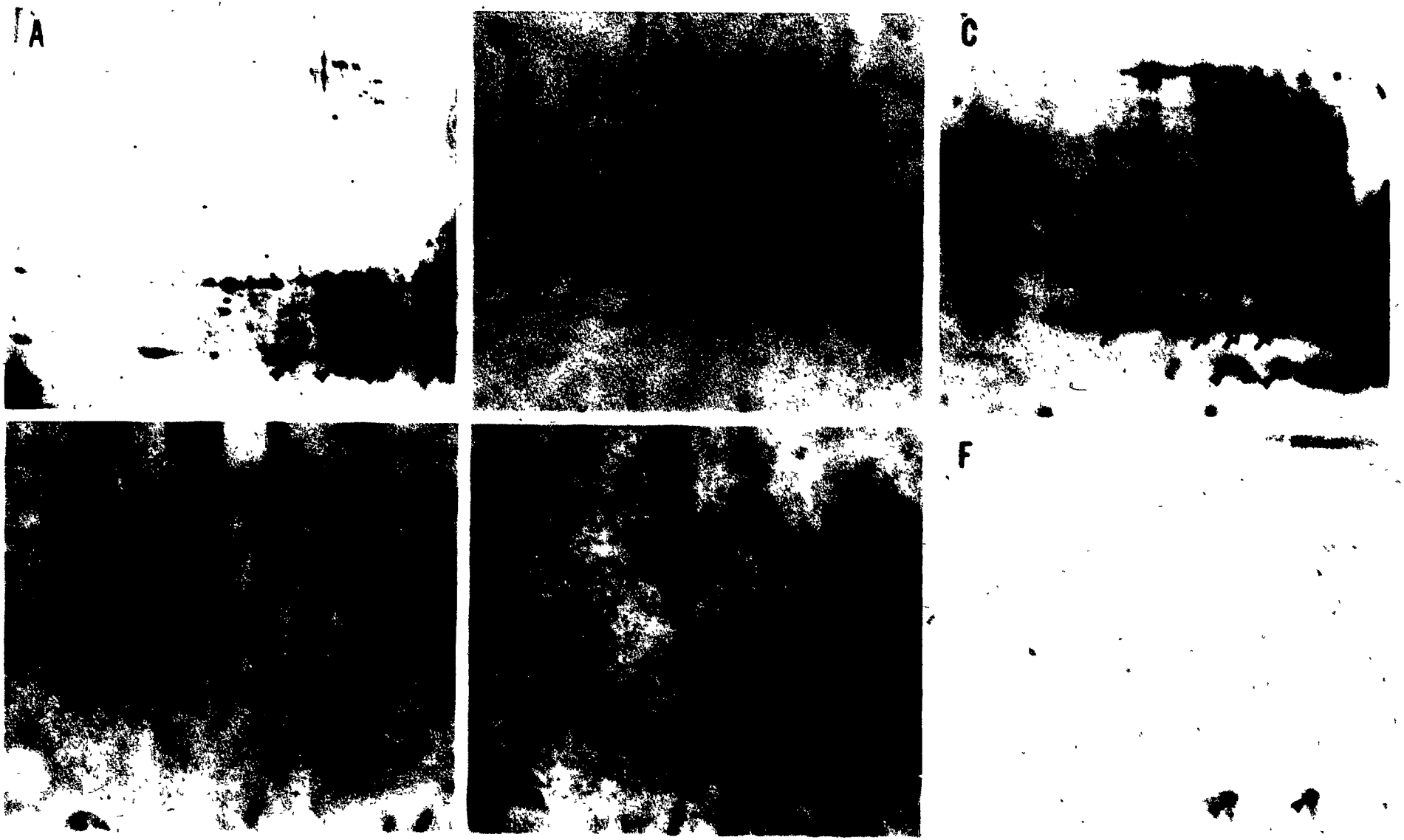

a
b
c

Figure 29 represents a multiple immunoreplica of the cytoplasmic proteins from nodules, following their purification by Sevag's method and Bio-Gel P-200 filtration (0-20,000 MW cut of the same fraction). Panel A in Fig. 29 shows an electrophoregram of these proteins stained with silver, to visualize possibly all polypeptides present in this fraction. A nitrocellulose replica of these proteins was first reacted with antibodies against the cytoplasm of uninfected roots (panel B), then with antibodies against the nodule cytoplasm (panel C). To examine if all of these proteins are of plant and not bacterial origin, the same replica was also reacted with antibodies against the nitrogenase-induced R. japonicum strain 61A76 (panel D). As indicated by arrowheads in panels A and C of Fig. 29, at least 3 relatively abundant polypeptides were identified as "nodule-specific" in this purified fraction of proteins. These polypeptides, molecular weight of which is approximately 12,000, are absent from uninfected roots (Fig. 29B) and Rhizobium (Fig. 29D), and thus are considered "nodule-specific" plant products. In addition to the polypeptides indicated by arrowheads, there are approximately 8 other proteins which also appear to be "nodule-specific", however those were found to be leghemoglobins (panel E).

Despite the lack of reactivity of the three "nodule-specific proteins with rhizobial antibodies (Fig. 29D) it remained possible that these polypeptides are secreted from bacteroids into the host cell cytoplasm. To obtain a convincing evidence for the host and not bacterial origin of these proteins, their isolation was also carried out using nodules which were in vivo labeled with ^{35}S-methionine in the presence of 100 μg of cycloheximide per milliliter of incubation medium. The synthesis of nodule cytoplasmic proteins was inhibited by

FIGURE LEGEND ON OTHER SIDE

Fig. 29. Screening of the low molecular weight polypeptides, isolated as shown in Fig. 26, for the presence of "nodule-specific" polypeptides by means of multiple immunoreplica technique: two-dimensional PAGE. Following purification procedure, the low molecular weight proteins were resolved by two-dimensional PAGE, transferred to nitrocellulose, and reacted with antibodies against cytoplasmic proteins from uninfected roots (B), root nodules (C), R.japonicum strain 61A76 (D), and soybean leghemoglobins (E). Proteins in the gel were stained with silver (A) following the transfer to nitrocellulose. Panel F (fluorogram) shows immunoprecipitate of ^{35}S-methionine-labeled cytoplasmic proteins from 2 week-old nodules with the "nodule-specific" antiserum. Two of the three apparent "nodule-specific" polypeptides, indicated by arrowheads, were found to be synthesized at this developmental stage. Open arrows indicate some of the 8 leghemoglobin polypeptides (see text).


A
C
F

about 97.3%, where none of the three "nodule-specific" proteins were found to be synthesized in the presence of cycloheximide (data not shown). On the other hand, immunoprecipitation of *in vivo* labeled cytoplasmic proteins from nodules with the "nodule-specific" antiserum indicated that at least two of the three "nodule-specific" proteins are synthesized at this stage (2 weeks after infection) of nodule development (Fig. 29F).

CONCLUDING REMARKS

This study demonstrated that, in addition to leghemoglobin, there are a number of other "nodule-specific" plant proteins (nodulins) which are induced following infection of the legume plant (soybean) with Rhizobium. While nodulin-35, a 35,000 MW protein present in both effective and ineffective nodules, is accumulated in a significant (4% by weight) amount in the nodule cytoplasm, other nodulins represent a very small fraction of total cytoplasmic protein. Developmental studies on nodulin-35 suggest that this "nodule-specific" protein is synthesized very early in the nodule structure, and that the level of this protein does not change significantly between days 7 and 21 of nodule development. Since its synthesis and accumulation are not affected by mutations in Rhizobium strains examined, it is possible that nodulin-35 is involved in the formation rather than in symbiotic function of root nodules. That this protein may in fact be involved in the process(es) of nodulation and not in the actual nitrogen fixation, was also suggested by a very low level of its synthesis in older tissue, as evidenced by immunoprecipitation of in vitro translation products of polysomes and mRNA from mature root nodules.

The low molecular weight "nodule-specific" proteins appear to be very different from nodulin-35 and their function may be related to the process of symbiotic nitrogen fixation rather than nodulation. Although their synthesis is detectable in very young (7 day-old) nodules, it is also maintained in later developmental stages. In contrast to nodulin-35, the rate of synthesis of these proteins appears to be much higher than the rate of their accumulation in the host cytoplasm. Their expression seems to be strongly influenced by genetic changes in Rhizobium. As measured by immunoprecipitation

of ^{35}S-methionine-labeled *in vitro* translation products, the synthesis of nodulins in ineffective nodules formed by strains SM5 and 61A24 of *R.japonicum* is reduced by about 9% and 27%, respectively. Since the synthesis of leghemoglobin in the SM5 and 61A24 strains of *R.japonicum* was found to be reduced by about 60% and 96%, respectively, relative to the effective (strain 61A76-induced) nodules, there seems to be no apparent correlation between the synthesis of nodulins and leghemoglobin in these nodules. A single gene lesion in *R.japonicum* SM5, resulting in the lack of nitrogenase component II, was found to have a significant differential effect on the expression of both leghemoglobin and nodulins, suggesting a plaeotropic effect of *Rhizobium* genes on the host genome during the symbiotic process. Mutations in *Rhizobium* also appear to have a selective qualitative effect on the expression of nodulins, as about 6 of the "nodule-specific" proteins are not detectable in the SM5-induced nodules.

Whereas nodulin-35 is a hydrophobic protein, at least some of the low molecular weight nodulins appear to be hydrophilic polypeptides. A variety of techniques used in an attempt to isolate these proteins indicated that their physical and chemical properties may significantly differ.

In spite of the fact that immunoprecipitations with the "nodule-specific" antibodies of different *in vitro* translation products resulted in reproducible populations of nodulins, it is not known why the majority of these proteins are of small molecular weight. A preparative immunoprecipitation of the nascent peptide-polysome complexes encoding nodulin-35, followed by isolation of the mRNA and its translation *in vitro*, have not led to proteolytic degradation of this 35,000 MW polypeptide. Although no evidence is available to deny the possibility of a selective, rather than generalized, proteolysis in the wheat germ cell-free system, it seems unlikely that

the "nodule-specific" proteins of soybean nodules are specific targets for proteolysis in such a heterologous system. Moreover, none of the nodulins are detectable in the translation product of uninfected roots and thus these proteins are indeed unique to root nodule cytoplasm. Although the genetic involvement of the host in the development of root nodules was known for some time, this study provided the first evidence for the physical presence of "nodule-specific" gene products that may be involved in the process of symbiotic nitrogen fixation.

Except for leghemoglobin, nodulin-35 and other (20) nodulins, most of plant cytoplasmic proteins are present in both uninfected roots and root nodules, as judged by electrophoretic and immunological analyses. In contrast, transition of Rhizobium from free-living cultures to bacteroids is accompanied by dramatic alterations of their gene expression, leading to substantial changes of their total protein composition. Although a number of proteins were identified as common to the free-living and bacteroidal stages, a large proportion of them could be localized only in Rhizobium cells before or after infection. In addition to marked differences between the free-living and associative (bacteroid) states, strains 61A76 (effective) and 61A24 (ineffective) of R.japonicum significantly differ in their total protein composition both before and after infection. The effective strain of R.japonicum was also found to secrete at least two small polypeptides into the host cell cytoplasm, however, it is not known whether these proteins cross the membrane envelope or remain in the space between bacteroids and this membrane. Since it is possible that the observed secretions are part of the putative communication between the micro and macrosymbionts, studies in this area using various ineffective nodules could improve our understanding of the symbiotic association.

It is believed that the newly developed method, referred to as multiple immunoreplica technique, provided a powerful tool for immunological screening of a number of proteins, using one polyacrylamide gel. The use of multiple immunoreplica technique confirmed the low molecular weight of some nodulins, which was first observed using a different experimental approach, ie. immunoprecipitation of in vitro translation products.

Application of Sevag's method / ammonium sulphate fractionation / Bio-Gel P-200 filtration, in conjunction with the immunological techniques described in this study, may lead to isolation of at least some "nodule-specific" proteins on a preparative scale. Molecular cloning of the "nodule-specific" sequences, which is now in progress, as well as characterization of their products (nodulins) in the development of effective and ineffective symbioses, may largely contribute to our understanding and perhaps to practical application of biological nitrogen fixation in plants.

APPENDIX I

MULTIPLE IMMUNOREPLICA TECHNIQUE

A variety of immunological techniques developed in recent years have led to identification of proteins present in very low quantities in the cell. The most common method used for detection of particular polypeptide(s) is immunoprecipitation of radioactive products from solutions. Localization of the resulting precipitates, especially those of a very low abundance, among other proteins in a two-dimensional polyacrylamide gel may be difficult and requires highly reproducible profiles. Moreover, due to the length of time of the reaction (usually 24 hr) and drastic treatment of the sample prior to immunoprecipitation (TCA, boiling in 1% SDS) (Lingappa et al., 1978; Marcu et al., 1978), this procedure may in some cases lead to artifactual changes in protein structure (e.g. proteolysis or chemical modification) and therefore result in an altered migration of the proteins ~~from~~ in polyacrylamide gels. An alternative procedure involves transfer of proteins to nitrocellulose paper, followed by detection of specific polypeptides using ^{125}I-labeled antibodies (Towbin et al., 1979). The antigen-antibody complexes can also be visualized by iodinated protein A (Granger and Lazarides, 1979).

This chapter describes a procedure of obtaining multiple replicas (partial "western-blots") of one polyacrylamide gel on nitrocellulose paper, and using each replica for screening a number of proteins with different antisera applied in sequence. Following detection of specific protein(s) with one antiserum, the antibodies can be removed by a low pH treatment and the nitrocellulose replica either may be reacted with other antisera or stored for several months until further use. Using

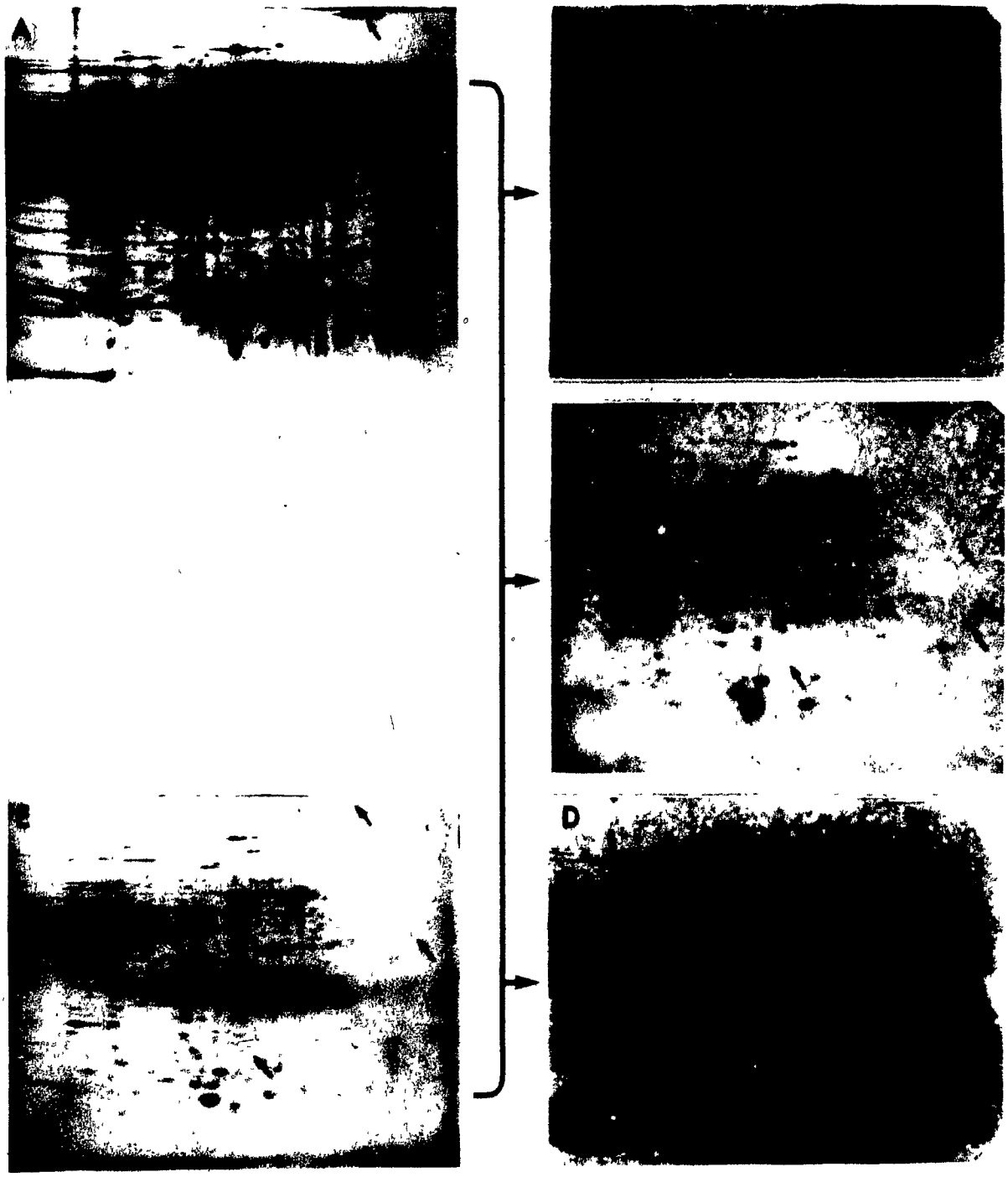
this technique, it is possible to react one gel with at least nine different antibodies, providing superimposable profiles to localize and identify various proteins.

This technique, referred to as multiple immunoreplica technique (Legocki and Verma, 1981), was developed and tested using cytoplasmic proteins of free-living Rhizobium and bacteroids. The cells were sonicated on ice two times for 30 sec in the presence of pancreatic ribonuclease (Sigma; 50 µg/ml), and extracts were treated with deoxyribonuclease (Sigma) according to a procedure by O'Farrell (1975). Following precipitation of proteins by saturated ammonium sulfate, samples were dissolved directly in the lysis buffer (O'Farrell, 1975) and subjected to two-dimensional electrophoresis (Methods). A detailed procedure for transferring proteins from gels to nitrocellulose and reacting the replicas with antisera is described in Methods.

In order to establish the optimum conditions for a series of partial transfers from a polyacrylamide gel to nitrocellulose paper, the electrophoretic transfer was carried out for various time lengths using variable amounts of current, and nitrocellulose replicas were stained for proteins. Figure 30 shows that a two-dimensional gel containing approximately 200 µg protein comprising 350-450 major polypeptides can be transferred to two or three nitrocellulose paper sheets in quantities sufficient for visualizing proteins by staining. Within 1 hr of electrophoresis at 300 mA all peptides detected in the gel are partly transferred to nitrocellulose (Fig. 30). It appears that some polypeptides transfer slightly faster than others, regardless of their molecular weight (Fig. 30, arrows). Since the transfer of proteins is directed toward one side of the gel, it is important to place

FIGURE LEGEND ON OTHER SIDE

Fig. 30. A series of partial electrophoretic transfers of proteins from a polyacrylamide gel to nitrocellulose papers. A two-dimensional gel containing approximately 200 μg protein identical to that in panel A was subjected to a series of three electrophoretic transfers at 300 mA for 1 h each, and the resulting nitrocellulose replicas were stained for proteins (panels B,C and D). An extensive staining of the original gel after the transfers shows a substantial amount of protein remaining in the gel (panel E). Some polypeptides, indicated by arrows, appear to transfer more efficiently than others and cannot be detected in the later replicas and original gel following the transfer. Proteins were extracted from free-living Rhizobium japonicum strain 61A76, and prepared for electrophoresis as outlined under Materials and Methods.

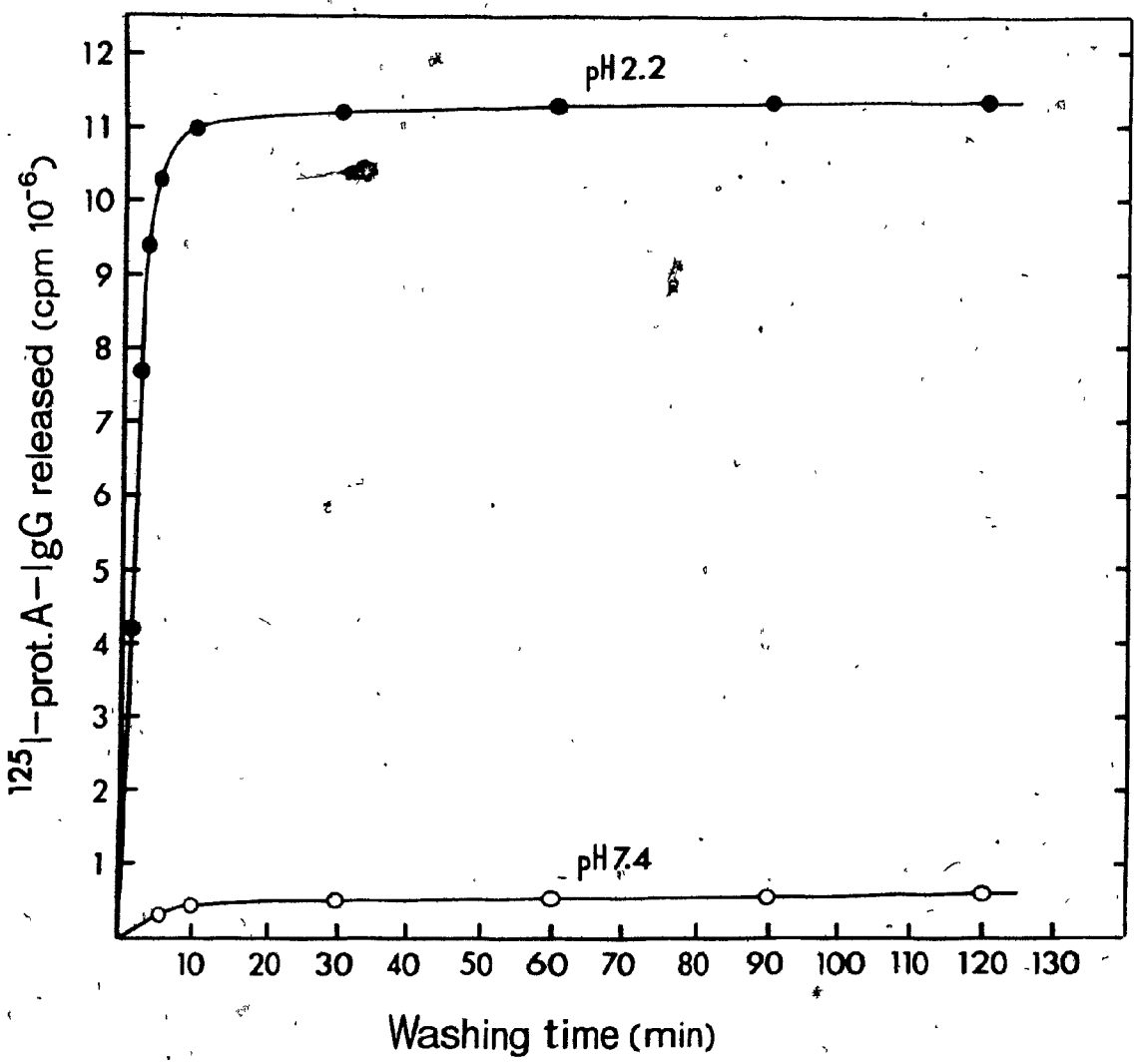

D

nitrocellulose paper on the same (anodic) side of the gel during consecutive transfers. To avoid mistakes, the upper right-hand corners of the gel and the nitrocellulose paper were notched prior to the transfer.

Following incubation of a nitrocellulose replica with antibodies and ^{125}I-protein A, the paper was washed thoroughly in buffer A (pH 7.4), dried briefly, and exposed to the X-ray film. When this paper was placed in 100 ml of buffer A and shaken gently for 2 hr, no release of ^{125}I-protein A from the nitrocellulose replica was observed. However, when the same paper was placed in a buffer of pH 2.2 (buffer B), a rapid release of radioactivity occurred (Fig. 31). The results indicate that after 5 min of such treatment approximately 90% of ^{125}I-protein A bound to nitrocellulose is released and after 90 min of further washing the release of radioactivity appears to be complete. That the removal of ^{125}I-protein A from nitrocellulose paper at pH 2.2 is indeed thorough can be directly examined by exposing the paper to an X-ray film (Fig. 32B). It was essential, however, to determine whether the treatment with pH 2.2 causes a complete release of IgG from proteins and not just the removal of ^{125}I-protein A associated with it. Following washing at pH 2.2, the nitrocellulose replicas were therefore neutralized to pH 7.4 and re-exposed to ^{125}I-protein A. No IgG was detected (Fig. 32C), suggesting that the removal of ^{125}I-protein A at pH 2.2 parallels the release of IgG from proteins. Immediately after removal of ^{125}I-protein A at pH 2.2, the regenerated nitrocellulose paper was neutralized by a brief washing in buffer A at pH 7.4. At this stage the replicas can be dried on a Whatman filter paper and stored in Saran Wrap or Seal-a-Meal bags at 4°C, or can be incubated with a second antiserum (see Methods).

FIGURE LEGEND ON OTHER SIDE

Fig. 31. Kinetics of removal of IgG-Protein A from a nitrocellulose replica by treatment with pH 2.2. Rhizobium proteins were transferred electrophoretically from a polyacrylamide gel to nitrocellulose paper and incubated with a homologous antibody and ^{125}I-Protein A, as described under Methods. Following exposure to X-ray film the paper was first incubated at pH 7.4 (open circles) for 2 h and then a pH 2.2 (dark circles). One-milliliter aliquots were taken and radioactivity was determined in 10 ml Aquasol.


pH 2.2
pH 7.4
^{125}I–prot.A–IgG released (cpm 10^{-6})
Washing time (min)
1
2
3
4
5
6
7
8
9
10
11
12
10
20
30
40
50
60
70
80
90
100
110
120
130

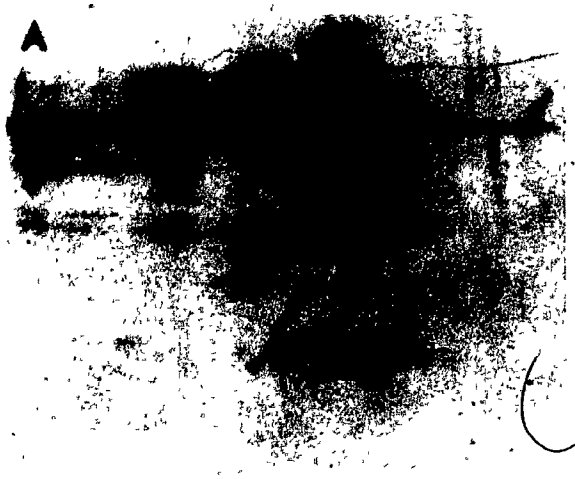
Depending upon the titer of the antisera and the overall handling of the nitrocellulose paper throughout the procedure, each replica may be treated with at least 3-6 different antisera (Fig. 32). Although the immunoreactivity of proteins does not seem to be affected throughout the treatment, the generalized background of radioactivity associated with nitrocellulose may increase as larger numbers of antisera are used. The original procedure for binding IgG to proteins on nitrocellulose paper (Towbin et al., 1979) involves a 40°C incubation with BSA and antiserum. I find that lowering the temperature at 30°C does not affect the efficiency of the reaction. Since some of the replicas are subjected to a series of incubations with different antisera, the lower temperature may prevent possible damage to antigens during the treatment.

Using this procedure, a number of proteins specific to either the wild-type or the ineffective strain of Rhizobium japonicum (strains 61A76 and 61A24, respectively) were detected in a single replica of proteins, resolved on a two-dimensional gel. The same replica was also used to identify subunits of ATP-ase (Fig. 32E).

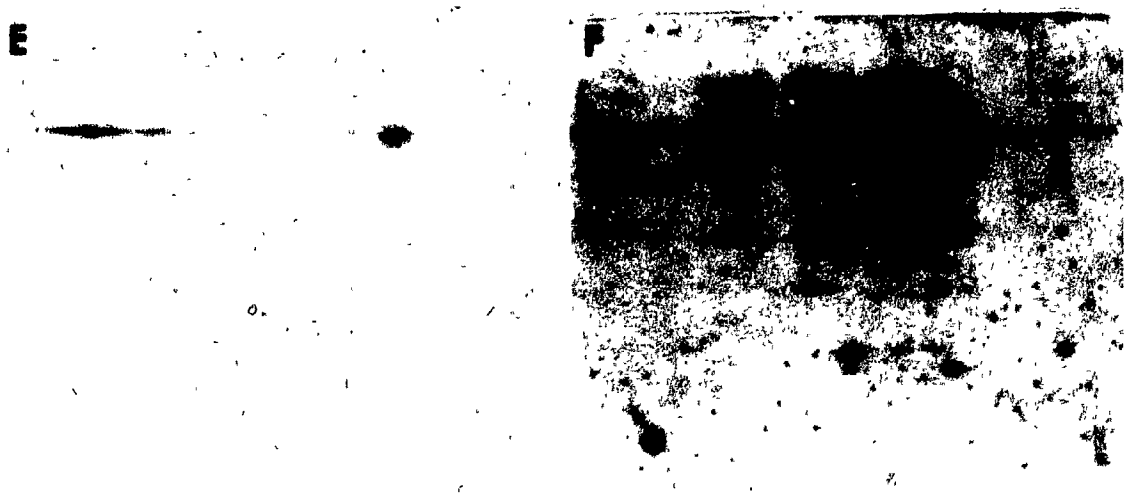
The multiple immunoreplica technique presented here generates several identical copies of the original gel, each of which can be reacted with at least three antisera and stored until further use (Fig. 33). After the electrophoretic transfer from a gel containing approximately 200 μg of protein to three nitrocellulose sheets, a virtually complete pattern of proteins is still detectable by staining of the original gel (Fig. 30E). Having identified and/or localized a particular polypeptide in the immunoreplica, it is possible to obtain microgram amounts of a specific protein from the original gel. The

FIGURE LEGEND ON OTHER SIDE

Fig. 32 . Multiple immunoreplica of proteins identified with a series of different antibodies. Proteins extracted from bacteroids of Rhizobium japonicum strain 61A76 were resolved in a two-dimensionl polyacrylamide gel and transferred to nitrocellulose paper. The nitrocellulose replica was first incubated with antiserum against total proteins of Rhizobium japonicum strain 61A76 and resulting antigen-antibody complexes were visualized by ^{125}I-Protein A (panel A). After a 90-min wash at pH 2.2, the removal of IgG-^{125}I-Protein A from the replica is complete (panel B). Subsequent exposure of the replica to ^{125}I-Protein A confirmed the absence of IgG from the nitrocellulose paper (panel C). The regenerated replica was incubated with antiserum against total protein from another strain of Rhizobium (strain 61A76).


A
B


C


E
F

number of the nitrocellulose replicas obtained from a gel is in proportion to the amount of protein applied on the gel and its rate of electrophoretic elution.

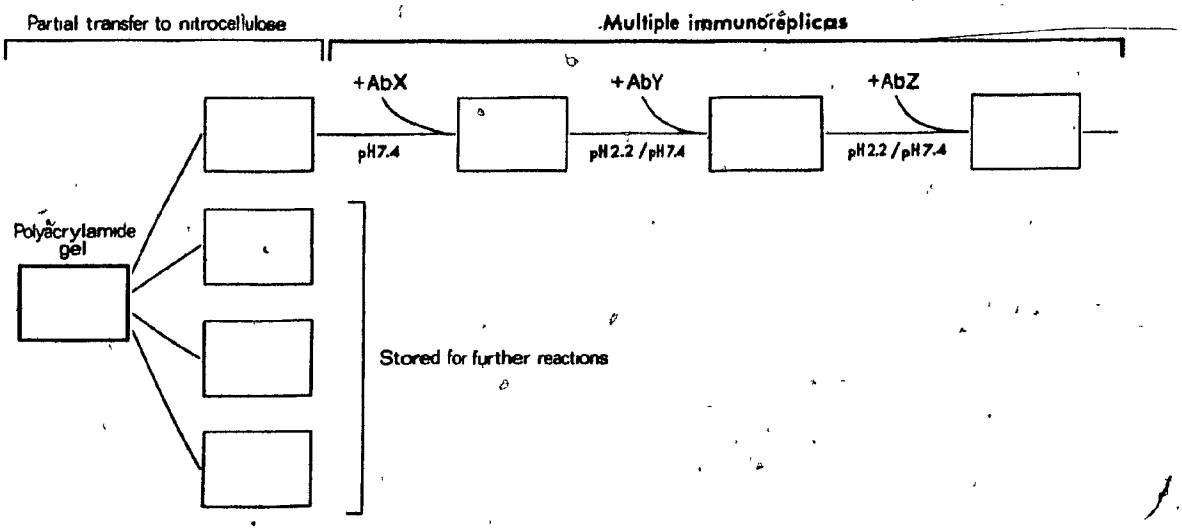
The kinetics of dissociation of IgG from proteins immobilized on nitrocellulose paper at pH 2.2 indicate that the release of antibodies from some polypeptides may not always be complete under the described conditions (data not shown). Although an extended (2-4 hr) wash at pH 2.2 appears to dissociate more strong IgG-protein complexes, a long-time exposure of proteins to the low pH is not recommended. Thus, following the pH 2.2 treatment, it may be desirable to incubate the immunoreplicas with ^{125}I-protein A and expose to X-ray films in order to examine for the presence of residual IgG which could account for the consecutive reaction with a different antibody.

Analysis of the nitrocellulose replicas derived from a polyacrylamide gel indicates that some proteins transfer more efficiently than others. Although the observed differences apply to very few polypeptides (Fig. 30), there seems to be no correlation between the velocity of electrophoretic transfer and the molecular size of proteins under these conditions. Visualization of proteins on nitrocellulose paper by staining indicates that the transferred polypeptides are localized on the surface of the paper and only excess protein penetrates the inner matrix of nitrocellulose. Since only the protein molecules present on the surface of the nitrocellulose paper are reactive with antibodies, this procedure does not appear to be quantitative.

Prior to immunological identification of proteins transferred to nitrocellulose paper, it is important to assess the degree of

FIGURE LEGEND ON OTHER SIDE

Fig. 33. Schematic illustration of the multiple immunoreplica technique. Each nitrocellulose replica of the gel can be reacted with a series of different antisera, leading to identification and localization of specific proteins in the original gel. Three replicas can be obtained to visualize general population of proteins but abundant proteins can be transferred to as many as four or five replicas.


Partial transfer to nitrocellulose
Multiple immunoreplicas
+AbX
+AbY
+AbZ
pH7.4
pH2.2 / pH7.4
pH2.2 / pH7.4
Polyacrylamide gel
Stored for further reactions

non-specific binding of antiserum to the nitrocellulose replica. This is done by incubating the replica with a non-immune serum, followed by the treatment with ^{125}I-protein A and exposure to X-ray films. The labeling of any non-specifically bound IgG of non-immune serum can be prevented by treatment of the replica with unlabeled protein A prior to the incubation with immune serum and ^{125}I-protein A (data not shown). The fact that all the antigens can be relocalized with the same antibody after a series of incubations with other antibodies, and repeated low pH treatments, suggests that there is no apparent loss of proteins or their reactivity during this manipulations (Figures 32A and F).

The multiple immunoreplica technique represents a simple experimental approach to identification to specific proteins resolved electrophoretically. Since the nitrocellulose replicas can be used several times and/or stored until further use, this technique appears to be applicable to a variety of studies involving protein identification. It could also be useful in clinical screening of particular proteins as well as for testing the specificity of an antiserum.

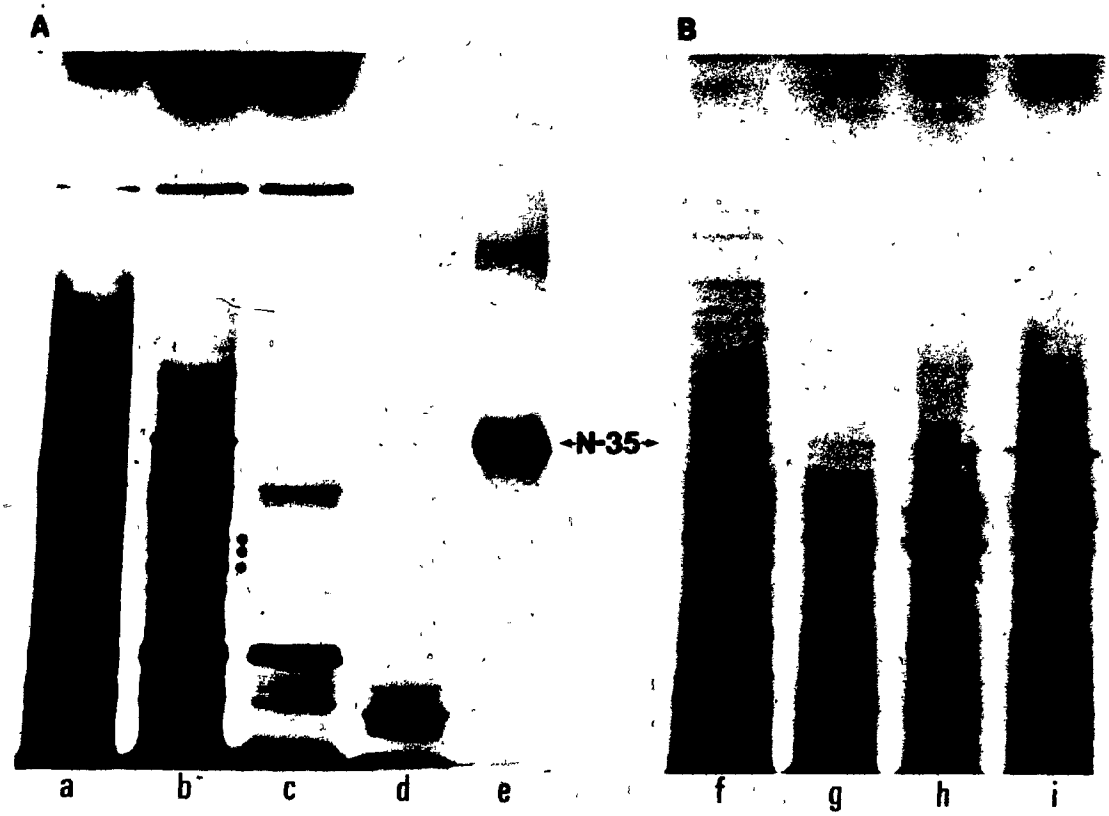
APPENDIX II

ISOLATION OF NODULIN-35 mRNA

To isolate mRNA specific to nodulin-35, total 80S-type polysomes were prepared from 1 g of 8 day-old nodules, and the polysomes containing nascent peptides were reacted with purified IgG to nodulin-35 (see Isolation of Specific mRNAs by Immunoprecipitation of Polysomes). The use of ribonuclease/protease-free IgG, as well as Na-heparine, were essential in this purification procedure. Following a 45 min incubation with IgG at 2°C, the polysomes were passed through a protein A-Sepharose CL-4B column, and the specific polysomes containing nodulin-35 nascent peptides were retained on the column. The mRNA was eluted from the column with 25 mM EDTA by dissociation of ribosomes from the protein A-IgG-nascent peptide-polysome complex. In vitro translation of the RNA isolated from immunoprecipitated polysomes in the presence of ^{35}S-methionine, followed by electrophoretic analysis of the translation product, indicated that this RNA fraction represents a highly enriched RNA encoding nodulin-35 (Fig. 34A). Analysis of the in vitro translation product of the total poly A(+) nodule mRNA (Fig. 34A, lane a) indicated a very low level of synthesis of nodulin-35. Indeed, immunoprecipitation of this product with antibodies to nodulin-35, corrected for a non-specific reactivity of the antibodies with the endogenous wheat germ translation product, suggested that the mRNA encoding nodulin-35 represents only about 0.6 - 1.4% of the total mRNA in 8 day-old nodules. Whereas the high enrichment of nodulin-35 mRNA isolated by the immunoprecipitation of polysomes is obvious (Fig. 34A, lane b), a direct comparison of its translation product with those from total nodule mRNA (lane a) and the wheat germ cell-free system (lane c),

FIGURE LEGEND ON OTHER SIDE

Fig. 34. SDS gel electrophoresis (fluorogram) of ^{35}S-methionine-labeled *in vitro* translation products of poly A(+) RNA encoding nodulin-35. Panel A: (a) total translation product of poly A(+) RNA from root nodules; (b) translation product of mRNA encoding nodulin-35, isolated by immunoprecipitation of nascent peptide-polysome complexes with antibodies to purified nodulin-35; (c) translation product of the undigested endogenous wheat germ mRNA (S23 translation background); (d) immunoprecipitation of (b) with antibodies to nodulin-35; (e) radioactive marker of nodulin-35. Panel B: (f) S23 translation background; (g to i) translation in the presence of increasing amounts of purified mRNA encoding nodulin-35. Dots in lane (b) indicate the presence of at least three translation products whose mRNA's co-purified with the mRNA for nodulin-35 (see text for details; see also Methods). N-35, nodulin-35.


A
B
←N-35→
a
b
c
d
e
f
g
h
i

indicated the presence of at least 3 other mRNA species co-isolated using this method (lane b, dots). Since the immunoprecipitation of the translation product from lane b with the antibodies to nodulin-35 did not yield any of these contaminant mRNA products (lane d), it was assumed that they do not share any antigenic homology with nodulin-35. In a similar experiment, where the protein A-Sepharose column was washed more extensively following the application of polysomes, no contaminant mRNAs were detected (Fig. 34B), thus indicating the potential for isolating specific mRNAs by this method. Using increasing amounts of an exogenous mRNA, particularly in quantities comparable to those of the undigested endogenous mRNA of the cell-free system, the overall background of translation increases (Fig. 34B). Although this observation is not relevant to this study, it should be mentioned that similar results were obtained using mRNAs from different sources, e.g. those encoding the 7S and 11S storage proteins of soybeans (Champa Sengupta, personal communication).

APPENDIX III

PROCEDURES ATTEMPTED TO ISOLATE "NODULE-SPECIFIC" PROTEINS OTHER THAN LEGHEMOGLOBIN

(i) Ammonium Sulphate-Fractionation of Root Nodule Cytoplasm

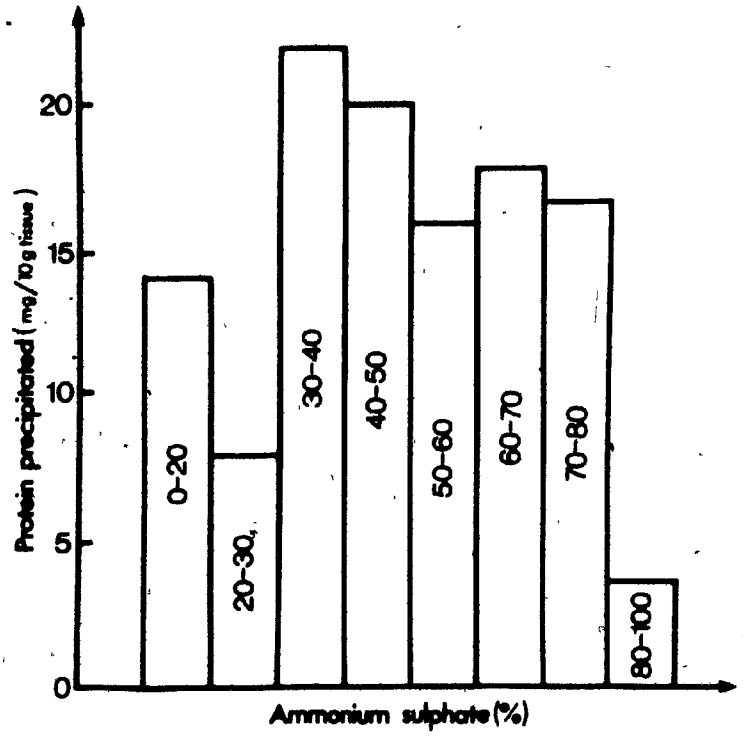
In order to determine if low molecular weight proteins, nodulins in particular, can be enriched by a differential fractionation with ammonium sulphate, about 30 ml of the host cytoplasmic proteins (4.3 mg/ml) was subjected to a step-wise ammonium sulphate fractionation. To measure the protein content, each precipitated sample was washed with its respective solution of ammonium sulphate, dissolved in 1 ml H_2O, and desalted on a Sephadex G-25 column. Following protein determination, about 200 µg of each sample were precipitated with TCA and analyzed electrophoretically on high Tris/urea polyacrylamide gels (Goldsmith et al., 1979; see Methods), which provide a good separation of small molecular weight proteins.

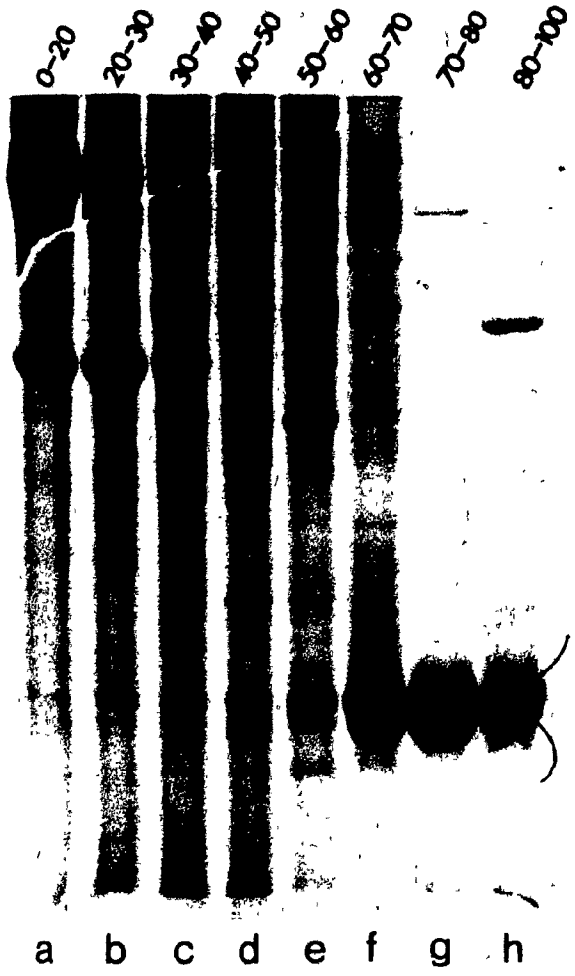
Figure 35 shows the quantitative distribution of cytoplasmic proteins from nodules, precipitated with increasing concentrations of ammonium sulphate (AS). Electrophoretic analysis of these fractions (Fig. 36) suggested that the fraction representing the most hydrophilic proteins (80-100% AS; lane h) may be enriched in low molecular proteins up to 20,000 size. It was apparent, however, that some small polypeptides are also removed at lower concentrations of AS, as well as that a substantial amount of leghemoglobin is present in the 80-100% AS fraction. Although the reactivity of this fraction with nodule antibodies suggested that at least some nodulins may be hydrophilic proteins, the low yield of protein obtained by this procedure (approximately 3% of the total cytoplasmic protein) could not fully justify this technique as an efficient method for isolation of small molecular weight proteins.

FIGURE LEGENDS ON OTHER SIDE

Fig. 35. Ammonium sulphate-fractionation of soluble cytoplasmic proteins from root nodules: a diagram. About 10 g of three week-old nodules, induced by the effective (61A76 or wild type) strain of R.japonicum, were homogenized in 30 ml of the isolation buffer (Methods), and soluble cytoplasmic proteins (4.3 mg per ml) were fractionated with increasing concentrations of ammonium sulphate, dissolved in 1 ml of H_2O, and de-salted on a Sephadex G-25 column. Protein was estimated according to the method of Lowry et al., 1951.

Fig. 36. Ammonium sulphate-fractionation of soluble cytoplasmic proteins from root nodules: SDS-PAGE. Protein fractions were obtained and processed as outlined in legend to Fig. 35 and Methods. Prior to electrophoresis, approximately 200 µg of each protein sample were precipitated with 10% (w/v) TCA, washed with ether, and dissolved in the equilibrium buffer containing 1% (w/v) SDS, 10% (v/v) β-mercaptoethanol and 0.063 M Tris-HCl pH 6.8 (Goldsmith et al., 1979). Composition of the high Tris/urea gel used here is described under Electrophoresis of Small Molecular Weight Proteins (Methods).


Protein precipitated (mg/10g tissue)
0
5
10
15
20
0-20
20-30
30-40
40-50
50-60
60-70
70-80
80-100
Ammonium sulphate (%)


0-20
20-30
30-40
40-50
50-60
60-70
70-80
80-100
a b c d e f g h

(ii) Chromatography on DEAE-Cellulose

Since the column chromatography using DEAE-52-cellulose has proven a successful method for isolation of the leghemoglobin (Lb) components from root nodules (Appleby et al., 1975; Verma et al., 1979), an attempt to isolate proteins other than Lb using a similar procedure was made. Preliminary results obtained from the chromatography of a total fraction of nodule cytoplasmic proteins using a 5-100 mM K-acetate buffer, pH 5.2 gradient indicated that, except for leghemoglobin, the majority of proteins did not fractionate under these conditions. Electrophoretic analyses of the eluted proteins by one-dimensional PAGE showed a poor resolution of proteins whenever the total, i.e. unfractionated, population of cytoplasmic proteins was applied to this chromatography. Further studies indicated, however, that the resolution of protein may be improved when only hydrophilic proteins from the nodule cytoplasm were applied to the DEAE-cellulose column. A series of chromatographies using samples pre-fractionated with ammonium sulphate (30-100%, 50-100%, and 70-100% AS), followed by their analysis by one-dimensional PAGE (data now shown), resulted in a good resolution of proteins from the 70-100% AS fraction only. Moreover, if the 70-100% AS fraction was used following a 0-50 mM K-acetate gradient applied to the column, a small amount of protein was eluted step-wise with 100 mM and 200 mM K-acetate (Fig. 37). Lanes g and h show that the proteins eluted from DEAE-cellulose at a high ionic strength (100 mM and 200 mM K-acetate, respectively) are enriched in low molecular weight polypeptides, but significant amounts of leghemoglobin are still associated with these fractions. A quantitative analysis of the eluted proteins indicated that the 100 mM and 200 mM fractions combined contain as little as about 5.2% of the protein present in the 70-100% AS fraction. Since the

70-100% AS fraction represents approximately 17.2% of the total cytoplasmic protein in root nodules (Fig. 35), these strong-DEAE-binding polypeptides represent less than 1% of the total nodule cytoplasic protein. Immunological screening for the presence of "nodule-specific" proteins among the strong-DEAE-binding polypeptides did not indicate any enrichment of "nodule-specific" proteins in this fraction. Also, the presence of significant amounts of leghemoglobin after DEAE-cellulose chromatography, was not desirable for application of this method for purifying the "nodule-specific" proteins of low molecular weight.

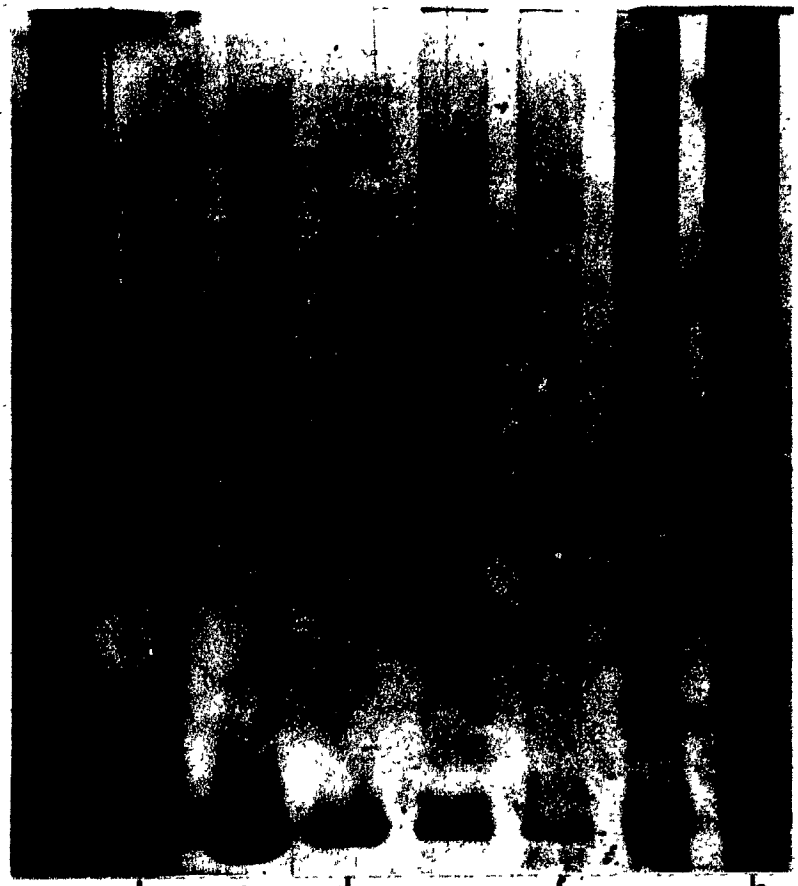
(iii) Hydrophobic Chromatography on Phenyl-Sepharose

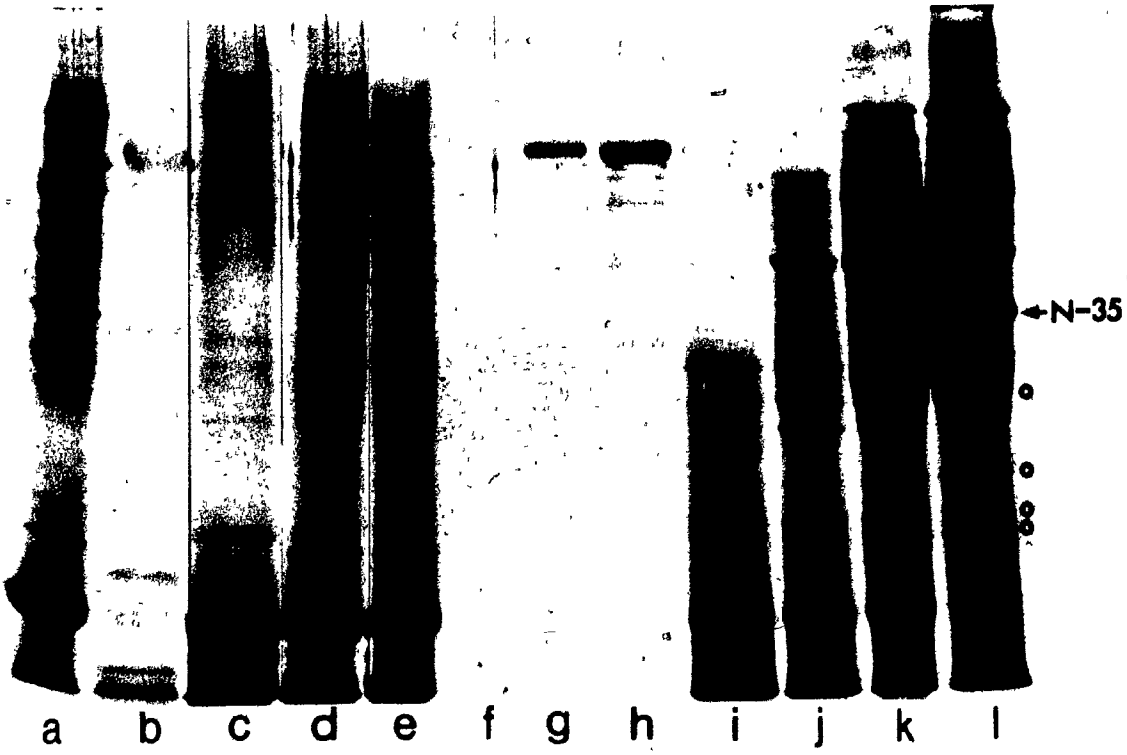
Preliminary results indicated that Phenyl-Sepharose efficiently binds almost all (about 99% by weight) of the soluble cytoplasmic protein from nodules in 1 M ammonium sulphate, and thus an attempt was made to fractionate nodule proteins using this chromatographic procedure. The unbound material (about 0.7% of total protein) was analyzed spectrophotometrically and electrophoretically, and it was found to contain large quantities of nucleic acids as well as some proteins of mostly acidic isoelectric points. Elution with decreasing concentrations of ammonium sulphate (0.5 M, 0.1 M and 0 M AS) resulted in the removal of approximately 74% of protein from the column, including leghemoglobin. Following a thorough washing of the column with 10 mM Na-phosphate pH 7.6, the remaining proteins were eluted with 10 M urea at room temperature. A subsequent application of 0.1 N NaOH to the column did not yield any proteins, suggesting that their elution with urea was complete. Figure 38 shows electrophoretic analysis by one-dimensional SDS-PAGE of the eluted fractions. Whereas the unbound

FIGURE LEGENDS ON OTHER SIDE

Fig. 37. Chromatography of soluble cytoplasmic proteins from nodules on a DEAE-cellulose (DE-52) column: analysis of selected fractions by SDS-PAGE. Prior to the chromatography, a 70–100% ammonium sulphate-precipitated fraction of cytoplasmic proteins (14 mg protein) was isolated and equilibrated to 5 mM K-acetate pH 5.2 by dialysis. Chromatography was carried out in a 5 to 50 mM gradient of the same buffer (lanes a, b, c, d, e, and f represent fractions number 18, 22, 33, 46, 55, and 64, respectively), followed by a step-wise elution with 100 mM and 200 mM K-acetate (lanes g and h). Elution of protein was monitored at A_{280}, and fractions were collected and processed as outlined in Methods.

Fig. 38. Fractionation of soluble cytoplasmic proteins from root nodules on a Phenyl-Sepharose column: analysis of selected fractions by SDS-PAGE. (a) Total cytoplasmic proteins prior to fractionation; (b) unbound material; (c), (d) and (e) proteins eluted with decreasing concentrations of ammonium sulphate (0.5 M, 0.1 M and 0 M, respectively); (f), (g) and (h) proteins eluted with 0.01 M, 0.1 M and 0.5 M $CaCl_2$, respectively; (i), (j), (k) and (l) proteins eluted with increasing concentrations of urea (1 M, 3 M, 6 M and 10 M, respectively). Circles in lane (l) indicate some polypeptides present in all four urea-eluted fractions. N-35, nodulin-35.


a b c d e f g h


N-35
a b c d e f g h i j k l

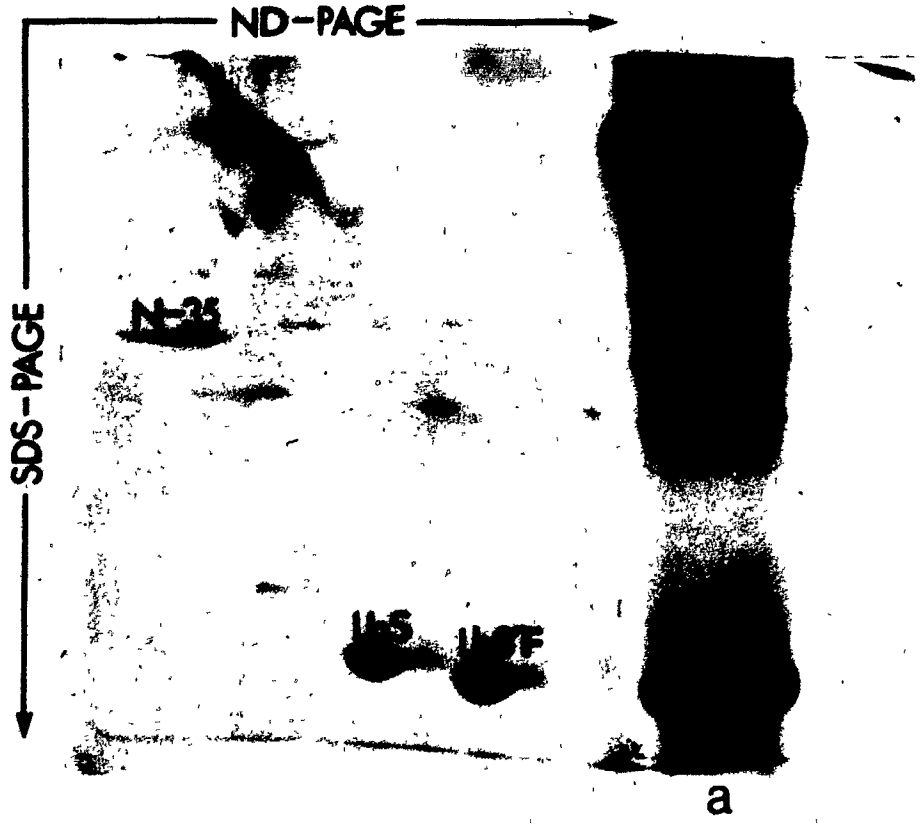
material contains trace amounts of wide size range polypeptides (lane b), application of a decreasing ionic strength of salt to the column causes elution of leghemoglobin and some fractionation of other proteins (lanes c, d and e). Following a thorough washing of the column with the 10 mM Na-phosphate buffer, Phenyl-Sepharose was treated with a strong chaotropic salt ($CaCl_2$), which is known to disrupt hydrophobic interactions, and hence could cause elution of some proteins from this matrix (Von Hippel and Schleich, 1969). Lanes f, g, and h of Fig. 38 show proteins eluted with 10 mM, 100 mM, and 500 mM $CaCl_2$. It is clear that application of Ca^{2+}, and possibly other chaotrophic ions, e.g. Ba^{2+} or Mg^{2+} (Pharmacia Fine Chemicals, 1976), may lead to a specific elution of some polypeptides (Fig. 38, lane h). To determine if the 10 M urea-eluted fraction of proteins can be differentially sub-fractionated, the column was subsequently eluted with 1 M, 3 M, 6 M, and 10 M urea (lanes i,j,k and l, respectively). A substantial enrichment of the low molecular weight polypeptides was observed, particularly in fractions eluted with the low urea concentrations (e.g. 1 M; lane i). There appears to be a correlation between the size of enriched polypeptides and the concentration of urea used for their elution. It is also apparent that nodulin-35 strongly binds to Phenyl-Separose and that it is eluted only at high concentrations of urea (lane l). Its tight association with Phenyl-Separose clearly suggests that nodulin-35 is a hydrophobic protein, supporting previous observations obtained by the ammonium sulphate fractionation and Sevag's method (Results and Discussion). The gradual removal of small proteins from a population of large polypeptides during the treatment with urea suggests that these proteins may form a complex which consists of a large number of polypeptides. Since a number of proteins, particularly of small

molecular weights, are present throughout the entire range of fractionation with urea (circles in Fig. 38), it is possible that at least some hydrophilic proteins are bound to the column through interactions with other, hydrophobic, polypeptides. That nodulin-35, for example, may indeed be associated with other proteins and, as a result, be part of a larger complex, was suggested by the observation that this polypeptide was found among the slow-migrating large molecular weight proteins in non-denaturing electrophoresis. Figure 39 shows a two-dimensional PAGE which was specifically designed to localize nodulin-35 in a non-denatured sample of the root nodule cytoplasm. Total cytoplasmic proteins from mature nodules were first resolved in a non-denaturing cylindrical gel, which was subsequently layered over a slab SDS-polyacrylamide gel. To localize nodulin-35 and leghemoglobin in the gel, the sample was run next to the non-denaturing gel in the second dimension. Despite a rather small size of nodulin-35, this protein was found among the slow-migrating large polypeptides. It should be noted, however, that the rate of electrophoretic migration of proteins under non-denaturing conditions is not only dependent on the molecular weight, but also on the shape and charge of protein molecules (Ornstein, 1962). Although the non-denaturing electrophoresis was carried out at 4°C using a low voltage, it is alsopossible that some protein complexes dissociated during the run (Boyack and Giddings, 1960; Mysels and Scholten, 1962).

Quantitative analysis of the Phenyl-Sepharose chromatography indicated that the proteins eluted with urea represent approximately 12.0% by weight of the total cytoplasmic protein from nodules. On the other hand, the low molecular weight polypeptides (below 20,000 MW)

FIGURE LEGEND ON OTHER SIDE

Fig. 39. A two-dimensional (non-denaturing / denaturing) PAGE of total cytoplasmic proteins from root nodules. Proteins were first electrophoresed in cylindrical gels under non-denaturing conditions at 4°C (Davis, 1964) and, following equilibration with SDS-sample buffer, electrophoresis was continued in the second dimension using a slab SDS-polyacrylamide gel (Laemmli, 1970). The same protein sample was run as a marker in the second dimension only (lane a). Since the application well for proteins electrophoresed in lane (a) was formed in the stacking gel about 3-4 mm below the position of the cylindrical gel, these proteins ran slightly ahead of the second dimension of non-denatured sample (compare front lines at the bottom of gel). ND-PAGE, non-denaturing polyacrylamide gel electrophoresis; LbF, LbS, the fast and slow-moving components of leghemoglobin, respectively.



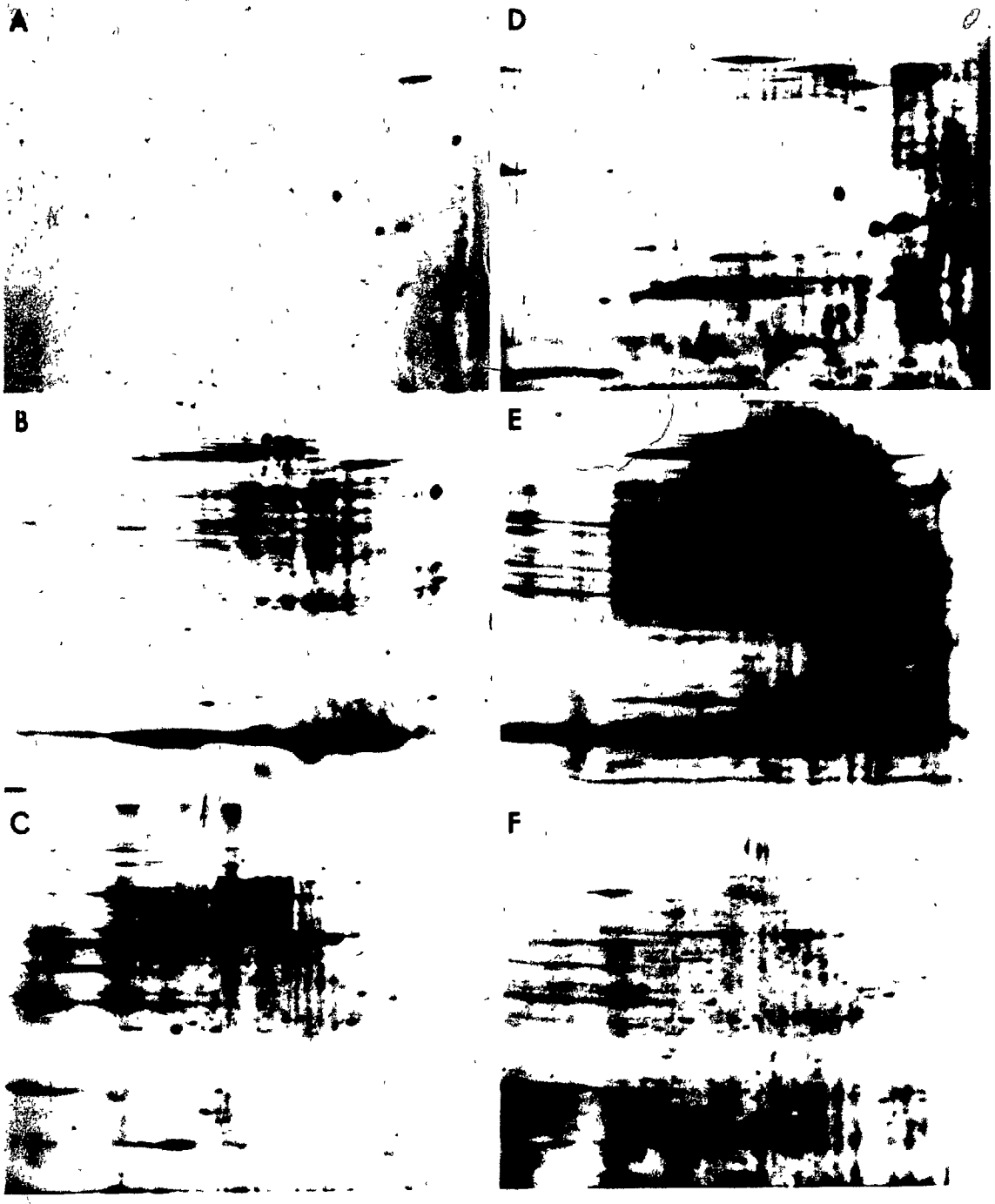
a

represent about 36.% of protein in this fraction, as determined spectrophotometrically by gel scanning (ORTEC Scan). It thus appears that the small molecular weight proteins, isolated by Phenyl-Sepharose followed by a Bio-Gel P-200 filtration (see Methods) represent up to 4.3% by weight of the total soluble cytoplasmic protein from root nodules.

Protein samples were taken at each purification stage and examined by two-dimensional PAGE (Fig. 40). Each gel was stained with both Coomassie blue (Fig. 40, panels A,B and C) and silver (panels D,E and F), as well as examined immunologically, according to the multiple immunoreplica technique (see Appendix I for detailed description). The unbound material, representing only 0.7% by weight of the total cytoplasmic protein, contains some small polypeptides (Fig. 40A and 40D), but their reactivity with nodule antibodies was very poor (not shown). As mentioned above, this fraction was found to contain large quantities of nucleic acids, however, there is no evidence for their structural association with proteins found in this fraction. Figures 40B and 40E represent proteins eluted with decreasing (1 M to 0 M) concentrations of ammonium sulphate. It is clear that in addition to the abundant leghemoglobins this fraction contains a substantial amount of other polypeptides, including some small proteins other than leghemoglobin. Considering the fact that leghemoglobin constitutes more than half of the protein content in this fraction, and that its molecular weight is similar to that of at least 4 nodulins, this fraction could not be used for isolation of nodulins on a preparative scale. Proteins eluted from Phenyl-Sepharose with 0-10 M urea are shown in Fig. 40C and 40F. This population of proteins contains a large number of small molecular weight polypeptides and only a small amount of leghemoglobins.

FIGURE LEGEND ON OTHER SIDE

Fig. 40 . Two-dimensional PAGE of soluble cytoplasmic proteins from nodules fractionated by Phenyl-Sepharose. Unbound material stained with Coomasie blue (A) and silver (D); proteins eluted with decreasing concentrations (1 M to 0 M) of ammonium sulphate stained with Coomasie blue (B) and silver (E); proteins eluted with 0 M to 10 M urea stained with Coomasie blue (C) and silver (F).


A
D
B
E
C
F

In order to examine for the presence of "nodule-specific" proteins in the Phenyl-Sepharose fractions, proteins shown in Fig. 40 were transferred to nitrocellulose papers and reacted with the anti-root and anti-nodule sera (for description of antisera see Methods), according to the multiple immunoreplica technique (Appendix I). Whereas the unbound material (Fig. 40A and 40D) showed a poor reactivity with antibodies, proteins eluted with 1 M to 0 M ammonium sulphate (panels B and E) and 10 M urea (panels C and F) strongly reacted with both anti-root and anti-nodule sera (data not shown). As mentioned above, due to the abundance of leghemoglobins in the 1 M ammonium sulphate-eluted fraction, immunological screening for "nodule-specific" proteins other than leghemoglobin was difficult, particularly in the low molecular weight region. A substantial absence of leghemoglobin from the 10 M urea-eluted fraction allowed to detect several (up to 7) small molecular weight polypeptides which appeared to be "nodule-specific", and whose molecular weights and isoelectric points were different from those of leghemoglobins. The relative concentration of these polypeptides in the 10 M urea-eluted fraction, however, was found to be very low and hence their isolation on a preparative scale would be difficult.

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