Development of Discontinuous Size Classes of Nodules of *Glycine max*

R. Stripf and D. Werner

Botanisches Institut der Philipps-Universität Marburg, Abt. Zellbiologie und Entwicklungsphysiologie der Pflanzen, Lahnberge, D-3550 Marburg

Z. Naturforsch. **35 c,** 776–782 (1980); received May 30, 1980

Rhizobium japonicum, Glycine max, Nodules Size Classes, Nitrogenase, Leghaemoglobin

In order to characterize developmental stages of nodules of *Glycine max* nitrogenase activity in the variety Caloria, infected with *Rhizobium japonicum* 61-A-101, was studied in atmospheres with 19 and 40% O_2. By the enhancement effect at 40% O_2 four stages of nodule development could be separated. Nitrogenase activity depends also among other things on nodule size. 3–4 maxima in nodule number and nodule weight were found by fractionating nodules in 12 different size classes. Discontinuous size distribution was found with medium aged and old effective nodules. Nodule number (20 to 30) of effective nodules per plant remains constant. Ineffective nodules remain far smaller than the effective ones, the number increases to more than 140 per plant and only one discontinuous size classe was observed.

Bacteroids and plant cytoplasm from nodules of some size classes are characterized by their leghaemoglobin content and specific activity of enzymes of nitrogen metabolism such as aspartate aminotransferase (E.C. 2.6.1.1), glutamate dehydrogenase (E.C. 1.4.1.2) and alanine dehydrogenase (E.C. 1.4.1.1). The data in the various size classes are similar both in the bacteroids and the plant cytoplasm, however, leghaemoglobin content and specific activity of the bacterial enzyme alanine dehydrogenase are positively correlated with the increasing nodule size.

Introduction

Developmental stages of legume nodules are not easy to define. This symbiosis requires the interaction between the plant genome and the *Rhizobium* cell. Because this new plant organ only develops after a complicated infection process by the rhizobia, the age of the nodule is usually given in days after infection. Formation and development of the nodules depend also on the stage of the primary and secondary roots and the root hair development at the time of infection. By use of seedlings of a certain age and a defined titer of *Rhizobium* cells from a log-phase culture, a constant number of effective nodules per plant was found between 15 and 45 d after infection [6]. With the same infection method we also got a linear relationship between a rising *Rhizobium* titer (10^3 to 10^7 ml^{-1}) and the nodule number per plant [2]. Mague and Burris [3], Lawrie and Wheeler [4] and Nash and Schulman [5] have already shown a correlation between intermediate size nodules and maximum nitrogenase activity. Nodule populations in these studies was separated only in three or four size classes by diameter (*Glycine max*) or by weight (*Vicia faba*). A discontinuous spectrum therefore could not be observed, which needs at least ten to twelve size classes.

Reprint requests to Prof. Dr. D. Werner.

0341-0382/80/0900-0776 $ 01.00/0

Materials and Methods

Seedlings of *Glycine max* var. Caloria, var. Mandarin and var. Chippewa 64 were infected with *Rhizobium japonicum* strain 61-A-101 and 3I1b85 (both effective) or 61-A-24 (ineffective) obtained from Dr. J. C. Burton, Nitragen Comp., Milwaukee, USA). Cultivation of *Glycine max* and *Rhizobium japonicum,* isolation of bacteroids and preparation of cell free extracts of bacteroids and nodules cell cytoplasm, enzyme, leghaemoglobin and protein assays were as described [1, 2, 6]. For the oxygen enhancement effect of nitrogenase activity, oxygen concentration was increased from 19 to 40%. For the experiments for Fig. 1 and 4 with a higher nitrogenase activity a larger part of the plants were incubated together with the root system than in experiments for Figs. 2 and 5. To evaluate the size distribution, nodules of 10 to 50 plants of a certain day after infection was sieved through a serie of sieves with the following data: 4000, 3800, 3600, 3300, 3000, 2800, 2600, 2400, 2200, 2000, 1800, 1600 µm mesh.

Results

Nitrogenase activity per mg nodule fresh weight of *Glycine max* var. Caloria, infected with *Rhizobium japonicum* 61-A-101 has a maximum just before 20 days after infection with about 48 nmol $C_2H_4 \cdot h^{-1} \cdot$ mg nodule fresh weight^{-1} (Fig. 1). During this time the total fresh weight of nodules per 50 plants has increased to about 7 g. In a second stage (day 22–35) a decrease in nitrogenase activity to about 6 nmol C_2H_4 is observed while the nodule weight doubles to 14 g in 50 plants. The third stage with a constant low nitrogenase activity follows. The separation of these nodules stages is even more obvious by the oxygen enhancement effect of nitrogenase activity (Fig. 2). At stage I there is no enhancement effect. At stage II we find a constant enhancement of 4 to 6 nmol C_2H_4 h^{-1} mg nodule fresh weight^{-1}. At stage III the effect is reduced and at the senescent stage IV disappeared. The average fresh weight of a nodule in this symbiosis increases from 3 mg at 15 d to 14 mg at 45 d after infection, while the nodule number remains constant [6]. It was assumed that there is a continuous growth of the small and young nodules to larger and older nodules. This is apparently not the case. Nodules of a certain day after infection were sieved and subdivided into 10 to 12 size classes, depending on the age of the root system. The smallest size class No. 1 includes all nodules smaller than 1.6 mm diameter, the largest size class all nodules larger than 4.0 mm per root. The weight of the nodules, falling through the increasing mesh opening of the twelve size classes are shown in Fig. 3. We find a continuously rising weight from 2 mg in the size class No. 2 to about 30 mg fresh weight in the size class No. 12. The mesh openings of the sieves were also checked with calibrated glass beads. Nodules of 50 plants 20 and 25 d after infection were harvested and separated into 10 or 12 size classes as shown in Fig. 4. At d 20 we find the size classes No. 4 and 7 with 2 to 3 times as much nodule weight as all other size classes. 5 days later a third peak is very obvious in the size class No. 9 to 10. The decrease in the size classes No. 1 to 4 at this stage is not followed by an increase in size classes No. 5 and 6 but only an increase in size classes No. 7 and 9 to 10. We followed the development of the total nodule number and nodule weight in the twelve size classes between 15 and 45 days after infection in another series of ex-

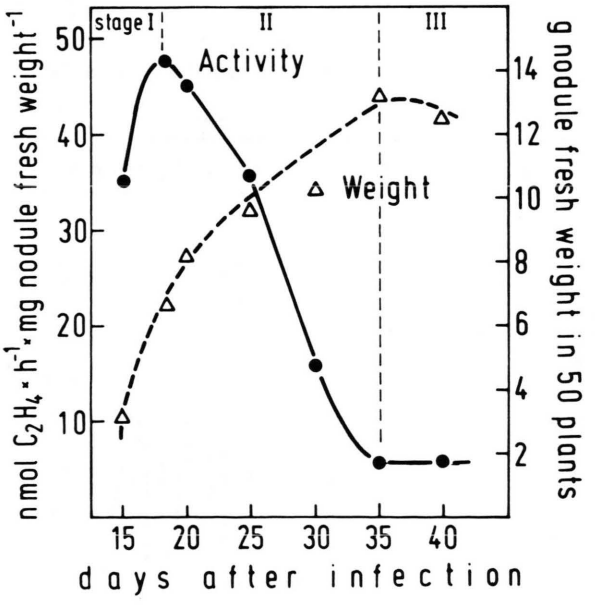

Fig. 1. Nitrogenase activity (–●–) and nodule weight (–△–) in *Glycine max* var. Caloria, infected with *Rhizobium japonicum* 61-A-101 at days after infection; $n = 50$ plants used per day.

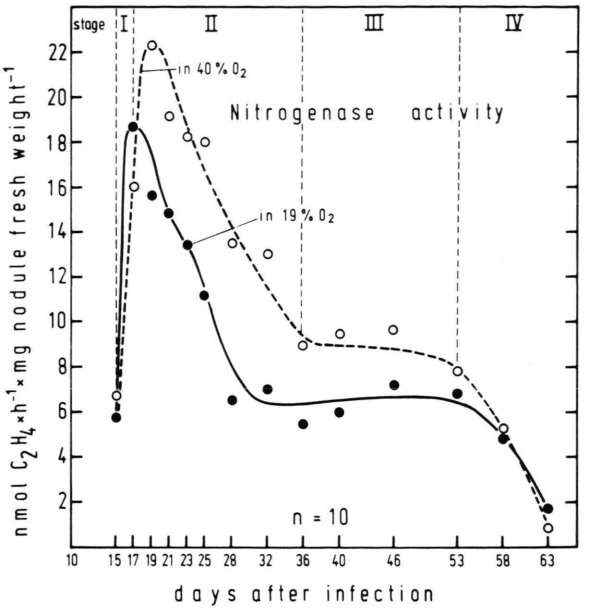

Fig. 2. Separation of four stages of nodule development by the oxygen enhancement effect of nitrogenase activity. Nodules of *Glycine max* var. Caloria, *Rhizobium japonicum* 61-A-101; test of the separated root system with nodules under 19% O_2 (–○–) and under 40% O_2 (–●–) with 10% acetylene in the gas phase, brought up to 100% with N_2; $n = 10$ plants used per day.

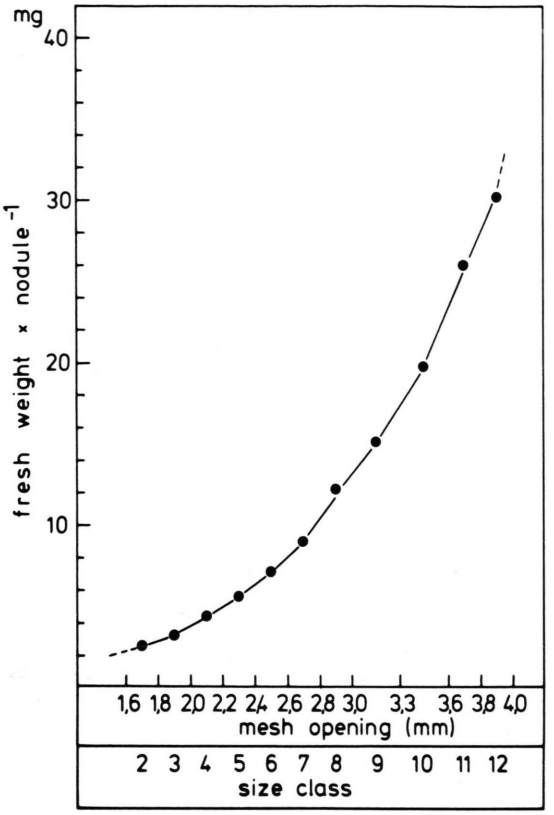

Fig. 3. Fractionation of nodules into 12 size classes by sieving through a cascade of sieves from 4.0 to 1.6 mm mesh (perlon gauze with squares). Fresh weight per nodule in the size classes No. 2 to 12.

periments with ten plants per day (Fig. 5). For those parameters we observed the development of three different size classes beginning at day 20. In nodules with the size more than 3 mm diameter there is, of course, a significant difference in the percentages of nodule weight and number. As also shown in this figure, the total number of nodules in the ten plants remains constant with about 300 nodules. Total nodule weight increases during the same time from 0.58 g to about 2 g. Very similar results were found with two other soybean varieties, Mandarin and Chippewa 64 and also by use of the *Rhizobium japonicum* strain 3I1b85.

Nodules of *Glycine max* var. Caloria infected with the ineffective strain of *Rhizobium japonicum* 61-A-64 were fractionated with the same method (Fig. 6). Total number of ineffective nodules increases from 281 in ten plants at d 15 after infection to 2147 at d 45. Total nodule weight, on the other side, increases very similarly to the effective nodule system: from 0.41 g at d 15 to 2.41 g at d 45. More than 80% of the nodule number remains in the two smallest size classes during the whole development. However, also with ineffective nodules we can observe in the percentage of the total weight an increase in the size class No. 4, larger than in size class No. 3 between 25 and 35 d after infection. This effect is by far not as significant as in the effective nodules in Fig. 5. For physiological characterization of some nodule classes we chose the smallest class No. 1

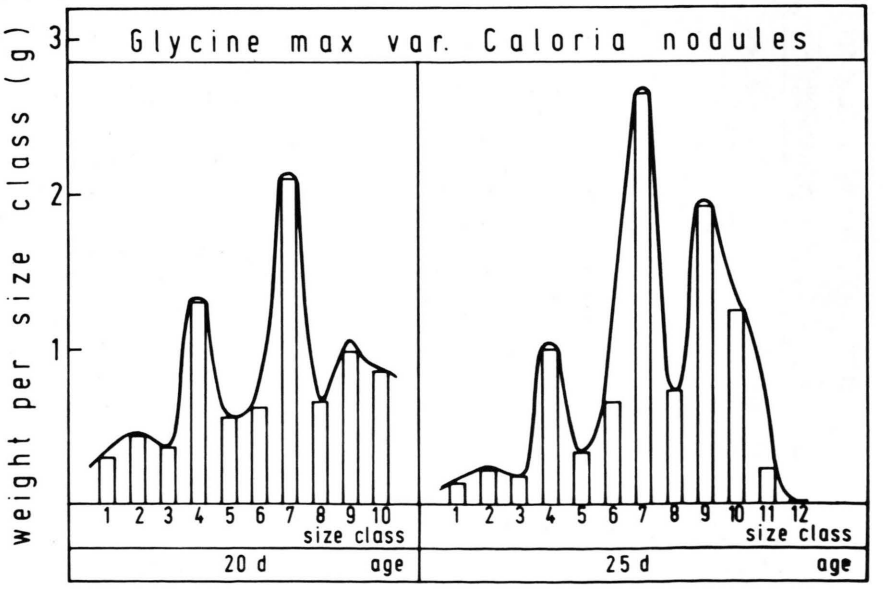

Fig. 4. Fractionation of nodules of 50 plants (s. Fig. 1) into 10 to 12 size classes by weight per size class, 20 and 25 d after infection.

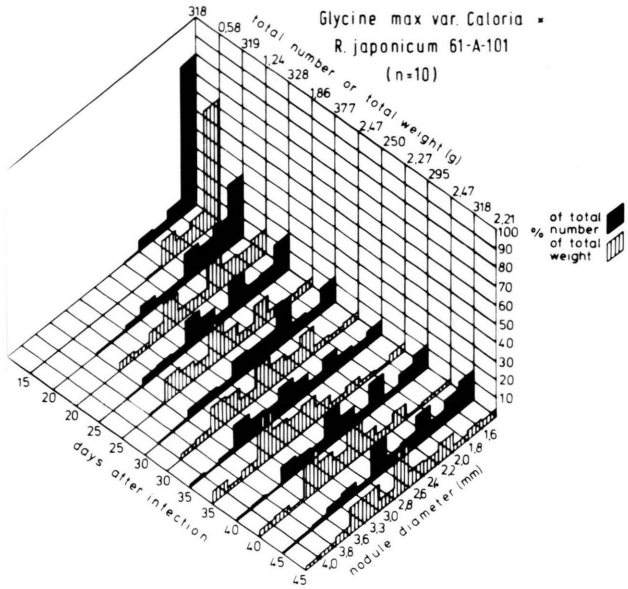

Fig. 5. Fractionation of effective nodules of 10 plants (s. Fig. 2) into 12 size classes by weight and number per size class, 15 to 45 d after infection. Total number and total weight of nodules in 10 plants during this development are given at the top of the columns.

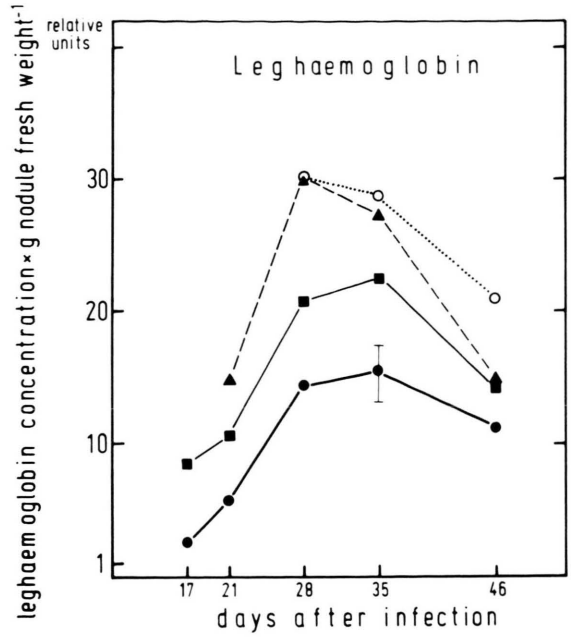

Fig. 7. Leghaemoglobin concentration in size classes of effective soybean nodules at days after infection. –●– < 1.8 mm; –■– 2.2–2.6 mm; –▲– 3.0–3.6 mm; –○– > 4.0 mm nodules diameter.

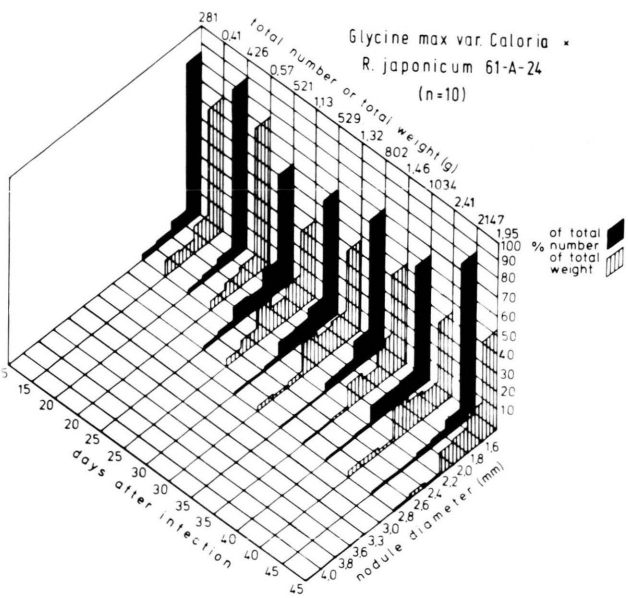

Fig. 6. Fractionation of ineffective nodules of *Glycine max* var. Caloria infected with the ineffective strain *Rhizobium japonicum* 61-A-24, into 12 size classes by weight and number per size class.

(smaller 1600 µm diameter) and size class No. 13 (nodules larger than 4000 µm diameter) and compared them with intermediate classes, nodules with 2200 to 2600 µm (size classes No. 4 to 6) and nodules with 3000 to 3600 µm (size classes No. 9 and 10). During the whole nodule development there is a significant size class effect on leghaemoglobin concentration, as found by other authors [5]. It is interesting to note that the fraction of nodules ageing as very small organs increase and decrease their leghaemoglobin content in a similar way as the very large nodules (Fig. 7). This statement of course holds only for those infection systems when no increase in nodule number complicates the interpretation of the results. A rather small but still significant size effect is found for the specific activity of aspartate aminotransferase in the plant cytoplasm of the nodules (Fig. 9). However, here the specific activity is somewhat larger in the smallest nodule size class compared with the larger ones. As already mentioned earlier [6], specific activity of this enzyme and the plant cytoplasm follows very closely the nitrogenase activity in the nodules. No size effect is established for specific activity of glutamate

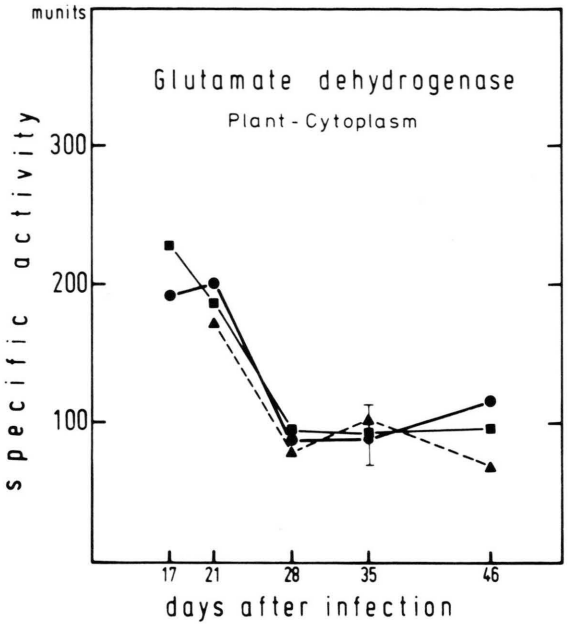

Fig. 8. Specific activity of glutamate dehydrogenase (E.C. 1.4.1.2) from plant cytoplasm fraction in size classes of effective soybean nodules at days after infection. Symbols as Fig. 7.

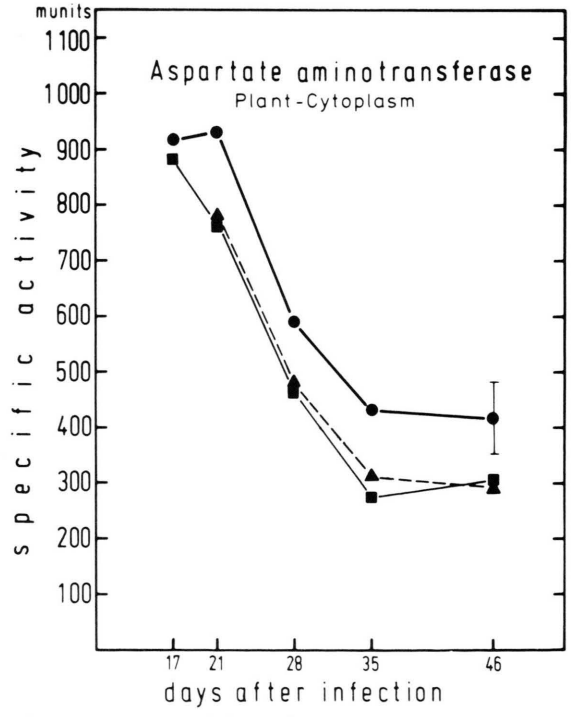

Fig. 9. Specific activity of aspartate aminotransferase (E.C. 2.6.1.1) from plant cytoplasm fraction in size classes of effective soybean nodules at days after infections. Symbols as Fig. 7.

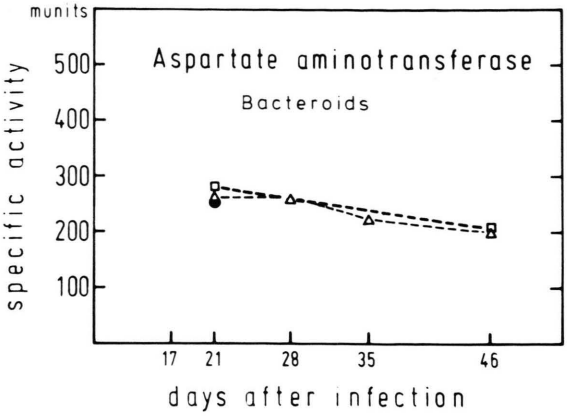

Fig. 10. Specific activity of aspartate aminotransferase from the bacteroid fraction in size classes of effective soybean nodules at days after infection. –●– < 1.8 mm; –□– 2.2–2.6 mm; –△– 3.0–3.6 mm nodules diameter.

Table I. Specific activity (nmol substrate · mg protein^{-1} · min^{-1} = m units) of alanine dehydrogenase (E.C. 1.4.1.1) from bacteroids in different size classes of nodules of *Glycine max* infected with *Rhizobium japonicum* 61-A-101.

Size class	Days after infection	Activity [m units]
< 1800 µm	21	60
2200–2600	21	120
2200–2600	46	280
> 4000	28	310
> 4000	46	510

dehydrogenase in the plant cytoplasm between 17 and 46 d after infection (Fig. 8).

We did not expect to find any size effect of specific activities in bacteroids from nodules from the various size classes. This is in fact the case for aspartate aminotransferase (Fig. 10). However, there is a significant effect of the size classes on specific activity of alanine dehydrogenase in bacteroids (Table I). Bacteroids from larger nodules of the same age have about twice the activity of bacteroids from smaller nodules. In addition, the activity of this enzyme increases with the age of all nodules sizes as already shown earlier for the total nodules population of soybeans [8].

Discussion

When we started to fractionate nodules of *Glycine max* in ten to twelve different size classes during the development, we expected to find a continuous in-

crease and decrease in nodule weight or number in the size classes. The maximum should shift with the age from smaller to larger size classes. However, we found with the sieve system calibrated with the nodules and with glass beads three to four maxima of size classes beginning at day 20 after infection. These size classes are maintained up to stage IV (senescent stage) (Fig. 5). We interpret this result by assuming three to four different sites at the root system of the soybeans, allowing preferentially the development of nodules in the size classes 2, 4, 7 and 9 to 10. By technical reasons the size classes No. 9 and 10 increased by 0.1 mm more than the other size classes. Nevertheless, the increase of nodules weight in size class No. 9 by more than 150% compared to size class No. 8, 25 d after infection (Fig. 4) is much larger than the additional increase of 20% of the area of the sieve mesh.

The gradient hypothesis of nodule formation [9, 10] with a development of 80 to 90% of the nodules opposite xylem poles would explain a type of normal distribution of the size classes. Most studies in this respect were done with the non-spherical root nodules of *Pisum sativum*. We do not know at present whether the three to four size maxima in our studies with nodules of *Glycine max* are the result of the distribution of nodules at three to four different root classes at which the infection has occurred. From the data in Fig. 4 and 5 we can conclude that age and size of nodules are two rather distinct parameters. By the oxygen enhancement effect of nitrogenase activity (Fig. 2) we separated four developmental stages of the whole population of nodules. Together with the eight maxima and minima of nodule size distribution, we found altogether about thirty different combinations of nodule sizes and ages. Three examples for physiological activities of some size classes were given in Figs. 7–9. The increasing leghaemoglobin concentration per mg nodule fresh weight was positively correlated with the largest nodules, aspartate aminotransferase was, statistically significant, most active in the smallest nodule size and glutamate dehydrogenase was the same in all size classes. The concentration of the two enzymes and of leghaemoglobin therefore must be different in the zones of the nodules. There seems to be no unusual difference between nodules from a size class at a minimum frequency (size class No. 5 to 6) compared to those from a maximum (size class No. 9 to 10). Aspartate aminotransferase from bacteroids in different size classes has the same specific activity of about 250 m-units during the whole nodule development. Therefore the increase by about 100% of the activity of alanine dehydrogenase in bacteroids from larger nodules cannot be a consequence of contamination by protein in the bacteroid fractions used as reference value for specific activity. As already shown earlier [1, 8] this enzyme is positively correlated with nitrogenase derepression in bacteroids and in free-living cells of *Rhizobium japonicum*. From the data in Table I we have to conclude that bacteroids can be different in enzyme activity from large and small nodules, in young and in old stages.

Dependency of nitrogen fixation from a certain optimum O_2 concentration has been demonstrated already in earlier studies with *Glycine max* [11] and *Pisum sativum* [12]. A decrease in variation of the oxygen enhancement effect of nitrogen fixation by using nodules of the same age was demonstrated by Bergersen [13]. As shown by this author nodule respiration and N_2 fixation increase up to 50% O_2. Respiration has a second maximum at 100% O_2 while nitrogen fixation decreases significantly. Enhancement of nitrogenase activity with detached soybean nodules is very sensitive and was found only up to a PO_2 of 0.2 [15]. The oxygen enhancement effect of nitrogenase activity certainly depends on several important physiological and cytological parameters of the nodules: nitrogen fixation capacity as limited by nitrogenase synthesis and regulation, reducing equivalents and ATP [14], nodule respiration, size of the bacteroid zone, status of the membrane envelope of the infection vacuoles in infected cells [8], leghaemoglobin concentration and location and supply of photosynthates from the plants. To characterize soybean nodules for developmental studies therefore, this oxygen enhancement effect of nitrogenase activity to characterize nodule age after infection of a root system of a seedling of a defined age and the fractionation of nodule sizes in at least ten different classes are recommended.

Acknowledgements

We thank the Deutsche Forschungsgemeinschaft for the continuing support in Sonderforschungsbereich "Zellenergetik und Zelldifferenzierung".

[1] D. Werner and R. Stripf, Z. Naturforsch. **33 c,** 245 (1978).
[2] D. Werner, Ber. Deutsch. Bot. Ges. **89,** 563 (1976).
[3] T. H. Mague and R. H. Burris, New Phytol. **71,** 275 (1972).
[4] A. C. Lawrie and C. T. Wheeler, New Phytol. **74,** 429 (1975).
[5] D. T. Nash and H. M. Schulman, Can. J. Bot. **54,** 2790 (1976).
[6] R. Stripf and D. Werner, Z. Naturforsch. **33 c,** 373 (1978).
[7] R. Stripf and D. Werner, Ber. Deutsch. Bot. Ges. **91,** 683 (1978).
[8] D. Werner, E. Mörschel, R. Stripf, and B. Winchenbach, Planta **147,** 320 (1980).
[9] D. A. Philipps, Physiol. Plant **25,** 482 (1971).
[10] K. R. Libbenga, F. van Iren, R. J. Bogers, and M. J. Schraag-Lamers, Planta **114,** 29 (1973).
[11] R. H. Burris, W. E. Magee, and M. K. Bach, Ann. Acad. Sci. Fenn. **60,** 190 (1955).
[12] G. Bond, Z. Allg. Mikrobiol. **1,** 93 (1961).
[13] F. J. Bergersen, J. Gen. Micorbiol. **29,** 113 (1962).
[14] C. Laane, H. Haaker, and C. Veeger, Eur. J. Biochem. **87,** 147 (1978).
[15] B. Koch and H. J. Evans, Plant Physiol. **41,** 1748 (1966).