

Differentiation of *Rhizobium japonicum*.

II. Enzymatic Activities in Bacteroids and Plant Cytoplasm during the Development of Nodules of *Glycine max*

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Phytotron grown plants of *Glycine max* var. Caloria infected with *Rhizobium japonicum* 61-A-101 under controlled conditions as 14 d old seedlings develop a sharp maximum of nitrogenase activity of $13 \pm 3 \text{ nmol C}_2\text{H}_4 \cdot \text{h}^{-1} \cdot \text{mg nodule fresh weight}^{-1}$ 19 d after infection, followed by a long period of reduced activity (3–5 nmol) between 30 and 45 days after infection. A higher maximum activity ($18 \text{ nmol C}_2\text{H}_4 \cdot \text{h}^{-1} \cdot \text{mg nodule}^{-1}$, lasting 7 days was found in *Glycine max* var. Mandarin, with a similar period of low activity (3–4 nmol) following, between 35 and 50 d after infection. Nitrogenase activity in the varieties infected with *Rhizobium japonicum* strain 311 b 85 is very similar. In both varieties, the leghaemoglobin continues to increase (3 fold) after the maximum nitrogenase activity is reached and starts to decline only after 35 to 40 d.

Specific activities of the three enzymes aspartate aminotransferase (E.C.2.6.1.1.), glutamate dehydrogenase (E.C.1.4.1.2.) and alanine aminotransferase (E.C.2.6.1.2.) in the plant cell cytoplasm are changing very similarly to nitrogenase activity in the bacteroids, whereas the specific activities of the three enzymes in the bacteroids decrease only very slightly between 19 and 45 d. For these 3 enzymes the specific activity in the bacteroids during the phase of maximum nitrogenase activity is only 20–40% of the specific activity in the cytoplasm.

A constant low activity (0.350 units) of glutamine synthetase (E.C.6.3.1.2.) is found in bacteroids from 19 to 45 d old nodules, whereas the specific activity in the plant cytoplasm increases from about 1.2 units at 19 d to more than 6 units at 45 d. The specific activity of GOGAT (E.C.1.4.1.3.) in bacteroids is 2–3 times higher than in the plant cell cytoplasm and increases slightly. Alanine dehydrogenase (E.C.1.4.1.1.) and 3-hydroxybutyrate dehydrogenase (E.C.1.1.1.30.) activities in bacteroids increase between 19 and 35 d after infection by a factor of 2–3. In 35–45 d old nodules, the specific activity of alanine-dh in bacteroids of the same *Rhizobium japonicum* strain (61-A-101) from plant var. Caloria is significantly smaller than in bacteroids from plant var. Mandarin, whereas for 3-hydroxybutyrate-dh the activity in bacteroids from var. Caloria is enhanced compared to bacteroids from var. Mandarin.

Introduction

Symbiosis development in root nodules of legumes is one of several intensively studied symbiotic relationships between microorganisms and higher plants or animals [1]. It is distinguished by the development of several new biochemical qualities, such as nitrogenase activity and leghaemoglobin synthesis and by typical morphogenetic events such as bacteroid and nodule cell differentiation [2]. In a previous study [3] we compared nitrogenase of derepressed free living cells of *Rhizobium japonicum* with bacteroids from nodules of *Glycine max* at peak N_2 -fixation, with regard to specific activities of some enzymes of N-metabolism.

A correlation of alanine dehydrogenase, aspartate aminotransferase, glutamine amide 2-oxoglutarate aminotransferase (GOGAT) and of glutamine synthetase with nitrogenase activity in the free living

Rhizobium cells and in the isolated bacteroids was found. We continue this work by measuring these enzyme activities and other parameters in the differentiation of the bacteroids and the plant cell cytoplasm during nodule development. Both symbiosis partners during this development, have to be analyzed separately after isolation from nodules of a distinct age. With these results we are able to decide, whether free living *Rhizobium* cells with nitrogenase activity resemble in all measured enzymes bacteroids from nodules with peak nitrogenase activity or in some parameters bacteroids from other stages or, perhaps, even the plant cell cytoplasm.

Materials and Methods

Cultivation and infection of *Glycine max*

Seedlings of *Glycine max* var. Caloria, var. Mandarin and var. Chippewa 64, were infected with pure cultures of *Rhizobium japonicum* str. 61-A-101 or 311b85 as described [3].

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Isolation of bacteroids and preparation of cell-free extracts from bacteroids and plant cell cytoplasm

After separation and purification from the plant cell material [3], the homogeneity of the bacteroid fraction was checked in an interference microscope. We prepared the cytoplasm fraction by centrifugation of the supernatant of the bacteroid pellet once at $4500 \times g$ (15 min at 4°C) and once at $38\,000 \times g$ (15 min at 4°C). Less than 1% of the activity of the bacteroid marker enzymes (3-hydroxybutyrate dehydrogenase, alanine dehydrogenase) was left in the cytoplasm fraction.

Enzyme assays

Nitrogenase activity and the other enzyme tests were performed as in the previous study [3]. Glutamine synthetase from the cytoplasm fraction was studied according to [4] with the following variation: the final buffer concentration was 50 mM and the pH 6.5. The pH optimum of 6.5 for the Mn^{2+} dependent transferase assay and of 7.5 for the Mg^{2+} dependent assay was determined and identical with the values of McParland *et al.* [4].

Protein determination

Although we used insoluble polyvinyl pyrrolidone the removal of phenolic compounds from the plant cell supernatant [5] was incomplete. After running the plant cytoplasm fraction through a Sephadex G25 column, the apparent values of protein found with the method of Lowry *et al.* [6] were significantly reduced compared with the method of Bradford [7]. However, we do not know to which extent phenolic compounds or other low molecular weight substances are responsible. Therefore, the protein in the cytoplasm fraction and in the bacteroids was determined according to [7], although for the bacteroids the method of Lowry gave almost identical results.

Leghaemoglobin

Leghaemoglobin in the cytoplasm fraction was determined by the pyridine haemochrome test [8]. The reduced haemochrome, which is not very stable [9], was measured within 5 min at 556 nm. ϵ_{mM} (556 nm) = 34.6 [8]. The leghaemoglobin concentration in Fig. 3 is given in relative units, which correspond to $10^{-2} \mu\text{mol}$.

Results

Nodules become visible on the roots of the soybean varieties used in this study 10–12 d after inoculation. The mean nodule fresh weight in the variety Caloria, infected with *Rhizobium japonicum* 61-A-101, increases rapidly to an average of 7 mg per nodule 21 d after inoculation and doubles again during the following 20 d period (Fig. 1). Then the weight (average of 30 plants) starts to decline. Nodule number per plant remains constant 15 d after infection (Fig. 1). The same results are found with the soybean variety Mandarin.

Nitrogenase activity (Fig. 2A) in the varieties Caloria and Chippewa 64 increases rapidly between 15 and 21 d to a maximum of $13 \pm 3 \text{ nmol C}_2\text{H}_4 \cdot \text{h}^{-1} \cdot \text{mg nodule fresh weight}^{-1}$. During the following 6–8 d it decreases to 5 nmol. In the variety Mandarin, activity increases to a higher value of $18 \pm 4 \text{ nmol}$ and does not begin to decrease before d 27. In all three varieties a stage with low (3–5 nmol) activity 30–45 d after inoculation follows. Flowering starts in all varieties a few days before peak N_2 -fixation and ends at the peak in the varieties Caloria and Chippewa 64, but 1–2 d later in the variety Mandarin. Infection with another strain of *Rhizobium japonicum* (311b85) gives the

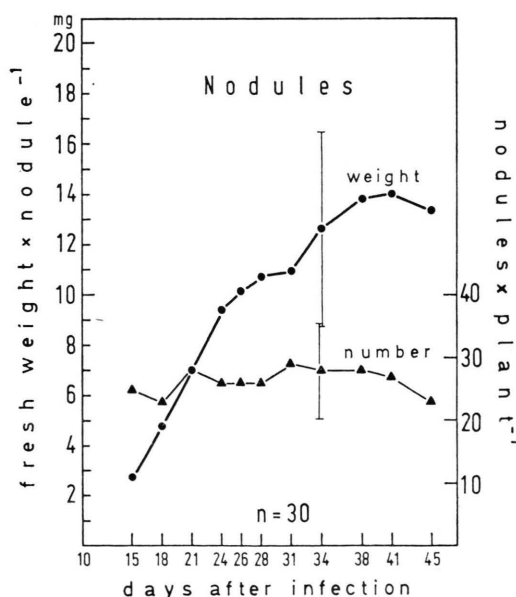


Fig. 1. Development of nodule number and weight per plant in *Glycine max* var. Caloria, infected with *Rhizobium japonicum* 61-A-101 as 14 d old seedlings. The verticals give the standard deviation ($n=30$).

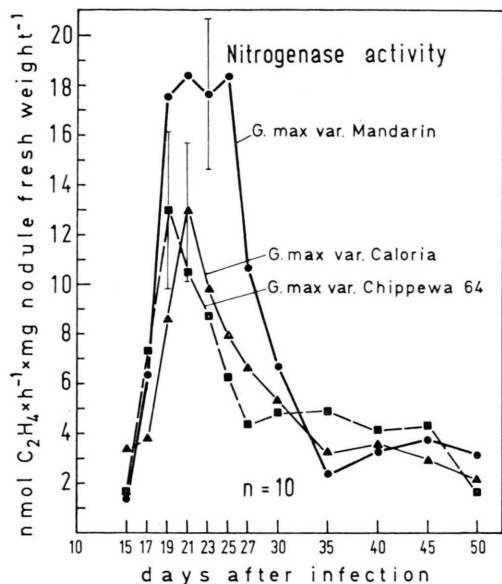


Fig. 2 A. Nitrogenase activity in the development of nodules in three varieties of *Glycine max*, infected with *Rhizobium japonicum* 61-A-101 as 14 d old seedling. The verticals give the standard deviation ($n=10$).

same results (Fig. 2 B). A plot of nitrogenase activity per plant (Fig. 2 C) shows the same steep increase between 17 and 21 d (var. Caloria), then a somewhat reduced decrease compared to the plot

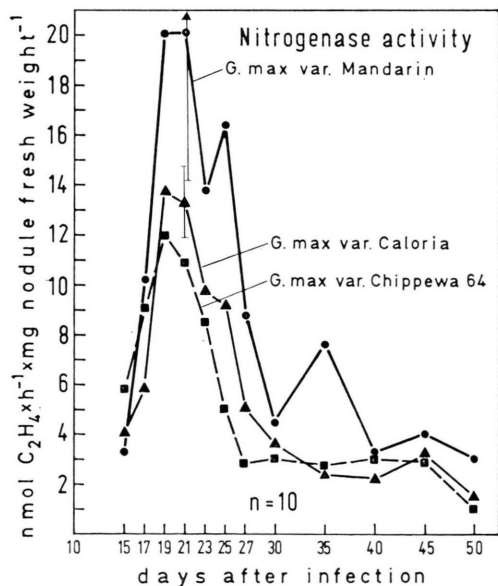


Fig. 2 B. Nitrogenase activity in the development of nodules in three varieties of *Glycine max*, infected with *Rhizobium japonicum* 311 b 85 as 14 d old seedlings. The verticals give the standard deviation ($n=10$).

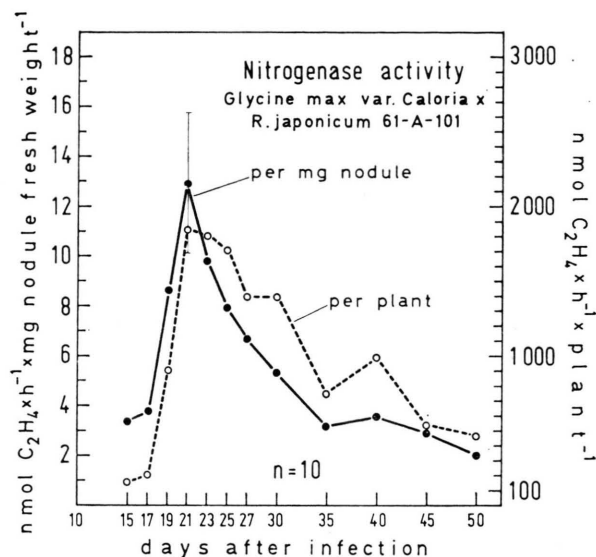


Fig. 2 C. Nitrogenase activity per mg nodule and per plant in the development of *Glycine max* var. Caloria, infected with *Rhizobium japonicum* 61-A-101 as 14 d old seedlings.

per mg nodule. The difference reflects the increase in the nodule weight per plant. For the following analyses we used the two varieties Caloria and Mandarin because of their different peak and length of nitrogenase activity.

Leghaemoglobin concentration (Fig. 3) increases in both varieties in a very similar way. After the maximum of nitrogenase activity (19 d), leghaemoglobin increases further by a factor of 2 to a maximum not before 30–35 d after infection. Then breakdown of the pigment starts. The maximum concentrations of $0.222 \mu\text{mol} \cdot \text{g nodule fresh weight}^{-1}$ in the variety Mandarin (30 d) and $0.212 \mu\text{mol}$ in the variety Caloria 35 d after inoculation are somewhat less than the concentrations found by Bergersen and Goodchild [36] in another variety with $0.293 \mu\text{mol}$ in 36 d old nodules and $0.363 \mu\text{mol}$ at day 43. Specific activities of aspartate aminotransferase (Fig. 4) in the plant cell cytoplasm of the nodules has a maximum 19 d after infection in both varieties with 1000–1100 m units. The activities decrease to one third at 35 d to about the same values found in uninfected root tissue. In the bacteroids a low and slowly decreasing activity of this enzyme is found, also between 35–45 d it is significantly lower than in the plant cell cytoplasm. As expected, alanine aminotransferase (Fig. 5) develops very similarly to aspartate aminotransferase in the plant cytoplasm and in the bac-

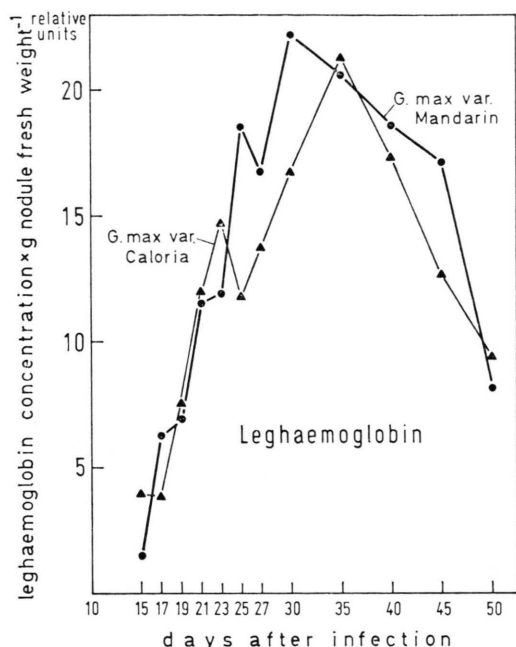


Fig. 3. Leghaemoglobin concentration (relative units) per g nodule fresh weight in the development of *Glycine max* var. Caloria and var. Mandarin, infected with *Rhizobium japonicum* 61-A-101 as 14 d old seedlings.

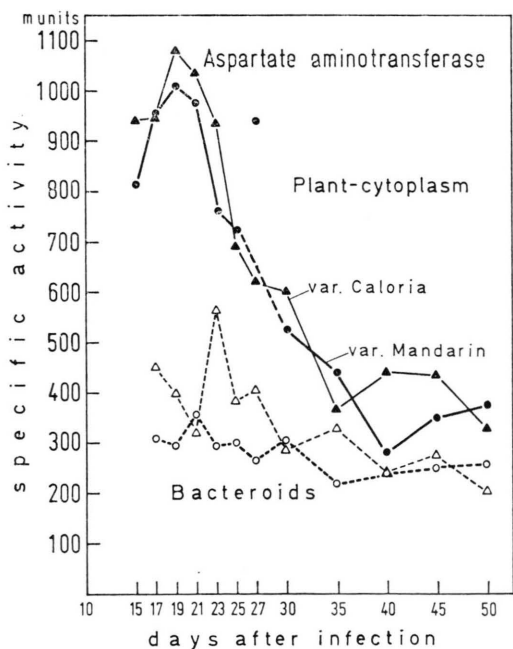


Fig. 4. Specific activity ($\text{nmol substrate} \cdot \text{mg protein}^{-1} \cdot \text{min}^{-1}$) of aspartate aminotransferase in bacteroids (----) and plant cytoplasm (—) in the development of nodules of *Glycine max* var. Caloria ($\blacktriangle, \triangle$) and var. Mandarin (\bullet, \circ), infected with *Rhizobium japonicum* 61-A-101 as 14 d old seedlings.

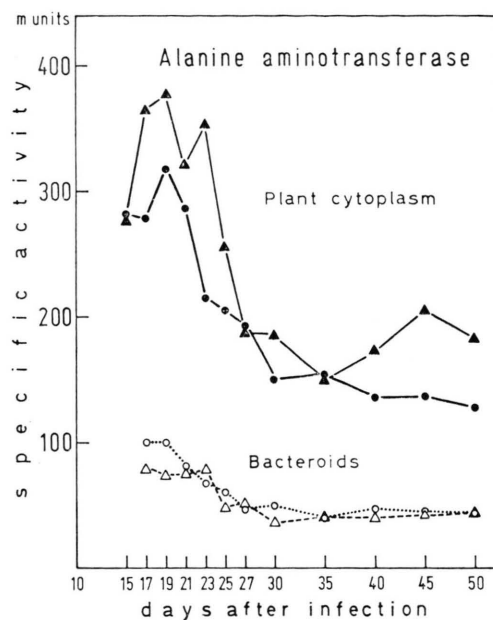


Fig. 5. Specific activity of alanine aminotransferase in the development of nodules of *Glycine max*, signs as in Fig. 4.

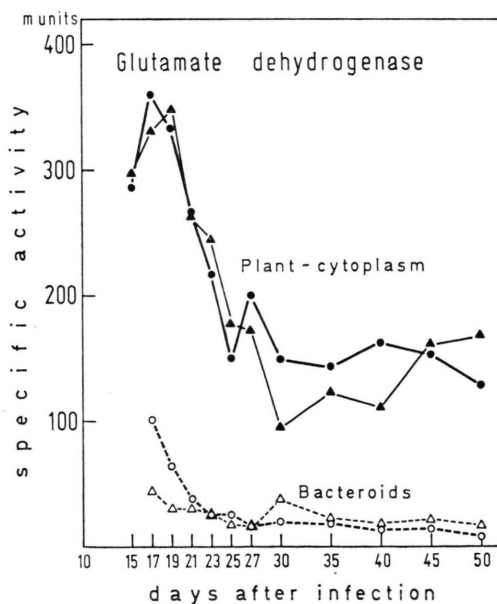


Fig. 6. Specific activity of glutamate dehydrogenase in the development of nodules of *Glycine max*, signs as in Fig. 4.

teroids, but specific activities are in general only about one third. NAD dependent glutamate dehydrogenase (Fig. 6) in the plant cytoplasm also develops similarly to the transaminases an early maximum of about 350 m units 17–19 d after infection falling to 100–170 m units between 30 and 45 d. The

very low activity of this enzyme in the bacteroid fraction is only a small part of the plant cytoplasm activity at any time (Fig. 6). In uninfected root tissue a specific activity of 80–120 m units was found. A completely different development of the specific activity of glutamine synthetase in the plant cell cytoplasm in both varieties compared to the enzymes described above is found (Fig. 7a). From less than 200 m units at day 15 the specific activity increases rapidly 20 fold at 23 d and further to 6000–7000 m units 45 to 50 d after infection. These activities were determined with the Mn^{2+} dependent transferase assay. In the uninfected root tissue from 37 d old plants corresponding to 23 d after infection of nodulated plants the activity of this enzyme was higher (1400–1700 m units) than in very young (15–17 d) old nodule cells (var. Mandarin). Compared to these unusually high specific activities in old nodule cells we find in bacteroids from both soybean varieties only 300–400 m units during the whole development (Fig. 7B). The ratio of the Mn^{2+} (0.3 mM) dependent test to the Mn^{2+} (0.3 mM) plus Mg^{2+} (60 mM) dependent assay remains with 1.4 ± 0.2 almost constant during the whole development. NADH dependent

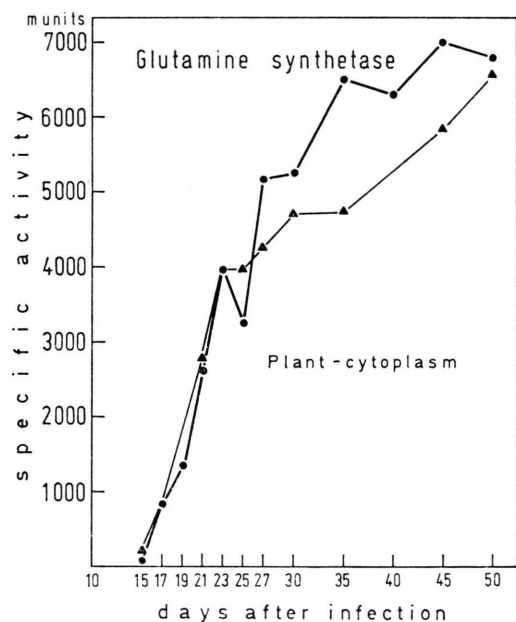


Fig. 7A. Specific activity ($\text{nmol substrate} \cdot \text{mg protein}^{-1} \cdot \text{min}^{-1}$) of glutamine synthetase in the plant cytoplasm during the development of nodules of *Glycine max* var. Caloria (▲) and var. Mandarin (●), infected with *Rhizobium japonicum* 61-A-101 as 14 d old seedlings.

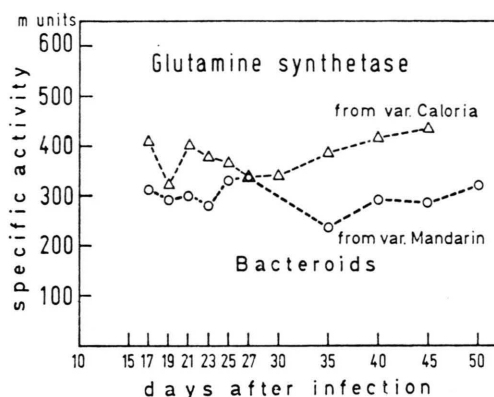


Fig. 7B. Specific activity of glutamine synthetase from bacteroids, during the development of nodules of *Glycine max*, infected as in Fig. 7A.

glutamate synthase activity is 2–3 times higher in bacteroids than in the plant cell cytoplasm of both varieties with a slight tendency for an increase from 17 to 50 d after infection (Fig. 8). No activity in both cell fractions in the NADPH dependent test could be measured.

Alanine dehydrogenase (NADH dependent) is only present in bacteroids. We find a sharp increase of the specific activity between d 15 and d 30 to about 280 m units in bacteroids from both varieties (Fig. 9). Then the specific activities in bacteroids from var. Mandarin remains at a higher level of 300 m units until d 50 whereas the activity in bacteroids from var. Caloria drops to about 200 m units. Also 3-hydroxybutyrate dehydrogenase is exclusively located in the bacteroids. As with alanine dehydrogenase, this activity also increases signifi-

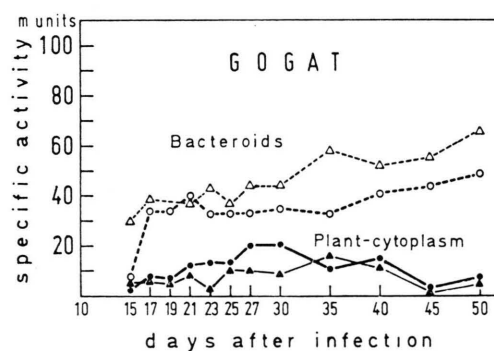


Fig. 8. Specific activity ($\text{nmol substrate} \cdot \text{mg protein}^{-1} \cdot \text{min}^{-1}$) of glutamine amide 2-oxoglutarate aminotransferase in bacteroids and plant cytoplasm in the development of nodules of *Glycine max* var. Caloria (▲, △) and var. Mandarin (●, ○), infected with *Rhizobium japonicum* 61-A-101 as 14 d old seedlings.

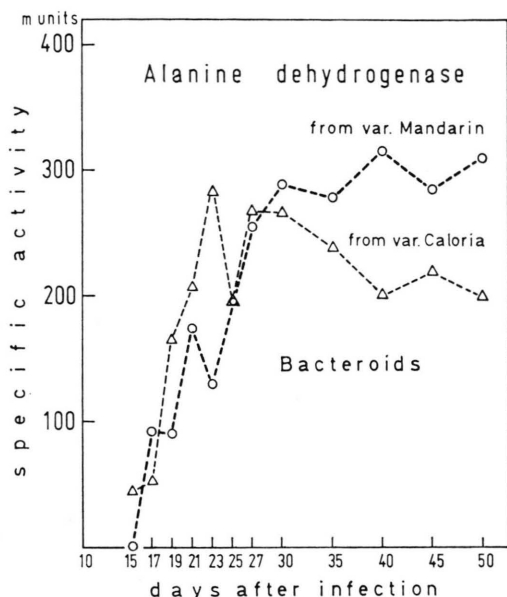


Fig. 9. Specific activities ($\text{nmol substrate} \cdot \text{mg protein}^{-1} \cdot \text{min}^{-1}$) of alanine dehydrogenase in bacteroids during the development of nodules of *Glycine max* var. Mandarin (O) and Caloria (Δ), infected with *Rhizobium japonicum* 61-A-101 as 14 d old seedlings.

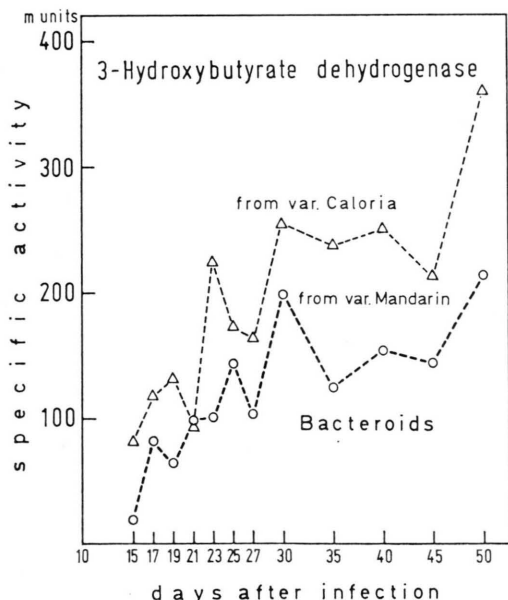


Fig. 10. Specific activities of 3-hydroxybutyrate dehydrogenase in bacteroids during the development of nodules of *Glycine max*, signs as Fig. 9.

cantly after the peak of N_2 -fixation with a maximum at 50 d after infection (Fig. 10). But for this enzyme, the specific activity in bacteroids from var. Caloria is significantly higher than in bacteroids

from var. Mandarin. By comparing the data from Fig. 9 and Fig. 10 it is clear, that the difference between the bacteroid enzymes from both plant varieties is not caused by a significantly different total protein value in the two varieties.

Discussion

In vivo activity of nitrogenase in nodules of legume plants is used in this and other studies [3, 10] from our laboratory as a relative parameter for characterization of enzymatic and fine structural differentiation. The steep increase to maximum N_2 -fixation in three different varieties of soybeans differing by not more than 1–2 days, between 17 and 19 d after inoculation with rhizobia (Fig. 2 A, 2 B) is considered as a central physiological event for the further nodule development. By the inoculation method, used in our studies, the number of nodules per plant has reached a plateau (Fig. 1) before nitrogenase activity is significant (15 d). Differences in the metabolic activities of legume nodules found in other studies [11, 12] may be a consequence of using nodules of not sharply defined age. We find no difference in the nodule development using our strains of *Rhizobium* as others have done [13–15] using distinct strains. The decrease in nitrogenase activity per mg nodule and per plant in our soybean varieties is observed at the same time as flowering and the beginning of seed formation, as also reported for green house grown *Pisum sativum* [16]. On the other hand, there are reports with *Glycine max* and *Arachis spec.* [17] of an increase in nitrogen fixation in field experiments at the time of seed formation. Of the many factors, different in phytotron and field experiments, the light dark regime alone influences in addition to nitrogenase activity transpiration and the amino acid and sugar contents as well [18].

There are recent reports [19, 20] of a continuing leghaemoglobin increase when nitrogen fixation starts to decline, whereas in other earlier reports [21, 22], a quantitative relationship between nitrogen fixing efficiency and leghaemoglobin content seemed to be established. The specific activities of aspartate aminotransferase, alanine aminotransferase and glutamate dehydrogenase in the plant cell cytoplasm (Figs 4–6) follow the nitrogenase activity in the bacteroids, tested *in vivo* in the intact nodules (Figs 2 A–B). However, in early stages (15 d after infection) these enzymes proceed nitrogenase activity.

High [23] and lower [24] specific activities for the same enzymes in *Pisum sativum* nodules may also come from the use of different nodule stages. However, at no nodule stage do we find (using both varieties of soybeans) as Ryan and Fottrell [25], a higher specific activity of aspartate amino transferase in the bacteroids compared to plant cytoplasm. For alanine aminotransferase we find, as Fottrell and Grimes [37], a four times higher specific activity in the plant cell cytoplasm compared to the bacteroids. As seen in Fig. 5, this ratio does not change very much during nodule development. For both transaminases, Klucas [38] found in the senescent state activities in bacteroids and plant cytoplasm without much change. A significantly higher specific activity of both transaminases in unfractionated nodules compared to uninfected root tissue and to free living *Rhizobium japonicum* cells have been already reported [35].

The maximum activity of glutamate dehydrogenase in the plant cytoplasm in both our soybean varieties (Fig. 6) is significantly higher than those reported by other authors [11, 25], whereas those in bacteroids (Fig. 6) are about the same [11, 12]. The mechanism by which a close correlation between these three enzymes in the plant cytoplasm and the nitrogenase in the bacteroids is established has to be studied further.

A central position in the regulation of nitrogenase is seen for the enzyme glutamine synthetase [4, 11, 12, 27, 28]. A high specific activity in free living diazotrophic bacteria is regulating nitrogenase activity by its adenylation. However, in bacteroids and in free living, nitrogenase derepressed *Rhizobium* cells much lower activities are found [3], which may be too low, to function in the same regulatory manner as in the free living cells. The same specific activity of this enzyme (Fig. 7 B) during stages with high and low nitrogenase activities further support the idea (also found with *Rhizobium leguminosarum* bacteroids [26]) that this enzyme in bacteroids does not function similarly as in free living diazotrophic cells. Even a negative correlation between both enzymes, a falling glutamine synthetase with an increasing nitrogenase, was found in bacteroids of *Rhizobium leguminosarum* (Planqué and Kijne, personal communication) during nodule development. Furthermore, we have to realize, that a close but varying association of the much higher glutamine synthetase activity of the plant cell cyto-

plasm with the bacteroids [27] could cause some variations in the bacteroid fraction. As many other authors [4, 11, 12, 27, 28] we find the major part of the enzyme activity of the nodule in the plant cytoplasm. The most important result from our experiments is the thirtyfold increase in the specific activity in the plant cell cytoplasm from 15 d to 50 d old nodules. With a specific activity of 1.5 units of this enzyme McParland *et al.* [4] concluded after purification that 2% of cytoplasm protein was glutamine synthetase. With a specific activity of 6–7 units in our nodules of defined age (45–50 d), this figure could perhaps be four times higher. The transferase assay of this enzyme, used in our and other studies, gives higher activities than the biosynthetic assay [4, 28–30]. On the other hand, the biosynthetic test is much more easily disturbed, *e.g.* ADP formed inhibits glutamine synthesis [4, 31]. Also a high ATP-ase activity in nodule extracts gives some problems [31, 32]. The biosynthetic assay gives the same activity at optimum pH with Mg^{2+} and with Mn^{2+} , whereas the transferase assay with Mn^{2+} at pH optimum of 6.5 has several times the activity of the Mg^{2+} dependent test at pH 7.5.

In contrast to the enzymes described so far glutamate synthase (GOGAT) has a 2–3 times higher specific activity in the bacteroids compared to the plant cytoplasm (Fig. 8). In bacteroids from other slow growing rhizobia this was also found [11, 25, 27] but there was no activity in bacteroids of *Rhizobium leguminosarum* [26, 27].

A significant increase in the activity of this enzyme in the cytoplasm of lupin nodules was seen [28] after separation of low molecular weight compounds such as glycine and glutamate by filtration through a Sephadex G 25 column [28]. We obtained no increase in the cytoplasm enzyme activity of our soybean varieties after the same procedure. However, we found a much higher instability of the activity in the crude plant cytoplasm extract compared to the bacteroid extract. After 8 h storage (at 0–4 °C) the activity in the plant cytoplasm decreased to one third, whereas in the bacteroid extract 100% was recovered.

Alanine dehydrogenase (Fig. 9) was found exclusively in the bacteroids as by Dunn and Klucas [11]. A report of high activities of this enzyme in the plant cytoplasm of lupin nodules [33] could be explained by a leakage of the bacteroids during the separation and extraction. In both varieties of

soybeans we find a five fold increase of the specific activity of this enzyme between day 16 and day 30, then a significantly higher activity in the bacteroids from var. Mandarin compared to those from var. Caloria. The development of 3-hydroxybutyrate dehydrogenase (Fig. 10), also only located in the bacteroids, is in good agreement with the increase of poly- β -hydroxybutyric acid (PHBA) in the bacteroids of *Rhizobium japonicum* [10], to fill finally 50% or more of the bacteroid volume and is also about 50% of the bacteroid dry weight [39]. More important for the mutual interaction of *Rhizobium* and plant cell differentiation is the significantly higher specific activity in bacteroids from the same *Rhizobium* strain in the variety. Caloria, whereas the opposite is the case in the specific activity of alanine dehydrogenase between d 35 and 50.

In the present study we emphasize more the symbiosis differentiation than the consequences for the pathway of ammonia and amino acid assimilation by changing enzyme activities. From our data, two main groups of differentiation events can be discerned, corresponding in the bacteroids and in the plant cell cytoplasm during nodule development:

1. In the early stages (15–30 d after infection) a close relationship between nitrogenase activity

located in the bacteroids, and the plant cytoplasm enzymes asparatate aminotransferase, alanine aminotransferase and glutamate dehydrogenase.

2. In the later stages (25–50 d after infection), during a significantly decreased nitrogenase activity, a marked increase of glutamine synthetase in the plant cytoplasm and of two bacteroid enzymes (alanine dehydrogenase and 3-hydroxybutyrate dehydrogenase) with an effect of the plant variety on the specific activities of these enzymes.

The mechanism of these mutual interactions of the symbiosis partners, mediated perhaps by varying pool sizes of metabolites of carbohydrate and N-metabolites and by a different energy charge will be studied in forthcoming papers, using ineffective *Rhizobium* strains and a further fractionation of the *Glycine max* nodules.

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