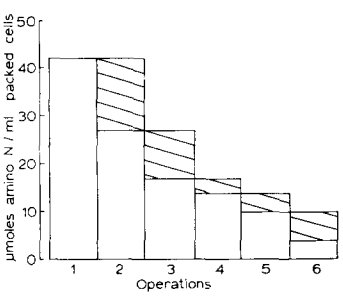
BBA 23304

The probable site of nitrogen fixation in root nodules of *Ornithopus sativus*

It has been suggested[1] that the legume root nodule symbiosis has evolved from rhizosphere associations between the plant and a free-living nitrogen-fixing bacterium. The agent of nitrogen fixation in the nodule might therefore be expected to be the bacterium rather than the plant. However, previous investigations[2,3] have suggested that the site of nitrogen fixation is not in the bacterial cells. This paper presents evidence favouring the bacterial cell as the site of nitrogen fixation.

Ornithopus sativus (Brot.) (serradella) plants inoculated with *Rhizobium lupini* strain D 25 were grown in solution culture and nodules were harvested immediately prior to use. Weighed samples of nodules, usually about 7 g, were flushed with argon and exposed to an $^{15}N_2$ enriched gas mixture. Exposures were terminated by crushing the nodules in a mortar cooled to —20°, subsequent manipulations were performed at 4°. The crushed material was filtered with suction and the filtrate, undiluted nodule sap, was centrifuged at 2000 × *g* for 10 min and then 18000 × *g* for 15 min. It was established by electron microscopy that the 2000 × *g* and 18000 × *g* pellets contained bacteroids and membrane structures, respectively, as their major components. The pellets obtained were extracted 3 times with 10 ml water. All samples were de-



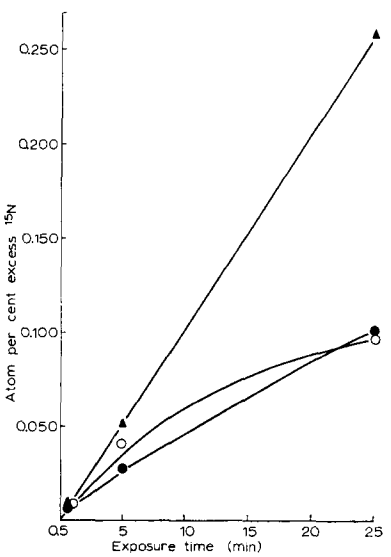


Fig. 1. Extraction of amino nitrogen from the bacteroid fraction of serradella nodules. Operations: 1, bacteroids fractionated by centrifugation at 4000 × *g* for 20 min; 2, 3 and 4 extracted with equal vol. of water; 5, extracted with cold 80% ethanol; 6, extracted with hot 80% ethanol. The cross-hatched region corresponds to the quantity of amino nitrogen extracted at each operation and the remainder is unextracted amino nitrogen. Amino nitrogen was determined by a colorimetric method[6].

Fig. 2. Time course of the ^{15}N enrichment in fractions of *O. sativus* nodules exposed to $^{15}N_2$. The enrichments shown are those in total N of water extracts from the 2000 × *g* (bacteroid) fraction, ▲—▲; 18000 × *g* (membrane) fraction, ●—●; and supernatant fraction, □—□. All extracts were deproteinized prior to analysis.

proteinized by heating at 80° for 10 min and subjected to Kjeldahl digestion prior to mass-spectrometric analysis.

The relative ease with which amino nitrogen is extracted from the bacteroid fraction of crushed nodules is illustrated in Fig. 1. It was thus obvious that in the preparation of nodule fractions this loss of amino nitrogen must be minimized and resulted in the fractionation procedure described in the preceding paragraph.

The rate of increase of atom per cent excess ^{15}N in the total nitrogen of aqueous extracts of nodule fractions is shown in Fig. 2. A relatively high rate of ^{15}N incorporation occurs in the bacteroid fraction. While this suggests an active role of the bacteroid fraction in nitrogen reduction it is necessary to localise the ^{15}N in particular compounds to provide a more critical assessment of this. In particular, it is required to know which of the ^{15}N-labelled compounds are of primary importance. For *O. sativus* nodules the sequence: $N_2 \rightarrow NH_3 \rightarrow$ glutamic acid has been proposed on the basis of pulse-labelling experiments[4]. The compounds of particular interest in determining the site of fixation are therefore ammonia and glutamic acid. Because of the mobility of ammonia and the small quantities involved, it has not been possible to examine the distribution of this compound in nodule fractions.

The atom per cent excess ^{15}N in glutamic acid in nodule fractions has been measured after short-term exposures to $^{15}N_2$. It has been found that it is the bacteroid fraction which contains the highest enrichment of ^{15}N in glutamic acid (Table I). It has also been shown that this fraction contains isocitric dehydrogenase[4] and glutamic dehydrogenase (unpublished). The presence of these two enzymes suggests the capacity of the bacteroid fraction to supply the necessary carbon skeleton, α-oxoglutaric acid, and to carry out the reductive amination to yield glutamic acid.

TABLE I

INCORPORATION OF $^{15}N_2$ INTO GLUTAMIC ACID ISOLATED FROM FRACTIONS OF SERRADELLA NODULES

Fraction	*Atom per cent excess* ^{15}N
2000 × *g*	0.285
8000 × *g*	0.184
18000 × *g*	0.084
Supernatant	0.212

The possibility that membranes associated with the bacteroid fraction contain the site of $^{15}N_2$ incorporation[3] could explain the experimental results obtained. However, electron micrographs indicate that a large proportion of bacteroids do not possess the enclosing membranes seen in sections of nodule tissues[5]. Correspondingly, there is an abundance of membrane structures in the 18000 × *g* fraction, but some of this material probably has other origins such as disrupted plant mitochondria. However, the critical comparison to be made is the relative rates of enrichment of ^{15}N in the 2 fractions (Fig. 2). It should be stressed that this comparison is only useful for linear rates of ^{15}N incorporation. The comparison made in this work is consistent with this criterion. A further point to be stressed is that the results obtained here depend, critically, upon the method of preparation of the nodule fractions.

In the absence of cell-free nitrogen-fixing activity in separate nodule fractions it is not possible to show unequivocally that the nitrogenase enzyme is present in the bacteroid fraction. However, the experimental support presented here for the bacteroids as the site of fixation must shift current emphasis to this fraction in the investigation of symbiotic nitrogen fixation.

It is considered that previous evidence[3], to the contrary may have resulted from the loss of ^{15}N-labelled compounds from the bacteroid fraction during preparation.

Department of Soil Science and Plant Nutrition,
Institute of Agriculture, University of Western Australia,
Nedlands (Australia)

I. R. KENNEDY*
C. A. PARKER
D. K. KIDBY

1 C. A. PARKER, *Nature*, 179 (1957) 593.
2 F. V. TURCHIN, *Soviet Soil Science*, 10 (1959) 1141.
3 F. J. BERGERSEN, *J. Gen. Microbiol.*, 22 (1960) 671.
4 I. R. KENNEDY, *Ph.D. Thesis*, University of Western Australia (1965).
5 D. K. KIDBY AND D. J. GOODCHILD, *J. Gen. Microbiol.*, 45 (1966) 147.
6 H. ROSEN, *Arch. Biochem. Biophys.*, 67 (1957) 10.

Received August 15th, 1966

* Present address: Department of Biological Sciences, Purdue University, Lafayette, Ind., U.S.A.

BBA 23309

Effects of glucose concentration on incorporation of [^{3}H]leucine into insulin using isolated mammalian islets of Langerhans

It has already been shown that there is an enhanced rate of incorporation of [^{3}H]leucine into insulin when slices of ox or rabbit pancreas are incubated *in vitro* with increasing concentrations of glucose or mannose[1]. So far, however, effects of this type have not been studied in mammalian islet cells separated from acinar tissue.

The method used for the isolation of the islets was based on that of MOSKALEWSKI[2] in which the acinar tissue of guinea-pig pancreas was separated from the islets of Langerhans by the action of collagenase. This technique has been successfully adapted by KOSTIANOVSKY AND LACY[3] for use with rat pancreas.

In the present studies rabbit pancreas was used. In early experiments it was found possible to cannulate the pancreatic duct and infuse a bicarbonate-buffered salt solution[4] by a method similar to that already described for rat pancreas[3]. Subsequently, however, it was found more convenient to remove the splenic portion of the organ from the animal into a petri dish, and merely to distend it subperitoneally by local injection of bicarbonate-buffered medium through a fine needle. By this means the tissue appears to be rendered more susceptible to the action of collagenase. The pancreas was then cut into small pieces with scissors and incubated with con-