The Metabolism of Starch in Effective and Ineffective Nodules of Soybean

by

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ABSTRACT

The relative levels of starch and the specific activities of amylase, starch phosphorylase and starch synthase were examined in effective (fix^+) and ineffective (fix^-) soybean root nodules at 14 and 21 days after infection with relevant strains of Bradyrhizobium japonicum. Starch levels decreased between 14 and 21 days post-infection in fix^+ nodules but increased in fix^- nodules. In effective nodules, the specific activities of amylase and synthase were found to positively correlate with starch levels, while phosphorylase activity was negatively correlated. In the three types of ineffective nodules, the correlations with starch levels were as follows:; amylase, positive or negative; synthase, positive or negative and phosphorylase, positive. A 105 kDa form of starch phosphorylase was present in nodule extracts, but not in extracts of uninoculated root. Sucrose synthase was localized in the cytoplasm of both infected and uninfected cells of effective nodules. Starch was present in uninfected cells of all nodule types. At 14 days post-infection, starch was present in the infected cells of effective nodules, although, it was not present in this cell type by 21 days. In situations where the bacteroids did not enter or disappeared from the nodule cells, large, distinctly starch free cells were apparent.

Résumé

Les quantités relatives d'amidon et les activités spécifiques de l'amylase, la phosphorylase d'amidon et l'amidon synthétase ont été mesurées dans les racines nodulaires de la fève de soja pouvant (Fix^+) ou ne pouvant pas (Fix^-) fixer l'azote après 14 et 21 jours d'infection avec des souches appropriées de *Bradyrhizobium japonicum*. Les niveaux d'amidon diminuent du 14ième au 21ième jours dans les nodules Fix^+ contrairement aux nodules Fix^-. Pour les nodules efficaces, l'amylase et la synthétase ont été en corrélation positive avec les niveaux d'amidon, tandis qu'on observe l'inverse pour l'activité de la phosphorylase. Dans les trois types de nodules inefficaces, la corrélation avec le niveau d'amidon était comme suit: amylase, positive ou négative; synthétase, positive ou négative; phosphorylase, positive. La phosphorylase d'amidon (espèce au poids moléculaire de 105 kDa) est présente dans les extraits de nodules mais non dans les racines non-infectées. Dans les nodules efficaces, la synthétase de sucrose est localisée dans le cytoplasme, autant dans les cellules infectées que celles qui ne l'étaient pas. L'amidon est présent dans les cellules non-infectées dans tout les types de nodules. Après 14 jours d'infection, on détecte de l'amidon dans les cellules infectées de nodules efficaces, bien que, celui ci ne soit plus présent au 21ième jour après l'infections. Dans les cas où les bactéroides n'ont pu pénétrer ou sont disparus des cellules nodulaires, on aperçoit de grandes cellules sans amidon.

Traduit par Francis Ouellette et Mario Filion.

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LIST OF ABBREVIATIONS

ADP	adenosine 5' diphosphate
ATP	adenosine 5' triphosphate
ADPG	adenosine 5' -(α-D-glucopyranosyl pyrophosphate)
bad^-	lacking bacteroid development
bad^+	having bacteroid development
^0C	degrees celsius
d	day
EDTA	ethylene-diaminetetra acetic acid
fix^+	nitrogen fixing
fix^-	non-nitrogen fixing
g	gram
GS	glutamine synthetase
h	hour
kDa	kilodalton
Lb	leghemoglobin
Lc	legcholeglobin
μm	micrometers
μmol	micro mole
mg	milligram
M	molar
min	minute
ml	millilitres
mM	millimolar
mRNA	messenger ribonucleic acid
NADP	nicotinamide adenine dinucleotide phosphate
nm	nanometers
PAGE	polyacrylamide gel electrophoresis
pbf	peribacteroid fluid
pbm	peribacteroid membrane
PBS	phosphate buffered saline
PEP	phosphoenolpyruvate
PFK	irreversible phosphofructokinase
PFP	reversible phosphofructokinase
PGA	3-phosphoglycerate
Pi	orthophosphate
PHB	poly-β-hydroxybutyrate
PMSF	phenylmethylsulfonyl fluoride
PPi	pyrophosphate
SBSS	starch bound starch synthase
SDS	sodium dodecylsulphate
SSS	soluble starch synthase
TBA	tertiary butyl alcohol
TBS	tris buffered saline
TCA	trichloroacetic acid
temp	temperature
UDP	uridine 5' diphosphate
UDPG	uridine 5'-(α-D-glucopyranosyl pyrophosphate)
UMP	uridine 5' monophosphate
UTP	uridine 5' triphosphate
UV	ultra violet

INTRODUCTION

Nitrogen fixation from symbiotic interactions between plants and microbes contributes significantly to the supply of fixed nitrogen in the global nitrogen cycle (Postgate, 1978). Successful symbioses can occur between plants and diazotrophic bacteria or mycorrhizal fungi (see Verma and Long, 1983).

Research has been concentrated on elucidating the mechanism of biological nitrogen fixation in plant/microbe interactions since it has potential to improve agriculture. Bacterial symbionts, primarily of the genus Rhizobium, have been in focus in the resulting scientific literature.

Overview of Rhizobium/Legume Symbiosis

The soil bacterium, Rhizobium spp., is capable of forming a tightly co-ordinated interaction with a wide range of higher plants leading to an endosymbiotic relationship (for reviews see Bauer, 1981; Verma and Long, 1983; Sutton, 1983; Verma and Nadler, 1984; Rolfe and Shine, 1984; Kondorosi and Kondorosi, 1986 and Long et al., 1986). As a result of this interaction, a differentiated and highly specialized structure, known as a nodule, is formed. It is inside the nodule that the rhizobia fix nitrogen [the conversion of atmospheric nitrogen (N_2) into ammonia (NH_3)]. Ammonia can be used as a source of nutrition by the plant, whereas, dinitrogen cannot. These nodules are usually found on roots, although there are some examples of photosynthesizing stem nodules (Lajudie and Huguet, 1987).

The various, naturally occurring species and strains of Rhizobium (fast growers) and Bradyrhizobium (slow growers) are usually host specific. This means that nitrogen fixing nodules (fix^+ or effective) are only produced when the bacteria infect homologous hosts. Interaction with heterologous

hosts either does not result in nodule formation or induces the formation of ineffective nodules which do not fix nitrogen (fix$^-$) (see Verma and Long, 1983; Long et al., 1986).

(i) Nodule Development: An Ultrastructural Perspective

Extensive ultrastructural analyses have elucidated the series of morphological events involved in nodulation, nitrogen fixation and nodule senescence. This work has provided a basis for metabolic and molecular studies.

Immediately following attachment of an appropriate Rhizobium spp. to the root hair cell, root hair curling occurs. The bacteria then enter the cells of the "crook" of the curled root hair (Vincent, 1980). An infection thread is formed as the endosymbiont makes its way into the root hair interior. A certain rhizobial exopolysaccharide is known to have a role in the induction of this infection thread (Long, et al., 1986).

Meristematic cell division occurs in the root inner cortex prior to the entry of the infection threads. This early stimulation of plant cell proliferation requires a homologous interaction and, therefore, seems to be at a point in the nodulation process when recognition of the host occurs (Long et al., 1986).

Based on the temporal nature of the meristematic divisions, legume nodules can either be determinate as is the case with spherical nodules (e.g. soybean) or indeterminate as is the case with elongate nodules (e.g. alfalfa). In the latter nodules, meristematic growth continues thoughout the entire life of the nodule in which there are clearly defined meristematic, mature and scenescent zones.

The bacteria are released upon entry of the infection thread into the dividing cells of the root cortex. Immediately afterwards, the Rhizobium pass into the cytoplasm as "unwalled" droplets enveloped by the plant cell plasma membrane. In this specialized role, the envelope plasma membrane develops new characteristics and is called the peribacteroid membrane (pbm). The fluid inside the pbm envelope is called the peribacteroid fluid (pbf) (see Verma and Long, 1983). Once enveloped by the pbm, the Rhizobium spp. differentiate into bacteroids which are different from the free living bacteria in that they have a modified outer membrane and a less defined cell wall (Long et al., 1986).

Provided all relevant conditions are favourable, the bacteroids begin fixing nitrogen inside the pbm. The processing of the resultant ammonia requires an interaction between the infected and uninfected cells both of which are present in mature nodules. The initial steps of nitrogen assimilation occur in the infected cell while the final steps happen in the peroxisomes of the uninfected cell (see Goodchild, 1977; Newcomb et al., 1985)

The infected cells are interspersed in with the uninfected cells in such a way that every infected cell is in physical contact with at least one uninfected cell (Selker and Newcomb, 1985; Dart, 1977; Newcomb, 1981). Numerous plasmodesmata exist between infected and uninfected cells and between two uninfected cells. Plasmodesmata between two infected cells are relatively uncommon (see Goodchild, 1977).

There are two types of uninfected cells: those at the periphery of the nodule and those which are interspersed with the infected cells in the nodule interior. The former are also called the portal band of cells and

are thought to have a role in determining the entry and exit of metabolites (Tjepkema and Yocum, 1974).

Uninfected cells are ultrastructurally different from the infected cells. As well as lacking bacteroids, the uninfected cells are smaller and both the starch granules and peroxisomes are larger and more numerous (Selker and Newcomb, 1985; Van den Bosch and Newcomb, 1986). The mature infected cells contain quantitatively less starch than uninfected cells in a variety of nodule types (Dandeard, 1926; Lechtova-Trnka, 1931; Biebdorf, 1938; Allen and Allen, 1940 and Bond, 1948). Passage of carbohydrates from one cell type to another has been suspected, but unproven. Ineffective nodules of soybean have more starch than effective nodules (Morrison and Verma, 1987). The reason for this is unknown.

Prior to senescence, the nodules receive up to 30% of the plant's photosynthates (Minchin et al, 1981; Mahon, 1983). But later, when the plant forms its pods, photosynthates are diverted from the nodule to the pods. It has been proposed that as a result of the decreased carbohydrate supply, the nodules senesce (Sutton, 1983).

As senescence advances, the infected cells eventually lose their turgor and begin to collapse in between the still turgid uninfected cells (Vance et al., 1980). The successive stages of nodule senescence (in the infected cells) are as follows: (1) increased electron density of the cytoplasm (2) the pbm develops small breaks resulting in the formation of numerous small vesicles, together with an increasingly abnormal appearance of the bacteroids, (3) the nucleus develops lobes and disintegrates and eventually (4) the host cell walls begin to breakdown (Cohen et al.,1986).

Rhizobium strains which cause the formation of ineffective nodules can

mimic the stages of senescence similar to the natural sequence of events (Werner et al., 1980).

(ii) Nodule Development at the Molecular Level

As described in the above section, nodule development comprises a multi-step cascade of events involving rhizobial root colonization, root hair adhering and curling, infection thread development, nodule initiation and development. This sequence of events requires a co-ordinated regulation and expression of several rhizobial and plant genes.

The isolation of different classes of bacterial mutants is possible through transposon mutagenesis. Genetically mutated Rhizobium often can cause the formation of nodules with varying developmental arrests depending upon which gene was acted upon by the transposon (Rostas et al., 1984). Such mutants are a useful aid in studying Rhizobium/plant interactions.

Transposon mutagenesis has been used to establish a number of B.japonicum mutants, strains T5-95 and T8-1 being examples. T5-95 induces the formation of nodules which are ultrastructurally similar to the wildtype, but are fix$^-$. T8-1 infected nodules are fix$^-$ and deficient in bacteroid development (bad$^-$) meaning that the bacteroids are not released from the infection thread (Rostas et al., 1984; Morrison and Verma, 1987).

A naturally occurring strain of B. japonicum (61A24) also causes the formation of ineffective nodules. The most striking characteristic of 61A24 nodules is the degradation of the pbm 18 to 20 d post-infection (Werner et al., 1980, 1984).

T5-95, T8-1 and 61A24 infected nodules each provide unique situations of developmental arrest: fix$^-$; fix$^-$, bad$^-$ and fix$^-$, pbm$^-$, respectively. Table 1 is a summary of all the characteristics of the three ineffective

nodule types as well a comparable effective nodule infected with wildtype B. japonicum strain 61A76.

Mutants similar to those mentioned above have been used to examine the step-by-step molecular interactions between plant and bacterium leading to the development of a functional nodule.

Initial contact between free-living Rhizobium spp. and the legume root hair are in the form of a chemical plant factor, known as a flavone (Bauer, 1981; Long et al. 1986). This factor is released from the growing host root hair and results in the induction of bacterial nodulation genes, provided the interaction is homologous (Long et al.,1986). After having been activated by the plant factor, the bacteria approach the root hair (due to chemoattraction) and become attached via a specific receptor molecule (Dazzo and Hubbell, 1981; Dazzo and Gardiol, 1984).

Several Rhizobium encoded proteins (bacteroidins) play an important role in the symbiotic functioning of the nodule. The bacteroidin, nitrogenase, is essential because it carries out the conversion of atmospheric nitrogen to ammonia (see Verma and Long, 1983). Nitrogenase establishes its role in soybean nodules gradually, from 9 to 16 d post-infection (post-infection means the number of days after the rhizobia have contacted the root). At 9 days post-infection, 0.1% of the maximum level of nitrogen fixation is evident; at 10 days this increases to 1%. By 16 days post-infection, the maximum level of nitrogen fixation is established and is maintained until pod formation (Sutton, 1983; Fuller and Verma,1984).

In the study of the plant genes and gene products essential to nodule development and functioning, the progress has been slow. Expression of certain plant genes result in the production of nodule-specific proteins or nodulins. There are 2 types of nodulins: structural and metabolic.

Table 1: Characteristics of nodules formed by different strains of B.japonicum in terms of nitrogen fixing ability, macroscopic appearance and ultrastructure.

Strain of B.japonicum	Nitrogen Fixation	Macroscopic Appearance at 21 d	Ultrastructure
61A76 (wildtype)	fix^+	-large nodules clustered on main root -deep red interior	-infected and uninfected cells -intact pbm
T5-95	fix^-	-small nodules throughout root -pink interior	-same as 61A76
T8-1	fix^-	-small nodules throughout root -green interior	-bacteroids are not released from infection thread
61A24	fix^-	-small nodules throughout root -green interior	-pbm breaks at 18-20 d post-infection

Structural nodulins are those which actually contribute to the building of the nodule. Metabolic nodulins are involved in the functioning of the nodule. To date nodulins involved in the oxygen, nitrogen and carbohydrate metabolisms have been studied (Verma and Long, 1983).

The nodulin, leghemoglobin (Lb), carries oxygen to the actively respiring bacteroids while maintaining a low level of free oxygen to which nitrogenase is sensitive (Appleby, 1984). Leghemoglobin is similar to animal hemoglobin and myoglobins, in that it has oxygen-binding capacity (Hunt et al., 1978). It is Lb which gives the interior of nodules the red coloration, similar to blood. The heme moiety of Lb is thought to originate from the bacteroids, although in soybean, it may be produced by the plant (see Verma and Nadler, 1984; Guerinot and Chelm, 1985). Table 2 shows the relative amounts of Lb mRNA in effective and 3 types of ineffective nodules of soybean.

During senescence, the properties of Lb change such that it becomes a different substance known as legcholeglobin (Lc). Legcholeglobin (Lc) differs from Lb in that the heme porphyrin ring is cleaved and the central iron atom is more easily released. A green colored interior is a characteristic of nodules containing Lc (Virtanen et al., 1947; Virtanen and Meitinen 1949).

Nitrogen assimilation (processing of ammonia) involves nodulins and follows one of two alternative pathways in the legume/Rhizobium interaction: one leading to the production of amides and the other leading to the production of ureides. Amides and ureides are carbon-nitrogen compounds which are easily utilized by the plant's metabolism. These two compounds are made in the nodule and are then transported to the plant via the phloem. In tropical legumes, such as soybean, it is ureides which are

Table 2: Characteristics of nodules formed by different strains of *B.japonicum* in terms of Lb mRNA levels (Morrison and Verma, 1987).

Strain of *B.japonicum*	Relative Lb mRNA in 21 d nodules
61A76 (wildtype)	100%
T5-95	37%
T8-1	8%
61A24	6%

produced and transported (Schubert, 1986).

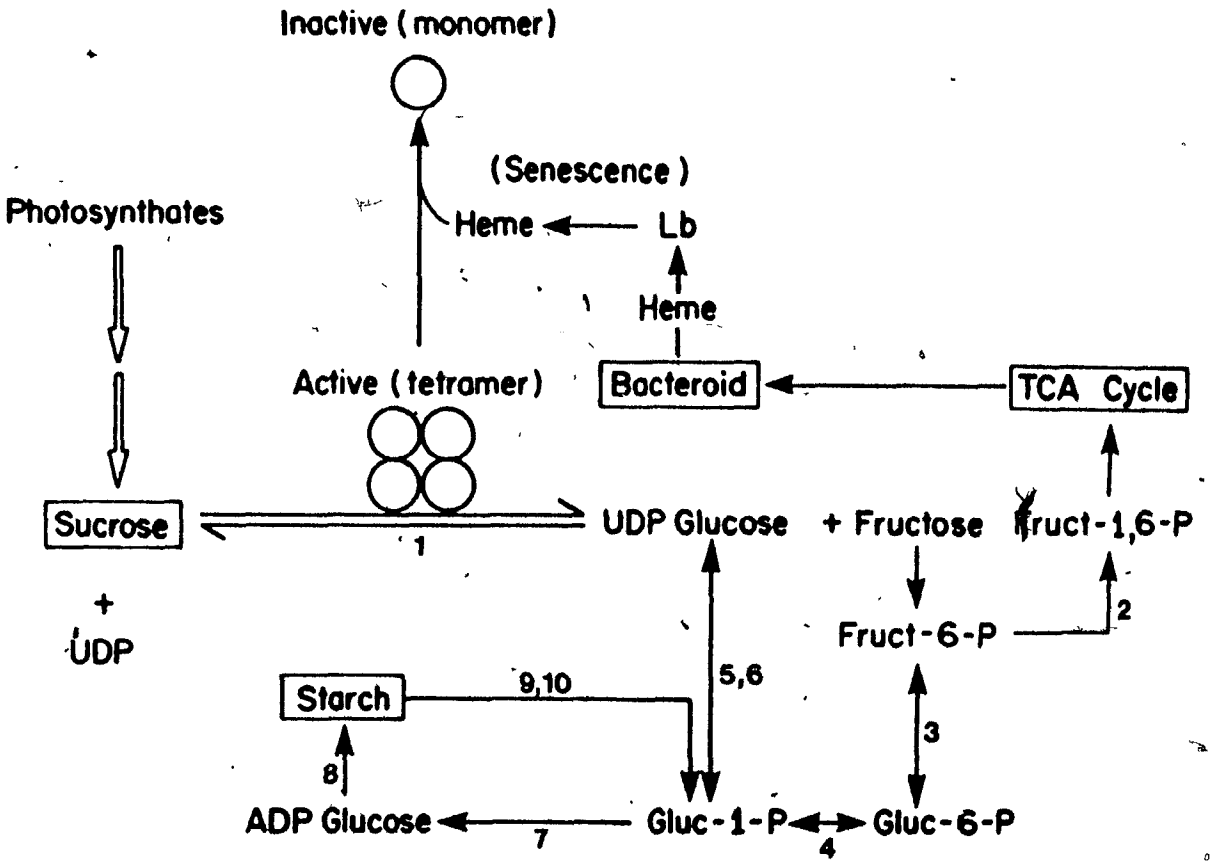
In the ureide pathway, the action of nodulins such as glutamine synthetase (GS), xanthine dehydrogenase, purine nucleosidase and a number of other enzymes catalyze the conversion of ammonia to uric acid (an intermediate in the ureide pathway). All catalytic steps occurring up to the formation of uric acid occur in the infected cells of the nodule (see Verma and Delauney, 1987). Once formed, the uric acid is then transferred to the peroxisome of the uninfected cell where uricase (another nodulin) and other enzymes carry out the final reactions which result in the formation of ureides. Newcomb et al. (1985) and Van den Bosch and Newcomb (1986) have reasoned that uricase acts in the uninfected cells because of the microaerophilic nature of the infected cell.

Carbohydrate metabolism is essential to nitrogen fixation since it provides energy to the bacteroids. Much work has been carried out on symbiotic nitrogen fixation, while the corresponding carbohydrate metabolism has received little attention. One of the soybean nodulins, Nodulin 100, has recently been identified as a nodule-specific form of sucrose synthase, an enzyme which can degrade sucrose. It is the only nodulin identified to date to be involved in carbohydrate metabolism (Thummler and Verma, 1987).

Carbohydrate Metabolism in Root Nodules and in Plants

Before more extensive work can be done at the molecular level, the nodule carbohydrate metabolism must be understood at the biochemical and physiological level. Figure 1 (Thummler and Verma, 1987) is a model of carbohydrate metabolism in root nodules based on what is known in nodules and what generally happens in plants.

Figure 1: Proposed model of the carbohydrate metabolism of soybean root nodules, including the possible role of heme in regulating the activity of sucrose synthase (Thummler and Verma, 1987). The numbers represent the following enzymes: (1) sucrose synthase, (2) phosphofructokinase, (3) phosphoglucoisomerase, (4) phosphoglucomutase, (5) UDPG pyrophosphorylase, (6) phosphodiesterase, (7) ADPG pyrophosphorylase, (8) starch synthase, (9) amylase and (10) starch phosphorylase.


Inactive (monomer)
(Senescence)
Photosynthates
Heme
Lb
Heme
Active (tetramer)
Bacteroid
TCA Cycle
Sucrose
UDP Glucose
+ Fructose
Fruct-1,6-P
1
+
UDP
Fruct-6-P
2
Starch
9,10
5,6
3
8
ADP Glucose
7
Gluc-1-P
4
Gluc-6-P

The carbohydrate supply to the nodule affects nitrogen fixation, although a controversy still exists as to how this influence is exerted (Sutton, 1983). The association between nitrogen fixation and photosynthate supply was first examined in the 1930's when two different views were propounded. Allison (1935, 1939) and Allison and Ludwig (1934) proposed that the amount of carbohydrate reaching the nodule was the dominant influence on the resultant amount of nitrogen fixation. Another group, Wilson et al. (1933) and Wilson and Fred (1939), have suggested that both the carbohydrate supply and existing ammonia level in the nodule are important factors in limiting the production of fixed nitrogen. So far, a consensus has not been reached between these two schools of thought, but some progress has been made in the study of involved enzymes and reserve carbohydrates.

(i) Sucrose Degradation

Sucrose is the major transported photosynthate in plants. This disaccharide is made in the leaves and is transported through the phloem to the non-photosynthetic tissues (Giaquinta, 1980 a,b). Once the sucrose reaches its target tissue, it is usually broken down into compounds more easily utilized by the plant (see Stryer, 1981).

Breakdown of sucrose can be carried out by three enzymes each able to complete the process itself. Alkaline and acid invertases catalyze reaction (1) (Rees, 1984). The reverse reaction of sucrose synthase (see Preiss, 1982 a,b; Morell and Copeland, 1984; Thummler and Verma, 1987) is shown in reaction (2). These reactions have been studied in nodules as well as in plants.

(1) sucrose $\rightleftharpoons$ D-glucose + fructose

(2) sucrose + UDP^{3-} + H^+ ⇌ UDP^{2-}-D-glucose + D-fructose

During early development of soybean nodules (0-10 d post-infection), the specific activity of acid invertase has been found to steadily decline, while that of the alkaline form of the enzyme increases and levels off (Morell and Copeland, 1984 and Anthon, personal communication). This type of activity during the early phase of nodulation conforms with the reported high levels of invertases during rapid development of plants in general (see Avigad, 1982).

Sucrose synthase activity increases over time at the same rate as Lb and nodule-specific GS in effective nodules of soybean [Lb and GS activities increase and stabilize in parallel with nitrogenase (Anthon, personal communication)]. Thus, in comparison with the invertases, sucrose synthase is more important during the period of active nitrogen fixation (10 d post-infection until senescence) (Thummler and Verma, 1987; Anthon, personal communication).

Thummler and Verma (1987) have suggested that nodule-specific sucrose synthase may be regulated by free heme which is released from leghemoglobin during senescence. In vitro experiments have shown that free heme is able to break down the active tetramer form of sucrose synthase to an inactive monomer. A model of this proposed regulation is shown in Figure 1.

(ii) Directing of Sucrose Breakdown Products

The products of sucrose breakdown can enter one of many metabolic pathways. The regulated sequence of events leading towards the appropriate partitioning of these products has been studied extensively in a number of plants (see Preiss, 1982 a,b), but only to a very limited degree in nodules.

Once the sucrose is degraded, the products can enter either the glycolytic pathway or be directed towards starch synthesis, depending on the energy needs of the cell. One key enzyme involved in determining the fate of sucrose breakdown products is phosphofructokinase which catalyses the conversion of fructose-6-phosphate to fructose-1,6-diphosphate (see Stryer, 1981).

An irreversible form of phosphofructokinase (PFK) is inhibited by high levels of ATP, while the reversible form of phosphofructokinase (PFP) is pyrophosphate dependent and is regulated by fructose-2,6-bisphosphate. Fructose 2,6-bisphosphate is known to stimulate respiratory utilization of sucrose and starch; low levels correlate with starch synthesis (Stitt, 1987). Both PFK and PFP are located in the cytoplasm of plant cells (Huber, 1986; Stitt, 1987).

In soybean root nodules, PFK activity is found to increase early in nodule development with a time course similar to alkaline invertase, whereas, root nodule PFP follows the same activity time course as acid invertase. In view of these findings, Anthon (personal communication) has suggested that PFK is of greatest importance during nitrogen fixation.

In plants, PFK is allosterically regulated by cellular ATP concentration. When ATP levels in the cell are low, PFK catalyses the conversion of fructose-6-phosphate to fructose-1,6-diphosphate which then can enter glycolysis. When cellular ATP levels are high, PFK is inhibited and fructose-6-phosphate will build up (see Stryer, 1981). Thummler and Verma (1987) have suggested that this type of allosteric regulation of PFK also occurs in nodules.

Excess fructose-6-phosphate favours the formation of glucose-6-phosphate via the enzyme phosphoglucose isomerase. Next, in a reaction

catalysed by phosphoglucomutase, glucose-6-phosphate can be converted to glucose-1-phosphate. Glucose-1-phosphate is a substrate for starch synthesis (see Stryer, 1981). Phosphoglucoisomerase and phosphoglucomutase have not been studied in nodules.

Glucose-1-phosphate can also be formed in another way, directly from the sucrose breakdown product, UDPG, via UDPG pyrophosphorylase. It has been shown that the pyrophosphate (PPi) concentration in plant cell cytoplasm is quite high, thereby, making it possible for this pyrophosphorylysis to occur (Stitt, 1987; Stitt et al., 1987; Rees et al., 1985; Black et al., 1985 a,b; Huber and Akazawa, 1986).

Recent findings have shown that UDPG pyrophosphorylase activity is high in both effective and ineffective nodules of soybean (Anthon, personal communication).

Breakdown of UDPG can also be carried out by a phosphodiesterase which carries out the following reaction:

(3) UDPG + H_2O $\rightleftharpoons$ glucose-1-phosphate +UMP

Either way, once glucose-1-phosphate is produced it can enter one of two pathways: towards glycolysis or in the direction of starch synthesis (see Preiss, 1982 a,b). The direction taken is controlled by a number of factors which will be described in the next section.

(iii) Factors Involved in the Control of Starch Biosynthesis

As well as being limited by the amount of available photosynthate, starch biosynthesis is also controlled by the allosterically regulated enzyme, ADPG pyrophosphorylase (see Avigad, 1982; Preiss, 1982 a,b). This enzyme has not been studied in root nodules.

ADPG pyrophosphorylase catalyzes the following reaction which results in the formation of ADPG, the substrate for starch synthase (an enzyme which synthesizes starch).

(4) ATP + Glucose-1-phosphate $\rightleftharpoons$ ADPG + PPi

ADPG pyrophosphorylase is located in the amyloplast and because of the relatively low concentrations of PPi usually found in this organelle, the reaction proceeds in the direction of ADPG synthesis (Gross and Rees, 1986).

The breakdown of ADPG, when conditions favour this pathway, is most likely carried out by ADPG phosphorylase (Dankert et al.,1964). Although it is not allosterically regulated, ADPG phosphorylase plays an important role in putting ADPG back into the hexose phosphate pool (as glucose-1-phosphate) with relatively little loss of energy (Feingold, 1982). Therefore, the combined activities of both pyrophosphorylase and phosphorylase contribute to the ADPG concentration in the amyloplast (de Fekete and Cardini, 1964).

The controlling element in the allosteric regulation of ADPG pyrophosphorylase in plants is the ratio of 3-phosphoglycerate (PGA) / orthophosphate (Pi) (Ghosh and Preiss, 1965; Heldt et al., 1977; Preiss, 1982 a,b). When this ratio is high, as a result of a build up of glycolytic intermediates and a high ATP level (indicated by a low Pi pool), the ADPG pyrophosphorylase activity is enhanced. When the same ratio is low, indicating a need for ATP in the cell, the pyrophosphorylase activity is inhibited and the ADPG remaining may be converted back to glucose-1-phosphate by the action of ADPG phosphorylase (Feingold, 1982).

Allosteric control of ADPG pyrophosphorylase varies considerably with cell type. For example, in Zea mays L. the ADPG pyrophosphorylase found in

the bundle sheath cells is more easily activated by PGA as compared to the form of the enzyme found in the mesophyll cells. Bundle sheath cell ADPG pyrophosphorylase also has a higher affinity for PGA and a lower affinity for Pi than the mesophyll cells. These cell-specific characteristics of the pyrophosphorylase make it more likely for starch synthesis to occur in the mesophyll than in the bundle sheath cells (Spilatro and Preiss, 1987).

Non-photosynthetic plant tissue has been documented to be less sensitive to allosteric regulation of ADPG pyrophosphorylase. It has been suggested that plant storage tissues have evolved towards a state of insensitivity to any regulation. Therefore, any carbohydrate substrates entering the cell would be converted to starch rather than be used for other functions (Spilatro and Preiss, 1987).

(iv) Location of Starch Synthesis and Degradation

Starch metabolism in plants is compartmentalised: some stages occurring in the cytoplasm and others in the plastids (Stitt, 1987). This aspect of carbohydrate metabolism has not been studied in root nodules.

Starch is synthesized in plastids (including chloroplasts and amyloplasts) while sucrose breakdown occurs in the cytoplasm. Starch synthesis is, therefore, dependant on the transport abilities of the plastid membranes for the provision of substrates and removal of waste products (Stitt, 1987).

The envelopes of amyloplasts and chloroplasts are similar in lipid composition and ability to form galactolipids (Fishwick and Wright, 1980). In vitro experiments have shown that the two plastid types are interconvertible (Aquettaz et al., 1987). Jenner (1980) has suggested that

all features of carbohydrate metabolism in the chloroplast also apply to the amyloplast, including transport ability.

There are two translocators in chloroplasts: one for phosphate and one for sugars. The phosphate translocator mediates the exchange between Pi and triose phosphates. The sugar translocator provides for the passage of glucose and other sugars (but not sucrose) into and out of the plastid (Stitt, 1987).

Glucose-1-phosphate may be transported into the amyloplast, although it is equally possible that it is converted to triose phosphates before it enters the amyloplast since triose phosphates are known to traverse the amyloplast membrane (Heldt, 1976; Walker, 1976). It is generally believed that the plastids and cytoplasm each have their own glycolysis, oxidative pentose phosphate pathway and reversal of these pathways, thereby, making it possible for the triose phosphates to be converted to glucose-1-phosphate in either location (see Preiss, 1982b).

(v) Starch Metabolism

Relatively little work has been done on the starch metabolism of nodules. However, it has been well studied in potato, spinach and maize (see Preiss, 1982 a,b; Jenner, 1982).

The major enzymes involved in the biosynthesis and degradation of starch are the following: ADPG pyrophosphorylase, starch synthase, amylase and starch phosphorylase. The two former enzymes are usually involved in synthesis, while the latter two are generally involved in degradation (Okita et al., 1979; see Preiss, 1982 a,b). Phosphorylase can also be involved in starch synthesis, a point which will be discussed in detail later in this section.

ADPG pyrophosphorylase and starch synthase have been localized in the plastid, while amylase and phosphorylase are found in both the plastid and the cytoplasm (Okita et al.,1979). In Dunaliella and Chlamydomonas, amylase and phosphorylase are found only in the chloroplast (Goyal et al., 1987).

The earliest report of in vitro catalysis of (1,4)-α-glycosidic linkage formation in plants was by Hanes (1940) who elucidated the following reaction catalysed by starch phosphorylase.

(5) Glucose-1-phosphate + α-glucan primer ⇋

Pi + (1,4)-α-glucosylglucan

The next significant study was carried out by Leloir et al., (1961). The production of the α-glucosidic linkages was catalysed from either ADPG or UDPG. ADPG has been found to be a better substrate for the starch synthesizing enzyme in view of the Km and Vmax. The enzyme, in this case, is starch synthase and it has since been found in a large number of plant extracts (Preiss and Levi, 1979, 1980; Preiss, 1982 a,b).

The activity of the enzyme starch synthase also results in the formation of starch from the substrates UDPG and/or ADPG. The reaction catalysed by starch synthase is shown below in (6).

(6) ADPG + (1,4)-α-glucan ⇋

ADP + α-4-glucosyl-α-1,4-glucan

Starch synthase has been studied in a wide range of plants (Preiss and Levi, 1979; 1980; see Preiss, 1982 a,b). This enzyme is present in plant cells in two forms: starch bound (SBSS) and soluble (SSS), each having somewhat different properties, although both require sugar nucleotides and a primer (amylose, amylopectin, glycogen or starch granules). Both forms of the enzyme have 15 to 30 times more affinity for ADPG than for UDPG and both are localized in the plastid (Cardini and Frydman, 1966 and Frydman

and Cardini, 1971). Neither SBSS nor SSS is likely to use UDPG *in vivo* because UDPG cannot enter the amyloplast (Anthon, personal communication).

Starch phosphorylase has been reported to be involved in both synthesis (Hanes, 1940; Schneider et al., 1981; Sivak et al., 1981 and Slabnik and Frydman, 1970) and in degradation of starch (see Preiss, 1982b). In order for starch phosphorylase to operate in the direction of starch synthesis, a certain concentration of glucose-1-phosphate is required, the Km for glucose-1-phosphate being 1-50 mM (see Preiss, 1982b). Often these high levels of glucose-1-phosphate are not present in the photosynthesizing plant cell, where the concentrations can be 1000-fold lower than that required for starch phosphorylase to operate in the direction of starch synthesis (Heldt et al., 1977). Phosphate concentrations have not been extensively studied in non-photosynthetic tissues.

In a number of plants, starch phosphorylase has been localized in both plastids and cytoplasm (de Fekete, 1966; Okita et al.,1979). In young potato tubers which are actively synthesizing starch, starch phosphorylase has been found in the amyloplast, while in mature potato tubers the enzyme is found only in the cytoplasm (Brisson, personal communication).

A starch phosphorylase inhibitor has been found which is ubiquitous in plants. In sweet potato, this inhibitor has been localized in the amyloplast, but the role (if one exists) in the regulation of starch phosphorylase has not yet been determined (Chang and Su, 1986). Starch phosphorylase in potato can also be inhibited by UDPG and amylase (see Halmer and Bewley, 1982).

Amylase catalyses the breakdown of starch into smaller, linear oligosaccharides which can then be acted on by starch phosphorylase. Interestingly, phosphorylase and amylase have been localized in both the

cytoplasm and plastids in many plants. In view of this, it has been proposed that some of the oligosaccharides are transported out of the amyloplast and degradation continues in the cytoplasm by both hydrolysis and phosphorylysis (Okita et al., 1979).

Starch metabolism of root nodules has been studied to a limited extent in alfalfa (Duke and Henson, 1984). In alfalfa nodules, changes in amylase activities were found to correlate inversely with the changing levels of starch. At the same time, phosphorylase activities showed a positive correlation with starch. Duke and Henson (1984) interpreted these observations as indicating that amylase is the most important enzyme for the degradation of starch.

Starch is not the only type of reserve carbon in the nodule. The bacteroids can also store carbon in the form of poly-β-hydroxybutyrate (PHB), although it has been shown that PHB cannot be used to support nitrogen fixation (Wong and Evans, 1971). Because of this, it has been proposed that starch is the most important reserve carbon source in the nodule (Yin and Sun, 1947).

Aims of this Study

Carbohydrate metabolism in root nodules is understood only to a very limited degree. Prior to extensive molecular studies, it is necessary to examine the physiology and biochemistry of the carbohydrate metabolism and the related morphological changes in order to obtain a solid background of information.

One very evident contrast between effective and ineffective nodules and between infected and uninfected cells is the accumulation of starch (ineffective nodules having more starch than effective and uninfected cells

having more starch than infected). This clear difference provides a good starting point for a comparative analysis of the biochemical sequence of events of nodule carbohydrate metabolism.

The underlying aim of this study was to explore the physiology and the biochemistry of nodule starch metabolism as well as the associated morphological changes. In the first stage of the work, whole nodules were examined in terms of their starch content followed by a study of the possible enzymes involved in synthesizing or in degrading the starch. The second stage involved looking again at the starch content and its metabolism, but this time in terms of the roles that specific cells play.

In view of what is known about the starch metabolism of nodules and of plants, the following hypotheses were made:

(1) Starch accumulation in the nodule is related to the energy source/sink ratio, both at the level of the whole nodule and in terms of the individual cells.

(2) The starch content of the nodules is related to the varying activities of the enzymes of starch metabolism in the nodule.

(3) There are two isoenzymes of starch synthase in the nodule: one for starch synthesis and the other for its degradation.

In the present study, the soybean/B. japonicum interaction was chosen as the experimental system because determinate nodules provide tissue with more uniform stages of development than that found in meristematic nodules like those of alfalfa.

Three ineffective nodules, T5-95, T8-1 and 61A24, allow one to look at factors influencing starch metabolism by examining situations where various aspects of the symbiosis are altered. The three situations which I have

been able to study are the following: (1) T5-95, fix$^-$ (2) T8-1, fix$^-$ bad$^-$ (3) 61A24, fix$^-$, pbm$^-$ (at 18 to 20 d) (see Tables 1 and 2).

The first part of the study involved quantifying the starch levels in all three ineffective nodules as well as the wildtype, 61A76, which is the genetic parent of T5-95 and T8-1. The specific activities of amylase, starch phosphorylase, and the two forms of starch synthase were examined in these nodule types in order to elucidate events underlying the accumulation and degradation of starch. Sucrose synthase has been localized with immunocytochemistry, in order to determine the cell type(s) in which this pathway operates. Finally, using light microscopy, starch was localized in the effective and ineffective nodules.

MATERIALS AND METHODS

Bacterial strains and microbiological methods

B. japonicum strain 61A76 and 61A24 were obtained from Nitragin Co. (Milwaukee). Strains T5-95 and T8-1 were isolated following random Tn5 mutagenesis of strain 61A76 (Rostas et al.,1984). The bacteria were grown on liquid yeast extract, mannitol medium (0.2 g K_2HPO_4, 0.2 g $MgSO_4$, 0.5 g NaCl, 1 g yeast extract, 10 g mannitol per litre of distilled water, pH adjusted to 6.9) and 1% agar with 0.1% bromothymol blue indicator at room temperature.

Plant growth conditions

Glycine max cv Prize were obtained from Strayer Seed farms (Iowa). The seeds were treated with 10% sodium hypochlorite solution for 3 min and rinsed in running sterile distilled water for 15 min before inoculation. Bacterial inoculum was prepared by harvesting late exponential phase cultures grown in liquid medium on a rotary shaker, washing the bacteria in sterile water and concentrating 15 times before applying to seeds. Every set of 180 seeds was inoculated with the rhizobia resulting from four days of bacterial growth in 1L of liquid medium as described above. About 200 nodules would eventually establish themselves on the plant, be the inoculum fix^+ or fix^-. Plants were grown in a controlled environment chamber as described by Fuller and Verma (1984) using autoclaved vermiculite in sterile pots.

Sampling of Plant Material

Nodules were harvested only from the oldest part of the root, since the nodule age and size in that area are approximately uniform (Morrison, personal communication). In the starch quantification experiment and the

enzyme activity assays, the experiment in question was carried out three times for each tissue type. The nodules used in each of the three cases came from a different harvest. The value of the mean and standard error of the mean were calculated.

Quantification of starch

Relative levels of starch (expressed in terms of glucose units/mg protein) were determined for 14 and 21 d post infection nodules and uninoculated root. To convert starch to glucose units, two aliquots of the sample were analysed: one in which the starch was left intact and another in which the starch was broken down to glucose by amyloglucosidase. The difference between the two aliquots in terms of the presence of the reducing sugar, glucose, represented the amount of starch in glucose units. The following solutions were employed: (A) (extraction buffer) dimethylsulfoxide:25% HCl, 4:1 (v/v), (B) 0.2M sodium citrate buffer (pH 10.6), (C) 0.05mM sodium citrate buffer (pH 4.6), (D) amyloglucosidase suspension [3.2 mg in 300 µl of (C)], (E) triethanolamine buffer (pH 7.6), (F) (co-enzyme solution) 6mM NADP, 40mM ATP. A 5 ml aliquot of solution (A) was used to extract the starch from 120 mg (about 10 to 12 nodules of uniform size) of tissue which had previously been ground to a powder in liquid nitrogen. This mixture was left at 60°C for 30 min. It was then centrifuged at 12,000 X g for 5 min at 4°C. To 1 ml of the supernatant 6 ml of solution (B) was added. From this 7 ml mixture two aliquots were taken, one 300 µl sample was incubated at 57°C for 20 min with 300 µl of solution D, while another 300 µl sample was left on ice with 300 µl of solution C. The reaction mixture contained the following: 200 µl of the extract, 400 µl distilled water, 300 µl solution (E), 100 µl solution (F) and 10 µl of a

suspension of hexokinase/glucose-6-phosphate dehydrogenase (Boehringer - Mannheim). Each sample was incubated at room temp for 1 h. Extinction was measured at 340 nm (using quartz cuvettes) both before and after addition of the hexokinase/glucose-6-phosphate dehydrogenase (Schafer, 1983).

Enzyme assays

Amylase:

The specific activity of amylase was determined in 14 and 21 d nodules and uninoculated root (units = μmol glucose produced per min). The assay was carried out as described by Okita and Preiss (1980). One hundred twenty mg of tissue were used in this protocol, again representing 10 to 12 nodules. The activity was measured in 1 ml reaction mixtures containing the following: 5 mg amylopectin, 40 μmol sodium acetate buffer (pH 6.0) and 50 to 200 μl of the enzyme sample. The assay mix was incubated at 37°C for 0 to 30 min. Aliquots were removed at 5 min intervals. The amount of reducing sugar was quantified according to the procedure of Nelson (1944) using glucose as a standard.

Starch phosphorylase:

The specific activity of starch phosphorylase was examined in the same tissue allotments as for the amylase assay. The phosphorylase assay was carried out in the direction of synthesis of starch. A 2.5 g sample of tissue (200 to 250 nodules) was ground to a powder in liquid nitrogen and resuspended in 5 ml of the following extraction buffer: 100 mM sodium citrate (pH 6.8), 0.05% sodium bisulfite, 0.2 mM PMSF and 0.6 μl 2-mercaptoethanol. Immediately after resuspension in the extraction buffer the samples were mixed and placed on ice. The suspension was then

centrifuged at 12,000 X g for 5 min. The resulting supernatant was used for this assay. For each 0.2 ml sample of extract the following substrate solution was prepared: 0.1 M sodium citrate (pH 6.8), 2% starch, and 0.15 M glucose-1-phosphate. The starch was dissolved in the buffer by heating in a 100°C water bath. A 0.2 ml sample of the extract and a 0.2 ml sample of the substrate were each preincubated separately for 1 min at 30°C. After 1 min, the two solutions were mixed and returned to 30°C. At time "0" a 20 µl sample was taken and sampling continued every min for 10 min. Samples were placed directly into a tube containing the following mixture: 1.5 ml water, 1.5 ml metavanadate/molybdate solution and 3 ml n-butanol. The extinction of the organic phase was measured at 310 nm using quartz cuvettes. Potassium phosphate was used as a standard. The protocol was modified by Brisson (personal communication) from that in Parvin and Smith (1969).

Starch synthase:

The assay of starch synthase was carried out in the direction of starch synthesis. One ADP unit is produced per addition of one glucosyl to starch. Upon addition of PEP and pyruvate kinase to the reaction mixture, the PEP phosphorylates ADP to ATP. Of PEP, pyruvate remains: the concentration of pyruvate being in a 1:1 ratio with the amount of ADP. The following assay is designed to measure the relative concentrations of pyruvate as an indication of starch synthase activity.

The extraction procedure involved the grinding of 0.2 g of tissue (about 18 nodules) to a powder in liquid nitrogen. The powder was then resuspended in 0.4 ml of water, filtered through two layers of Miracloth and centrifuged at 2,500 X g for 10 min. The supernatant was then removed

and saved (as it contains the SSS). The pellet was resuspended in 0.4 ml water (containing the SBSS) (see Baxter and Duffus, 1971).

The reaction mix contained the following: 0.3 μmol ADPG, 0.1 μmol EDTA, 40 μmol glycine buffer (pH 8.4), 0.1 ml enzyme preparation and water to 0.4 ml (Hawker et al., 1974). Phosphoenolpyruvate (PEP) in 0.4 M KCl (0.025 ml) was then added to the reaction mixture. Immediately following, 1 unit of pyruvate kinase in 0.1 M $MgSO_4$ was added and mixed. The reaction mixture was incubated at 37°C for 0, 5, 10, 15 and 30 min. Pyruvate was used as a standard with a range of 10 to 60 μg (per tube). To each reaction tube, 0.15 ml of 0.1% 2,4-dinitrophenylhydrazine in 2N HCl was added, mixed and left at room temp for 5 min. Finally, 0.2 ml of 10M NaOH with 1.1 ml 95% ethanol was added, mixed and centrifuged to remove debris. The absorbance was measured at 520 nm using plastic disposable cuvettes. The protocol for estimation of ADP was modified from Leloir and Goldemberg (1960) excluding any steps necessary for blood (Farkas, personal communication).

Light microscopy:

(A) Paraffin-embedded material

Whole or half nodules were fixed in 5 ml of formalin-acetic acid-alcohol (FAA) (50% ethanol, 90 ml; glacial acetic acid, 5 ml; 38% formaldehyde, 5 ml) for 24 h at 4°C. The tissue was then rinsed 3 times in 50% ethanol and treated for a minimum of 6 h with each of following series of solutions: TBA 1 (50 ml distilled water, 40 ml 95% ethanol, 10 ml tertiary butyl alcohol(TBA)), TBA 2 (30 ml distilled water, 50 ml 95% ethanol and 20 ml TBA), TBA 3 (15 ml distilled water, 50 ml 95% ethanol and 35 ml TBA), TBA 4 (45 ml 95% ethanol and 55 ml TBA) and TBA 5

(75 ml TBA and 25 ml 100% ethanol). After exposure to this series of solutions, the tissue was placed in pure TBA at 37°C, pure TBA at 61°C, TBA: paraffin wax (1:1) at 61°C and, finally, pure paraffin wax at 61°C repeated 2 times. At this point the tissue was embedded in paraffin wax. The blocks were cut into 8 µm sections on a A.O. Spencer Rotary 820 microtome. The sections were mounted onto glass slides by first placing 2 drops of Haupt's adhesive on the clean slide followed by 6 drops of 4% formalin. The ribbons produced by sectioning were placed onto this mixture and the slides were placed on a warming tray at 60°C for a few seconds. The excess fluid was subsequently drained and the slides were then placed in a 37°C oven to dry for 12 to 24 h.

The sections were then deparaffined by placing the slides in the following solutions, for a period of 5 min each: 2 times 100% xylene, xylene/ethanol (1:1), 100% ethanol, 95% ethanol, 70% ethanol, and 50% ethanol. The sections were then stained specifically for starch in the following manner: Gram's iodine, 15 min; 2 times 10-min iodine rinse (2% iodine in 100% ethanol); hematoxylin, 10 min, 10 s in acidified water and aqueous safranin 5 min. Gram's iodine is specific for starch (see Pearse, 1954). Hematoxylin and safranin were used for background staining. The sections were then cleared: 50% ethanol 5 min, 70% ethanol 5 min.,95% ethanol 1 min, 100% ethanol 30 s, xylene/ethanol (1:1) 30 s, 100% xylene 30 s. The cover slip was placed on slides still wet with xylene using D.P.X. (BDH) to secure it. The slides were then placed on a 60°C warming tray overnight and then observed under the microscope.

(B) Plastic embedding for high magnification studies.

Small pieces of tissue (0.5-1 mm were fixed in 3% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.2). The specimens were then washed in cold

(4°C) buffer 6X, each wash lasting 10 min. The tissue was then dehydrated in ethanol and embedded in Spurr's epoxy resin (Spurr, 1969). The blocks were sectioned on a Porter-Blum ultramicrotome MT-1 (Sorvall) to obtain 1.5 µm thick sections using a glass knife. Sections were mounted individually onto water droplets on glass slides. After drying, the sections were stained with a mixture of Azure II and methylene blue (Selker and Newcomb, 1985) at 70°C. The slides were then rinsed with distilled water and a cover slip was placed on top as described above.

Electron microscopy:

The tissue was fixed as described above, for plastic embedding, the only difference being that it was done on ice. Three plastics (Spurr, IR white and Lowicryl) were tested in order to find the one best suited to the antigenicity of the given antigen under examination. Lowicryl K4M was found to give the best results when used as outlined in Lacoste-Royal and Gibbs (1985). The first stage was dehydration of the tissue using the following series of ethanol solutions: 25% ethanol, 2 times 15 min at -5°C; 50% ethanol, 3 times 20 min at -18°C; 75% ethanol, 3 times 20 min at -18°C and 95% ethanol, 3 times 30 min. at -18°C. The infiltration series was carried out next: 95% ethanol:resin 2:1, 1 hour; 1:1, overnight; 1:2, 2 times 120 min. and pure resin overnight all at -18°C. The tissue was then embedded in pure resin (first equilibrated at -18°C for 15 min) in BEEM capsules. Polymerization was carried out in the following way: 24 hours at -18°C and 1 to 2 d at room temp, both durations being under UV light (General Electric lamp# F1).

The immunocytochemical analysis was carried out as described below. Pale gold sections were picked up on Formvar-coated nickel grids and placed

(section side down) on the following series of solutions (1 drop each): phosphate buffered saline (PBS), 15 min; antibody diluted 1:1000 in PBS, 30 min; protein A gold diluted 1:10 in PBS, 30 min. All steps were done at room temp. The sections were post stained in 2% uranyl acetate prior to electron microscopy. The procedure described above is from Mangeney and Gibbs (1987). The antibody made against nodule-specific sucrose synthase or nodulin 100 (anti-nodulin 100) was made as described in Thummler and Verma (1987). The anti-potato starch phosphorylase (anti-PSP) was a gift from N. Brisson of l'Université de Montréal.

Western blotting and immuno-detection of proteins:

Proteins were resolved on denaturing polyacrylamide gradient gels (ranging from 7.5 to 15% acrylamide) (Laemmli, 1970). Each lane was loaded with 60 μg of protein. Two gels were prepared, one stained with Coomassie brilliant blue and the other used for the Western blotting. The procedure for the Western blot involved transfer of proteins onto nitrocellulose (0.45 μm) and using the buffer system described by Burnette (1981). Incubation of antisera (diluted 1:100 in tris buffered saline (TBS)) with blots and detection of the antigen/antibody complexes with iodinated protein A were done as described by Davis et al. (1983). Staphlococcus aureus protein A (Boehringer Mannheim) was iodinated using the procedure of Langone (1980).

RESULTS

Starch quantification:

The starch quantification data for effective and ineffective nodules and of uninoculated root are shown in Figure 2. In 14 d wildtype nodules, 26 µmoles glucose equivalents of starch were present. In all types of 14 d ineffective nodules, the levels of starch were lower than levels found in the 14 d wildtype nodules, T5-95, T8-1 and 61A24 each having 12, 8 and 24 µmoles of glucose equivalents, respectively. From 14 to 21 d post-infection, the starch levels decreased in wildtype nodules by about 80%. On the other hand, it increased in all ineffective nodules, by 80% in T5-95, by 165% in T8-1 and by about 1200% in 61A24 infected nodules. The starch level of uninoculated root was 70 µmoles of glucose equivalents and was, thus, higher than that in any nodule except in 21d nodules of 61A24 infected roots. The values shown in Figure 2 represent the mean of three replicates each of which deviated from the mean by less than 10%.

Enzyme assays:

(i) Amylase

Specific activities of amylase of effective and ineffective nodules and of uninoculated root are shown in Figure 3. The amylase specific activity decreased from the 14 to the 21 d samples by just over 40% in 61A76, by 20% in T5-95 and by just over 65% in T8-1 nodules. The only nodule type which showed an increase was 61A24, of about 35%. One striking aspect of the amylase data was the relatively high levels of the enzyme found in 14 d T8-1 nodules, about 330% higher than that found in the wildtype nodules of the same age. The other 2 ineffective nodules, T5-95 and 61A24, also had levels higher than that found in the wildtype at 14 d, but only by 35% and 30%, respectively. At 21 days, the level of amylase activity in the

Figure 2: Relative starch content (expressed in glucose units/mg protein) in nodules 14 and 21 d post-infection and in uninoculated root. The tissue types are as follows (referring to the infecting bacterium in each case): A=61A76, B=T5-95, C=T8-1, D=61A24 and R=uninoculated root.

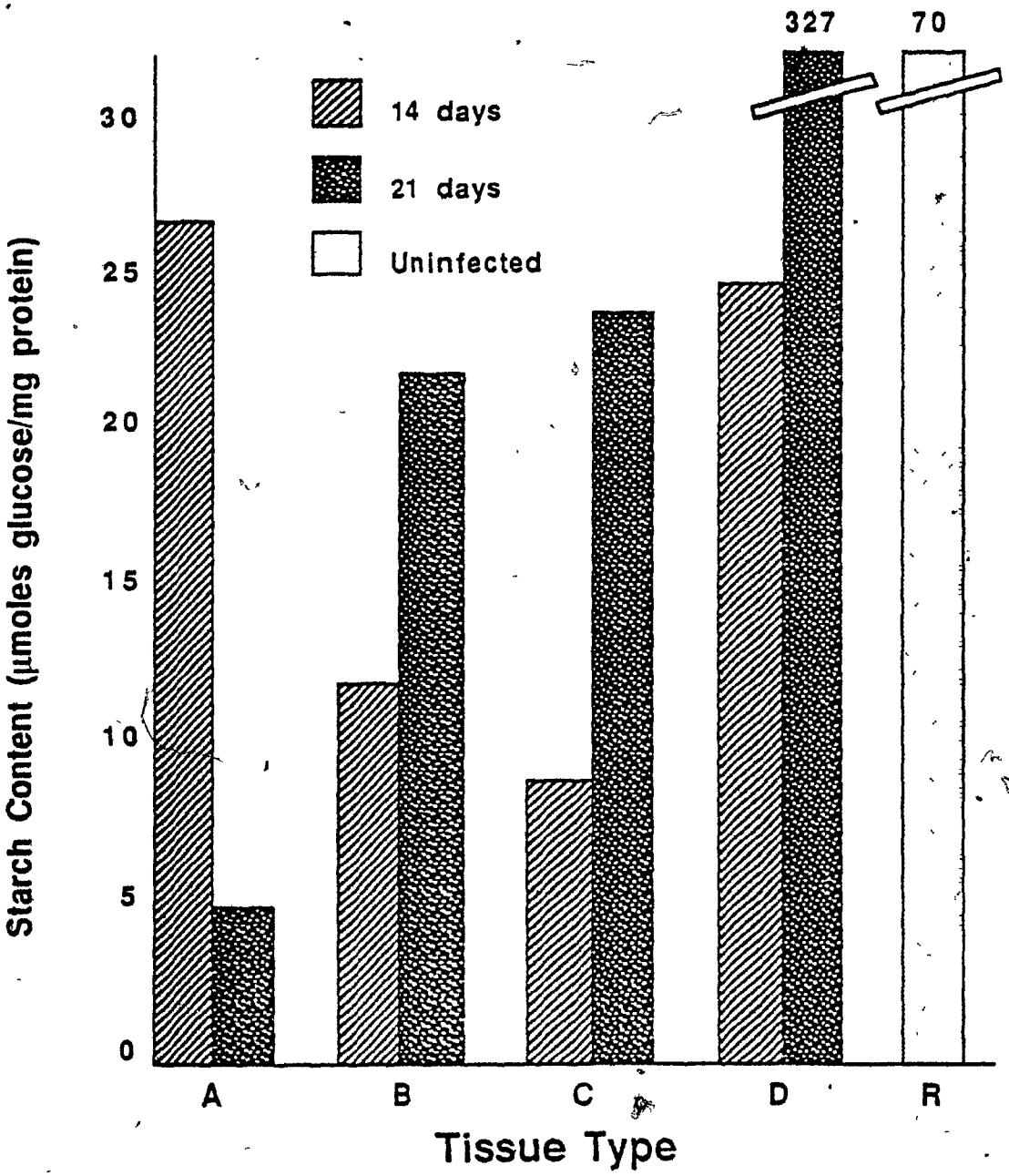
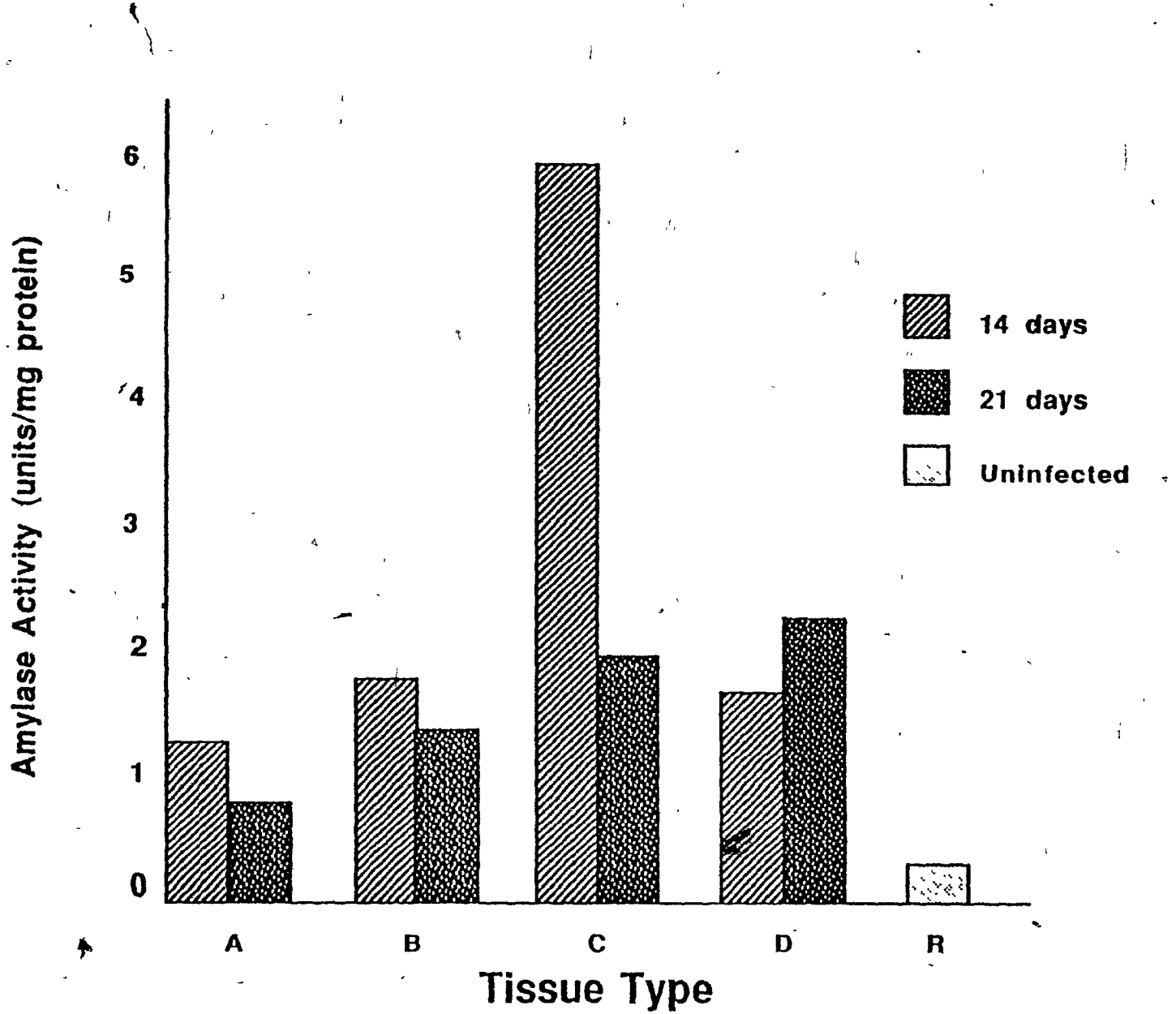

327
70
14 days
21 days
Uninfected
30
25
20
15
10
5
0
Starch Content (µmoles glucose/mg protein)
A
B
C
D
R
Tissue Type

Figure 3: Specific activity of amylase in 14 and 21 d nodules and uninoculated root (units= μmol glucose produced per min per mg protein). The tissue types are as follows (referring to the infecting bacterium in each case): A=61A76, B=T5-95, C=T8-1, D=61A24 and R=uninoculated root.


Amylase Activity (units/mg protein)
0
1
2
3
4
5
6
14 days
21 days
Uninfected
A
B
C
D
R
Tissue Type

ineffective nodules was again higher than that found in the effective nodules of the same age by: 90% in T5-95, just over 150% in T8-1 and 200% in 61A24 nodules. The specific activities of uninoculated root were very low, being about 1/3 of that found in the 21 d wildtype nodules. The values shown in Figure 3 represent the mean of three replicates each of which deviated from the mean by less than 10%.

(ii) Starch Phosphorylase

Specific activity of starch phosphorylase in effective and ineffective nodules and uninoculated root is presented in Figure 4. Starch phosphorylase specific activity increased from the 14 to 21 d samples by about 80% in the wildtype, by more than 980% in T5-95, by 135% in T8-1 and by more than 540% in 61A24 nodules. At 14 d, the wildtype had the highest relative activity, 70% more than that found in T5-95, about 70% more than in T8-1 and about 20% more than that found in 61A24 nodules. At 21 d, this situation was reversed for T5-95 and 61A24 nodules, each having about 70% and 180% more phosphorylase activity than the wildtype counterpart, respectively. The 21 d T8-1 nodules had about 1/2 the activity of the wildtype nodules of the same age. The level in uninfected root was also low, having roughly 1/2 the activity of the 21 d wildtype nodules. The values shown in Figure 4 represent the mean of 3 replicates each of which deviated from the mean by less than 10%.

(iii) Starch Synthase

(a) SBSS

The specific activities of SBSS of effective and ineffective nodules and uninoculated root are shown in Figure 5. During the time between 14 and 21 d post-infection, the wildtype and T8-1 nodules showed decreases (from 14 to 21 d) of about 75% and 25%, respectively. Conversely, the T5-95 and

Figure 4: Specific activity of starch phosphorylase in 14 and 21 d nodules and uninoculated root (units= n mol phosphate released per min per mg protein). The tissue types are as follows (referring to the infecting bacterium in each case): A=61A76, B=T5-95, C=T8-1, D=61A24 and R=uninoculated root.

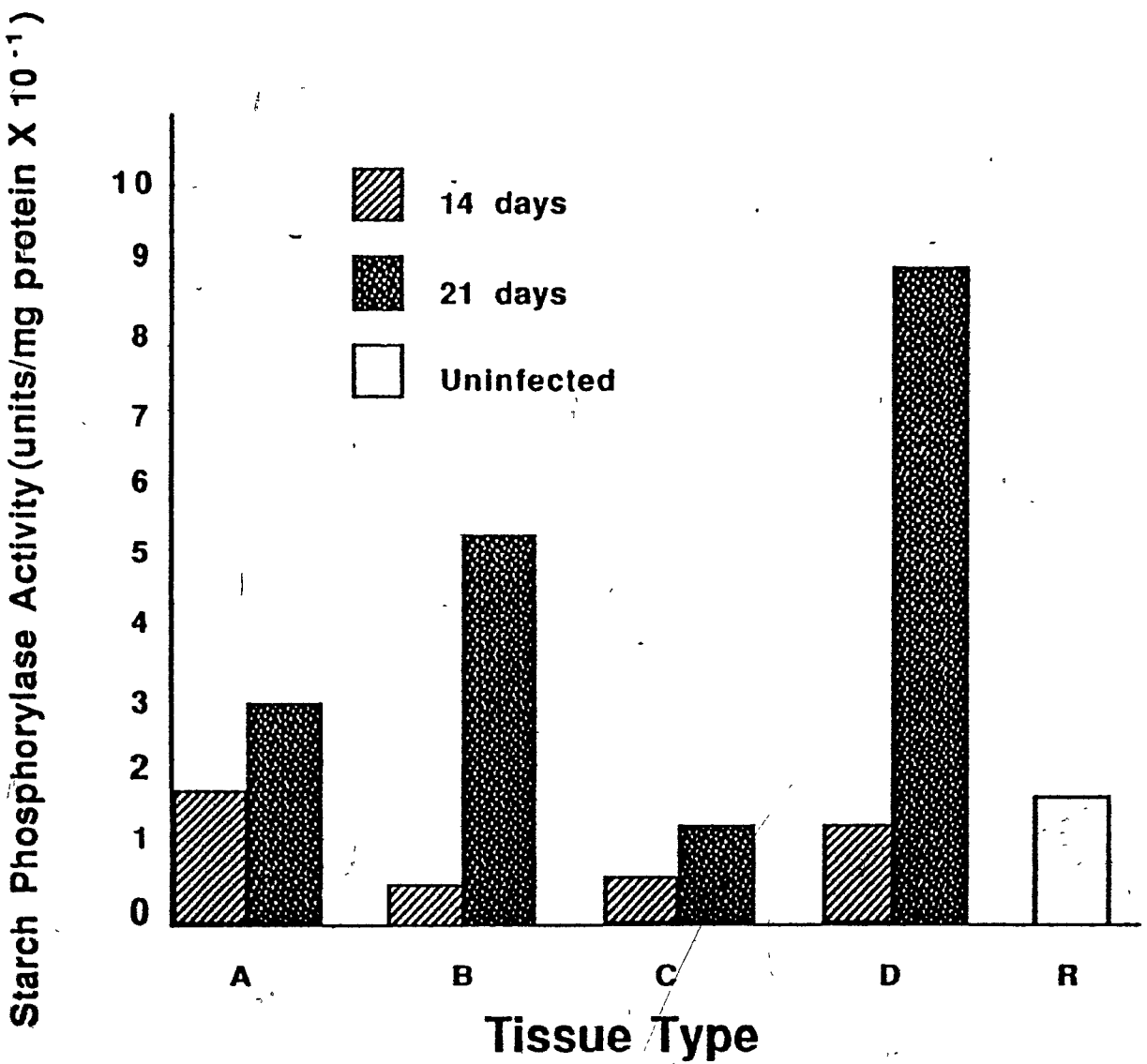
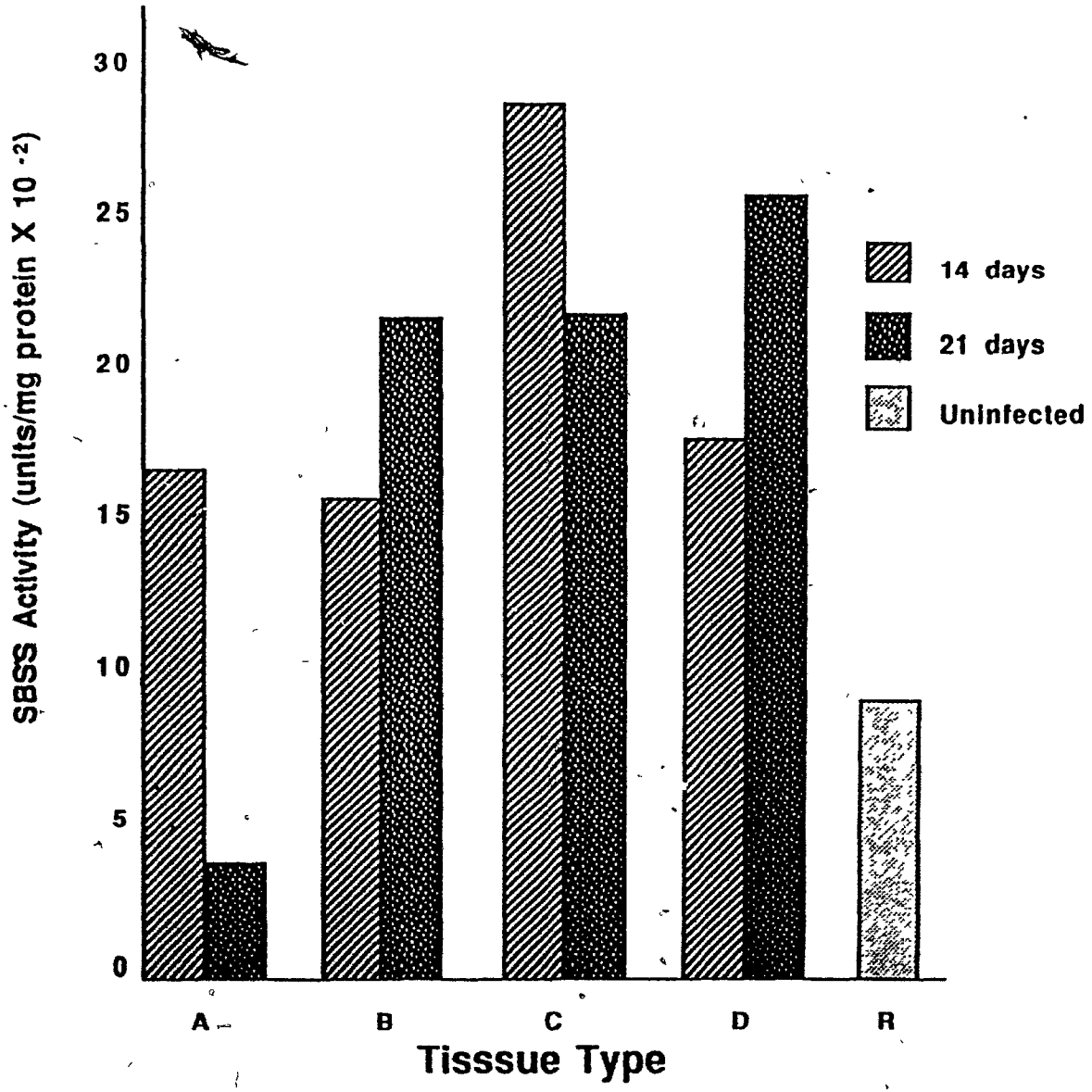

Starch Phosphorylase Activity (units/mg protein X 10⁻¹)
10
9
8
7
6
5
4
3
2
1
0
14 days
21 days
Uninfected
A
B
C
D
R
Tissue Type

Figure 5: Specific activity of SBSS in 14 and 21 d nodules and uninoculated root (units= μmol pyruvate produced per min per mg protein). The tissue types are as follows (referring to the infecting bacterium in each case): A=61A76, B=T5-95, C=T8-1, D=61A24 and R=uninoculated root.


SBSS Activity (units/mg protein X 10 -2)
30
25
20
15
10
5
0
A
B
C
D
R
Tisssue Type
14 days
21 days
Uninfected

61A24 nodules showed increases (from 14 to 21 d) of about 40% and 45%, respectively. At 14 d, both the T5-95 and 61A24 nodules had levels of specific activity similar to the wildtype nodules of the same age. The T8-1 nodules, at 14 d, had an activity level 70% higher than the wildtype nodules of the same age. At 21 d post-infection, all the ineffective nodules had higher activity levels than the wildtype of the same age by: 450% for T5-95 and T8-1 and 550% for 61A24. The uninfected root level was intermediate between the effective and ineffective situation at 21 d, being 125% more than that of the 21 d wildtype. The values shown in Figure 5 represent the mean of 3 replicates each of which deviated from the mean by less than 10%.

(b) SSS:

Specific activity of SSS of effective and ineffective nodules and uninoculated root is shown in Figure 6. An increase of about 65% in the specific activity of SS synthase was observed from the 14 to 21 d samples of wildtype nodules. A decrease of just over 15% was observed in T5-95 nodules from 14 to 21 d post-infection. Both T8-1- and 61A24- infected nodules appeared to maintain constant levels of activity at both 14 and 21 d. At 14 days, both the T5-95 and 61A24 nodules had higher levels of activity than the wildtype, by about 50% and about 65%, respectively. The 14 d wildtype nodules had about 25% more specific activity than that found in T8-1 nodules of the same age. At 21 d, the wildtype had about 25% and 55% more activity than T5-95 and T8-1 nodules, respectively. The 21 d 61A24 nodules had a similar enzyme activity as in the wildtype nodules of the same age. The 21 d wildtype nodules had about 70% less activity than did the uninoculated root. The values shown in Figure 6 represent the mean of 3 replicates each of which deviated from the mean by less than 10%. The data presented in Figures 2 to 6 are summarized in Table 3.

Figure 6: Specific activity of SS6 in 14 and 21 d nodules and uninoculated root (units= µmol pyruvate produced per min per mg of protein). The tissue types are as follows (referring to the infecting bacterium in each case): A=61A76, B=T5-95, C=T8-1, D=61A24 and R=uninoculated root.

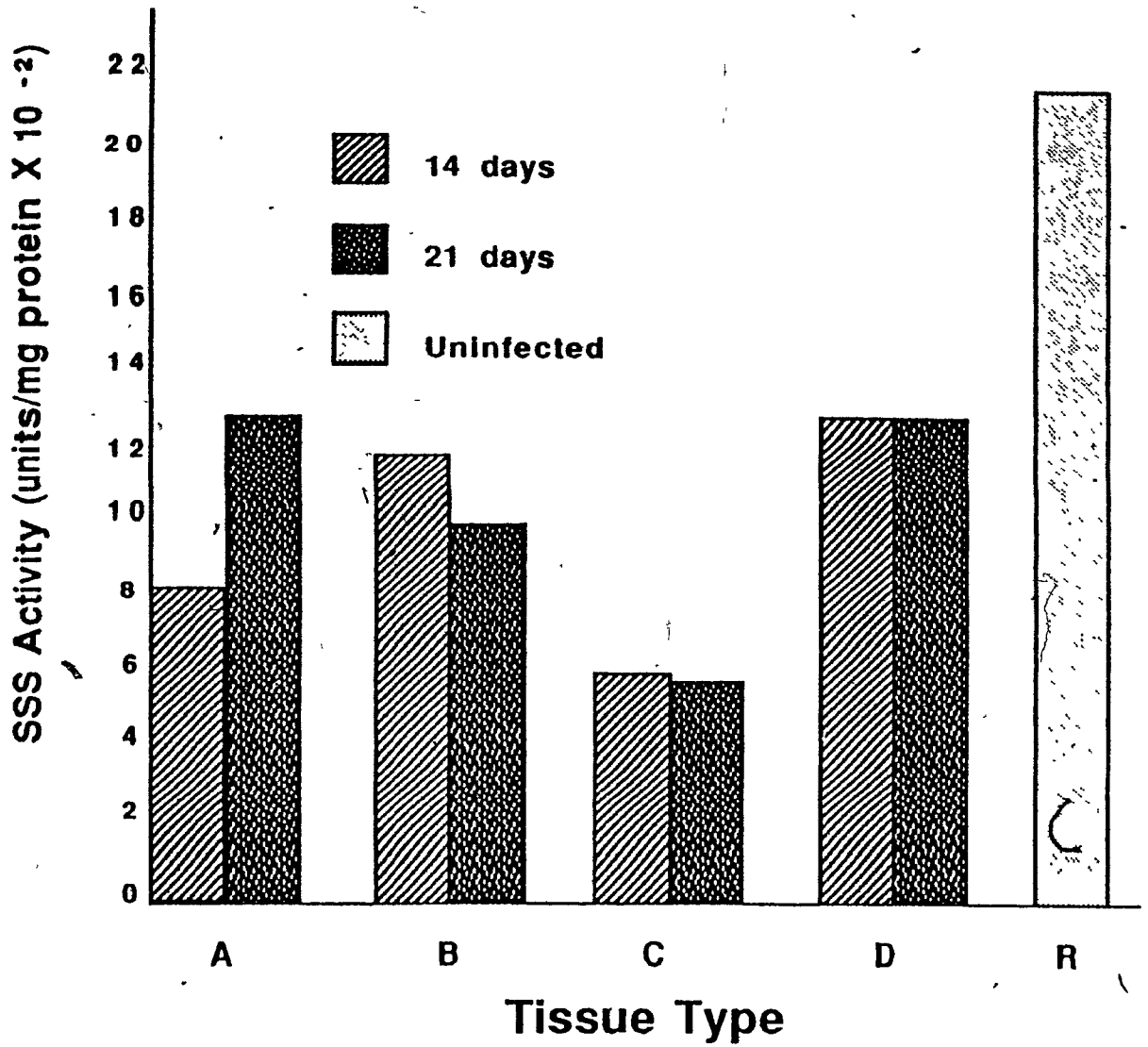

SSS Activity (units/mg protein X 10 -2)
22
20
18
16
14
12
10
8
6
4
2
0
14 days
21 days
Uninfected
A
B
C
D
R
Tissue Type

Table 3. A summary of the results presented in Figures 2 to 6.

Table 3: Summary of the levels of starch and the specific activities of amylase, starch phosphorylase, SBS synthase and SS synthase in nodules formed by different strains of B.japonicum and uninoculated root. All levels are expressed relative to that found in 21 d nodules infected with wildtype B.japonicum (strain 61A76) which represents one unit. Any value going beyond the 6th level is accompanied by an arrow (↑).

Strain of B.japonicum		St	Am	SP	SBSS	SSS
61A76	14 d	5	2	<1	4	<1
	21 d	1	1	1	1	1
T5-95	14 d	2	2	<1	4	1
	21 d	4	2	2	6	<1
T8-1	14 d	2	6(↑)	<1	6(↑)	<1
	21 d	5	3	<1	6	<1
61A24	14 d	5	2	<1	5	1
	21 d	6(↑)	3	3	6(↑)	1
uninoculated root		6(↑)	<1	<1	2	2

Identification of specific proteins in effective and ineffective nodules

Protein profiles obtained with SDS-PAGE and staining with Coomassie Brilliant Blue are shown in Figure 7. Leghemoglobin (Lb) bands are indicated (as "leg"). All the proteins refered to in the autoradiograph presented in Figure 8 are also indicated in Figure 7.

Reaction of protein bands with the antibody was identical for effective and ineffective nodule extracts. For this reason the ineffective lanes are not shown in Figure 8.

In Figure 8 (lane b) faint bands are apparent at 52 and 59 kDa which seem to correspond to bands at the same molecular weights in the potato lane (d). The 66 kDa band (fourth arrow from the top) was distinctly present in the 14 d soybean nodule lanes and there was also a faint band at 66 kDa in lane b (containing the 21 d wildtype nodule extract). The 76 kDa (third arrow from the top) band was present again, in the 14 d soybean nodule lanes and very faintly in the 21 d lanes. The 87 kDa band (second arrow from the top) was present at relatively high levels in all nodule lanes and at a lower level in the uninoculated root lane. The 105 kDa band (top arrow) was present at a low level in lanes A and B (14 and 21 d nodules, respectively) and at a high level in the potato lane (D). This band was extremely faint in the uninoculated root lane (C).

Localization of sucrose synthase

Figures 9 a and b show nodule specific sucrose synthase localized in 18 d effective nodules using protein A gold. The grouping of gold particles indicated that the enzyme is present in the cytoplasm of both infected and uninfected cells.

Location of starch

(i) Whole nodule:

Figure 10 shows cross sections of 21 d effective (a) and ineffective

Figure 7: SDS-PAGE gel stained with Coomassie brilliant blue. Markers shown in the far left hand lane represent the following molecular weights, starting at the top ($x10^3$): 92, 66, 45, 31, 21.5, and 14.4. Each lane was loaded with 60 μg of protein from the following tissue types (the first eight lanes being nodule) 1: 14d 61A76, 2: 21d 61A76, 3: 14d T5-95, 4: 21d T5-95, 5: 14d 61A24, 6: 21d 61A24, 7: 14d T8-1, 8: 21d T8-1, 9: uninoculated root and 10: mature potato tuber. Arrow heads represent proteins of the same molecular weight as those refered to in Figure 8 and in the text.

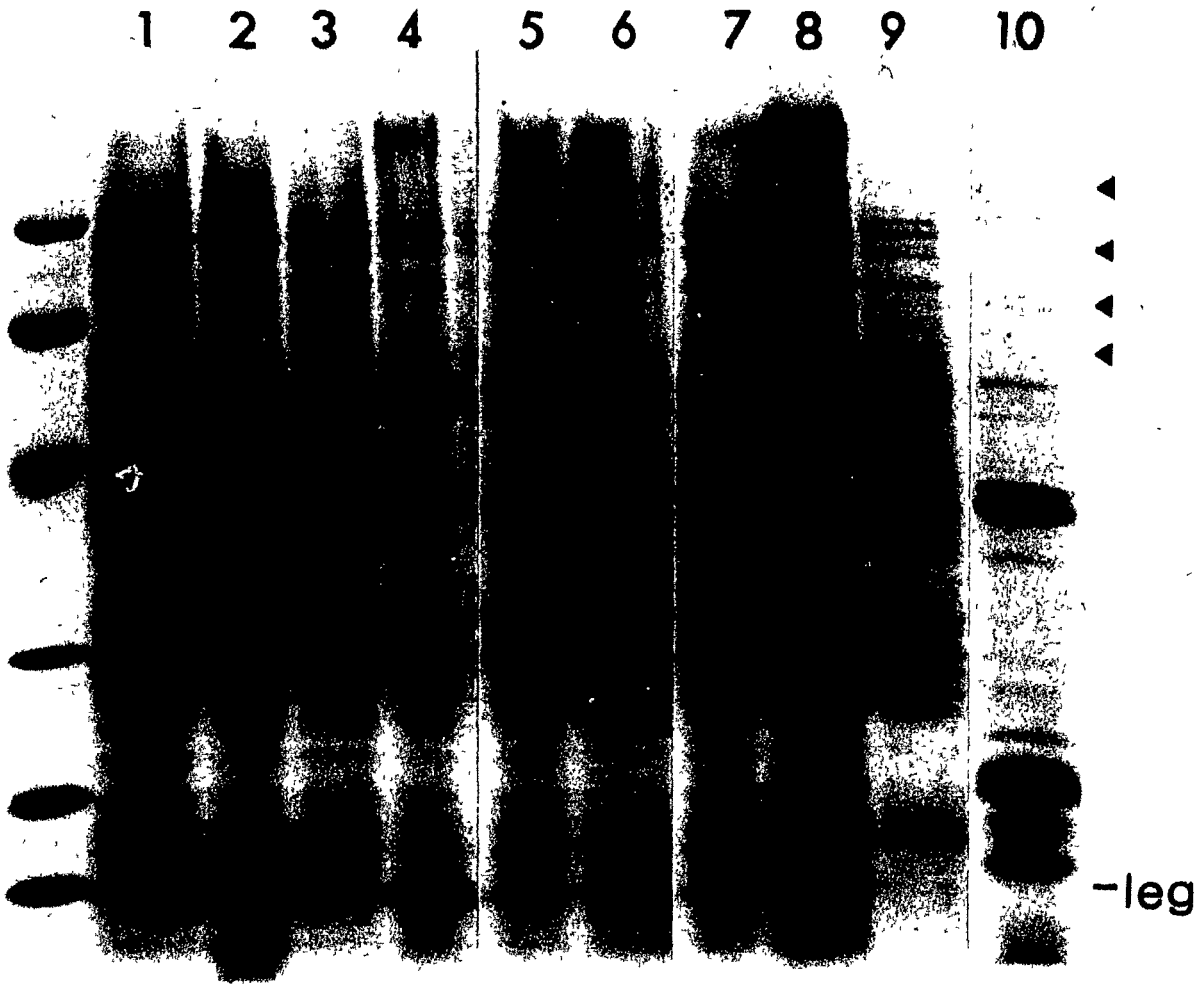

1
2
3
4
5
6
7
8
9
10
-leg

Figure 8: Detection of protein homologous to potato starch phosphorylase. SDS-gel pattern of proteins (prepared exactly as shown in Fig.7, but without Coomassie staining) identical to those present in lanes 1,2,9 and 10 now represented as a,b,c and d, respectively, each lane containing protein from the following nodules as listed: a, 14 d 61A76; b;21 d 61A76; c, uninoculated root and d, mature potato tuber. These proteins from the gel were transferred to nitrocellulose and incubated with antisera made against PSP. Antigen-antibody complexes were detected using [^{125} I] protein A and autoradiography. Arrow heads represent protein bands discussed in the text and correspond to similarly ordered arrowheads in Figure 7. The results from the ineffective lanes were not included because the protein from the 14 and 21 d reacted to the anti-PSP in exactly the same way as the 14 and 21 d effective nodules, respectively.

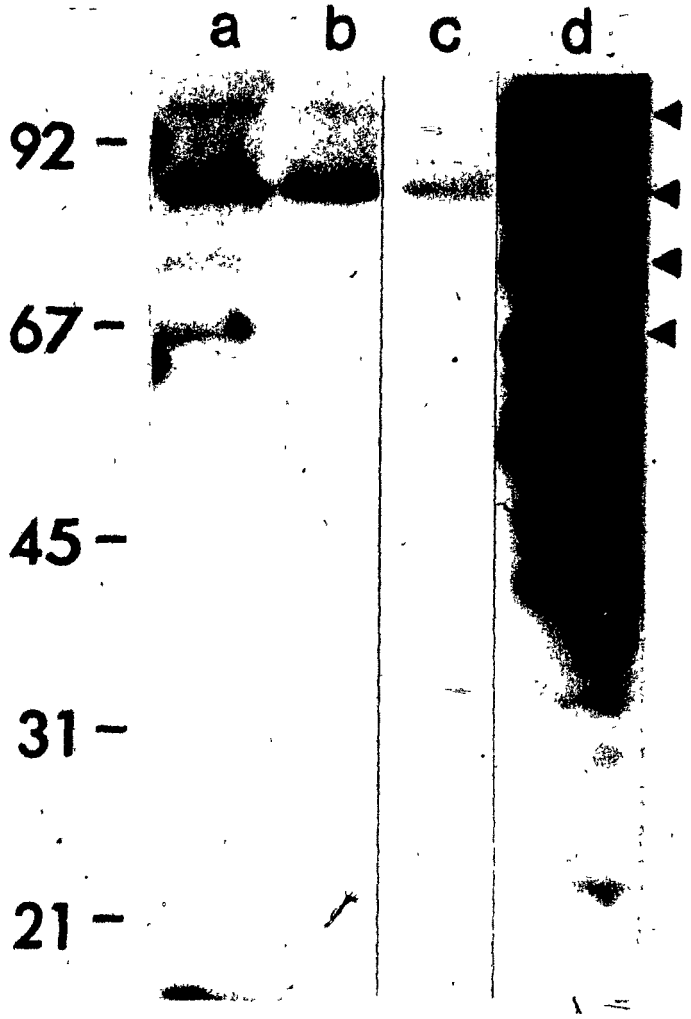

a
b
c
d
92 -
67 -
45 -
31 -
21 -

Figure 9: Immunocytochemical localization of nodule-specific sucrose synthase on thin sections of 18 d wildtype nodules. Sections were incubated with anti-nodulin 100. Specific labelling with gold particles was observed in the cytoplasm of both infected (IC) and uninfected (UC) cells. Gold was sparse or absent in the pbm, pbf, bacteroids (b), vacuoles (V), peroxysomes (p), amyloplasts (A) or starch grains (St). Panel (a), an infected and uninfected cell side by side and (b), the intercellular space (IS). Bar=1 μm.

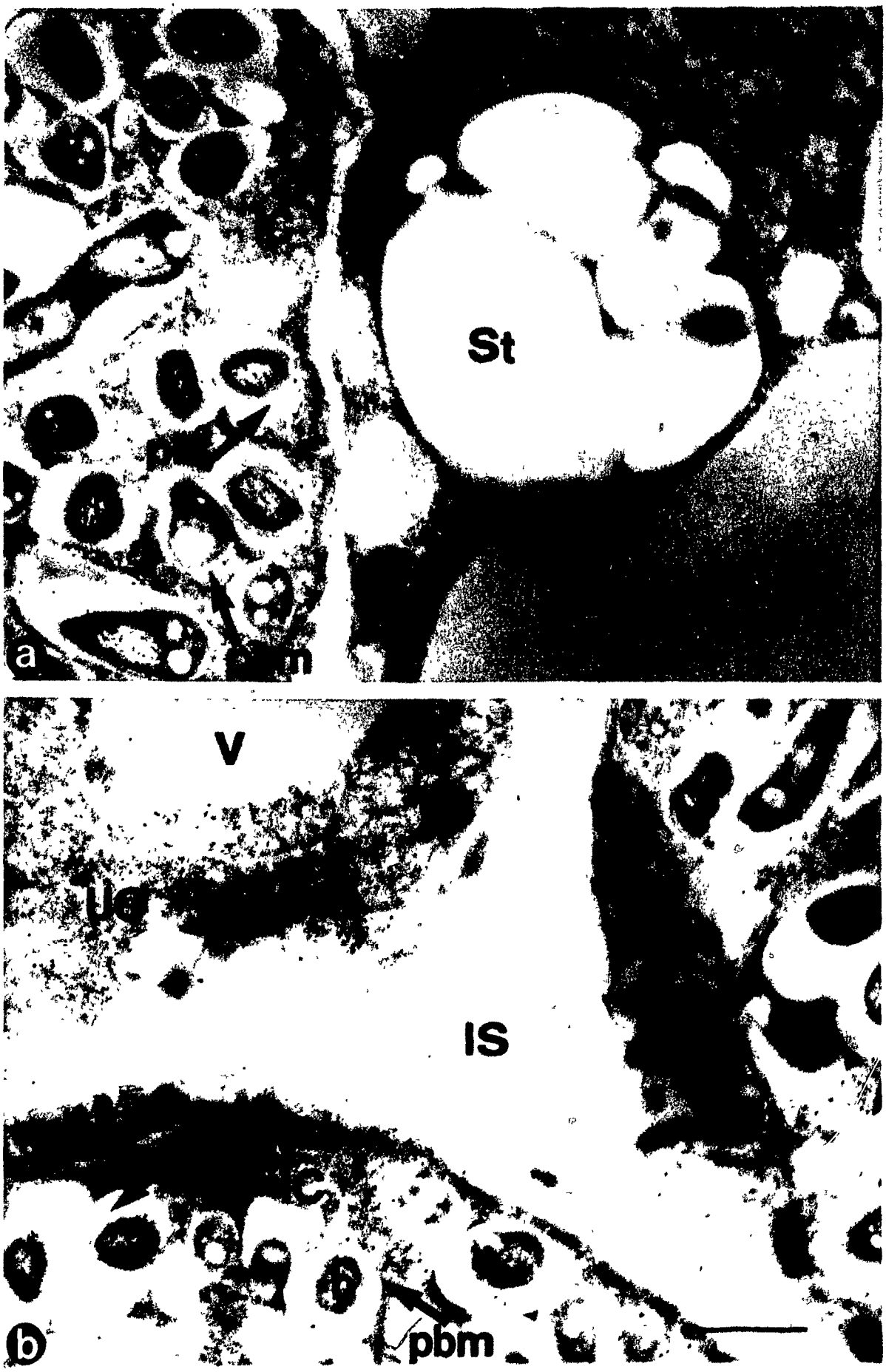
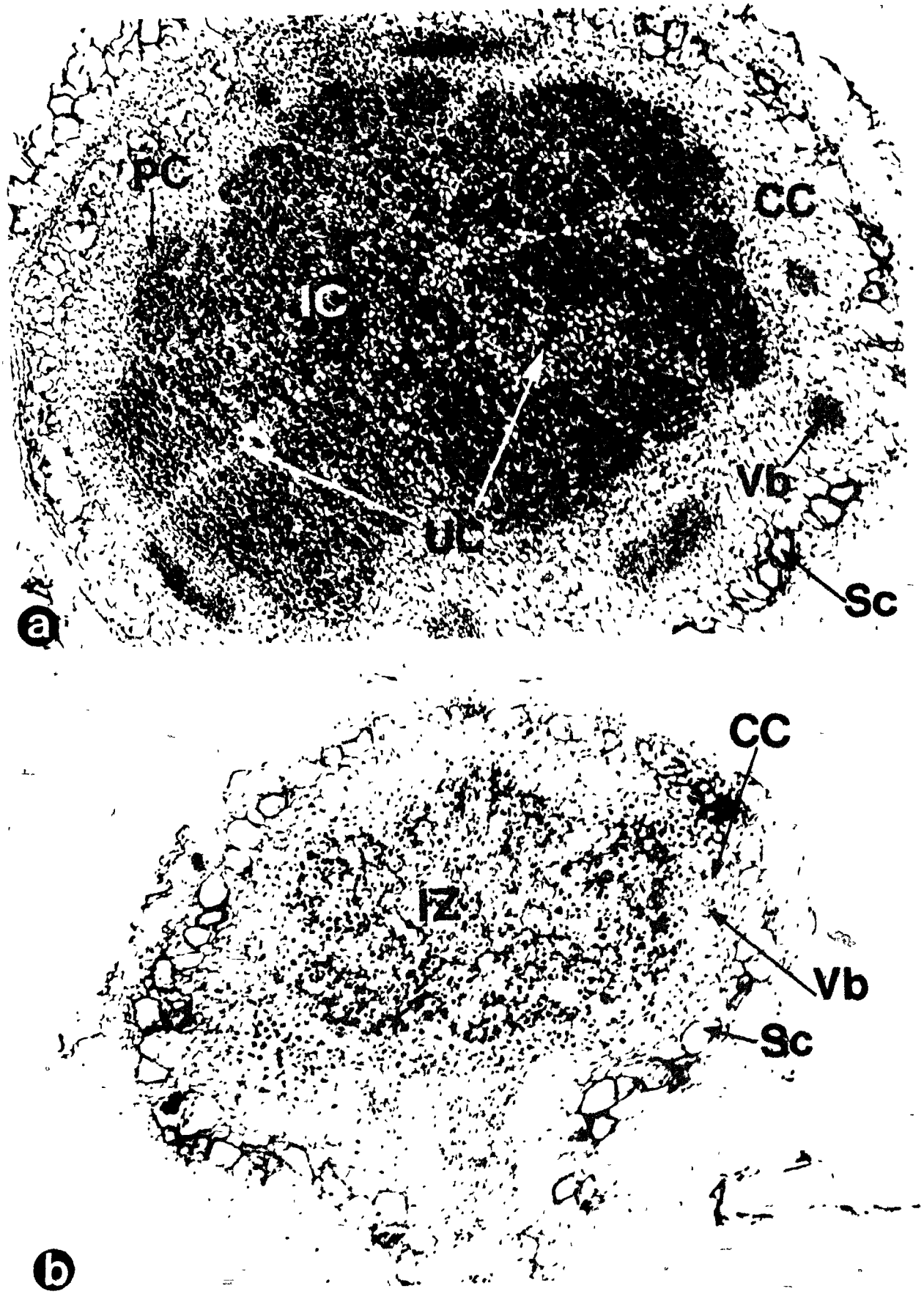

St
a
n
V
Ug
IS
b
pbm

Figure 10: Cross sections of 21 d effective (a) and ineffective (b) nodules. The following cell types are shown: cortical (CC), interstitial uninfected (UC), peripheral (PC), infected (IC) and the following: vascular bundles (Vb), infected zone (IZ) and thick walled sclerenchyma (Sc). Magnification= 50 X.


PC
CC
IC
UC
Vb
Sc
a
CC
IZ
Vb
Sc
b

(infected with the 61A24 strain of B.japonicum) (b) nodules. The most visible difference was the absence of bacteroids in (b). Whole nodule sections were stained with hematoxylin/safranin

(ii) Low magnification studies:

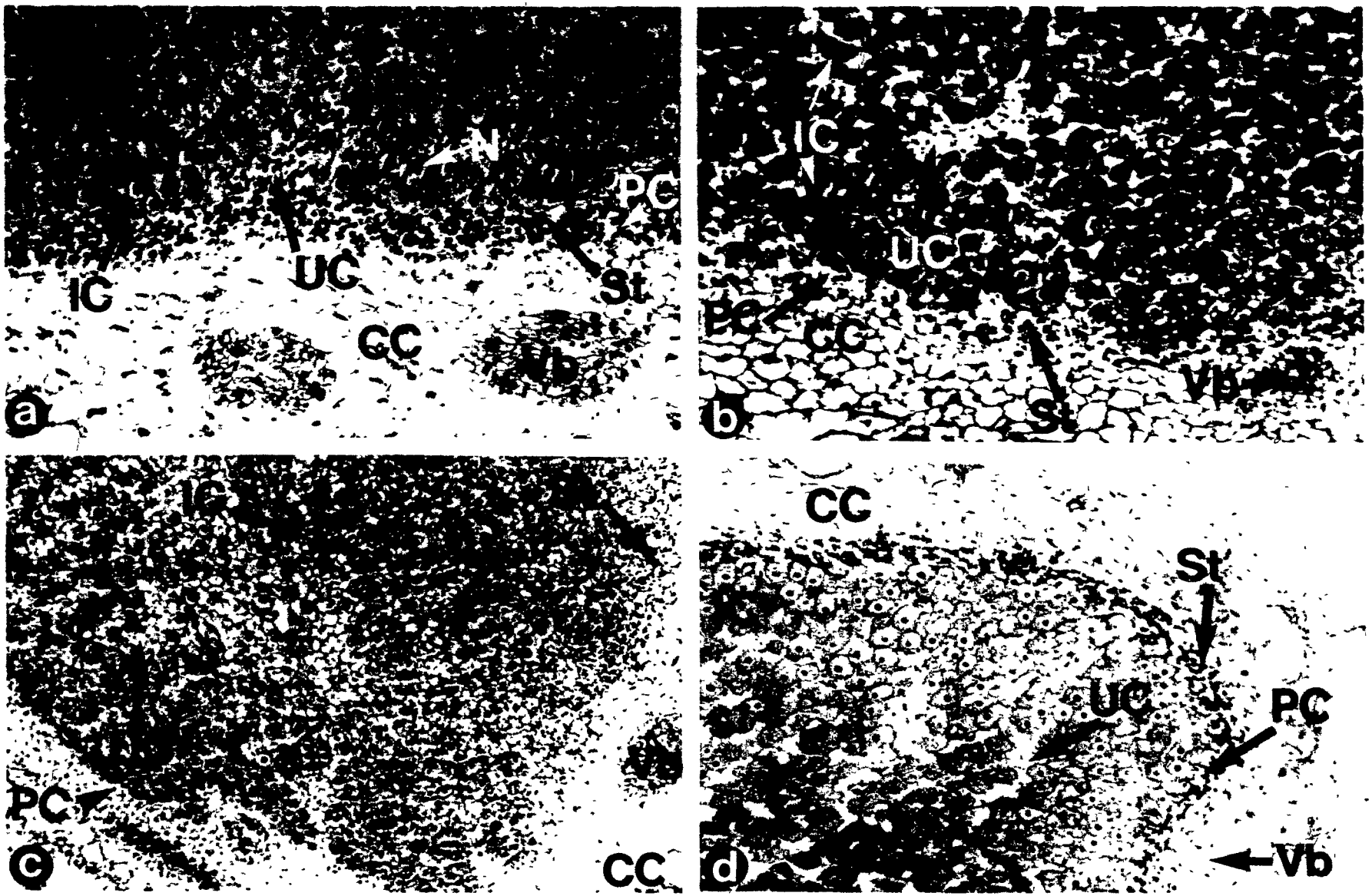
Sections made from paraffin embedded material are shown in Fig 11 a - h. Thick sections of this material showed large populations of cells for determination of the location of starch in the nodule as a whole.

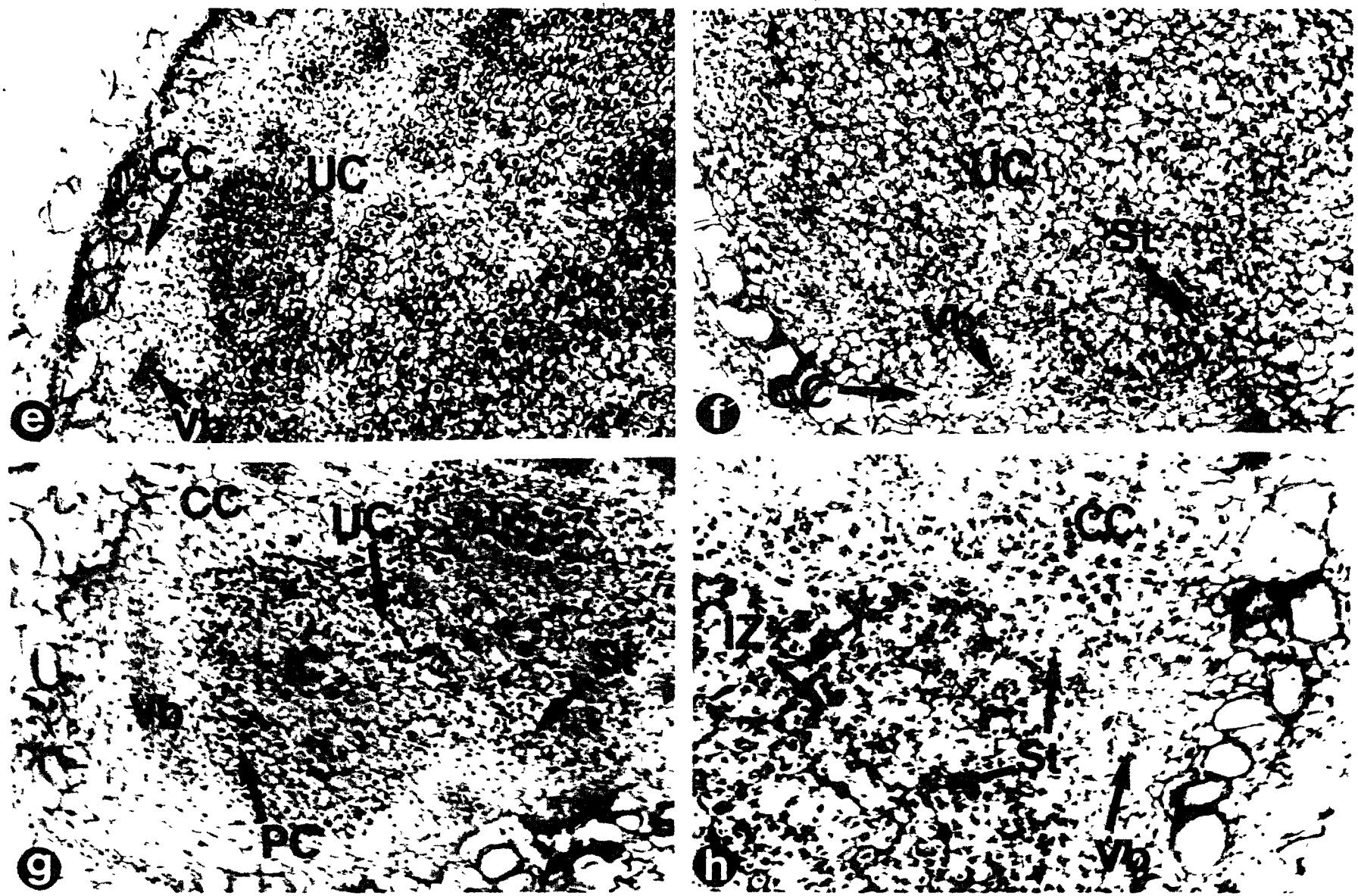
The sections of the 14 d 61A76 nodules (a) indicated that starch was present in uninfected cells both at the outer edge of the central group of infected cells and in cells interpersed amongst the infected cells (see Figure 9 for definition of these areas). Very little starch was apparent in the cortical cells and in the immediate vicinity of the vascular bundles. The 21 d wildtype nodules (b) showed a similar pattern except there was much less starch in the interstitial uninfected cells and again very little starch was found in the cortex of the nodule.

At 14 d, the T5-95 nodules (c) did show an accumulation of starch, mainly in uninfected cells close to the large vascular bundles. Starch was apparent both at the outer edge of the core and interstitially in patches. At 21 d, these nodules (d) showed a very distinct absence of starch in the core cells and a clear layer of starch containing cells at the outer periphery. Some starch was found in the cortical cells (at a higher level than that found at 14 d), although, the amount was low relative to the other nodule types.

In 14 d T8-1 nodules (e) there was little starch accumulation. Sections of the 21 d T8-1 nodules (f) showed an accumulation of starch mainly in the cortical cells. There was an abundance of relatively large cells containing sizable nuclei in the center of the nodule which were found to be devoid of starch.

Figure 11: Cross section of paraffin-embedded tissues of 14 and 21 d nodules stained with a combination of hematoxylin/safranin and Gram's iodine. The following legend applies: infected cell (IC), uninfected cell (UC), peripheral cells (PC), vascular bundle (Vb), cortical cells (CC), nucleus of infected cells (N), infected zone (IZ) and starch (St). (a) 14 d 61A76 (b) 21 d 61A76 (c) 14 d T5-95 (d) 21 d T5-95 (e) 14 d T8-1, (f) 21 d T8-1 (g) 14 d 61A24 and (h) 21 d 61A24 infected nodules. Magnification= 140 X.


N
PC
UC
IC
St
CC
Vb
a
IC
UC
PC
CC
Vb
St
b
IC
PC
CC
c
CC
St
UC
PC
Vb
d


CC
UC
e
Vb
UC
St
f
CC
UC
g
Vb
PC
CC
PZ
St
h
Vb

The 61A24 nodules had a large amount of starch in the uninfected cells at the periphery of the 14 d nodules (g). A much lower level of starch was apparent in the interstitial uninfected cells. Starch was also found in the cortical cells. At 21 days these nodules (h) had an abundance of starch in all cell types.

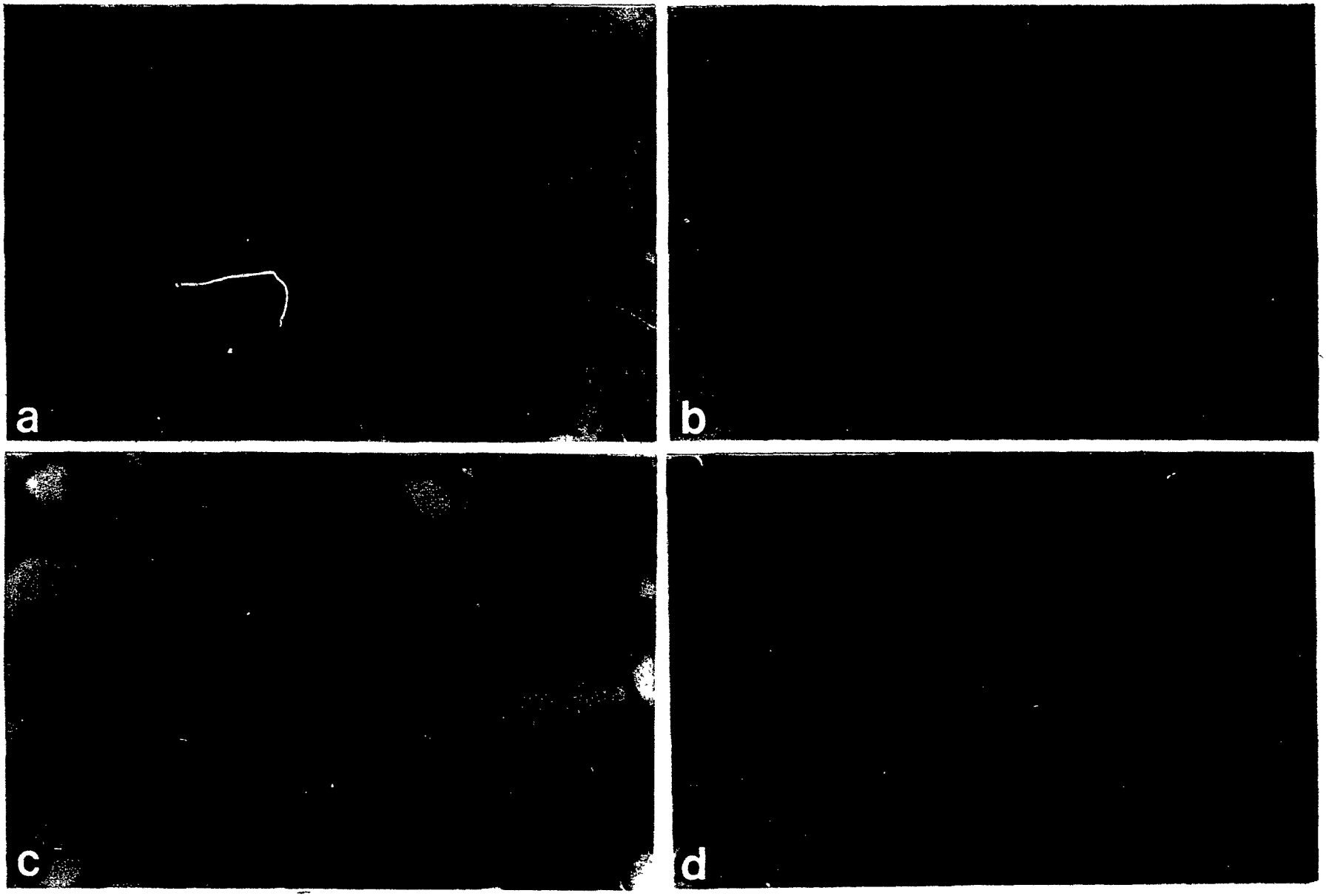
(iii) High magnification studies:

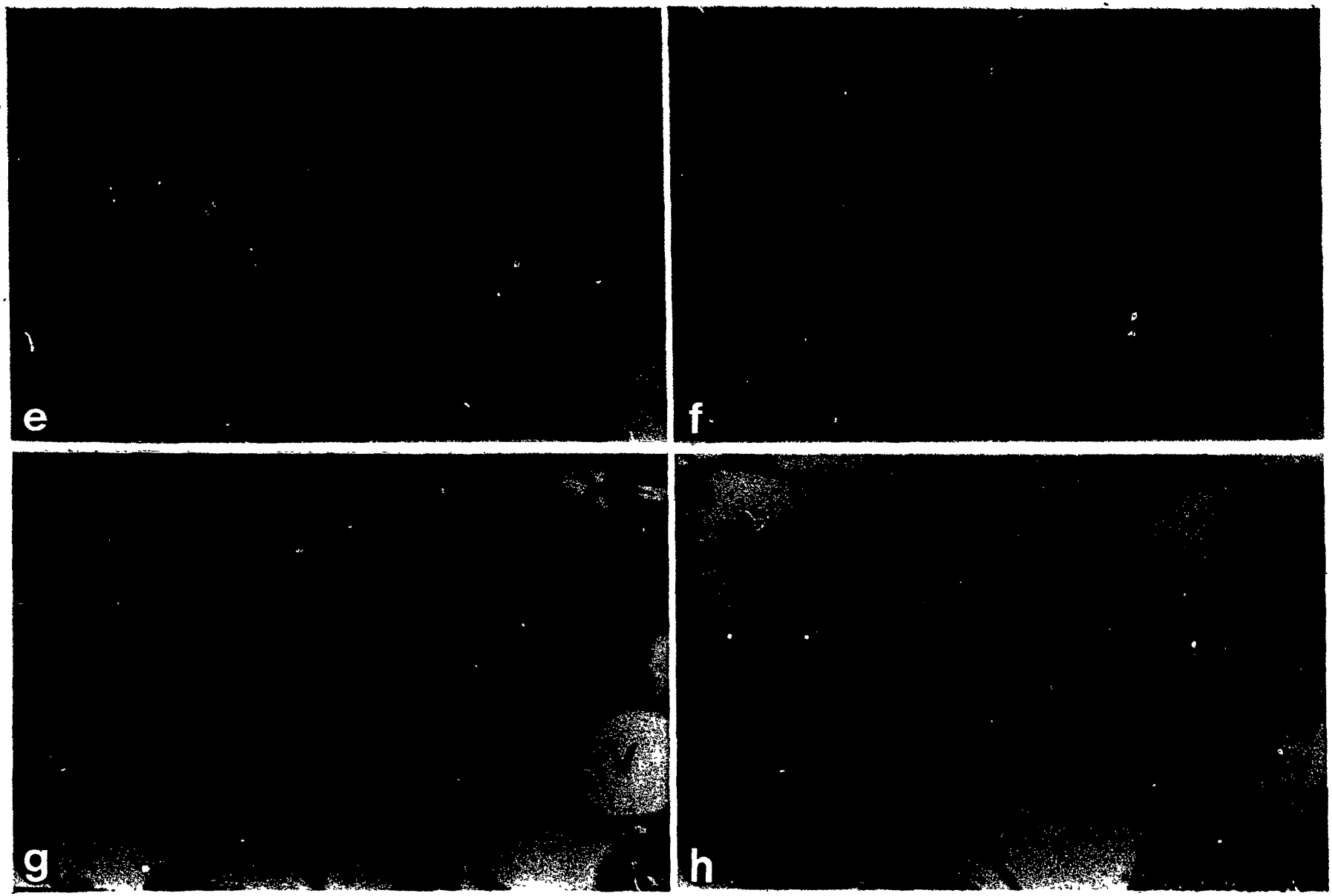
Figures 12 a - h are micrographs of cross sections of plastic-embedded tissue magnified 1,400 X. Plastic-embedding allows thinner sections which can be observed at high resolution. The stain used in this case, differentiates the following: starch (light or dark blue) in contrast to the bacteroids (dark purple) and the nuclei (light purple).

At 14 d, the wildtype nodules (a) had large amyloplasts in the uninfected cells. There was also a substantial number of these "starch positive" (blue) bodies, although smaller, at the outer periphery of the infected cells. At 21 days these nodules (b) have a distinct absence of starch in the infected cells. Upon observing a number of sections from different nodules, only very rarely were small starch positive bodies found at the outer edge of the 21 d nodules. Another difference between the 14 and 21 d wildtype nodules is that the amyloplasts in younger nodules were stained a light blue with scattered, interior deep blue granules while at 21 d most of the amyloplast was deep blue.

Very little starch was apparent in the 14 d T5-95 nodules (c). At 21 d, the level of starch in the T5-95 nodules appeared to have increased somewhat, although, as in the wildtype nodules, there was a distinct lack of starch in the infected cells. The starch in 21 d nodules (d) stained light blue and very few deep blue staining regions were evident.

Figure 12: Cross sections (1.5 μm thick) of plastic-embedded material. Staining was carried out using a combination of azure II and methylene blue. The following legend applies: infected cell (IC), uninfected cell (UC), nucleus (N), starch (St), vacuoles (V), bacteroids (b) and nucleolus of the infected cell (No). (a) 14d 61A76 (b) 21d 61A76 (c) 14d T5-95 (d) 21 d T5-95 (e) 14 d T8-1 (f) 21 d T8-1 (g) 14 d 61A24 (h) 21 d 61A24 infected nodules. Magnification= 1400X.


a
b
c
d


e
f
g
h

Relatively little starch was present in the 14 d T8-1 nodules (e), while in the 21 d nodules (f) starch was present in the cells with both large and small nuclei. The 61A24 nodules had relatively large amounts of deep blue staining starch in the uninfected cells of the 14 d nodules (g). At 21 d (h), a similar pattern is evident, although some cells seemed to be distinctly empty. Starch was absent even in cells with signs of pbm breakdown.

DISCUSSION

Accumulation of starch in nodules

Starch levels decreased in effective nodules and increased in ineffective nodules from 14 to 21 d post-infection. This difference between effective and ineffective nodules may be interpreted in terms of the energy sink and source present in each nodule type.

Effective nodules, in which nitrogen fixation is known to be maximal from 16 d post-infection until senescence, have a well-established energy sink by 21 d (Lawn and Brun, 1974). The carbohydrate requirements of this energy sink could explain the disappearance of the starch from these particular nodules.

The increase in starch content of the ineffective nodules could have been due to a relatively small energy sink because of the lack of nitrogen fixation. Excess sucrose (beyond that required for cellular maintenance) in these nodules was probably converted to starch rather than being directed towards the TCA cycle as it would be in the nitrogen fixing nodules.

The amount of available carbohydrate in nodules is partly determined by the activity of sucrose synthase (Anthon, personal communication). Thummler and Verma (1987) have shown that sucrose synthase activity is high in 10, 14 and 21 d effective and ineffective nodules, but is low in senescent nodules. All the nodules used in this study were presumed to have had high sucrose synthase activity. The possibility that 61A24 nodules, due to senescent-like behaviour, had low sucrose synthase activity is unlikely, since it accumulated high levels of starch.

The increase of 1200% in starch content of 61A24 nodules from 14 to 21 d post-infection was much larger than the increases found in the other

ineffective nodules. It may be that either the 61A24 nodules received more sucrose or the T5-95 and T8-1 nodules metabolised some of the excess sucrose instead of storing it as starch. The band of peripheral cells (Tjepkema and Yocum, 1974) may have had a role in this differentiated storage of starch by allowing more sucrose through in the 61A24 nodules or by limiting the entry of sucrose into the T5-95 and T8-1 nodules.

The starch content of the uninoculated root was relatively high (Fig 1). This is not unusual since starch is commonly found to accumulate in the roots of most plants (see Preiss, 1982b) and soybean is not an exception (Yin and Sun, 1947).

Starch quantities in 14 d ineffective nodules were found to be lower than their wildtype counterparts probably because of the generally slower development of the ineffective nodules (Morrison, personal communication).

Enzymes of Starch Metabolism

Table 3 is a summary of the levels of starch and specific activities of amylase, starch phosphorylase, and two forms of starch synthase found in this study . In interpreting the activity patterns in relation to the starch content of these nodules each nodule type was considered separately. Soluble starch synthase activity in the nodule was interpreted apart from the other enzymes because of its consistently low activity in nodules compared to uninoculated root.

In effective (wildtype) nodules, specific activities of amylase decreased from 14 to 21 d post-infection, while phosphorylase activity increased. At the same time, starch levels decreased by 80%. This pattern is contrary to that found in effective alfalfa root nodules where amylase activities were found to correlate inversely with starch, while

phosphorylase activities have a positive correlation (see Duke and Henson, 1984). This discrepancy in enzyme activity patterns between soybean and alfalfa nodules may be because the former are determinate and the latter meristematic.

The changes in starch content and the associated activities of amylase and phosphorylase in effective nodules are in conformity with observations on other plants (Okita et al., 1979). According to a theory developed by Okita et al (1979), amylase acts first on the starch to break it down into smaller oligosaccharides; phosphorylase then continues the process finally producing glucose-1-phosphate. This may well explain the pattern of amylase and phosphorylase activity in effective nodules.

The decrease in SBSS activity from 14 to 21 d in the wildtype nodules is positively correlated with the concurrent depletion of starch. If reduced activity of SBSS reflects reduced synthesis, then it may have been to the cell's advantage since the enzyme's substrate supply is probably limited by the allosterically controlled activity of ADPG pyrophosphorylase (which is inhibited when the cell needs ATP). Energy would, therefore, not be wasted on the production of an enzyme which was not being used.

In T5-95 nodules, the increased SBSS activity from 14 to 21 d post-infection, may explain, at least in part, the increase in starch content over the same time period. The large increase in phosphorylase activity was also in positive correlation with the starch level change implicating a role for starch phosphorylase in starch synthesis--a possibility which will be further clarified in the discussion of the 61A24 nodules. The decreased amylase activity from 14 to 21 d post-infection could have contributed to a decrease in starch degradation leading to the observed increase in starch content.

The relatively high levels of amylase activity in 14 d T8-1 nodules could have been due to the absence of a control mechanism for amylase production at the level of protein synthesis. The subsequent decrease of amylase activity at 21 d may have been due to decreased synthesis of the enzyme possibly because of limited supplies of substrates for protein synthesis. Decreased production of the enzyme is energetically advantageous for the cell because, with the lowered energy sink characteristic of fix- nodules, starch breakdown was probably not needed anyway.

In all nodule types where the bacteroids were released from the infection thread (wildtype, T5-95 and T8-1), the level of phosphorylase activity at 21d post-infection was high, while it was much lower in the T8-1 nodules of the same age. Possibly, the presence of the bacteroids inside the cell may have been necessary for the activation of high levels of phosphorylase activity, although more work is required to prove this.

Similar to what has been proposed for the T5-95 nodules, the elevated levels of SBSS activity may have contributed to the high starch content in the T8-1 nodules at 21 d post-infection.

In 61A24 nodules, the activities of amylase, phosphorylase and SBS synthase increased along with elevation of starch levels from 14 to 21 d post-infection. Interestingly, the increase of SBSS activity in 61A24 nodules was not drastically different from that found in the other ineffective nodules despite the massive accumulation of starch in these naturally occuring nodules. Starch bound starch synthase, therefore, may not have been the only enzyme involved in starch synthesis in the 61A24 nodules. Phosphorylase may be involved since it synthesizes starch in other plants (Hanes, 1940; Schneider et al., 1981; Sivak et al., 1981; Slabnik and Frydman, 1970), whereas, amylase does not (see Preiss, 1982 a,b).

Starch phosphorylase exhibited intriguing behavior, its activity increasing in parallel to both increasing (in T5-95 and 61A24 nodules) and decreasing (in wildtype) levels of starch content from 14 to 21 d post-infection. The obvious reconcilation of this contradiction is that starch phosphorylase has two roles of both synthesis and degradation. At the moment, this statement is speculation and more work is needed to provide proof for the apparent double functioning of starch phosphorylase.

Phosphorylase may have promoted starch synthesis only if sufficient amounts of glucose-1-phosphate were present to satisfy the Km requirements of the phosphorylase enzyme (see Preiss, 1982b). Although a high concentration of glucose-1-phosphate was unlikely in the cytoplasm, it may have been possible in the contained environment of the amyloplast.

In all nodule types, SSS activity was consistently lower than that found in the uninoculated root which may indicate that SSS plays a larger part in starch synthesis in the root than it does in the nodule.

Although SSS activity was not high in the nodules, it could still be important. A basal level of the SSS may have been necessary in the nodule, possibly providing primers for the initiation of starch synthesis (Hawker et al., 1974; Preiss et al., 1982b).

Starch Phosphorylase: A Nodule Specific Form?

The amino acid sequence of starch phosphorylase is known and has been found to be conserved in a wide range of organisms. For example, the potato enzyme shows 51% and 40% homology with those of E.coli and rabbit muscle, respectively (Nakano and Fukui, 1986). Thus, it was not surprising that antibody made against potato starch phosphorylase reacted with the soybean protein.

In young potato tubers, a single band at 105 kDa reacts with the anti-PSP (Brisson, personal communication). Mature potato tuber samples, in contrast, contain a prominent 105 kDa band as well as a number of degradation products. Brisson (personal communication) has proposed that these degradation products are very specific for the various stages of progressive maturation of potato tubers. One can speculate that the lower bands in lanes a and b of Figure 7 represented degradation products.

A 105 kDa band was found in the nodule samples, but not in the sample from uninoculated root. Possibly, the 105 kDa band represented a nodule-specific form of starch phosphorylase, but more work is required to prove this.

Another speculation can be made along this line. Since the anti-PSP also reacted with an 87 kDa band in nodule and root samples as well as with the 105 kDa band in the nodules, the two proteins differing in molecular weight may represent two cross-reacting isoenzymes of starch phosphorylase.

The 87-kDa band reacted strongly with the antibody in the 14 day nodule tissue despite the low levels of starch phosphorylase specific activity at the same time point (see Fig.4). The 87 kDa band may, therefore, have been a degradation product of starch phosphorylase.

Protein samples from all types of ineffective nodules were found to react with anti-PSP in the same manner as the wildtype protein counterparts. Two possible explanations for this may be: (a) while the protein was present in all nodule types, it was active in some and inactive in others; (b) other isoenzymes may also exist which do not cross react with the anti-PSP.

There is also the possibility that one form of phosphorylase was specific to the amyloplast and one to the cytoplasm. This speculation is

substantiated by what happens in spinach leaf where there are two forms of starch phosphorylase, one form in the chloroplast and another in the cytoplasm, the two forms showing little cross-reactivity (Schachtele and Steup, 1986; Steup and Schachtele, 1986). The same may be true for soybean nodules.

Overall, the results from the Western blot suggest that there was more starch phosphorylase in nodules than in uninoculated root. At least part of this difference could be due to a nodule-specific form of this enzyme.

Subcellular Location of Sucrose Synthase

The nodule specific form of sucrose synthase (nodulin 100) was found to be present in the cytoplasm of both infected and uninfected cells (see Fig. 9a and b). This suggests that sucrose synthase acts in both cell types, but more cell-specific activity assays are required to prove this.

According to the immunocytochemistry, Nodulin 100 was not present in intercellular spaces, bacteroid, pbm, pbf, vacuoles, peroxisomes or amyloplasts. The apparent absence of the enzyme from these spaces suggests that nodule-specific sucrose synthase is a cytoplasmic enzyme which is the case in some plants (Preiss, 1982 a,b).

Relatively little work has been done in area of localization of sucrose synthase or any of the other enzymes involved in starch metabolism in nodules. Sucrose synthase has been found, however, in the cytoplasm of reserve tissues (see Preiss, 1982 b).

Location of Starch

In terms of amounts of starch, the light micrographs shown in Figures 10 and 11 support the data in Figure 1 for the wildtype nodules, namely, the 14 d cross sections had more starch than the 21 d cross sections.

Differences between the location of starch in the 14 and 21 d wildtype nodules were evident. Because these differences were easily discerned in the wildtype situation, the 14 and 21 d wildtype nodules were compared in detail so as to develop an explanation for the contrasting starch levels.

One reason for the decrease in starch from the 14 to 21 d wildtype nodules could be the disappearance of starch from the infected cells. At 14 d, there were substantial amounts of starch in the infected cells while at 21 d they were much reduced. This parallels morphological studies of alfalfa nodules, where younger infected cells contain considerable amounts of starch and mature infected cells contain only very little or none (Vance et al., 1980).

This disappearance of starch specifically from the infected cells can be explained by the interior physiology of these cells. Theoretically, the energy sink is lower at 14 d because nitrogen fixation has not yet reached maximum and because there was a decreased requirement for carbon skeletons needed to make ureides (Fuller and Verma, 1984). In the 14 d wildtype nodules, the combination of maximum sucrose synthase activity and a low energy sink may have led to the accumulation of the breakdown products of sucrose. These excess carbohydrates were probably converted to starch in the infected as well as the uninfected cells because both cell types would have a low energy sink and receive roughly the same amount of sucrose (Thummler and Verma, 1987).

There are other possible explanations for the reduced starch content of

the 21 d nodules. The 14 d nodules may have had different regulation of starch metabolism as compared to the 21 d nodules. And there is also the possibility that the starch storage or the nature of the starch metabolism enzymes themselves may have been cell specific.

The uninfected cells may have had more starch than the infected cells at both 14 and 21 d post-infection for the following reasons: (1) there are no bacteroids in the uninfected cells, thus, there is more space (2) less protein synthesis (Lb alone represents 30% of the soluble protein in the nodule and it was localized in the infected cell), thus, more carbon might be accumulated in starch rather than in protein (3) different regulation of starch biosynthesis (in comparison to that found in the infected cells).

Another way of looking at the differences in starch accumulation between infected and uninfected cells is in terms of cell roles. The morphological studies suggested that the uninfected cells had a role in starch storage, just as they do in nitrogen assimilation.

The uninfected cells may have a role in starch storage in both effective and ineffective nodules. In the T5-95 nodules, the uninfected cells had more starch than the infected cells. And in the T8-1 and 61A24 nodules, the smaller cells were found to contain more starch than the larger ones (the smaller cells possibly having represented the cells targeted to be uninfected before the nodule development was arrested).

Conclusions:

Many aspects of the original hypotheses have been supported by the data shown in this thesis. Certain parts of the hypotheses have not been fully proven but the results obtained in the effort have indicated new questions to be asked and have shown which direction future endeavours should take.

The first hypothesis stated that the starch accumulation in the nodule is related to the source/sink ratio. This hypothesis was supported by the starch accumulation data and by the microscopical examination of starch content and location when the two were considered in view of the known time-course of nitrogen fixation and other cellular processes. The second hypothesis called for a relationship between the starch content of the nodule and the varying activities of the enzymes of starch metabolism. The enzyme activity data depicted in this thesis supported this hypothesis, although, only at the level of correlation. Finally, the third hypothesis suggested that there are two forms of starch phosphorylase. The results indicated that the hypothesis may be true, but that more work is required to make a solid statement on this matter.

The results of the exploratory set of experiments described in this thesis provided enough background for the raising of new, more specific questions. Determination of cell roles in root nodule starch metabolism can now be addressed with the knowledge that starch accumulation is affected by the energy status of the cell in question. Elucidation of the cellular interactions involved in both the carbon and nitrogen metabolisms would increase the understanding of the nodule operation as a whole and, thus, provide the needed background to investigate the same operation at the molecular level.

The next stage of this research would be to separate the various cell types of the nodule and determine for each the starch content as well as the specific activities of amylase, phosphorylase and synthase. The regulation of starch metabolism could also be studied in the separated cells. The activity of ADPG pyrophosphorylase could be examined in relation to the effects of Pi and PGA.

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