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Heterogeneous Population of Mitochondria in Soybean Root Nodules

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ABSTRACT

Two fractions of mitochondria were obtained from soybean [*Glycine max* (L.) Merr.] root nodules, hypocotyls and cotyledons by Percoll discontinuous density gradient centrifugation, respectively. The positions of the two mitochondrial fractions banding in the gradient were similar in nodules and hypocotyls, but those of cotyledons were different from nodules and hypocotyls. A relative amount of two mitochondrial fractions in soybean nodules changed during nodule development. The upper fraction of mitochondria in the gradient isolated from soybean nodules showed slightly higher oxidative activities than those of the lower fraction. However, several enzyme activities of two mitochondrial fractions were almost identical, except malate dehydrogenase. Although the size and shape of mitochondria were hardly different between the two fractions of mitochondria isolated by Percoll discontinuous gradient centrifugation, two types of mitochondria which had highly condensed cristae and had ambiguous innermembrane structure were observed in the upper mitochondrial fraction of soybean nodules. These results indicated that population of mitochondria in soybean root nodules was heterogeneous.

INTRODUCTION

Nitrogen metabolism is mutually correlated with carbon metabolism. In legume root nodules, atmospheric nitrogen is fixed and is assimilated to amide or ureide. Nitrogen fixation of bacteroids requires carbon compounds, and ammonium assimilation in host plant cells demands carbon skeletons and energy. C_4-dicarboxylic acids of the TCA cycle intermediates are most likely to be carbon sources for supporting nitrogen fixation of bacteroids (Ronson et al. 1981, Finan et al. 1983). The synthesis

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of glutamine and glutamate which fixed N is initially incorporated into (Ohyama and Kumazawa 1980) depends upon the provision of α-ketoglutarate from the TCA cycle. Recently, mitochondria which had fully functional phosphorylative and oxidative activities have been isolated from soybean or cowpea root nodules (Day et al. 1986, Rawsthorne and LaRue 1986, Puppo et al. 1987, Suganuma and Yamamoto 1987). These suggest that mitochondria function for providing carbon compounds and energy required for nitrogen metabolism in root nodules though the oxygen tension in root nodules is low.

In the present paper, we describe that population of mitochondria in soybean root nodules is heterogeneous. The significance of heterogeneous population of mitochondria in soybean root nodules is discussed with respect to nitrogen metabolism.

MATERIALS AND METHODS

Plant culture

Soybean seeds [*Glycine max* (L.) Merr. cultivar T202] were sown in vermiculite and inoculated with *Bradyrhizobium japonicum* strain 009. After 7 days, plants were transferred and grown in N-free liquid culture media in a greenhouse under natural daylight conditions (Suganuma and Yamamoto 1987).

Preparation of mitochondria

Soybean nodules were homogenized in a grinding medium [0.05M Tris-HCl buffer (pH 7.5), 0.5M sucrose, 10mM EDTA, 1%(w/v) Na-isoascorbate, 0.1%(w/v) BSA] with Polyclar AT powder and crude mitochondria were prepared by differential centrifugation as described previously (Suganuma and Yamamoto 1987). The crude mitochondrial fraction resuspended in 3ml of washing medium [0.05M Tris-HCl buffer (pH 7.5), 0.4M sucrose, 0.1%(w/v) BSA] was loaded on Percoll discontinuous density gradient prepared by layering 13ml of 45%(v/v) Percoll, 14ml of 33%(v/v) Percoll and 12ml of 21%(v/v) Percoll solutions. Each Percoll solution contains 10mM Tris-HCl buffer (pH 7.5), 0.25M sucrose and 0.1%(w/v) BSA. The gradient was centrifuged at 7,500×g for 30min with an angle rotor. After centrifugation, the gradient was fractionated into 2ml each from the bottom. Mitochondria of hypocotyls and cotyledons from soybean seedlings incubated for 5 days at 30°C were isolated in a similar way except that potassium phosphate buffer was used instead of Tris-HCl buffer for hypocotyl mitochondria.

Oxygen uptake and enzyme assays

Two fractions of mitochondria aggregating at the interfaces of the 45% and 33% layers and of the 33% and 21% layers isolated from nodules of 6 week-old soybean plants were collected after Percoll gradient centrifugation, and were resuspended in a small amount of the washing medium for oxygen uptake measurements and enzyme assays. Oxygen uptake was measured polarographically at 25°C using a Clark-type oxygen electrode as described previously (Suganuma and Yamamoto 1987). The

following enzymes were assayed spectrophotometrically according to published procedures. Fumarase (Boland et al. 1982), isocitrate dehydrogenase (Suganuma and Yamamoto 1987), β-hydroxybutyrate dehydrogenase (Wong and Evans 1971), malic enzyme (Macrae 1971), malate dehydrogenase (Nawa and Asahi 1971), cytochrome *c* oxidase (Nawa and Asahi 1971), glutamate dehydrogenase (Yamaya et al. 1984), and aspartate aminotransferase (Murray and Kennedy 1980). Protein was determined by the modified Lowry procedure (Bensadoun and Weinstein 1976).

Electron microscopy

For electron microscopy, two fractions of mitochondria isolated from nodules of 5 week-old soybean plants were pelleted and fixed for 2h at 4°C with 4% glutaraldehyde in wash buffer [50mM Tris-HCl buffer (pH 7.5) and 0.4M sucrose], respectively. The pellets were then washed in wash buffer 3 times and postfixed with 1% OsO_4 in wash buffer for 1.5h. The samples were dehydrated with graded concentrations of ethanol followed by acetone, and embedded in Quetol 812. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with JEOL, JEM-2000FX electron microscope.

RESULTS AND DISCUSSION

Figure 1 shows a distribution of marker enzymes for mitochondria, fumarase and isocitrate dehydrogenase, and that for bacteroids, β-hydroxybutyrate dehydrogenase, after Percoll discontinuous density gradient centrifugation of crude mitochondrial fraction isolated from soybean root nodules. Mitochondria appeared at the interfaces of the 45% and 33% layers (peak 1) and of the 33% and 21% layers (peak 2) in the gradient, while most bacteroids sedimented in the bottom. Mitochondria isolated from soybean hypocotyls and from cotyledons were also separated into two fractions by the Percoll gradient centrifugation, respectively (Fig. 2). However, the positions which mitochondria appeared in the gradient were different between hypocotyls and cotyledons. In the hypocotyls, mitochondria aggregated at the interfaces of the 45% and 33% layers and of the 33% and 21% layers similar to nodule mitochondria, while mitochondria from cotyledons banded at the interface of the 33% and 21% layers and at the top of the gradient. These results indicate that the populations of mitochondria in the root nodules, hypocotyls and cotyledons of soybean are heterogeneous, and furthermore types of mitochondria present in the tissue are different depending on the organ.

Goldstein et al. (1980) have fractionated mitochondria in wheat seedlings into two bands by a linear Percoll gradient centrifugation. Morohashi et al. (1981) have shown by sucrose density gradient centrifugation that light and heavy mitochondria were present in imbibed peanut cotyledons. The Percoll discontinuous density gradient centrifugation described here could separate at least three types of mitochondria. The nodule and hypocotyl had two types of mitochondria aggregating at the interfaces of the 45% and 32% layers and of the 33% and 21% layers in the gradient. On the other

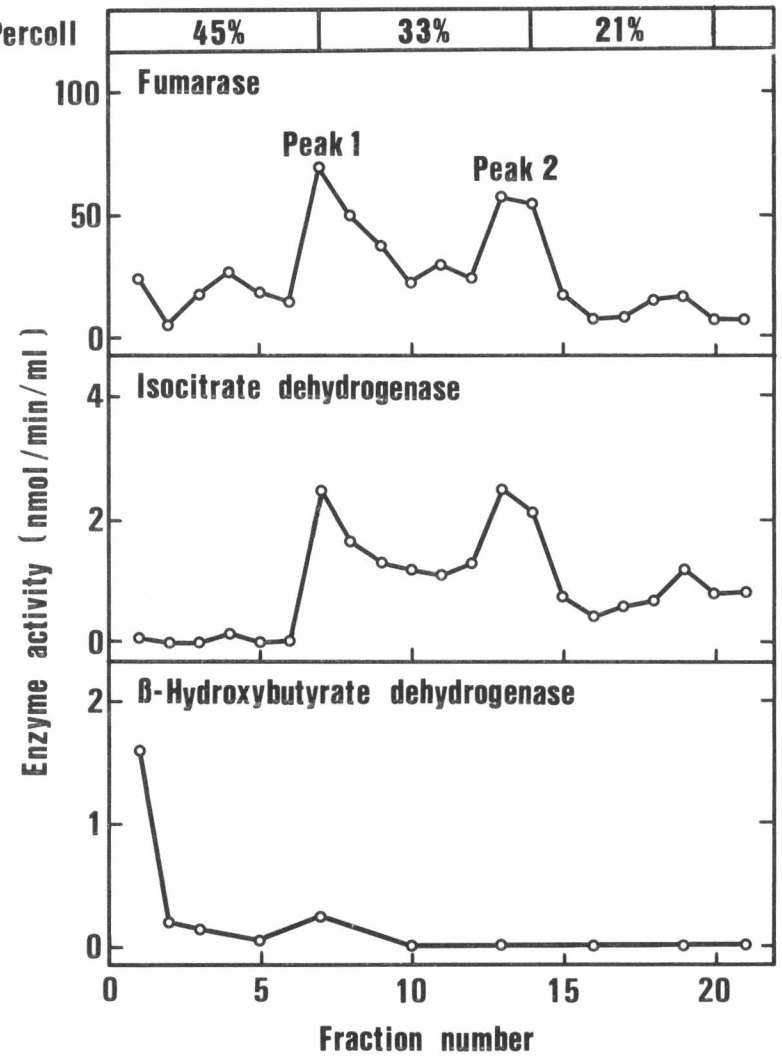

Fig. 1 Distribution of marker enzymes for mitochondria and bacteroids after Percoll discontinuous density gradient centrifugation of crude mitochondria isolated from soybean nodules.

hand, the cotyledons had one of the two types of mitochondria banding at the interface of the 33% and 21% layers and another type of mitochondria banding at the top of the gradient (Figs. 1 and 2). Although the physiological significance of these diversity is not known, the difference of these distribution of mitochondria in these three organs might be due to metabolism which will be active in the tissue or oxygen availability.

This is the first report that showed two types of mitochondria present in soybean root nodules. We then investigate the distribution pattern during nodule development, respiratory and enzyme activities, and ultrastructure of two types of mitochondria in

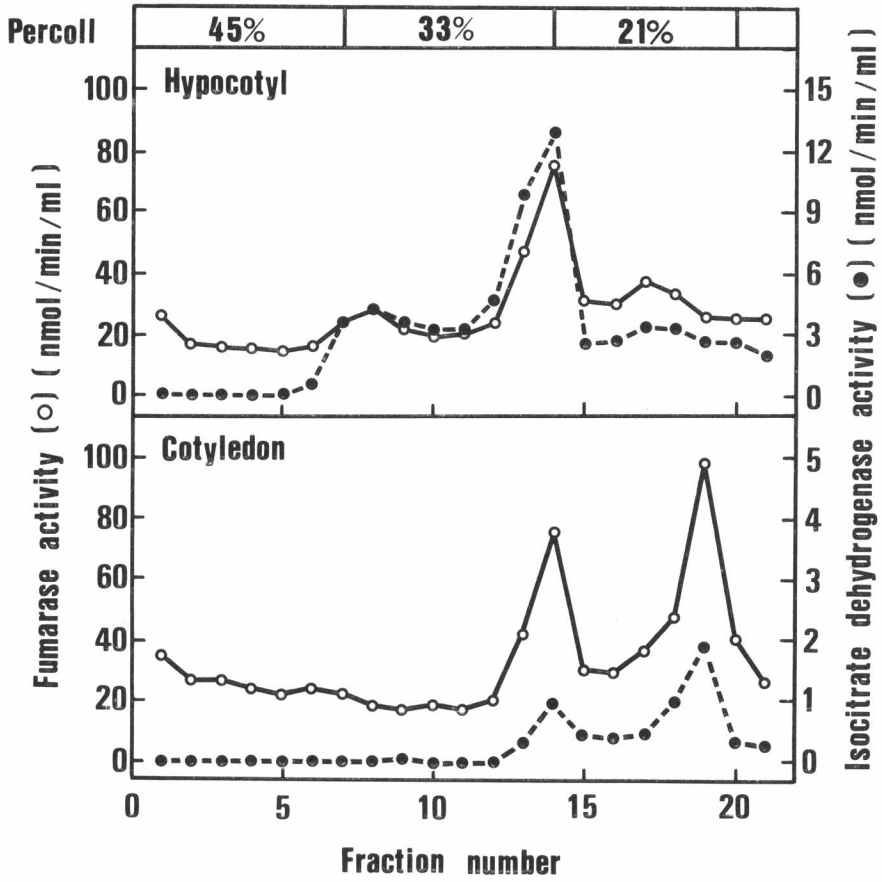

Fig. 2 Distribution of marker enzymes for mitochondria after Percoll discontinuous density gradient centrifugation of crude mitochondria isolated from hypocotyls and cotyledons of soybean seedlings.

soybean root nodules.

Figure 3 indicates the distribution of marker enzymes for mitochondria isolated from nodules of 3, 5, and 7 week-old soybean plants. All three different ages of nodules had two types of mitochondria. However, relative amounts of two mitochondrial fractions varied in the 3 week-old and the 5 and 7 week-old nodules. In the 3 week-old nodules, the activities of marker enzymes at the interface of the 33% and 21% layers were greater than those at the interface of the 45% and 33% layers. On the other hand, almost similar activities of marker enzymes were detected at the interfaces of the 33% and 21% layers and of the 45% and 33% layers in the 5 and 7 week-old nodules. When the activities of fumarase and isocitrate dehydrogenase around the two peaks were compared in the three different ages of nodules, the activities of the peak 2 mitochondrial fraction aggregating at the interface of the 33% and 21% layers decreased, and those of the peak 1 at the interface of the 45% and 33% layers increased as the nodules

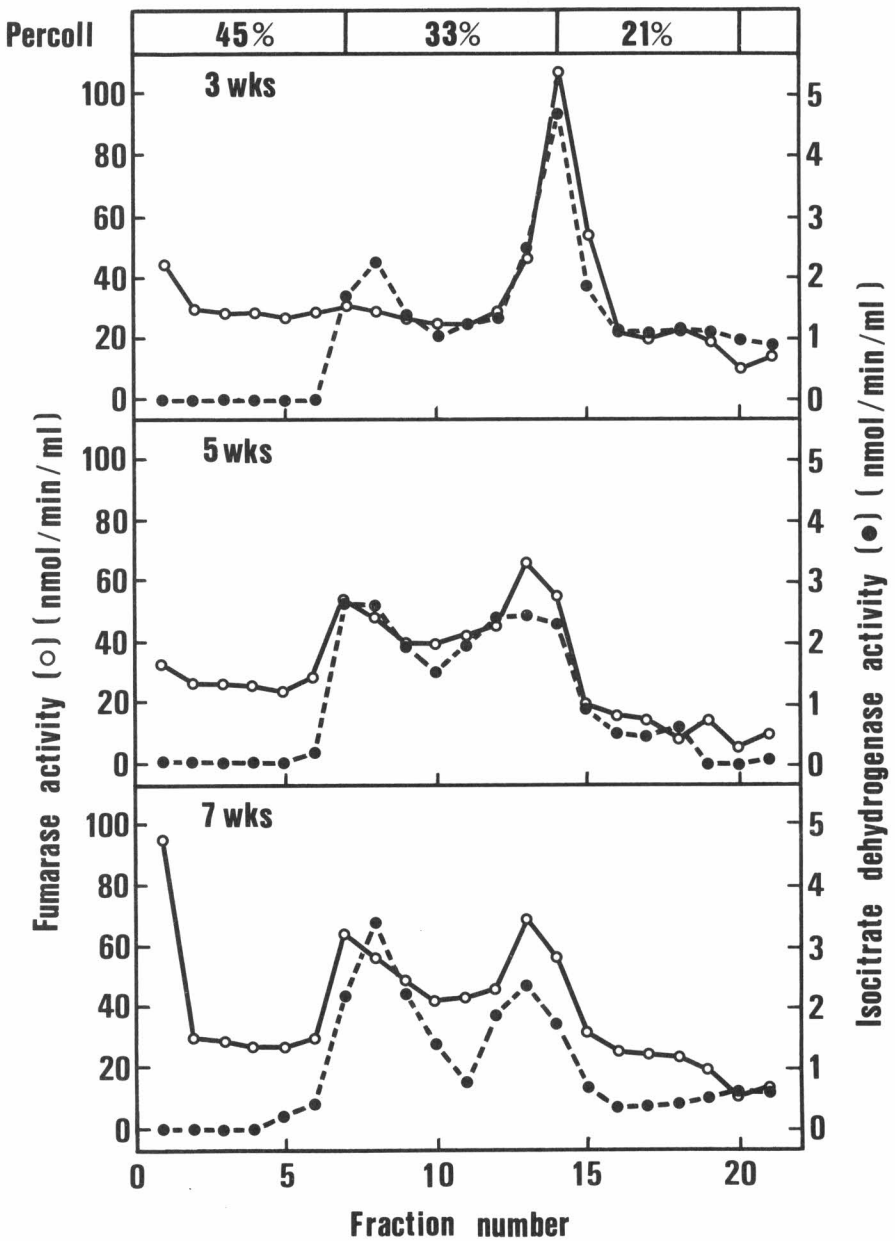

Fig. 3 Distribution of marker enzymes for mitochondria after Percoll discontinuous density gradient centrifugation of crude mitochondria isolated from different ages of soybean nodules.

developed from 3 to 5 and 7 weeks. These results suggest that the peak 2 mitochondria transform to the peak 1 mitochondria during the nodule development. In a previous paper, we showed that the amount of mitochondrial protein recovered from the nodules hardly changed during the nodule development (Suganuma and Yamamoto 1987). This paper shows that qualitative, not quantitative, changes of mitochondria will occur during the development of soybean nodules.

We next compared the two fractions of mitochondria isolated from soybean nodules to see if there were any functional or structural distinctions between the two mitochondrial fractions. The peak 2 mitochondrial fraction had slightly higher respiratory activities than those of the peak 1, though both mitochondrial fractions had similar phosphorylating activities (Table 1). Activities of several enzymes localized in mitochondria were compared between the two mitochondrial fractions (Table 2). They had almost identical activities in most enzymes investigated except malate dehydrogenase. The activity of malate dehydrogenase was slightly greater in the peak 2 mitochondrial fraction than that in the peak 1. This result coincides with the higher oxidative activity of malate in the peak 2 mitochondrial fraction (Table 1).

Components of the two mitochondrial fractions isolated from nodules of 5 week -old soybean plants were investigated by electron microscopy (Fig. 4). Mitochondrion was the major organelle in the both fractions though both fractions were contaminated with some membranous structures. The size and shape of mitochondria were hardly different between the two fractions of mitochondria. The peak 1 mitochondrial fraction showed homogeneous mitochondria with highly condensed cristae. However, the peak 2 mitochondrial fraction contained two types of mitochondria. The one of them was identical to mitochondria observed in the peak 1 fraction, and the other which appeared only in the peak 2 fraction had ambiguous innermembrane structure. These distinctive types of mitochondria were observed in the electron micrograph of soybean nodule mitochondria isolated by the centrifugation of Percoll gradient composed of 45% and 21% layers (data not shown). Moreover, these two types of

Table 1 Oxidative and phosphorylative activities of two mitochondrial fractions isolated from soybean nodules by Percoll discontinuous density gradient centrifugation

Fraction	Substrate	Respiratory rate	ADP/O	RCR
Peak 1	succinate	175±30	1.37±0.10	2.03±0.19
	malate	159+19	2.25±0.29	4.72±0.77
Peak 2	succinate	250±57	1.41±0.13	1.85±0.19
	malate	200±33	2.28±0.26	7.37±2.61

Peak 1 and peak 2 mitochondria were collected after Percoll discontinuous density centrifugation and were resuspended in a small volume of washing medium, respectively. Respiratory rates refer to state 3 respiration and were as nmoles O_2 absorbed per min per mg mitochondrial protein. Values are the averages ± standard deviations of 3 experiments.

Table 2 Enzyme activities of two mitochondrial fractions isolated from soybean nodules by Percoll discontinuous density gradient centrifugation

Enzyme	Activity (μmol/min/mg mitochondrial protein)	
	Peak 1	Peak 2
Isocitrate degydrogenase	0.069	0.069
Fumarase	0.449	0.454
Malate dehydrogenase	16.442	22.658
Malic enzyme	0.015	0.017
Cytochrome *c* oxidase	0.157	0.167
Glutamate dehydrogenase(NAD)	0.045	0.049
(NADH)	0.699	0.688
Aspartate aminotransferase	0.219	0.218

Peak 1 and peak 2 mitochondria were collected after Percoll discontinuous density gradient centrifugation and were resuspended in a small volume of washing medium, respectively. Enzyme activities are expressed as μmoles pyridine nucleotide oxidized or reduced, or substrate utilized per min per mg mitochondrial protein. Glutamate dehydrogenase was assayed in both directions, and NAD and NADH in the parenthesis are coenzymes used for reactions of deamination and amination, respectively.

mitochondria can also be observed in the electron micrograph of Percoll-purified soybean nodule mitochondria shown by Puppo et al. (1987).

Nitrogen fixing activity of soybean root nodules increased as plant grew and reached maximum about 6 weeks after sowing (Suganuma and Yamamoto 1987). The variations of the relative amount of two mitochondrial fractions from 3 to 5 and 7 weeks (Fig. 3) were correlated with nitrogen fixing activity, indicating that the increment of the peak 1 mitochondrial fraction was associated with nitrogen fixing activity or ammonium assimilation. Fletcher (1972) proposed that higher plant cells have two populations of mitochondria ; one associated primarily with respiration (respiratory mitochondria) and the other with the provision of carbon skeletons for subsequent biosynthesis (synthetic mitochondria). In this study, the peak 2 mitochondrial fraction showed higher respiratory activities than those of peak 1 (Table 1). However, it is not clear whether differences between the two mitochondrial fractions correspond with his proposal, since the activities of glutamate dehydrogenase and aspartate aminotransferase were scarcely varied between the two mitochondrial fractions (Table 2).

Electron microscopic studies showed distinctive types of mitochondria present in soybean root nodules (Fig. 4), besides two mitochondrial fractions obtained by the Percoll gradient centrifugation described here (Fig. 1). Further separation of those mitochondria in soybean nodules should be necessary for biochemical analysis. We tried to separate mitochondrial fraction by Percoll-self generating centrifugation, but it was unsuccessful (data not shown). In a previous paper, we showed that aerobic

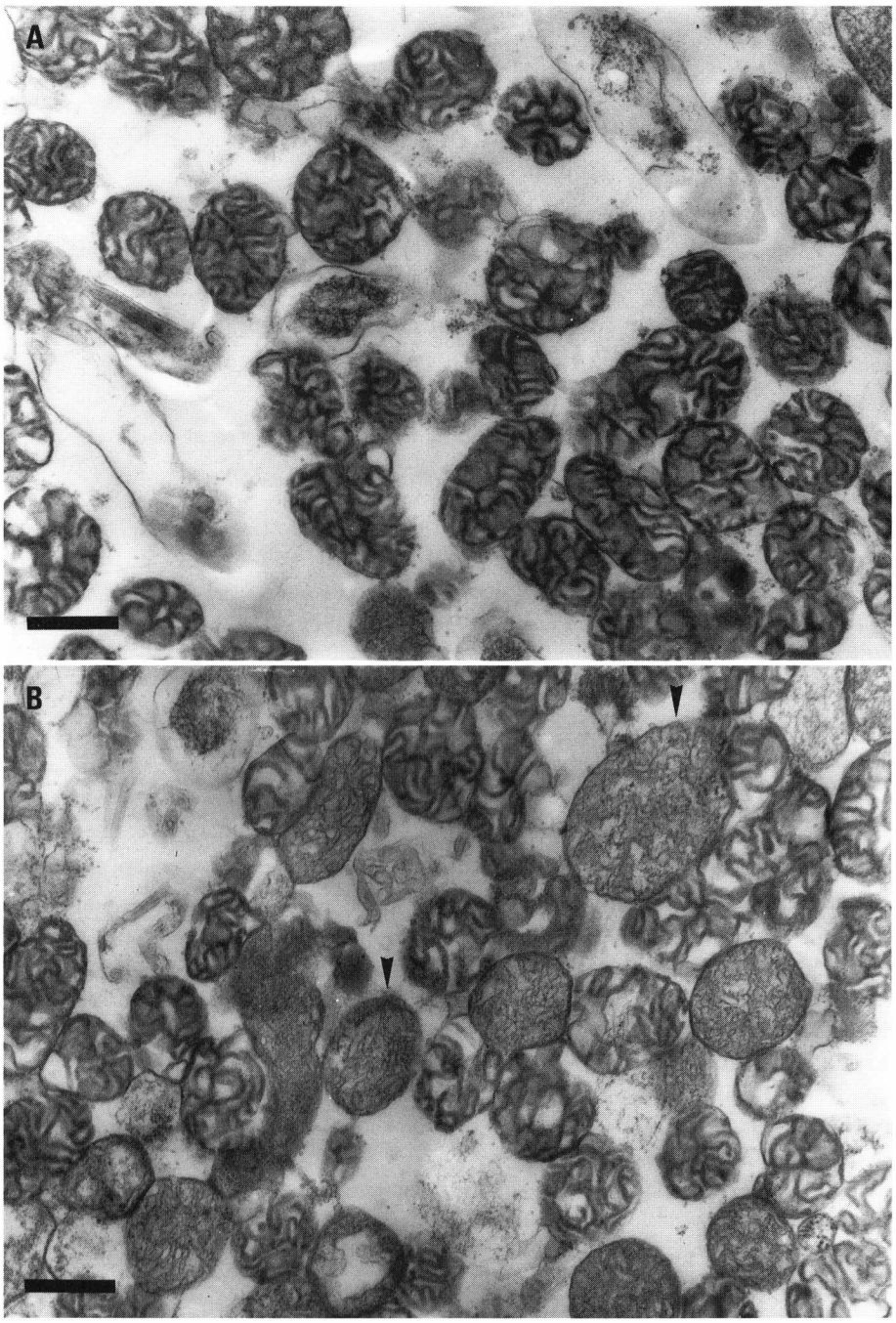

Fig. 4 Electron micrographs of two mitochondrial fractions obtained by Percoll discontinuous density gradient centrifugation of soybean nodules. Mitochondrial fractions of peak 1 (A) and peak 2 (B) were designated in Fig. 1. Arrowheads indicate representatives of mitochondria with ambiguous inner-membrane structure described in the text. Each bar represents 500nm.

metabolism is active in the infected cell fraction, and anaerobic metabolism is active in the uninfected cell fraction of soybean root nodules (Suganuma et al. 1987). Two types of mitochondria which were observed only in the peak 2 mitochondrial fraction might be responsible for the diversity of mitochondria present in the infected and uninfected cells in soybean nodules.

In this paper, heterogeneous population of mitochondria in soybean root nodules was shown by Percoll discontinuous density gradient centrifugation and by electron microscopy. Further separation and characterization of the heterogeneous population of mitochondria in soybean root nodules are under investigation.

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