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LABELLING OF LUPINE NODULE METABOLITES WITH $^{14}CO_2$ ASSIMILATED FROM THE LEAVES

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Key Word Index—*Lupinus luteus*; Leguminosae; nitrogen fixation; nodule; bacteroid; carbohydrates; amino acids; organic acids.

Abstract—On feeding $^{14}CO_2$ to the shoots of lupine (25 mCi per plant) 30 min was the minimal time needed to determine the incorporation of label into bacteroid compounds. The predominant incorporation, exhibited in all root, nodule and bacteroid samples after 30 min exposure, was into sucrose (45–90% of the corresponding fraction radioactivity) of the neutral fraction; into malate (30–40%) of the acid fraction; into aspartic acid and asparagine (60–80% in sum) of the basic fraction. The composition of carbon compounds containing the greatest amount of ^{14}C in the cytosol of nodules and in bacteroids was similar. Their radioactivity after 30 min exposure was for bacteroids (nCi per g of bacteroid fr. wt): sucrose 5.73, glucose 1.00, malate 0.15, succinate 0.11; for the nodule cytosol (nCi per g of nodule fr. wt): sucrose 200.00, glucose 8.40, malate 9.34, succinate 8.50. Thus it was demonstrated that in lupine, sucrose is the main photoassimilate entering not only into nodules but also into bacteroids. The biosynthesis of aspartic acid and asparagine occurs during nitrogen fixation in bacteroids.

INTRODUCTION

The passage of photosynthetic products from leaves into nodules is of great importance for symbiotic nitrogen fixation in legumes [1, 2]. Sugars, primarily sucrose, are the main transport form of photoassimilates for most higher plants [3, 4]. It is mainly sucrose that passes into nodules and thus serves as a source of carbon substrates necessary both for the formation of ATP and electron donor used by nitrogenase and for ammonia assimilation during nitrogen fixation [2]. However, the pathways of sucrose and other sugars in nodules have been studied insufficiently. It is important to elucidate the type of compounds by means of which photosynthetic products arrive at bacteroids. A study on incorporation and distribution of the label assimilated by leaves from $^{14}CO_2$ into metabolites of the nodule cytosol and bacteroids is a possible direct method. The experiments by Antoniw and Sprent with French-bean [5] and by Reibach and Streeter with soybean [6], where incorporation of ^{14}C arriving from leaves into nodule and bacteroid metabolites was being studied, are well known.

The aim of the present work was to study the incorporation of the label from $^{14}CO_2$, assimilated by lupine leaves, into carbon compounds of the nodule cytosol and bacteroids with a minimal exposure time. The lupine root system in the experiment was shielded from $^{14}CO_2$, as dark CO_2 fixation in the nodules of legumes is known to be active [7–9].

RESULTS AND DISCUSSION

Studying primary products, which pass from leaves into nodules and then into bacteroids, was one of the main tasks of our research. According to modern ideas, the rate of photoassimilate translocation through the phloem in the majority of herbaceous plants varies from 50 to 100 cm/hr [4]. Considering the distance from the lupine leaves to nodules, we chose 30 min as a minimal exposure time for the above-ground part of the plants in the $^{14}CO_2$ atmosphere. Preliminary experiments had shown that under these conditions radioactivity of no less than 20 mCi per plant is necessary for the analysis of the label incorporation into individual bacteroid compounds. Table 1 shows that at 25 mCi per plant the radioactivity of individual bacteroid fractions after 30 min exposure was about 1 nCi per plant, which corresponds to *ca* 7000 dpm/min per fraction since we used three plants simultaneously. It is sufficient for the reliable determination of the label incorporation into the main label-susceptible compounds of each fraction.

Fractional distribution of ^{14}C in time (Table 1) shows that photosynthesis and nitrogen fixation were sufficiently active as indicated by the fact that in 1.5 hr (from 30 to 120 min) the label incorporation into all fractions considerably increased, i.e. by 3–4 orders of magnitude. Moreover, active labelling of nodules and bacteroids as compared to the main root after 30 min exposure indicates that photosynthetic products entering the root system arrive first of all into the sites of nitrogen fixation and assimilation. Moreover, it should be borne in mind that bacteroid biomass is ten times less than the root biomass and that calculations of the label incorporation were made per plant. We carried out several similar experiments during three vegetative periods and they all gave the same results as in Table 1. Distribution of the label among individual compounds of each fraction was similar and almost time-independent, therefore we present the data of two experiments only with 30 min exposure for all the experiments (Table 2).

Table 1. Label incorporation into the roots and nodules of lupine at various durations of plant exposure to $^{14}CO_2$

	Fractions	Radioactivity (nCi per plant)		
		Exposure time (min)		
		30	60	120
Main root	Neutral	1.59	39.4	20500
	Acid	2.55	45.4	423
	Basic	3.45	41.5	664
Nodule cytosol	Neutral	181.00	—	11300
	Acid	48.40	80.9	3090
	Basic	48.40	786.0	3840
Bacteroids	Neutral	0.91	6.4	793
	Acid	1.05	3.6	106
	Basic	1.09	7.6	232

The nodules (2.4–2.7 g) and roots of three plants were used for the analysis of the material for each exposure time. Radioactivity was 25 mCi $NAH^{14}CO_3$ per plant. The data are the average of three replicates.

As is seen from Table 2 in the neutral fraction of all samples, the label was predominantly incorporated into sucrose and, to a lesser degree, into its hydrolysis products, glucose and fructose. The data on fructose and arabinose are combined, since in the experiments they were not readily separated. The sum of radioactivities of these 3–4 sugars amounted to over 90% of radioactivity of each neutral fraction. The label incorporation into other neutral alcohol-soluble compounds developed on chromatograms with both diphenylamine and silver nitrate was weak and hardly detectable after 30 min exposure. These data generally agree with those obtained earlier for French-bean [5] and soybean [6] and implicate sucrose as the main transport form of photoassimilates passing from leaves into roots and then into nodules, and, probably, from the host to the bacteroids.

Table 2 shows that in the organic acid fractions the greatest incorporation was into malate, and in some cases into succinate which confirms the literature data on the nodules of other legumes [5, 6, 10]. This indicates an active exchange of these compounds in the legume nodules, which appears to be associated with the biosynthesis of carbon skeletons which are necessary for assimilation of ammonia produced during nitrogen fixation [11]. We should note that in the bacteroids a spot is developed in the region of the start line which possessed a considerable proportion of radioactivity (45–60%) of the acid fraction.

The same observation was made for the bacteroids from French-bean nodules by Antoniw and Sprent [5] who came to the conclusion that the spot contained 6-phosphogluconate and, possibly, other hexose esters. In our case, the presence of phosphorus in this compound (or a group of compounds) was also shown by developing the chromatogram with ammonium molybdate followed by UV irradiation [12].

Table 2. Patterns of labelling of free sugars, amino acids and organic acids from the roots and nodules of lupine after the exposure of its above-ground parts to $^{14}CO_2$

	Radioactivity (nCi)*					
	Experiment 1, 1981			Experiment 2, 1982		
	Roots	Nodule cytosol	Bacteroids	Roots	Nodule cytosol	Bacteroids
Sucrose	93.7	91.3	87.5	71.3	40.5	77.5
Glucose	3.5	3.9	7.5	5.3	25.2	8.3
Fructose + arabinose	2.0	3.7	5.0	11.6	27.0	8.3
Fumarate + aconitate	6.0	16.6	10.4	6.9	8.9	7.7
Succinate	25.8	25.1	11.6	14.8	23.4	5.4
α-Ketoglutarate	2.7	3.0	3.7	9.0	4.4	3.1
Malate	35.1	38.4	15.0	32.8	33.7	29.8
Citrate + isocitrate	2.8	—	8.2	10.0	9.3	3.4
X ($R_f = 0$)†	24.5	7.2	45.7	26.4	8.5	50.4
Asparagine	38.0	31.9	37.6	21.4	41.1	4.8
Aspartate	44.0	26.4	43.6	46.8	35.7	70.5
Glutamate	12.0	10.2	9.1	14.9	9.0	10.2
Alanine	5.2	18.8	7.5	8.7	9.3	11.1
γ-Amino butyrate	—	5.2	1.9	3.9	3.0	2.1

*Values are the percentage of the corresponding fraction radioactivity.

†A spot at the start-line of the chromatogram of the organic acid fraction was always present. It exhibited radioactivity evidently corresponding to phosphorus esters of sugars.

Exposure time 30 min. Label incorporation 350 and 280 nCi/g nodule fr. wt in experiments 1 and 2, respectively. See the explanatory note to Table 1.

Table 3. Levels of ^{14}C in the major carbohydrates and organic acids from the lupine nodules at various duration of exposure of the overground parts of the plants to $^{14}CO_2$

		Radioactivity		
		Time (min)		
	Compounds	30	60	120
Nodule cytosol (nCi/g nodule fr. wt)	Sucrose	200.00	—	14000
	Glucose	8.40	—	575
	Malate	9.34	20.5	620
	Succinate	8.50	12.4	422
Bacteroids (nCi/g bacteroid fr. wt)	Sucrose	5.73	35.3	2970
	Glucose	1.00	4.3	189
	Malate	0.15	0.7	13
	Succinate	0.11	0.4	64

While Table 2 gives the relative radioactivity of compounds (values are the percentage of the corresponding fraction radioactivity) which enables one to estimate the patterns of labelling of metabolites, the data in Table 3 show precisely which carbon compounds contained the highest amounts of ^{14}C. One can see that the composition of the most highly labelled carbon compounds from the cytosol of the nodules and bacteroids was similar and did not change with exposure time. It follows from this that all of them (sucrose, glucose, malate and succinate) may pass into bacteroids from the plant part of the nodule. Preference, however, should be given to sucrose since, firstly, its labelling was considerably higher, and secondly, the label incorporation into sucrose of the bacteroids increased with time much more rapidly than into glucose or malate. The conclusion is supported by the fact that in one of the experiments at 30 min exposure the specific incorporation of ^{14}C into sucrose of bacteroids was three times higher than into glucose, i.e. 44.5 and 13.9 nCi/μmol, respectively.

The type of compounds that enter nodules as photosynthetic products and pass into bacteroids is a basic problem of the symbiotic nitrogen fixation. In the recent review by LaRue *et al.* [13] sugars are assigned a secondary role since the data obtained mostly with mutants of fast-growing *Rhizobium* species point to C_3 and C_4 organic acids as a primary carbon source in bacteroids. Yet, the fact has not been conclusively established for all the legumes.

It should be noted that at low concentrations of O_2, sucrose and glucose were the best energy substrates for nitrogen fixation in the bacteroids isolated from French-bean, soybean and pea nodules [14]. We had shown that the *Rhizobium lupini* bacteroids contain active invertase [15], and they can assimilate ^{14}C-glucose [16] and synthesize amino acids from sucrose [17]. The above facts, as well as the data on incorporation of ^{14}C, assimilated by the lupine leaves into sugars of the nodules and bacteroids (see Tables 2 and 3), and the results obtained for soybean [6] leave no doubt that sugars, and mainly sucrose, are the main carbon source for the bacteroids of *Rhizobium*, at least in the nodules of soybean and lupine.

The study on label distribution among amino acids indicated that in roots, cytosol of nodules, and particularly, in bacteroids, the main proportion of ^{14}C (60–85%) is observed in aspartic acid and asparagine (Table 2). The same results were obtained at 60 and 120 min exposure (data omitted). In similar experiments with French-bean and soybean the main proportion of the label was observed in glutamate [5, 6]. This may be ascribed to a considerable difference in the mechanisms of ammonia assimilation in the nodules of various legumes, e.g. in soybean and lupine [2]. The data presented in Table 2 emphasize the importance of aspartic acid and asparagine in the fixed nitrogen assimilation in the lupine nodules.

It has been earlier shown that the *R. lupini* bacteroids can synthesize aspartic acid due to direct ammonia assimilation catalysed by aspartase [18]. An extremely high content of ^{14}C in aspartate and asparagine of the

Table 4. Specific incorporation of the label into amino acids of the lupine nodules after exposure of the above-ground part of the plant to $^{14}CO_2$

	Radioactivity (nCi/μM)					
	Bacteroids		Nodule cytosol		Roots	
	Experiment 1	Experiment 2	Experiment 1	Experiment 2	Experiment 1	Experiment 2
Aspartate	2.22	2.90	8.22	1.77	2.98	19.57
Glutamate	3.93	1.70	5.23	1.77	11.05	25.97
Alanine	4.28	1.03	5.25	1.06	3.17	4.01

Exposure time 30 min. Label incorporation 280 nCi/g and 55 nCi/g of nodule fr. wt in experiments 1 and 2, respectively. See the footnote to Table 1.

bacteroids (60–80% of radioactivity of the basic alcohol-soluble fraction, Table 2) indicates that aspartic acid is synthesized in bacteroids. At the same time the specific incorporation of radioactive carbon into amino acids (with reference to the amount of a given amino acid in the sample), which may be synthesized, owing to direct ammonia binding by nitrogen-free precursors (Table 4), shows that in the lupine nodules both the 'aspartate' and 'glutamate' pathways of ammonia assimilation occur simultaneously. Apparently, the contribution of each pathway into assimilation of fixed nitrogen in the lupine nodules may only be estimated by using ^{15}N.

EXPERIMENTAL

Plant material. Plants of *Lupinus luteus* L. of the 'Bystrorastushchii 4' variety were inoculated with an effective strain of *R. lupini* 359a and grown in a greenhouse in vessels with quartz sand on the Pryanishnikov medium without nitrogen source. Conditions for seed inoculation and growth were described earlier [19].

^{14}C-Labelling of nodule metabolites. For the experiments selected plants were taken at the stage of the first flowering on the day of the experiment at 11 a.m. The plant tops were placed into separate 6 l. vol. hermetically sealed chambers and shielded from roots by a special rubber membrane, 3–4 plants being used for each exposure (total nodule wt of 2.4–4.2 g $NaH^{14}CO_3$ with radioactivity of 25 mCi) being placed into a special chamber cell. Exposure started by mixing $NaH^{14}CO_3$ with conc. H_3PO_4 (5 ml) and ended by cutting away the root from the overground part of the plant. Exposure was carried out at 3000 lx illumination at room temp.

Separation of the nodule cytosol and bacteroid fractions. After exposure, the roots with nodules were taken out of sand and washed in running H_2O. The nodules were separated from roots, weighed and ground in the cold in a medium of the following composition: 0.1 M K-Pi buffer, pH 7; 0.3 M sucrose; 0.1 M ascorbic acid; 1 mM $MgCl_2$; 3% PVP. The homogenate obtained was squeezed out through a Kapron fabric, brought up to 10 ml using the above medium and centrifuged at 3000 *g* for 8 min to remove large particles and starch granules. The supernatant was then centrifuged at 7000 *g* for 10 min to give host cell cytosol and bacteroids.

Extraction of cell carbohydrates, amino acids and organic acids. The bacteroid pellet, supernatant (the nodule cytosol fraction) and ground roots were fixed with 10-fold vol. of boiling 80% EtOH, 35–40 min elapsing from the separation of the root from the stem to bacteroid fixation. The samples obtained were extracted five times with boiling 80% EtOH; the extracts were then evaporated until dry and redissolved in double-distilled H_2O.

Separation of acidic, basic and neutral fractions from extracts. Separation of extracts into the fractions of amino acids, organic acids and sugars was carried out by chromatography on columns with the ion exchange resins Dowex 50 W × 8 and Amberlite IRA-401 in the anion and cation form, respectively. Desorption of the basic fraction was carried out with 2 M NH_4OH, and of the acid fraction with 2 M $(NH_4)_2CO_3$ in 2 M NH_4OH. Organic acids were transformed into the free form by repeated passage through Dowex 50 W × 8. All the samples were evaporated until dry and redissolved in 1–2 ml 40% EtOH.

PC. Different solvent systems were used for each group of metabolites: BuOH–HOAc–H_2O (4:1:5) for sugars; the same solvents but in the ratios 4:1:5, 8:3:1, 4:1:1 for amino acids; BuOH–HCOOH–H_2O (18:2:9) for organic acids [12]. In all the cases Whatman No. 1 paper was used.

Determination of metabolite radioactivity. A liquid scintillation counter SL-30 (Intertechnique, France) was used. Radioactivity of individual components was determined by cutting out the corresponding spots of the chromatograms and putting them into scintillation vessels without preliminary elution. Commercial preparation ZhS-8 (USSR) based on dioxane with addition of 10% EtOH was used as a scintillator.

Amino acid and sugar analysis. A quantitative content of sugars was determined using a Biotronic LC-200 sugar analyser and amino acids using Liquimat 'Labotron' amino acid analyser.

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