

OLEOSOMES (LIPID BODIES) IN  $N_2$ -FIXING PEANUT ROOT NODULES

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BY

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## ABSTRACT

Nitrogen-fixing peanut root nodules have oleosomes (lipid bodies) in infected and uninfected cells. The oleosomes were characterized and compared with their counterparts in the seed tissue. Measurements made from scanning electron micrographs of isolated oleosomes revealed that eighty percent of the nodule oleosomes were of smaller size (0.16-1.0  $\mu\text{m}$  in diameter [dia.]) while in the seeds the larger size (1.0-5.5  $\mu\text{m}$  dia.) dominated. The larger oleosomes in the nodules were exclusively found in the uninfected three layers of cortical cells adjacent to the infected zone. Morphometric analyses have shown significantly higher numbers of oleosomes covering about 8% of the infected cell area in immature (white) nodules, whereas the mature/old (pink) nodules had lesser numbers occupying about 4-5% of the cell area. The decrease in the number of oleosomes possibly reflects their utilization in mature/old nodules which effectively fix nitrogen. The oleosomes were distinctly stained by p-phenylenediamine (pPD) at both light and electron microscopic levels. Oleosomes are osmiophilic and spherical in structure. An electron-dense rim was observed around the nodule oleosomes, where lipolytic activity was also demonstrated using cytochemical methods. The rim was absent in the seed oleosomes. The defatted oleosomes were found to be surrounded by a 'half-unit membrane' and a non-extractable rim of possible proteinaceous substance. Gas chromatographic analyses of the lipid from the isolated oleosomes indicated the presence of

higher amounts of saturated fatty acids in the nodule oleosomes than in the seed. This study indicates that the nodule oleosomes differ from the seed oleosomes, with respect to the presence of (1) an electron-dense rim (2) showing lipolytic activity and (3) higher amounts of saturated fatty acids. Nodule oleosomes seem to be transient storage organelles to be metabolized, while in the seed they are meant for long term storage.

*For my mom & dad*

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# I. INTRODUCTION

Nitrogen ( $N_2$ ) is the fourth major elemental constituent of living organisms and is essential for their growth and development. It is a component of many bio-molecules such as nucleic acids and proteins. Eukaryotic organisms depend on a source of reduced or oxidized form of nitrogen, even though it covers about 78% of the earth's atmosphere. Nitrogen reacts only by a great expenditure of energy and hence was called *diazo* (meaning without life) by Lavoisier (Mora and Lara, 1988). Only a few prokaryotic organisms are able to utilize the atmospheric nitrogen by reducing it to ammonia ( $NH_3$ ), a process known as nitrogen fixation. These organisms are collectively termed as nitrogen fixers or *diazotrophs*. This fixed nitrogen can be assimilated by the plants. Animals are able to utilize nitrogen compounds assimilated by the plants either directly or indirectly.

## I.1. NITROGEN CYCLE

About 98% of the global nitrogen occurs in primary rocks and is largely unavailable to the biosphere. The atmospheric reservoir is estimated to be about  $3.9 \times 10^{15}$  tonnes, of which terrestrial living systems contain  $2.5 \times 10^5$  tonnes or 0.000000025%, the soil contains  $3.3 \times 10^{11}$  tonnes or approximately 33 times the nitrogen in living systems (Burns and Hardy, 1975). The marine biosphere has been estimated by

Martin (1970) to contain  $4 \times 10^9$  tonnes of nitrogen in various forms including the oceanic sediments. This limited amount of nitrogen is shuttled between various components of the biosphere, being continuously increased by *diazotrophs* and simultaneously depleted by processes which convert the fixed nitrogen back to atmospheric nitrogen. This shuttling of nitrogen between various components is customarily explained in the form of nitrogen cycle (Fig. 1).

In this cycle the atmospheric nitrogen is first fixed either by biological or natural nonbiological and commercial processes, which contribute to the reduced or oxidized forms of nitrogenous compounds to the biosphere, soil and water. Secondly upon death and decay of plants, animals and microorganisms the assimilated nitrogen is returned to the earth. Ammonia, the highly reduced form and nitrate, the highly oxidized form of nitrogen are interconvertible by action of various microorganisms. Lastly, the nitrogen cycle is completed by the reduction of nitrate to nitrogen by microorganisms, a process known as denitrification. One sixth of the total nitrogen is lost to the atmosphere per annum by the process of denitrification and is compensated for by atmospheric nitrogen fixation (Burns and Hardy, 1975).

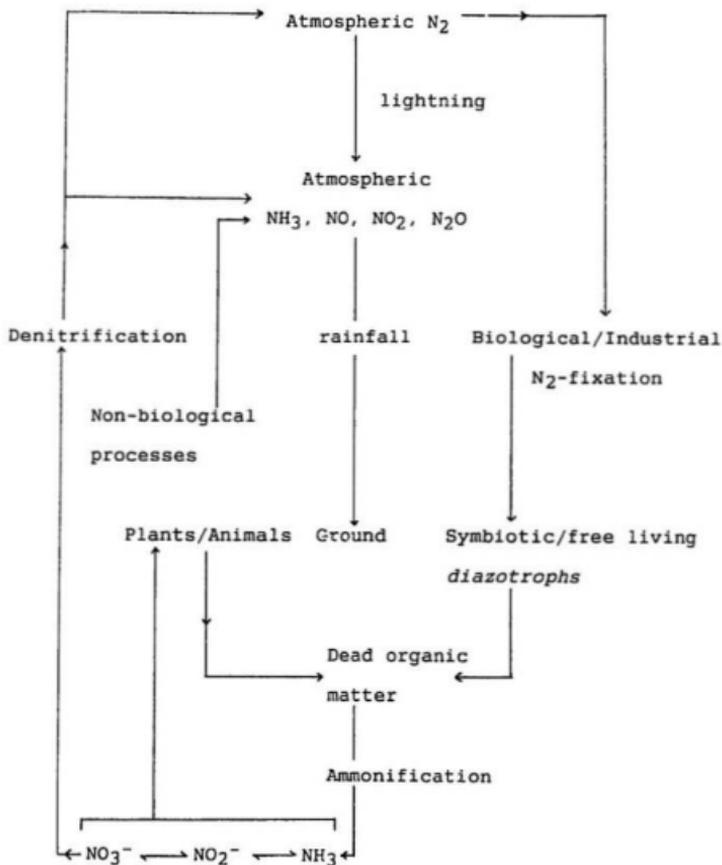


Fig. 1. The nitrogen cycle (modified from Gallon and Chaplin, 1987).

## 1.2. BIOLOGICAL NITROGEN FIXATION

Biological nitrogen fixation is the reduction of atmospheric nitrogen to ammonia by nitrogen fixing organisms. It is represented by the following equation:



This reaction is catalyzed by nitrogenase which can occur in two forms, EC 1.18.6.1 in free living *diazotrophs* and EC 1.19.6.1 in symbiotic *diazotrophs*. Nitrogenase requires the input of a large amount of energy. Only prokaryotes possess nitrogenase and are able to reduce atmospheric nitrogen. Based on the ribosomal RNA analyses, prokaryotes are classified into two main divisions, the eubacteria (including cyanobacteria) and the archaeobacteria (Stackebrandt and Woese, 1981). Both of these groups have nitrogen fixing representatives. These prokaryotic *diazotrophs* may be free living (for example, *Clostridium pasteurianum*, *Klebsiella pneumoniae* and *Azotobacter vinelandii*), or form casual associations (*Azospirillum*-grass) and symbiotic associations (*Rhizobium*-legume/non legume associations, *Frankia*-non legume associations, cyanobacteria-plant associations) with other organisms. Legumes have attracted particular attention due to their ability to enter into symbiotic associations with *Rhizobium*, *Bradyrhizobium* and *Azorhizobium*, collectively known as rhizobia. The symbiotic nitrogen fixation by legumes

contributes almost 50% to the global nitrogen fixed (Dixon and Wheeler, 1986). In addition, legumes are a major source of protein for human and animals.

### I.3. SYMBIOTIC NITROGEN FIXATION BY LEGUMES

The plant family, Leguminosae (Fabaceae), is the third largest Angiosperm family, present from the tropics to the arctic and includes forms varying from annual and perennial herbs to trees and shrubs. The wide distribution of legumes could be attributed to their ability to enter into a symbiotic association with nitrogen fixing rhizobia. Rhizobia are gram-negative rod shaped cells without endospores and are aerobic and motile. Infected by one or more of the specific bacterial genera *Rhizobium*, *Bradyrhizobium*, or *Azorhizobium*, legume roots undergo a complex series of alterations resulting in the formation of tumor like structures known as root nodules, which the bacteria inhabit. *Azorhizobium* forms stem nodules in many aquatic legumes. Eventually, the two organisms establish metabolic cooperation: the rhizobia reduce atmospheric nitrogen to ammonia, which is exported to the plant for assimilation and the bacteria are provided with a carbon source required for the high energy demanding process of nitrogen fixation by the host. Several factors in the legumes and the rhizobia are specifically expressed when they are exposed to one another and are responsible for recognition, infection, nodule formation and nitrogen fixation. Vincent (1980) has

divided the formation of root nodules into three main divisions which can be subdivided. These developmental stages can be blocked or inhibited discretely by using plant or rhizobial mutant strains. The three stages are:

- 1) Preinfection stage, in which rhizobia attach to the root hairs and root hair curling occurs.
- 2) Infection and nodule formation, rhizobia enter the root cells through the infection threads or through the large basal cells and are released in the plant cells and develop into bacteroids.
- 3) Nitrogen fixation, the functional stage of the root nodule.

#### I.3.1. PREINFECTION STAGE

##### I.3.1.1. RECOGNITION

Rhizobia exhibit species specificity and infect only one or at the most, a few species of legumes (Table 1). First of all rhizobia must come in contact with the legume roots. Rhizobia have been shown to be attracted by plant root exudates, flavones and root hair proteins. Flavones have been shown to specifically induce nodulation factors in rhizobia. Plant lectins and rhizobial cell surface polysaccharides may also be involved in specific recognition. These factors are discussed in the following sections.

Table 1. Rhizobia-Plant Associations (Long, 1989)

<i>Rhizobium</i>	Plant
<i>Rhizobium meliloti</i>	Alfalfa
<i>Rhizobium leguminosarum</i>	
biovar <i>viciae</i>	Pea, vetch
biovar <i>trifolii</i>	Clover
biovar <i>phaseoli</i>	Bean
<i>Rhizobium fredii</i>	Soybean
<i>Bradyrhizobium japonicum</i>	Soybean
<i>Rhizobium loti</i>	Lotus
<i>Rhizobium galegae</i>	Galegae
<i>Bradyrhizobium</i> sp.	Parasponia (non-legume)
<i>Bradyrhizobium</i> sp. 32H1	Peanut, cowpea, soybean parasponia?
<i>Azorhizobium caulinodans</i>	Sesbania (stem nodules)

## Root exudates and flavones

Rhizobia are chemotactic (Borgman *et al.*, 1988; Caetano-Anollés *et al.*, 1988) towards plant roots, probably due to the plant root exudates, particularly flavones (Sprent, 1989). Rhizobial nodulation (nod) genes are specifically induced by exuded plant flavonoids (Peters and Long, 1988). In alfalfa, the inducing compound has been isolated and identified as luteolin. It induces nod genes in *Rhizobium meliloti* (Peters *et al.*, 1986). Different flavonoids have been identified as nod gene inducers in exudates of clover (Redmond *et al.*, 1986), soybean and pea (Kosslak *et al.*, 1987; Zaat *et al.*, 1988). Terouchi and Syōno (1990a,b) have shown that flavone, secreted from oats, induced the hair curling genes in *R. leguminosarum* biovar *trifolii* as well as attachment of biovar *trifolii* and *Bradyrhizobium* sp. to monocots in the same manner as they attach to the host dicots. These authors have suggested that the formation of infection threads depend on host specificity much more than attachment and root hair curling and that it is one of the most important factors in the determination of specificity. Nodulation genes are defined by their ability to form nodules on the correct host. Several nodulation genes have been identified, some are host specific (nod N, M, L, F, E) and some are common (nod A, B, C, D). Nodulation genes from two species are shown in figure 2.

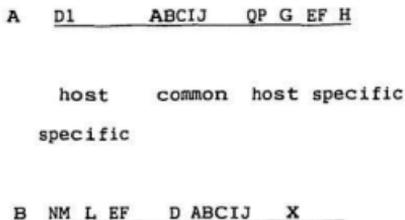


Fig.2. Map of nodulation genes in A) *R. meliloti*  
B) *R. leguminosarum* (Long, 1989).

Mutants in nod A, B or C are completely Nod<sup>-</sup> (no nodule formation). Nod A and B products are required to cause cell division for nodule formation (Dudley *et al.*, 1987) and nod C for deformation and curling of root hair (Kondorosi *et al.*, 1984; Bender *et al.*, 1987). Nod A and B products are located in the cytoplasm in *Rhizobium meliloti* and nod C has been identified as a transmembrane protein, which is also present during nodule development. John *et al.* (1988), have suggested that nod C protein may play an important role as a cell surface receptor in transducing the low molecular weight, diffusible growth factors of nod A and B from the bacterial to the plant cell. Nod A,B and C are highly conserved between rhizobia.

#### Root hair specific proteins

Root hair specific soluble and membrane bound proteins rich in calcium, cobalt and iron have been identified in peas and

soybeans (Werner and Wolff, 1987). Rhizobia have high requirements for these nutrients. These authors also suggest that besides being involved in the uptake of nutrients and water as in all root systems they may also be involved in the recognition and internalization of rhizobia.

#### I.1.3.2. RHIZOBIAL ATTACHMENT TO HOST ROOT

Rhizobia must come in contact with the legume roots and attach to the root hairs. Several factors such as plant lectins and rhizobial cell surface polysaccharides may be involved in specific binding or attachment of rhizobia to the legume root hairs. Other structures such as cellulose microfibrils, fimbriae or pili may also be involved in nonspecific attachment.

#### Lectins

Lectins are proteins produced by a wide variety of plants and bind specifically to carbohydrate moieties of the cell surface polysaccharides of rhizobia. The suggestion that lectins may play a role in the recognition of rhizobia was made as early as 1950 by Krüpe (Etzler, 1986). This proposal was later expanded by Bohlool and Schmidt (1974). They found a correlation between the binding of the rhizobial strains to lectin and their ability to infect the host plant. This is also supported by the presence of lectin at the tip of the growing root hairs and on epidermal

cells located just below the root hairs, the infection sites of rhizobia (Dazzo and Hubbel, 1975; Law and Stridjdom, 1984). Diaz *et al.* (1989), have shown that root lectin is a molecular determinant of specificity in the *Rhizobium*-legume interaction. Recently, two different types of lectin have been isolated from the peanut seeds and root nodules (Kishinivesky *et al.*, 1988). Law *et al.* (1988), have suggested that this may be vital to some aspect of the symbiotic relationship of peanut with *Bradyrhizolium* sp. The rhizobia-lectin interaction may trigger a chain of events leading to effective symbiosis (Bauer, 1981).

#### **Rhizobial surface polysaccharides**

Rhizobial surface polysaccharides include the extra-cellular polysaccharides (EPS), capsular polysaccharides (CPS), lipopolysaccharides (LPS) and  $\beta$ -glucans. The rhizobial surface polysaccharides possibly act as signals or substrates for signal production and also as osmotic material necessary during recognition and infection process. EPS and LPS may vary in a species specific manner and may react with lectins. CPS has been shown to be modified by plant cell enzymes in clover-*R. leguminosarum* biovar *trifolii* interaction (Dazzo *et al.* 1979), in which the CPS is digested except for a few spots and the rhizobia binds to the root hair in an end-on manner. Complete absence or alterations of surface polysaccharides result in the formation of empty nodules, due to the failure of rhizobial

mutants to infect (Finan *et al.*, 1985; Leigh *et al.*, 1987; Müller *et al.*, 1988). Carlson *et al.* (1987) have found that a mutant of *R. leguminosarum* biovar *phaseoli*, defective in LPS, is unable to form infection threads in bean.

#### Other factors

Surface structures such as fimbriae or pili and cellulose fibrils are also shown to be involved in the attachment of rhizobia to the host (Vesper and Bauer, 1986; Kijne *et al.*, 1988).

#### 1.3.2. INFECTION

The next step after recognition of the specific host by the rhizobia and attachment to the root hairs is infection of the host root. Infection of legumes by rhizobia primarily occurs through root hairs by the formation of an infection thread (Bauer, 1981). Rhizobia may also invade the roots through cracks/wound as described for *Arachis* (see section I.4) and *Stylosanthes* (Sprent, 1989). The plant cell wall is a formidable defense against invading organisms. It is not well understood how rhizobia recognize and penetrate the plant cell wall. Whether active degradation occurs or not is still not clear. Following the initial attachment to root hairs, rhizobia produce substances that cause root hair curling. Invasion occurs via the formation of an infection thread which seems to

invaginate from the root cell wall enclosing the bacteria and grows toward the root cortex (Robertson and Lyttleton, 1982). The synthesis and composition of the infection thread was thought to be similar to that of the cell wall. Higashi et al. (1986), have shown that the infection thread was not affected by the cell wall degrading enzyme driselase. The infection thread matrix contains a plant glycoprotein (Bradley et al., 1988). The infection thread attaches to the cell wall, which is facilitated by the formation of the funnel shape (Higashi et al., 1986) and then the bacteria in the infection thread may be acclued in the interspace between the cell wall and infection thread sheath. The rhizobia secrete specific enzymes (Higashi and Abe, 1980) which degrade the host cell wall but not the infection thread sheath. In the next cell a continuous infection thread sheath is formed. Recently Roth and Stacy (1989b) have shown, by using mutant strains of *Bradyrhizobium* that are not released from the infection thread, that the endoplasmic reticulum (ER) synthesis signal and the infection thread wall digesting vacuoles are essential for the release of the bacteria from the infection thread. The released rhizobia are enclosed within a membrane (peribacteroid membrane) of plant origin.

### Peribacteroid membrane envelope

The bacteroids in the infected cells are compartmentalized within the peribacteroid membrane envelope. The peribacteroid membrane is of plant origin (Verma *et al.*, 1978; Mellor *et al.*, 1985). Two views have been proposed on the origin of the peribacteroid membrane (Mellor and Werner, 1987). The first view is that the peribacteroid membrane is derived from the plasma membrane (Verma *et al.*, 1978; Blumvald *et al.*, 1985) and the other proposal is that it is synthesized by the ER directly or through the golgi apparatus (Robertson *et al.*, 1978; Mellor *et al.*, 1985). Brewin *et al.* (1985), have shown that monoclonal antibodies raised against antigens in the peribacteroid membrane cross react with the plasma membrane and golgi, suggesting common antigens. Roth and Stacey (1989a,b), have shown that the peribacteroid membrane is composed of membrane from the host infection thread membrane, ER and *denovo* synthesis at the time of release of rhizobia from the infection thread and suggest that the membrane synthesis is carried out by the ER and golgi apparatus.

The peribacteroid membrane is important in that it may protect the bacteroids from plant defense mechanisms (Vance and Johnson, 1983). This view is supported by experimental evidence presented by Mellor *et al.* (1985) and Werner *et al.* (1985), who observed that the naked bacteroids elicit a plant phytoalexin response. The peribacteroid membrane, due to its strategic

position, must play an important role in nitrogen fixation by controlling the passage of molecules between the symbiont and the host (Fortin *et al.*, 1985; Price *et al.*, 1987). A dicarboxylate transporter has been identified in the peribacteroid membrane (Udvardi *et al.*, 1988). The peribacteroid membrane also contains several nodulins (nodule-specific proteins), such as nodulin 24 and 26 (Fortin *et al.*, 1985, 1987; Verma *et al.*, 1986). Nodulin 26 is expressed independently of peribacteroid membrane formation, whereas other nodulins depend on the appearance of this membrane (Morrison and Verma, 1987). Based on studies using mutants of *Bradyrhizobium japonicum*, Werner *et al.*, (1988) and Mellor *et al.* (1989), suggest that the rhizobia are responsible for at least four different signals leading to the formation of peribacteroid membrane by the host plant.

The peribacteroid space, which lies between the peribacteroid and the bacteroid outer membrane houses several activities found in the vacuoles, such as proteases, alpha mannosidase (Mellor *et al.*, 1984), trehalase (Mellor, 1988) and protein protease inhibitors (Garbers *et al.*, 1988). Mellor (1989), proposed that the endosymbiont is within a specialized host plant lysosome based on the biochemical evidence that has been accumulating suggesting similarities between the endosymbiotic compartment and lysosomes.

## Leghemoglobin and Nodulins

The products of plant genes specifically expressed in the nodules are known as nodulins (Van Kammen, 1984; Verma *et al.*, 1986). The presence of over 20 nodulins in pea, soybean, alfalfa and some other legumes have been reported so far (Delauney and Verma, 1988). Some of these nodulins are specific for the peribacteroid membrane (Fortin *et al.*, 1985). Nodulins are also differentially and sequentially expressed in the infected, uninfected and cortical cells (Kouchi *et al.*, 1989; Scheres *et al.*, 1990) of soybean root nodules. They have been divided into early and late nodulins. Late nodulins are expressed after the infection and nodule structure formation. Early nodulins (ENOD) have been shown to be involved in the formation of the inner cortex of the nodule (Van de Wiel *et al.*, 1990) and in the infection process (Scheres *et al.*, 1990). The functions and sites of individual nodulins are largely unknown, except for Leghemoglobin proteins (Appleby, 1984; Van den Bosch and Newcomb, 1988), uricase (Van den Bosch and Newcomb, 1986) and glutamine synthetase (Verma *et al.*, 1986; Brangeon *et al.*, 1989), a few which have been well characterized. Leghemoglobin, which used to be the characteristic feature of legume nodules, is also reported to be present in non-legume roots (Bogusz *et al.*, 1988). Leghemoglobins are similar to animal hemoglobins (Appleby, 1984) and occur as several types in legumes and non-legumes (Appleby *et al.*, 1988).

### I.3.3. NODULE STRUCTURE

The nodule structure varies with different host species. In general they are divided into two groups (Sprent 1979; Bergersen, 1982).

1. Indeterminate nodules: Temperate legumes such as *Pisum sativum*, *Medicago sativa* and *Trifolium* sp. develop indeterminate nodules characterized by a defined meristem during nodule development with an open vascular system. Nodules are cylindrical and elongated and arise from the cortical tissue close to the endodermis in the root.

2. Determinate nodules: Tropical legumes such as peanut, soybean, lupins and cowpea nodules are spherical with determinate growth and a closed vascular system. These nodules arise from the outer cortical region just below the epidermis of the root.

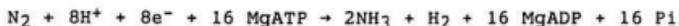
### I.3.4. NITROGEN FIXATION

Nitrogen fixation occurs in symbiotic nodules housing transformed bacteroids enclosed within the peribacteroid membrane. In some legumes the rhizobia are not released from the infection thread (Sprent, 1989). The enzyme nitrogenase (EC 1.19.6.1) located in the cytoplasm of the bacteroids catalyzes the conversion of atmospheric nitrogen to ammonia. Industrially the conversion of nitrogen to ammonia requires an iron catalyst, 300 atm pressure and 300° C, whereas nitrogenase

performs this reaction at room temperature and atmospheric pressure.

### Structure and reaction mechanism of nitrogenase

Nitrogenase complex is composed of dinitrogenase reductase and dinitrogenase. Nitrogenase is irreversibly inactivated by oxygen. Leghemoglobin regulates the oxygen level in legume root nodules (Appleby, 1984). Nodule structure has also been suggested as being involved in regulating the oxygen diffusion in legumes (Hunt *et al.*, 1987; Witty *et al.*, 1987; Dakora and Atkins, 1989). Dinitrogenase reductase is about 60 kD, is composed of two identical subunits (Postgate, 1982) and has an  $Fe_4S_4$  center capable of transferring single electrons. The dinitrogenase protein is about 218-245 kD. It is a tetramer composed of two identical subunits of ca 50 kD each and ca 60 kD each (Postgate 1982). Dinitrogenase contains ca 30 iron atoms, ca 32 sulfides and 2 molybdenum atoms per tetramer. The iron, molybdenum and sulfur atoms are thought to be arranged in four  $Fe_4S_4$  centers, two  $Fe_8S_6Mo$  centers (iron-molybdenum cofactor, FeMoco) and possibly two sulfur centers each containing two iron atom. The FeMoco is believed to be the active substrate-reducing site of nitrogenase. Hydrogen evolution is an inherent property of nitrogenase and the currently accepted stoichiometry for nitrogen fixation is:



A schematic representation of the nitrogenase reaction mechanism is shown in Fig. 3. Nitrogenase requires high energy input in the form of ATP and a source of  $H^+$  and  $e^-$  for dinitrogen reduction. Nitrogenase is a slow enzyme since after the transfer of one electron from the Fe protein to the FeMo cofactor it dissociates. The FeMo cofactor stores the electrons before transferring them to the substrate ( $N_2$ ). Hydrolysis of Mg.ATP to Mg. ADP and Pi occurs simultaneously with electron transfer. Nitrogenase accounts to 5-30% of total rhizobial cell protein (Haaker and Klugkist, 1987).

#### Energy source for nitrogenase

It has been well established that the photosynthate is converted into dicarboxylic acids in nodule cells and oxidized in the bacteroids via the citric acid cycle (Tajima *et al.*, 1986; Kouchi and Yoneyama, 1986; Streeter and Salminen, 1988). Organic acids also increase the rate of bacteroidal respiration and nitrogenase activity (Ramaswamy and Bal, 1986; Tajima *et al.*, 1986). Succinate and oxygen have been shown to stimulate the accumulation of ATP in the bacteroids (Tajima and Kouzai, 1989). The organic acids are demonstrated to be essential for bacteroid metabolism and effective symbiotic nitrogen fixation (Ronson *et al.*, 1981; Finan *et al.*, 1983; Humbeck and Werner, 1989). Khan *et al.*, 1985, have proposed that amino acids could also be used by the bacteroids as an additional source of energy for nitrogen fixation.

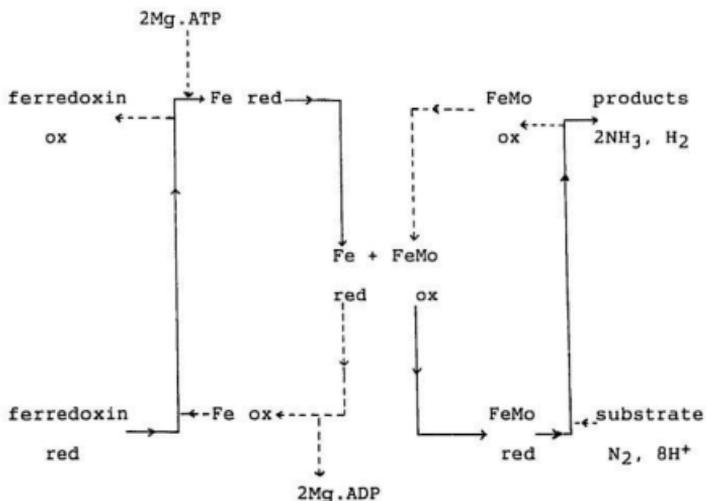


Fig. 3. Schematic diagram showing the nitrogenase reaction mechanism (Dixon and Wheeler, 1986). It is possible for two iron proteins (Fe) to bind to the iron-molybdenum protein (FeMo). The pathway for the electron is shown by the solid line. The oxidized (ox) or reduced (red) state of the components are also indicated.

#### I.3.5. NITROGEN ASSIMILATION

Nitrogen fixed by the *Rhizobium* bacteroids in legume root nodules is excreted as ammonia to the surrounding host cell. This ammonia is assimilated into the amide group of glutamine and then metabolized into the major transporting form, asparagine in temperate legumes such as lupin, pea, clover and peanut (Shelp and Atkins, 1984) and in the form of ureides, allantoin and allantoic acid in tropical species such as cowpea, soybean, mung bean and pigeon pea (Schubert, 1986; Atkins, 1987). The infected cells require NADH, oxoglutarate and energy in the form of ATP. Infected cells contain high cytosolic and mitochondrial malate dehydrogenase activity (Kouchi *et al.*, 1988; Day and Mannix, 1988) and Bryce and Day (1990) suggest based on their investigation of soybean nodule mitochondria that nodule mitochondria may operate a truncated form of TCA cycle and primarily oxidize malate and export ATP and oxaloacetate to the cytosol for ammonia assimilation. The assimilated products of nitrogen is exported through the xylem.

#### I.4. SYMBIOTIC N<sub>2</sub>-FIXATION BY PEANUT

The genus *Arachis* (peanut or ground nut) is a member of the family *Leguminosae* (*Fabaceae*), subfamily *Papilionoideae*, tribe *Aschynomenae*, subtribe *Stylosanthinae*. Wynne and Halward (1989) describe its members as perennial or annual herbaceous

plants with tri- or tetrafoliate, stipulate leaves, papilionate flower, tubular hypanthium and sessile ovary. The fruits are formed underground because of the formation of the unique pegs, which are formed by the elongation of the intercalary meristems closer to the basal ovule and between the remaining ovules. Peanuts flower and set pods over a period of several weeks and hence take a longer time to mature. Because of this it is also difficult to harvest over 80% of the potential crop produced (Hartwig, 1989).

Peanut or groundnut was discovered and domesticated by the original inhabitants of South America, probably the natives in the area of Peru and Brazil. Peanut was introduced to Europe by the Spanish and later in the 16th century carried over to Africa by the Portuguese. It was also introduced to the East Indies in the same era through regular trade routes between Mexico and the Philippines. Groundnut became an important crop during late 19th century and large scale production as an oil yielding crop followed in the twentieth century (Aykroyd *et al.*, 1982).

Peanuts are used directly as food, roasted, boiled or processed into peanut butter and refined peanut oil is used for cooking purposes in several countries. Apart from contributing nutritionally (high energy calorie source ca. 9 Kcal/g of triglyceride) peanut oil also increases the palatability, flavor, texture, mouth feel and structure of the food (Stauffer, 1989).

## Peanut root nodule formation

*Arachis hypogaea* L. var. Jumbo Virginia used in this investigation is a cultivated variety of peanut. The cultivated peanut is self pollinating and the occurrence of natural crosses are rare. Peanut interacts with *Bradyrhizobium* sp. 32H1 and forms root nodules. The infection process in peanut by rhizobia differs from cowpea or soybean where infection normally occurs through the formation of infection threads as described earlier (section I.3.2). In peanut, rhizobia cause curling, root hair deformations and enter the root through the opening caused by the emerging lateral roots. Chandler (1978) observed that infection occurred in the large basal cells found at the base of the root hairs. A non-nodulation trait is controlled by two recessive genes and seem to be associated with the absence of root hairs (Nigam *et al.*, 1982; Nambiar *et al.*, 1983) and is root controlled (Vance *et al.*, 1988). The invaded basal cells divide repeatedly, become smaller and are incorporated in the nodule tissue. The rhizobia infect the cortical cells through intercellular movement. The membrane-bound bacteria are released into the host cell through an altered cell wall. After release in the host cell, the rhizobia multiply rapidly, surrounded by the peribacteroid membrane. The infected cells also divide repeatedly. The transformation of rods into spherical-shaped bacteroids occurs only after the host cell ceases to divide. The rhizobia undergo drastic morphological changes during transformation into spherical

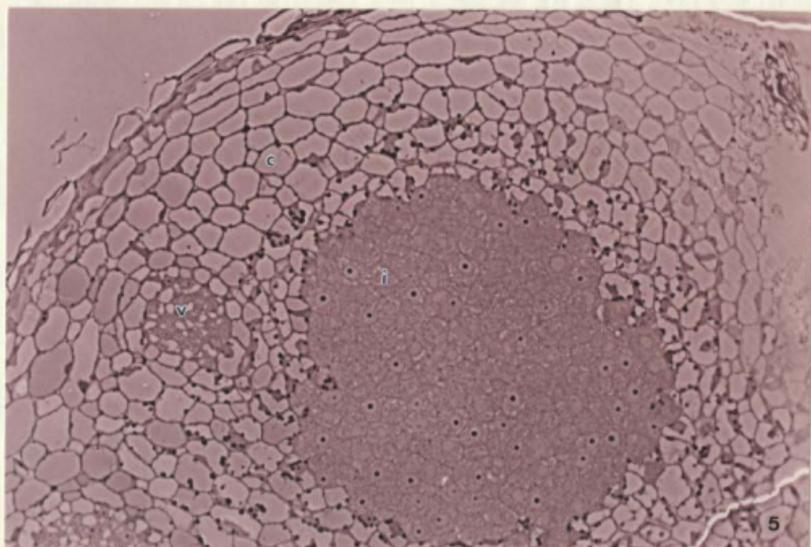
bacteroids. Bal *et al.* (1985), have reported that the rhizobia replace their outer membrane before they differentiate into bacteroids.

#### Nodule structure

Peanut root nodules are spherical in shape (Figs. 4 and 5), of determinate type, with a centrally-infected zone filled with spherical bacteroids and are surrounded by uninfected cortical cells. The surface of the peanut root nodules is smooth and continuous. Sen *et al.* (1986), have found remarkable differences between the nodule structure and organization of peanut and cowpea root nodules infected by the same strain, *Bradyrhizobium* sp. 32H1. The rhizobial bacteroids remained rod-shaped in cowpea but transformed into spherical shaped bacteroids in peanut (Staphorst and Strijdom, 1972). All the cells in the infected zone of peanut nodules were infected except for rays of uninfected cells dividing the infected zone into two or three compartments (Hameed, 1986). Whereas in cowpea, the infected zone was interspersed with uninfected cells. The infected cells of peanut have been found to be isodiametrical and uniform in size with a central vacuole and a nucleus surrounded by tightly arranged bacteroids enclosed singly in a peribacteroid membrane envelope (Bal *et al.*, 1985; Sen *et al.*, 1986). Dense bodies showing positive reaction with diaminobenzidine (DAB) staining is closely associated with the bacteroids and may be involved in the catabolism of oleosomes

Fig. 4. Peanut root system showing nodules.

Fig. 5. Cross section of a peanut root nodule showing Cortex (C); Infected cells filled with bacteroids(I) and the vascular bundles (V).



260 x

(Bal, 1986). Further investigation of dense bodies is required to form any firm conclusion. The oleosomes (lipid bodies) have also been reported to be present in the infected and uninfected cells of peanut root nodules (Hameed, 1986; Emerson and Bal, 1988) and absent in cowpea root nodules formed by infection of the same strain of *Bradyrhizobium*. It has been suggested by Sen *et al.*, (1986) that the hosts play the dominant role and control the differentiation of the nodular tissue and the morphogenesis of the bacteroids.

Nitrogenase activity of peanut root noudes have been shown to be significantly higher than those of cowpea inoculated with the same strain (Sen and Weaver, 1980, 1981, 1984a,b; Hameed, 1986). While the extreme modifications of the peanut bacteroids as such is not responsible for the higher specific activity of nitrogenase in the peanut root nodules (Sen and Weaver, 1984a) it has been suggested that the oleosomes observed in the peanut nodule could provide an additional source of energy apart from the direct supply of photosynthate to the bacteroids (Bal *et al.*, 1989).

#### I.4.1. OLEOSOMES

Oleosomes have been referred to as spherosomes (Mülethaler, 1955; Frey-Wyssling *et al.*, 1963; Hrsel, 1966; Sorokin, 1967), fat/lipid bodies (Ching, 1972; Lin and Huang, 1983) and

oil bodies (Roughan and Slack, 1982; Herman, 1987), but as an ubiquitous oil-storing body found in all tissues, the term "oleosome" coined by Yatsu *et al.* (1971), seems to be most appropriate. Therefore, this term is consistently used to mean fat/lipid/oil bodies or spherosomes. Most of our knowledge of oleosomes is derived from the study of oil storing seeds which contain storage lipids in the form of triacylglycerols comprising about 20-50% of the total dry weight of the seed (Appelqvist, 1975; Gurr, 1980; Roughan and Slack, 1982). The lipid reserve is synthesized during seed maturation to provide energy and carbon skeleton for the growth and development of the germinating embryo (Huang *et al.*, 1986). The presence of oleosomes in peanut root nodules is unusual (Bal *et al.*, 1989) and are not commonly observed in other temperate and tropical legumes. It is not known how they are synthesized in the peanut nodules. However, based on the evidence obtained from the oil rich seeds two models have been proposed as to the origin of oleosomes.

#### Ontogeny of oleosomes

The origin of oleosomes from the endoplasmic reticulum (ER) was first proposed by Frey-Wyssling *et al.* (1963) and later elaborated by Schwarzenbach (1971). There is little evidence to indicate that oleosomes contain enzymes required for triacylglycerol biosynthesis. Ninety percent of the lipid present in the oleosomes is in the form of triacylglycerol, the

remainder is mostly phospholipids (Gurr *et al.*, 1974). Fatty acids are known to be synthesized in plastids (Appelqvist, 1975; Gurr, 1980; Roughan and Slack, 1982; Harwood, 1989). In spinach leaves plastids have also been demonstrated as the site of triacylglycerol synthesis (Martin and Wilson, 1984). The triacylglycerol synthesis is shown in microsomes of safflower *in vitro* (Stobart *et al.*, 1986), which presumably represent the ER. Triacylglycerol synthesis is known to be associated with the rough ER in the scutella of maize, cotyledons of peanut and soybean and in the endosperm of castor bean (Huang *et al.*, 1986). The mechanism of transport of fatty acids from the plastids to the ER and from the ER to the oleosomes is not clearly known. Based on three dimensional reconstruction of sections from cotyledons of *Cuphea* embryonic cells, Deerberg *et al.*, (1990) have suggested that the polymorphic form of the plastids greatly increases their surface area and this in turn could guarantee an intimate contact with the ER and facilitate the transacylation of free fatty acids synthesized in the plastids to form triacylglycerol. It has been proposed that the newly-synthesized triacylglycerol is accumulated between the bilayers of the ER which then pinches off to form the oleosomes surrounded by a monolayer of phospholipids (Wanner and Theimer, 1978; Wanner *et al.*, 1981). Alternatively, oleosomes could arise in the cytoplasm by condensation of triacylglycerol molecules followed by formation of the membrane (Bergfeld *et al.*, 1978). Peanut root nodules seem to be well equipped to form oleosomes. All the compartments involved in the synthesis of

triacylglycerol and oleosomes from the photosynthate provided in the form of sucrose must be present in the infected and uninfected cells. The oleosomes are present throughout their developmental stage and even in senescent nodules.

#### I.5. OBJECTIVES

The unusual presence of oleosomes in the infected and uninfected cells, as well as the high nitrogenase activity of the peanut root nodules prompted this investigation of oleosomes. This is the first instance where, an attempt was made to characterize the oleosomes and to determine their role in the nodule. The oleosomes present in the peanut root nodules appear similar to that of the seed, however their characteristics are shown to be different by ultrastructural observations in these two functionally different organs. The distribution, composition and the functional involvement of oleosomes in the peanut root nodules are examined in this investigation and compared with seed oleosomes.

## II. MATERIALS AND METHODS

### II.1. PLANT MATERIALS

Peanut (*Arachis hypogaea* L. var. Jumbo Virginia) seeds were germinated on wet paper towels and inoculated with broth culture of *Bradyrhizobium* sp. 32H1 (Sen and Weaver, 1980). The germinated peanut seeds were transferred to pots filled with vermiculite and were grown in environmental chambers. Cowpea (*Vigna unguiculata* L. var. Black Eye) were washed in distilled water and planted directly in the pots of vermiculite. The plants were grown under day/night conditions of 16 h/8 h, 27° C/22° C, 70%/50% humidity and approximately 700  $\mu\text{mol m}^{-2} \text{s}^{-2}$  photon flux density. The plants were irrigated with nitrogen-free nutrient solution (Ellfolk, 1960). Plants grown in the fields of the International Crop Research Institute for Semi-Arid Tropics (ICRISAT), Patancheru, A. P., India, were also sampled by Dr. A. K. Bal during a visit to the institute, for ultrastructural observation and to study the distribution of oleosomes (morphometric analyses) in the field grown peanut root nodules.

### II.2. BRADYRHIZOBIUM CULTURES

*Bradyrhizobium* sp. 32H1 was obtained through the courtesy of Nitragin Co. Milwaukee, WI, USA. The cultures were maintained in yeast extract mannitol agar and cultured in a broth

containing 10 g mannitol, 0.5 g  $K_2HPO_4$ , 0.2 g  $MgSO_4 \cdot 7H_2O$ , 0.1 g NaCl, 0.4 g yeast extract in 1 l of distilled water at pH 6.8-7.0 with constant shaking in an Orbit Environs Shaker, Lab-Line Inc. at 28° C (Vincent, 1970).

### II.3. LIGHT AND ELECTRON MICROSCOPY

Thin slices of peanut root nodules at different stages of development (immature: 1-1.5 mm in dia., white; mature: 1.5-2.5 mm in dia., pink; mature/old: >2.5 mm in dia., pink) were fixed in a mixture of 5 ml of 50% (w/v) glutaraldehyde and 2 g of paraformaldehyde (Karnovsky, 1965) in 50 ml of 0.1 M cacodylate buffer pH 7.2 for 2 h, washed in the same buffer, and post fixed in 1% (w/v) osmium tetroxide ( $OsO_4$ ) in 0.1 M cacodylate buffer pH 7.2 for 1h at 4° C. The samples were then washed three times with buffer and dehydrated through an ethanol series 25-100% (v/v) and embedded in Spurr's embedding medium (Spurr, 1969). For lipid preservation, some of the samples were treated with 1% (w/v) p-phenylenediamine (PPD) in 70% ethanol for 30-60 min. during dehydration to preserve the lipids from being extracted (Bal, 1990). PPD also enhances lipid staining by reacting with the reduced and unreduced bound osmium (Ledingham and Simpson, 1972; Boshier *et al.*, 1984). As a control the samples were treated with hexane for 45 min. after fixation in the aldehyde mixture and subsequent dehydration in ethanol series prior to  $OsO_4$  treatment. This helped to differentiate the oleosomes from the dense bodies, which were not extracted,

whereas the oleosomes appeared as empty spaces. The control samples were then rehydrated, treated with  $\text{OsO}_4$  and 1% pPD and processed as described above. Cowpea root nodule slices were processed in the same manner for comparison.

The semithin sections (0.5-2.0  $\mu\text{m}$ ) of pPD treated samples were viewed with a Zeiss light microscope without further staining. Ultrathin sections were post-stained with uranyl acetate and lead citrate and viewed with a Zeiss EM 109 transmission electron microscope. For morphometric analyses, positive slides were made at a magnification of 100-160x using a Zeiss photomicroscope. The slides were viewed under a Wild Heerbrugg dissecting microscope (Wild Type 181300) with camera lucida at magnifications ranging from 2,000-10,000x. The measurements were made on 60-100 cells in each stage with a Houston Instruments Hipad digitiser on line with an Apple II computer.

#### II.4. TOTAL LIPID ESTIMATION

The amount of total lipid present in the peanut and cowpea root nodules was estimated by the gravimetric method described by Bligh and Dyer (1959). A mixture of chloroform and methanol in a ratio of 2:1 (v/v) was used to extract lipid from the nodule homogenate. The chloroform layer was separated by centrifugation at 400 g. A small amount of chloroform containing extracted lipid was taken in a pre weighed vial and

was evaporated under nitrogen gas. The vial with the lipid residue was weighed again by using a micro-balance to estimate the amount of total lipid. Protein present in the nodules were estimated according to Lowry *et al.*, (1951).

## II.5. OLEOSOMES

II.5.1. Isolation: Peanut root nodules were homogenized in 50 mM tris.HCl buffer containing 0.5 M NaCl, pH 7.2 (Yatsu and Jacks, 1972), centrifuged at 400 g for 5 min. or filtered through 12 layers of cheese cloth and the supernatant was centrifuged at 30,000 g for 20 min. A tiny fat pad floating on top contained the oleosomes.

II.5.2. Ultrastructure: The fat pad was smeared on a slide and stained with alcoholic Sudan III and observed with a light microscope. The fat pad was also immobilized in 2% (w/v) agar (Jacks *et al.*, 1967), fixed overnight in 6% (w/v) glutaraldehyde in 0.1 M phosphate buffer, pH 7.2 and post fixed in 2% (w/v) OsO<sub>4</sub> in the same buffer and processed for electron microscopy as described earlier. The control fraction of the fat pad was treated with hexane overnight after fixation in 6% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2 and dehydration through a series of ethanol up to 100%. After hexane treatment the control samples were rehydrated, post-fixed in 2% OsO<sub>4</sub> in 0.1 M phosphate buffer, pH 7.2 rinsed in the same buffer, dehydrated and embedded for electron microscopy. The fat pad

fraction was also smeared on a coverslip, fixed as above and treated with 1% pPD in 70% ethanol during dehydration. The samples were critical point dried in 100% ethanol, mounted on aluminium stubs and coated with gold for observation using a Hitachi S570 scanning electron microscope.

II.5.3. Fatty acid analyses: Lipids were extracted from the oleosome fraction to determine the fatty acid composition by using a solvent mixture of chloroform:methanol (2:1 v/v) (Bligh and Dyer, 1959). A small quantity (few crystals) of hydroquinone was added as an antioxidant to the solvent mixture. The chloroform was evaporated under nitrogen gas. The samples were transmethylated by the addition of 2 ml of sulfuric acid and methanol mixture (1:15 v/v) and heating at 55-60° C for 4-5 h. Then the methyl esters were extracted three times with 1.5 ml of hexane. The hexane layers were combined and rinsed twice with distilled water to remove residual sulfuric acid. This was followed by evaporation of hexane under nitrogen and the residual samples were reconstituted in carbon disulfide for gas chromatographic analyses. The samples were analyzed in a Perkin Elmer 8310 gas chromatograph at 185° C having a wall coated open tubular column. (Supelco sp-2330, 0.25 mm in dia., x30 m long). The fatty acid peaks were identified by comparison with known standards.

For comparison, the oleosomes from dry peanut seeds were also isolated, processed for scanning electron microscopy and gas

chromatographic analyses using similar methods as above.

## II.6. CYTOCHEMICAL LOCALIZATION OF LIPOLYTIC ACTIVITY

Lipolytic activity was localized following the cytochemical method recommended by Chard and Gay (1986), in which strontium chloride was used as the capturing agent. Other methods (Nagata, 1974) were also tried but did not produce consistent results. Thin slices of nodules and germinating peanut seeds soaked in distilled water for 5 days were incubated at 37° C for 3 h in a medium containing 1 ml of 5% (v/v) tween 80 (Polyoxyethylene 20 sorbitan mono-oleate, J. T. Baker Chemical Co., N. J., USA.), 2.5 ml of 0.2 M tris. maleate buffer pH 7.2, 1 ml of 20 mM strontium chloride, 2.5 ml of 2.5% (w/v) sodium taurocholate and 18 ml of distilled water, after fixation in aldehyde mixture (as above) at 0-4° C and washing in 0.1 M cacodylate buffer pH 7.2. The samples were then washed with 2% EDTA (w/v) in the same buffer, treated with 1% OsO<sub>4</sub> also in the same buffer and processed for electron microscopy as above. Control sections were incubated in the above medium without tween 80.

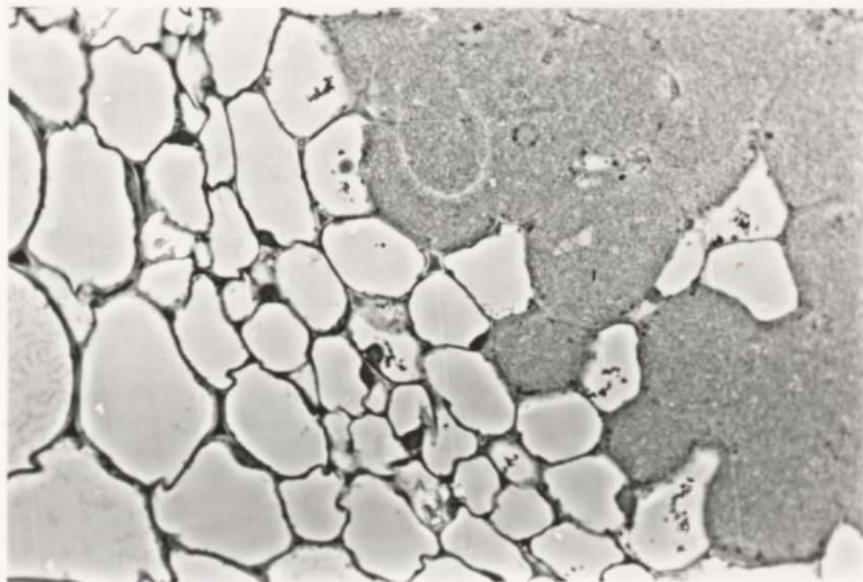
### III. RESULTS

#### III.1. DISTRIBUTION OF OLEOSOMES

The oleosomes were well preserved for both light and electron microscopy by *en bloc* staining with pPD. Oleosomes were not observed in cowpea root nodules (Fig. 6), however they were abundant in the infected and uninfected cells of peanut nodules (Fig. 16) formed by infection of the same bacterial strain (*Bradyrhizobium japonicum* Sp. 32H1). In peanut nodules, the oleosomes in the infected cells were very close to the peribacteroid membrane (Fig. 7). In many instances the oleosomes were found to be in contact with the peribacteroid membrane and adpressed to the bacteroid surface (Fig. 8). About 65.3% of the oleosomes had been estimated to be in close contact with the peribacteroid membrane from 22 electron micrographs (taken at a magnification of 3000x) of different nodule cells from seven plants. In other cases the oleosomes were in close proximity, within about 0.5  $\mu$ m of the peribacteroid membrane. The amount of total lipid estimated by the gravimetric method, was also comparatively higher in peanut nodules than in cowpea (Table 2). Morphometric analyses revealed about 4-8% of the peanut root nodule cell area to be occupied by oleosomes with significant differences in their numbers between immature and mature stages of nodule development (Table 3). The mature nodules from field grown plants also showed a similar distribution of oleosomes to that of mature/old nodules grown

Fig. 6. Semithin section of cowpea root nodule. Note the lack of oleosomes in the infected cells (I). Cf. Fig. 16, page 51.

Semithin section of peanut root nodule showing oleosomes.



410 x

Table 2

Amount of total lipid in peanut and cowpea root nodules as estimated by gravimetric method (Bligh and Dyer, 1959).

	Nodule size	Peanut	Cowpea
Total lipid $\mu\text{g}/\text{mg}$ protein	2-3mm	785.88	306.09
	<2mm	242.42	56.47
Total lipid mg/g dry wt. of nodule	1-3mm	40.43	8.25

Results presented are an average of two samples.

Figs 7-11. Electron micrographs of peanut root nodules.

Fig. 7. Electron micrograph of pPD-stained preparation showing oleosomes (L) in the vicinity of bacteroids (b). Note the higher electron density of the dense body (D) in the peribacteroid space.

Fig. 8. An oleosome (L) in contact with the peribacteroid membrane (pme) shown at a higher magnification. Note the lack of an electron-dense rim around the oleosome in this preparation without pPD staining. The bacteroid (b) cell wall is close to the peribacteroid membrane.

Fig. 9. Ultrastructure of hexane-treated nodule cells showing lack of some membrane profiles and the presence of oleosome ghosts (Lg) as empty spaces. Note that the dense body (D) remains unextracted by hexane.

Figs. 10 and 10a. Electron micrographs of a section of the hexane-treated fat pad (isolated oleosomes). The membrane and the electron-dense rim are not extracted. The oleosome ghosts (Lg) are packed together. In fig. 10a the "half-unit membrane" can be seen (arrow) where the two oleosome ghost membranes are not juxtaposed.

Fig. 11. Electron micrograph of oleosome (L) after staining by DAB reaction showing a membrane (arrow) at the periphery. Note also some possible extraction of lipid during preparations.

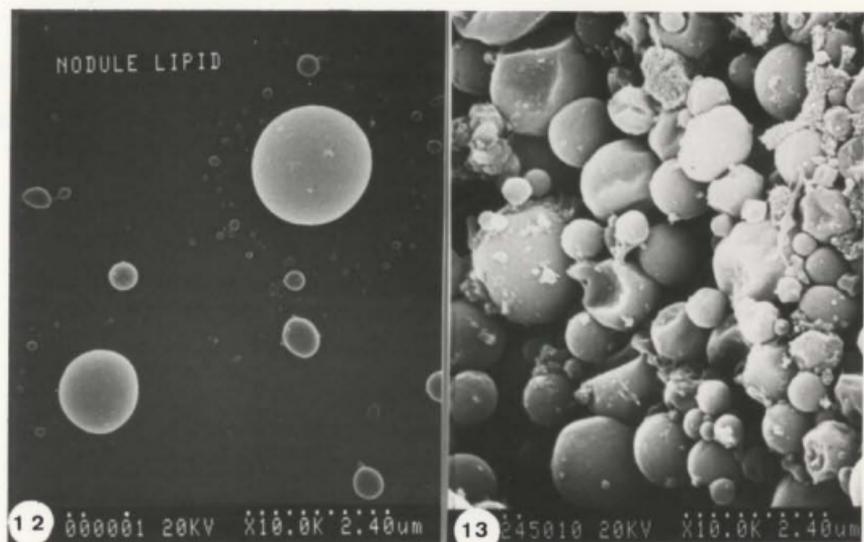


Table 3

Morphometric analyses of oleosomes in pPD treated peanut root nodule sections (1.5  $\mu\text{m}$ ).  $\pm$  s.d.

Nodule stage/size	% area of oleosomes/cell (1.5 $\mu\text{m}$ sections)	N <sup>o</sup> . oleosomes / $\mu\text{m}^2$ of cell area (1.5 $\mu\text{m}$ sections)
Immature nodules (white)/1-1.5 mm	7.840 $\pm$ 2.670	0.068 $\pm$ 0.024
Mature nodules/1.5- 2.5 mm	3.821 $\pm$ 1.252	0.032 $\pm$ 0.010
Mature/old nodules >2.5 mm	5.867 $\pm$ 1.803	0.041 $\pm$ 0.014
Field grown nodules from ICRISAT/3-4 mm	4.330 $\pm$ 0.450	0.049 $\pm$ 0.015

in environmental growth chambers.

Scanning electron microscopic observations made on oleosome fractions of nodules and seeds (Figs. 12 and 13) revealed some differences in the population classes determined by size, which varied from 0.16-5.5  $\mu\text{m}$  in dia.(Fig. 14). The greater proportion of seed oleosomes fall into the larger size class (2.0-5.5  $\mu\text{m}$  dia.) compared with the nodules. About 80% of the nodule oleosomes are between 0.16-1.0  $\mu\text{m}$  in dia. The larger oleosomes in the nodule fractions originated from the uninfected cortical cells (Fig. 15). Three distinct layers of such cells (Fig. 16) with oleosomes could be observed lying adjacent to the infected zone. In pPD treated cells the oleosomes appeared dark brown, denser than the other structures of the cell except the nucleolus (Fig. 16). The control preparations extracted with hexane revealed a clear difference between nucleoli and the oleosomes, by the disappearance of the latter (Fig. 17). In these preparations the bacteroids became more clearly visible, possibly due to the unmasking of lipids.

### III.2. ULTRASTRUCTURE OF OLEOSOMES

An electron-dense rim and some less electron-dense areas within could be seen in pPD-preserved sections of oleosomes (Fig. 7 and 15), which were not revealed in routine preparations for electron microscopy (Fig. 8). The peripheral rim of

Figs. 12 and 13. Scanning electron micrographs of nodule and seed oleosomes (isolated). Note the considerable size variations in both preparations.

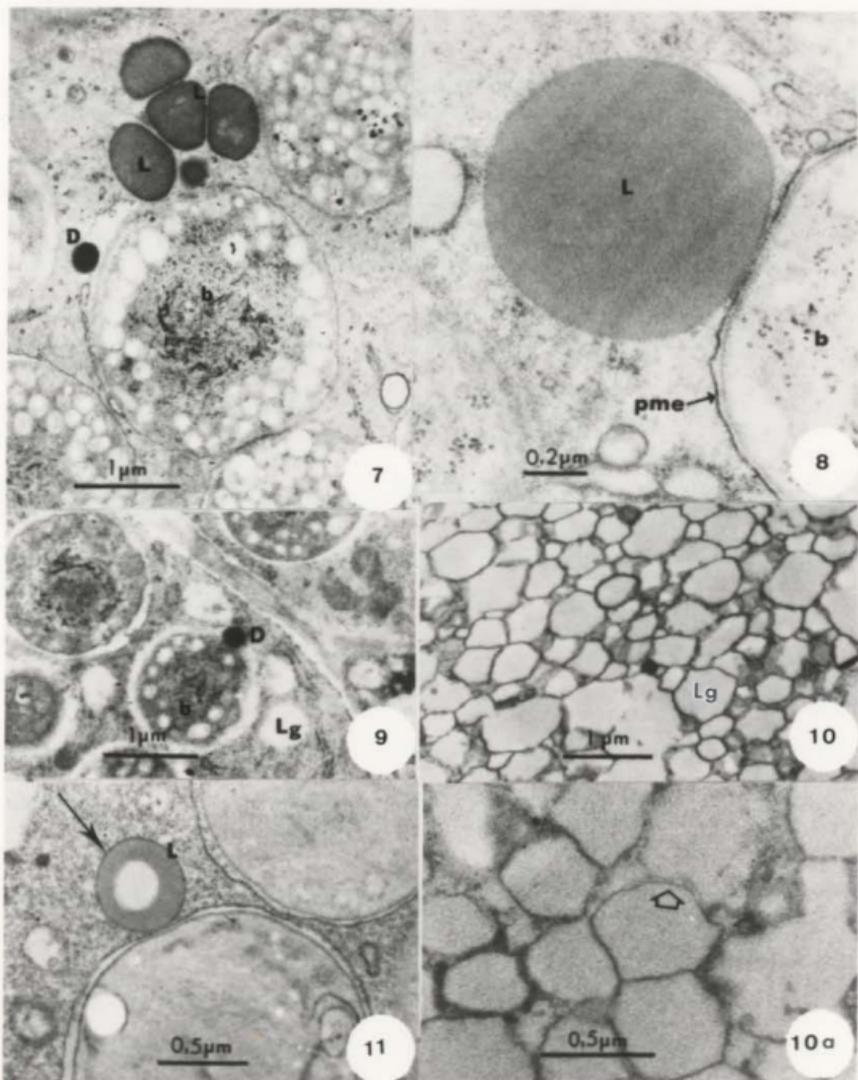


Fig. 14. A histogram showing the distribution of different size classes ( $\pm$  s. d.) of nodule and seed oleosomes.

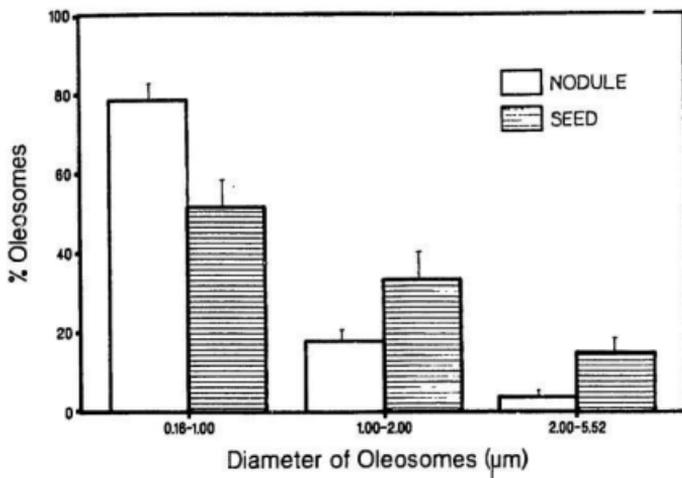


Fig. 15. An electron micrograph of pPD stained preparation showing the infected zone and the uninfected cortex and the distribution of oleosomes (L) of different sizes; the larger oleosomes are in the uninfected cells. The infected cell has spherical bacteroids (B) with dense bodies (D) in the peribacteroid space.

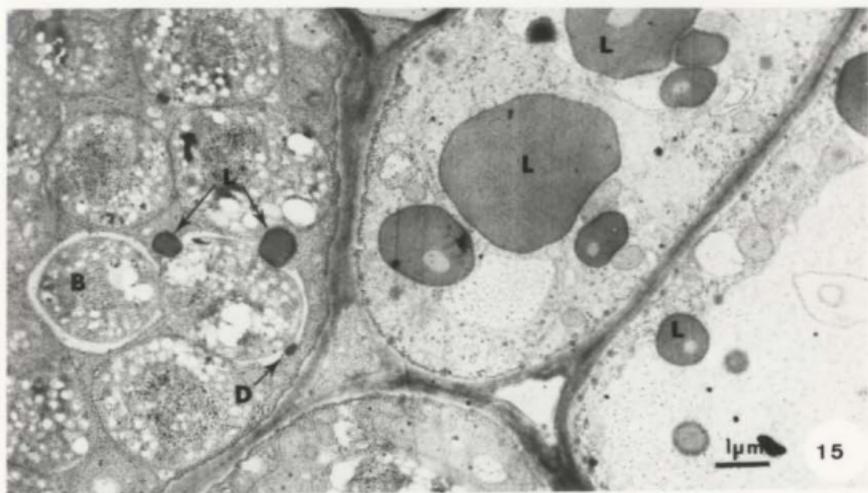
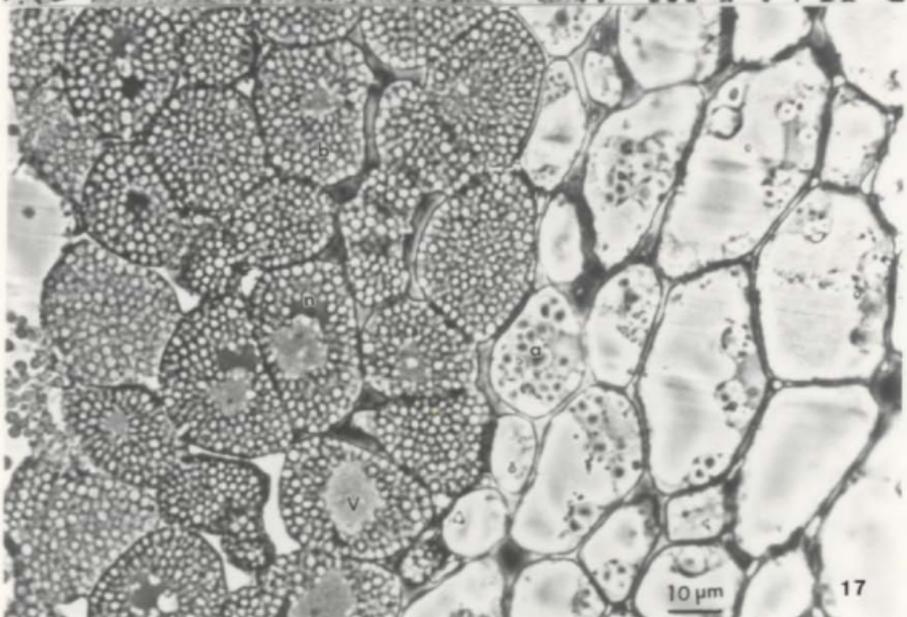
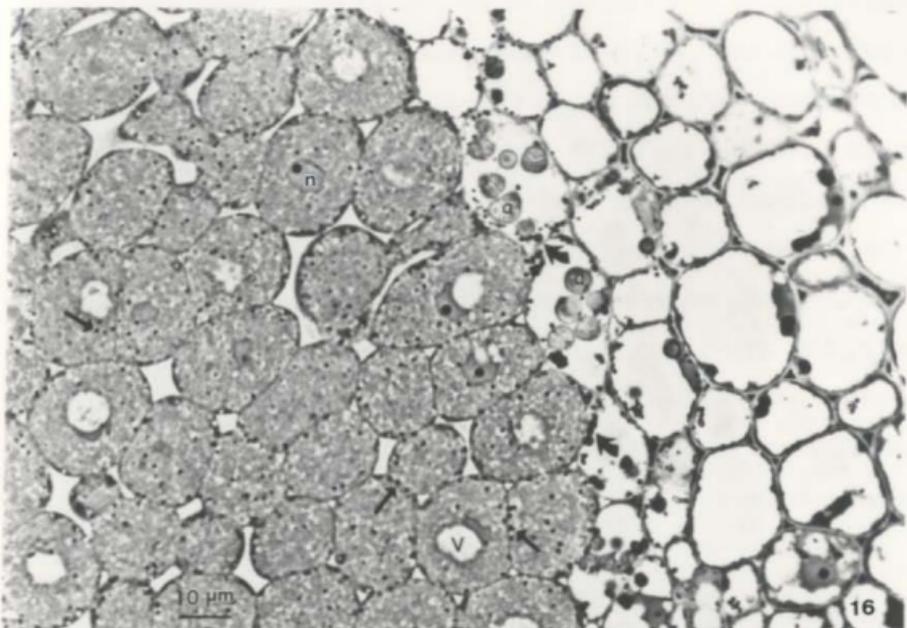


Fig. 16. Photomicrograph of a 1.5  $\mu\text{m}$  section of pPD-stained (*en bloc*) root nodule showing oleosomes (arrows), nucleus (n) with a dense nucleolus and vacuoles (v) in the infected cells. Oleosomes (arrows) are also present in the adjacent uninfected cortical cells, which are also characterized by the presence of amyloplasts (a). Note the three layers of cortical cells adjacent to the infected zone containing oleosomes, some of which are larger in size (large arrows).

Fig. 17. Photomicrograph of control preparation treated with hexane to solubilize the lipids. Note the lack of oleosomes. All other structures viz. nucleus with nucleolus (n), vacuoles (v) and the bacteroids (b) are clearly observable.



electron density was not observed in the seed oleosomes. The nodule oleosomes also tended to be irregular in shape (Figs. 7 and 15) rather than being spherical (Fig. 8). The staining of dense bodies in the peribacteroid space was also enhanced and appeared denser than the oleosomes (Figs. 7 and 24). Hexane treatment did not impair the electron density of the dense body (Fig. 9), but oleosome ghosts could be clearly identified as empty spaces. The oleosome ghosts were most convincing in hexane-extracted oleosome fractions (Figs. 10 and 10a). Formation of bilayers of oleosome membranes could be detected at the points of contact. The tripartite nature of the half-unit membrane (Yatsu and Jacks, 1972) was not very clear throughout due to the non-extractable rim surrounding the oleosomes. In certain configurations, where the oleosome membrane was not juxtaposed, a single layer measuring about 2.3 nm was apparent (Fig. 10a). The membrane surrounding the oleosomes became visible also in tissues prepared for the diaminobenzidine (DAB) reaction as shown in Fig. 11.

### III.3. LIPOLYTIC ACTIVITY

Lipolytic activity was localized by using tween 80 as the substrate and strontium chloride as the capturing agent. The activity appeared in the periphery of the oleosomes that were, in many instances, closely adpressed to the peribacteroid membrane (Fig. 18). Control preparations did not show any significant reaction product except for a few electron-dense

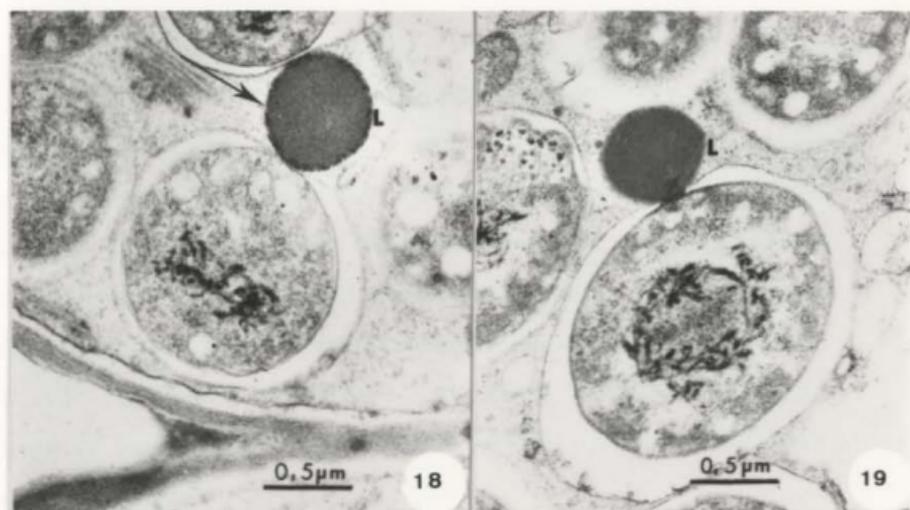
particles (Fig. 19). In the seed tissue the activity was found only in the glyoxysomes (Fig. 20). The control preparation of seed tissue did not show any reaction product (Fig. 21).

#### III.4. FATTY ACID ANALYSES OF OLEOSOMES

Fatty acid analyses of the peanut nodule oleosome and seed oleosomes were carried out on isolated fractions, which stained positive for lipids with Sudan III and also showed the presence of oleosomes when observed under the transmission electron microscope (Fig. 22) and scanning electron microscope (Figs. 12 and 13). They also retain their original configuration after isolation. The results of the analyses are shown in Table 4. The major fatty acids traced by the chromatographs were C16:0, C18:0, C18:1, C18:2, C18:3 and C20:0. Quantitative variations in the amounts of such fatty acids present in the two populations obtained from the two functionally different organs are apparent from the data. However, C22:0, which was present in relatively small amounts in the seed was totally undetectable in the nodule. C16:0, C18:0, C18:3 and C24:0 however, were present in higher amounts in the nodule oleosomes. Nodule oleosomes are comparatively richer in saturated fatty acids.

Figs. 18 and 19. Localization of lipolytic activity in peanut root nodule.

Electron micrographs of a nodule showing the granular electron-dense reaction product of lipolytic activity (arrow) around the periphery of the oleosome and the lack of it in the control preparation respectively.



Figs. 20 and 21. Localization of lipolytic activity in peanut seed cotyledon.

Electron micrographs of seed cotyledon showing lipolytic activity as a granular-dense reaction product (arrow) in the glyoxysomes (G). The control preparation (Fig. 21) does not show any such reaction products. Note the oleosomes (L) are also devoid of lipolytic activity. In such preparations the membranes of the glyoxysomes appear in negative contrast and are barely visible.

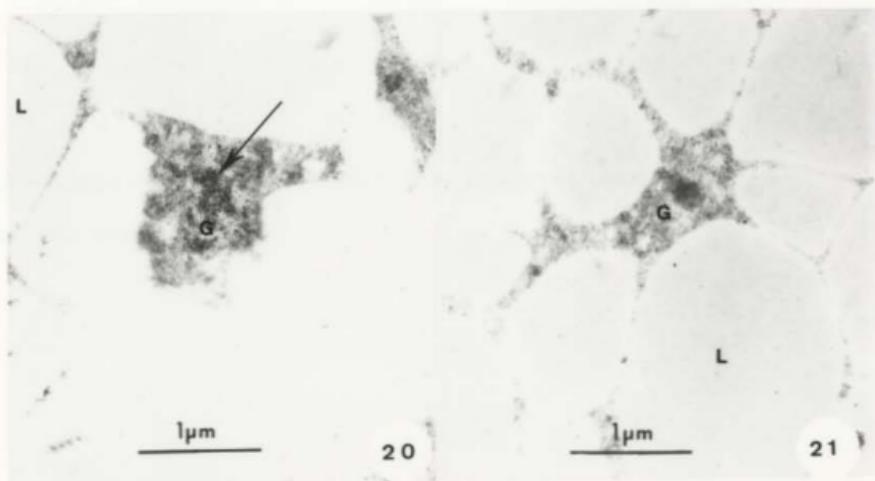


Fig. 22. Electron micrograph of the isolated nodule fat pad showing the oleosomes (L).

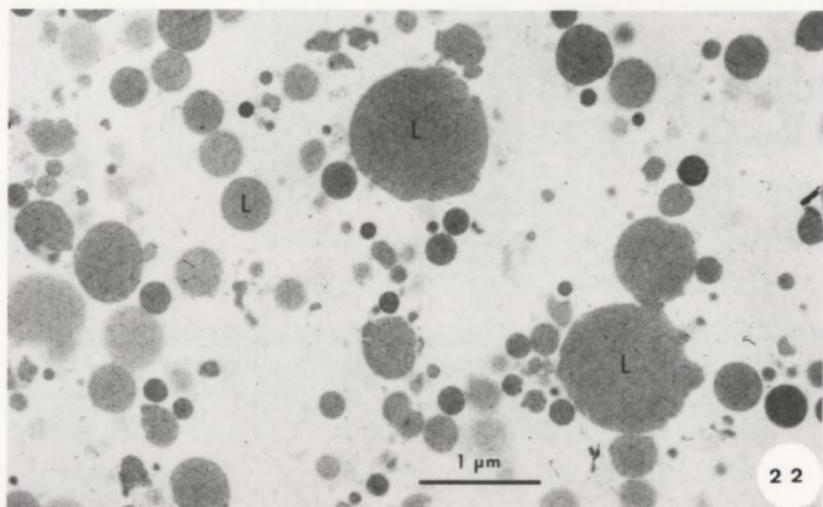


Table 4

Major fatty acid composition (% total lipid,  $\pm$  s.d. and n=4) of peanut root nodule and seed oleosomes.

Fatty acid	Nodule oleosomes	Seed oleosomes	Peanut oil*
C16:0	18.62 $\pm$ 1.88	11.92 $\pm$ 2.71	06.0 - 16.0
C18:0	28.01 $\pm$ 9.96	02.56 $\pm$ 0.88	01.3 - 06.5
C18:1	13.72 $\pm$ 3.80	52.51 $\pm$ 1.69	35.0 - 72.0
C18:2	20.47 $\pm$ 2.33	29.08 $\pm$ 3.81	13.0 - 45.0
C18:3	13.80 $\pm$ 1.64	01.93 $\pm$ 0.63	01.0
C20:0	02.51 $\pm$ 0.77	00.57 $\pm$ 0.35	01.0 - 03.0
C22:0	-	01.36 $\pm$ 0.97	01.0 - 05.0
C24:0	01.02 $\pm$ 0.65	00.16 $\pm$ 0.14	00.5 - 03.0
C24:1	-	00.21 $\pm$ 0.24	-

\*Taken from Codex (1979). Codex Alimentarius commission report, 13<sup>th</sup> session (Report of the 10<sup>th</sup> session of codex committee on Fats and oil, London 4-8 December, 1978). Alinorm 79/17.

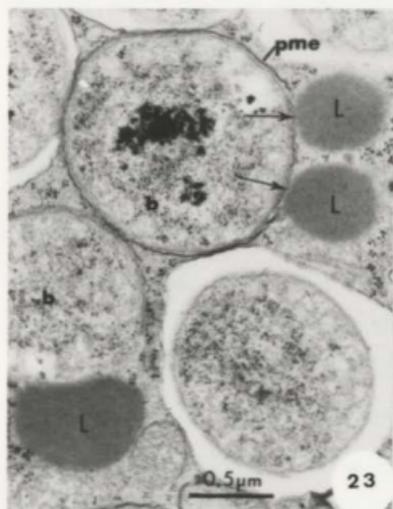
## IV. DISCUSSION

### IV.1. DISTRIBUTION OF OLEOSOMES

The presence of oleosomes in the infected and uninfected cells of peanut root nodules is unique. The oleosomes have not been observed in the infected cells of other tropical and temperate legumes. Cowpea nodules infected with the same strain of rhizobia lack oleosomes (Fig. 6). This suggests that the synthesis of oleosomes is probably under the control of the host legume. The three layers of uninfected cortical cells, in addition to the cells of the infected zone in peanut nodules, were distinctly identified in pPD treated preparations as having numerous oleosomes (Fig. 16). In some instances oleosomes appear to be in close contact with the peribacteroid membrane (Figs. 23 and 24). Oleosomes have been reported to be present in the uninfected and cortical cells as well as the newly infected cells of arctic legumes *Oxytropis* sp., *Astragalus* sp. (Newcomb and Wood, 1986) and sub-arctic legume *Lathyrus* sp. (Barimah-Asare and Bal, unpublished). Oleosomes may control the ability of the nodules in these arctic legumes to survive exposure to severe extremes of temperature (Somerville and Browse, 1991). However, well developed infected cells of these nodules lack oleosomes and are suggested to be used to support nitrogen fixation (Newcomb and Wood, 1986). Oil storing seeds predominantly contain oleosomes, which function as storage organelles. The peanut

Fig. 23. Electron micrograph of a nearly mature (28 days) nodule cell showing oleosomes (L) in intimate contact with the peribacteroid membrane (pme) and bacteroid (b). The peribacteroid membrane is not well defined at the point of contact (arrows). Not pPD treated.

Fig. 24. Electron micrograph of a mature (35 days) nodule cell, treated with pPD showing oleosome (L) in contact with the peribacteroid membrane (pme), which seem to be adpressed against the bacteroid cell surface at the point of contact (long arrow). Note that the oleosome in this preparation reveals more electron dense outer rim (outlined arrow).



root nodules are not storage organs like the seeds. The function of the nodules is related to all the activities of symbiotic nitrogen fixation. Therefore, the oleosomes present in the nodules may not be considered as serving a long term storage function, but possibly as metabolically dynamic organelles taking part in the symbiotic process.

Peanut nodules contain higher amount of total lipid than cowpea root nodules as estimated by the gravimetric method (Table 2). The oleosomes probably account for the observed higher total lipid content of the peanut root nodules. However, when compared with the seeds (Jacks *et al.*, 1967) peanut nodules contain relatively small number of oleosomes (Table 3). This has been one of the major constraints in this investigation. Morphometric analyses were found to be very useful, especially in quantitative measurements of oleosomes at different stages of nodule development. Oleosomes in nodules occupy only about 4-8% of the infected cells. In immature (white) nodules lacking leghemoglobin, oleosomes are present in significantly high numbers and also occupy more of the cell volume (Table 3). This is possibly due to the lack of nitrogen fixation in the absence of leghemoglobin (Bergersen, 1982). Leghemoglobin provides a steady low concentration of oxygen flow to the bacterial oxidases for energy generation required in the nitrogen-fixing process (Appleby, 1984, 1985; Suganuma *et al.*, 1987) and is essential for effective symbiotic nitrogen fixation (Werner *et al.*, 1984; Studer *et al.*, 1987; Pladys and

Rigaud, 1988). In the mature (pink) nodules, which are actively fixing nitrogen (Hameed, 1986; Bal et al., 1989) the oleosome number and their volume remain significantly lower. As nitrogen fixation slows down with aging of the nodules there is an increase in the number of oleosomes (Table 3). The data suggest that with active nitrogen fixation some of the oleosomes are utilized as observed in the case of the mature nodules, while maintaining a steady state in mature/old nodules, where nitrogen fixation is at a lower rate (Hameed, 1986). Accumulation of oleosomes has been observed in peanut nodules induced by fix<sup>-</sup> strains of *Bradyrhizobium* (639 and 7031) when compared with the wild type strain, NC92 (Bal and Siddique, 1991). They have reported that there is no detectable activity of nitrogenase (nitrogen fixation) in these nodules and hence the oleosomes are accumulated rather than being utilized.

Dark-treated and detopped peanut plants maintain nitrogenase activity for prolonged periods (up to 48 h) and a decrease in the number of oleosomes has been found (Siddique and Bal, 1991). In contrast to peanut, detopped or dark-treated white clover nitrogenase activity declined very rapidly, within four hours (Dabas et al., 1987). It has been suggested that the oxygen supply to the center of the nodules may be the limiting factor of nitrogenase activity (Hartwig et al., 1987, 1990; Carroll et al., 1987) in defoliated and dark-treated plants. In the nodules of the dark-treated and detopped peanut plants which lack the photosynthate supply, the correlation between

nitrogen fixation (acetylene reduction assay) and oleosome degradation has been clearly demonstrated (Siddique and Bal, 1991). The data from the developmental stages (Table 3) also support the hypothesis that the oleosomes serve as a supplementary source of energy in peanut root nodules.

#### IV.2. ULTRASTRUCTURE OF OLEOSOMES

Oleosomes from seeds vary in size, measuring 1-2  $\mu\text{m}$  dia. (Jacks *et al.*, 1967; Yatsu *et al.*, 1971). Our analyses of different populations of isolated oleosomes from peanut nodules and seeds indicate more of the large oleosomes (2-5  $\mu\text{m}$  dia.) in the dry seed, whereas in the nodule the smaller oleosomes (0.16-1.0  $\mu\text{m}$ ) dominate (Fig. 14). Oleosomes are spherical in shape and filled with a moderately electron-dense matrix delimited by a half-unit membrane (Yatsu and Jacks, 1972; Wanner and Theimer, 1978). Peanut nodule oleosomes appear similar in structure. The half-unit membrane is not always visible in all preparations as reported earlier (Yatsu and Jacks, 1972). In defatted, isolated fractions of oleosomes (Figs. 10 and 10a) and in tissues tested for DAB reaction (Bal *et al.*, 1989), a thin membrane around the oleosomes could be seen in both experimental and control preparations (Fig. 11). The oleosome membrane is not clearly seen in unextracted tissue due to its uniform osmiophilic nature and the presence of an electron-dense rim (Figs. 7 and 15) around the periphery of the oleosomes. The electron-dense rim is revealed by the pPD

staining procedure, which not only preserves the oleosomes during dehydration of tissues but also clarifies their presence at the light microscope level in semi-thin (1.5  $\mu\text{m}$  thick) sections facilitating the study of their distribution pattern in the nodule tissue. However, this rim of electron-dense material found around the nodule oleosomes was not revealed in the seed oleosomes using identical procedures. The half-unit membrane measured about 2.3 nm in defatted oleosomes, which is in accordance with measurements reported for peanut (Yatsu and Jacks, 1972), onion and cabbage oleosomes (Yatsu *et al.*, 1971).

The monolayer of oleosomes contains no unusual phospholipids (Huang *et al.*, 1986). The membrane proteins of the oleosomes have been isolated and identified from several species (Qu *et al.*, 1986). The electron-dense membrane remnants of the oleosomes after extraction in hexane (Fig. 10 and 10a) may represent such proteins. The protein patterns of the oleosome membrane is reported to be distinctly different in the 11 taxonomically diverse species examined (Qu *et al.*, 1986). The protein components of the oleosomes are also different from those of other subcellular organelles as revealed by gel electrophoresis (Bergfeld *et al.*, 1978; Moreau *et al.*, 1980; Slack and Roughan, 1980; Qu *et al.*, 1986) and immunocytochemistry (Herman, 1987). The accumulation of oleosome membrane proteins is shown to be regulated by abscisic acid in

the scutella of maize (Vance and Huang, 1988), in developing rapeseed (Murphy *et al.*, 1989) and microspore embryos (Taylor *et al.*, 1990). Genes coding for the L3 protein (abundant oleosome membrane protein) of maize have been identified (Vance and Huang, 1987) and a similar gene is also reported to be present in carrot (Hatzopoulos *et al.*, 1990). It would have been interesting to look at the protein composition of the peanut nodule oleosomes in comparison with the seed.

#### IV.3. LIPOLYTIC ACTIVITY

Accumulation of the reaction products of lipolytic activity at the periphery of the oleosomes is shown clearly (Fig. 18). The seed oleosomes did not respond in the same manner. The reaction product of lipolytic activity appeared clearly in glyoxysomes (Fig. 20). This also corresponds to the earlier reports of lipase activity found in glyoxysomes of peanut, soybean cotyledons and castor bean endosperm (Huang and Moreau, 1978; Lin *et al.*, 1982; Trelease and Doman, 1984; Maeshima and Beevers, 1985). Demonstration of lipolytic activity in the nodule oleosomes suggests that the oleosomes are being actively catabolized and free fatty acids are released from the storage triacylglycerol. The released fatty acids could be oxidized to generate energy yielding products (section IV.4). In maize, lipase is synthesized in free polyribosomes (Wang and Huang, 1987). Vance and Huang (1987) have suggested that the lipase has to bind to the oleosomes and

the oleosome membrane proteins either singly or in association may play a structural role and act as receptors for lipase. The electron-dense layer (Fig. 10), which was not extracted by hexane treatment in the oleosomes of peanut nodules, may represent such proteins, where lipase is also bound. Lipase is shown to be bound to the oleosomes in cotyledons of rape, mustard and the scutella of maize (Lin and Huang, 1983; Wang *et al.*, 1984).

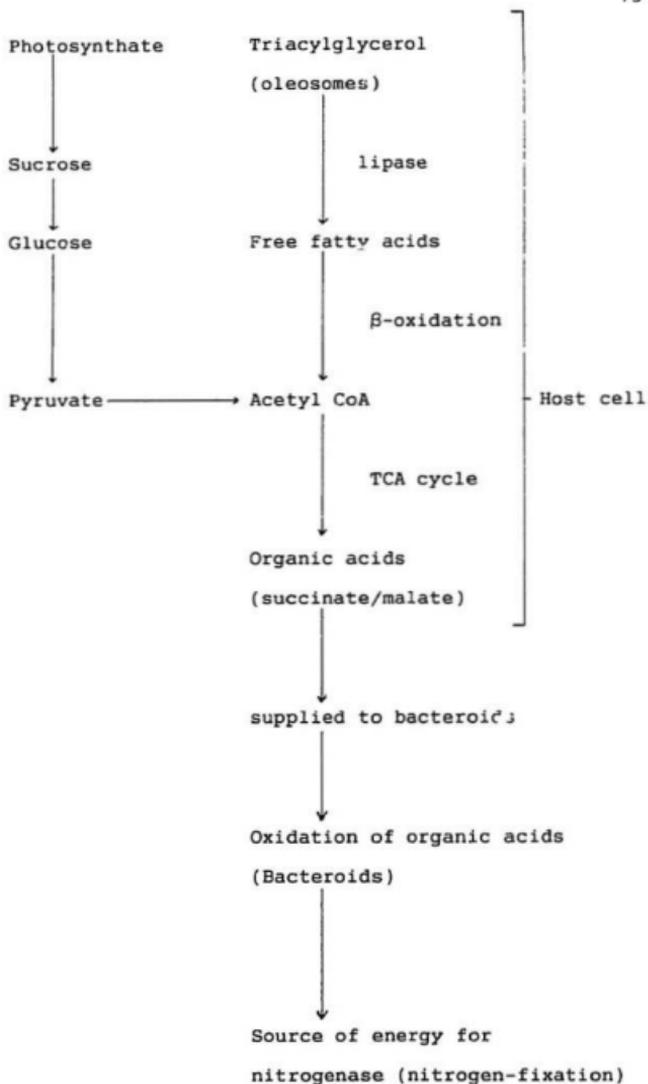
#### IV.4. FATTY ACID COMPOSITION OF OLEOSOMES

The fatty acid composition of nodule oleosomes shows striking quantitative differences (Table 4). They contain more saturated fatty acids (C16:0, C18:0, C20:0 and C24:0), amounting to about 50% of the total lipids, whereas seeds have only about 20%. Alterations in the fatty acid composition of soybeans inoculated with *Bradyrhizobium* have also been reported (Pacovsky and Fuller, 1986). These authors have found that the nitrogen-fixing soybeans had higher amounts of C16:0 and C18:0 than the non-nodulated nitrogen-fertilized plant in the seed as well as higher amounts of C18:1, 18:2 and 18:3. These authors have suggested that the nutrient requirement, amount of lipid storage material and membrane composition in symbiotically grown plants may have lead to different modes of metabolism favored by the host. The possible advantage of having saturated fatty acids is that the yield of ATP molecules during complete oxidation is higher than unsaturated fatty

acids (Lehninger, 1975). Therefore, peanut nodules with oleosomes should have a distinct physiological advantage in the highly energy demanding process of nitrogen fixation (Heytler *et al.*, 1985; Stam *et al.*, 1987). Fatty acids, as such, are not taken up by the bacteroids. Lipid catabolism in the infected cells could generate organic acids such as succinate or malate (Fig. 25) and provide an additional source of energy other than photosynthate.

Lipolytic activity demonstrated in the nodule oleosomes indicate that the oleosomes are catabolized and free fatty acids are released. Free fatty acids are then oxidized through the  $\beta$ -oxidation path way (Tolbert, 1981) which, has been shown to occur in the microbodies and bacteroids (Siddique and Bal, 1991) of the peanut root nodule where the presence of catalase activity has also been demonstrated. Dense bodies showing positive catalase activity may also be involved in lipid catabolism (Hameed, 1986). Acetyl CoA, released from the  $\beta$ -oxidation of free fatty acids may enter TCA cycle or glyoxylate cycle thereby generating organic acids. Evidence from studies of mutants of rhizobia lacking functional TCA cycle enzymes clearly demonstrates that these enzymes are required for effective symbiotic nitrogen fixation (Gallon and Chaplin, 1987). Dicarboxylic acids, such as succinate or malate, have been suggested as the most likely carbon source supplied to the bacteroids *in vivo* (Dilworth and Glenn, 1984; Day *et al.*, 1989).

Fig. 25. Schematic diagram showing the metabolic involvement of oleosomes in nitrogen fixing peanut root nodules.



It is well recognized that the photosynthate supplied in the form of sucrose is converted to organic acids, such as succinate or malate, play an essential role in the nitrogen fixation of bacteroids (Finan *et al.*, 1983; Streeter and Salminen, 1985; Dilworth and Glenn, 1985; Kouchi and Yoneyama, 1986; Humbeck and Werner, 1989). Organic acids have also been shown to support higher rates of respiration as well as nitrogenase activity in the bacteroids (Ramaswamy and Bal, 1986; Streeter and Salminen, 1988). Recently, a dicarboxylate transporter has been identified and described in soybean nodules (Udvardi *et al.*, 1988; Day *et al.*, 1989), which is capable of transporting succinate or malate at rates sufficient to support the measured nitrogenase activity. This is further supported by the identification of malic enzyme, a key enzyme for succinate or malate oxidation in bacteroids via tricarboxylic acid cycle (Day and Copland, 1988; McKay *et al.*, 1988; Kouchi *et al.*, 1988; Kimura and Tajima, 1989).

Evidence from the morphometric analyses of oleosomes during different stages of nodule development (Table 3), lipolytic activity (Fig. 18) and other evidence indicate that the oleosomes are being utilized in the peanut root nodules for nitrogen fixation. Oleosomes probably provide an additional source of energy in the form of organic acids to the nitrogen-fixing bacteroids and are metabolically involved in the nodule physiology.

## V. CONCLUSIONS

In the present investigation it has become clear that the oleosomes of peanut root nodule tissue differ from those of the seed with respect to the following characteristics:

1. The presence of an electron-dense rim surrounding the nodule oleosomes, which was not revealed in the seed.
2. Lipolytic activity was localized cytochemically around the periphery of the nodule oleosomes. Whereas, in the seed, the reaction product was localized in the glyoxysomes.
3. Saturated fatty acids made up about 50% of the total fatty acid in the nodule oleosomes, twice the amount present in the seed.

These findings suggest that the nodule oleosomes are transient, not long-term storage organelles as in the seed and their characteristics indicate metabolic involvement in the nodule physiology. Their association with the peribacteroid membrane and the bacteroids is also emphasized.

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