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Translocation of Photosynthates into Vacuoles in Spinach Leaf Protoplasts

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ABSTRACT

A method was developed for the isolation of vacuoles from the mesophyll protoplasts of spinach leaf, employing the discontinuous Ficoll density gradient centrifugation technique. Isolated vacuole preparations were judged to be free from other organellar fractions based on the assays of marker enzyme activities of individual organelles.

Using this isolation method, a time-dependent translocation of ^{14}C-labeled photosynthates into vacuoles was determined. In contrast to a significant transport of ^{14}C organic acids such as malate and citrate within 10 to 15 minutes ^{14}C neutral sugars and amino acids were barely transported into vacuoles during 40 minutes incubation, in spite of the fact that a relatively large amount of these compounds are found in the vacuoles. It was also found that a majority of [^{14}C]sucrose remains in the cytosol, apparently not actively moving into the vacuoles. Overall results appear to suggest that vacuoles are not actively engaged in photosynthetic carbon metabolism in spinach leaf protoplasts.

The isolation of central vacuoles from various higher plant tissues has been reported by numerous investigators who have shown that several solutes, *e.g.* salts, neutral sugars, amino acids, organic anions, and secondary metabolites, accumulate in vacuoles, and the mechanism(s) of transport of these substances across tonoplasts have been discussed (15). It has also been established that some lytic enzymes such as proteinase, DNase, phosphodiesterase, and α-mannosidase are localized in vacuoles (19) and that these enzymes are possibly involved in the hydrolytic breakdown of storage substances as well as cellular components (27). Therefore, in some aspects, the function of plant vacuoles is similar to animal lysosomes. An example of the dynamic function of plant vacuoles is provided by the diurnal rhythmic fluctuation in the contents of vacuoles isolated from *Sedum telephium* and the role of these vacuoles in the organic acid metabolism of this CAM plant (10). Active transport of sugars into vacuoles has been demonstrated using sugarbeet (3, 5), sugarcane (22), and rubber tree (2). The mechanism of ion transport into vacuoles is largely unknown, but experimental evidence is available showing the involvement of ATPase in the ion movement associated with vacuoles (11, 17, 24, 25).

In contrast to these biochemical properties of vacuoles of specific origins, little is known concerning the functional role of leaf vacuoles in photosynthetic carbon assimilation in C_3 and C_4 plants. Technical difficulties isolating vacuoles in pure form because of their morphological fragility has hampered elucidation of their functions in the metabolic flow in leaf tissues. In the work described in this communication, we have attempted to analyze the transport of various photosynthetic products into vacuoles derived from spinach leaf protoplasts. We have established a relatively simple method of isolating vacuoles from spinach leaf protoplasts. Various metabolites were fractionated from illuminated protoplasts after exposure to $^{14}CO_2$ and the distribution of radioactivity was determined. We have found that organic acids such as malate accumulate temporarily in the vacuoles. However, no measurable amount of sucrose, one of the final products of photosynthesis, was found in the vacuole.

MATERIALS AND METHODS

Protoplasts. Leaf of spinach (*Spinacia oleracea* cv Kyoho), freshly harvested in the field, was used throughout this investigation. Protoplasts were prepared according to the two-step enzymic digestion method described by Nishimura and Akazawa (18) after a slight modification; in the second step, 2% Driselase was added to 2% Cellulase Onozuka. Also, to avoid contamination with epidermal protoplasts, the final preparation of protoplasts obtained by the centrifugation at 100*g* for 3 min was washed twice with 0.7 M mannitol containing 3% Percoll. Epidermal protoplasts were not sedimented in 0.7 M mannitol containing 3% Percoll. The precipitate was suspended with 0.7 M mannitol and washed twice with 0.7 M mannitol so as to make protoplasts suspension to 1 to 2×10^6 particles/ml.

Isolation of Vacuoles. The preparative method employed was similar to that described by Buser and Matile (4) after some modifications, and the overall flow-sheet is illustrated in Figure 1. By treating the protoplasts with DEAE-dextran solution at room temperature, separated vacuoles were subsequently freed from cell debris by sedimentation at 100*g* for 3 min. The supernatant fraction was then layered on top of the step-gradient of 1% and 5% Ficoll solution containing 0.7 M mannitol, 10 mM Hepes buffer (pH 7.8 at 25°C), 2 mM EDTA, and 2 mM DTT, and centrifuged at 10,000*g* for 1 h. The vacuoles were recovered at the interface of two Ficoll layers. It took 1.5 h to prepare vacuoles from protoplasts. The yield of vacuoles was normally 5 to 6% of initially used protoplasts.

Photosynthetic CO_2 Assimilation. The light-dependent CO_2 fixation by the protoplasts (0.3–0.4 mg Chl) was measured in a reaction mixture containing 0.7 M mannitol, 40 mM MOPS[2]-KOH (pH 7.0), 4 mM $CaCl_2$, and 4 mM $NaH^{14}CO_3$ (28.4 Ci/mol) in a total volume of 3.5 ml. Light intensity was 90 klux and temperature was 25°C. Ordinarily, after 1 min preillumination, the reaction was started by adding $NaH^{14}CO_3$. Total $^{14}CO_2$

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[2] Abbreviations: MOPS, morpholinopropane sulfonic acid; PEPCase, phosphoenolpyruvate carboxylase; RuBisCO, ribulose 1,5-bisphosphate carboxylase/oxygenase.

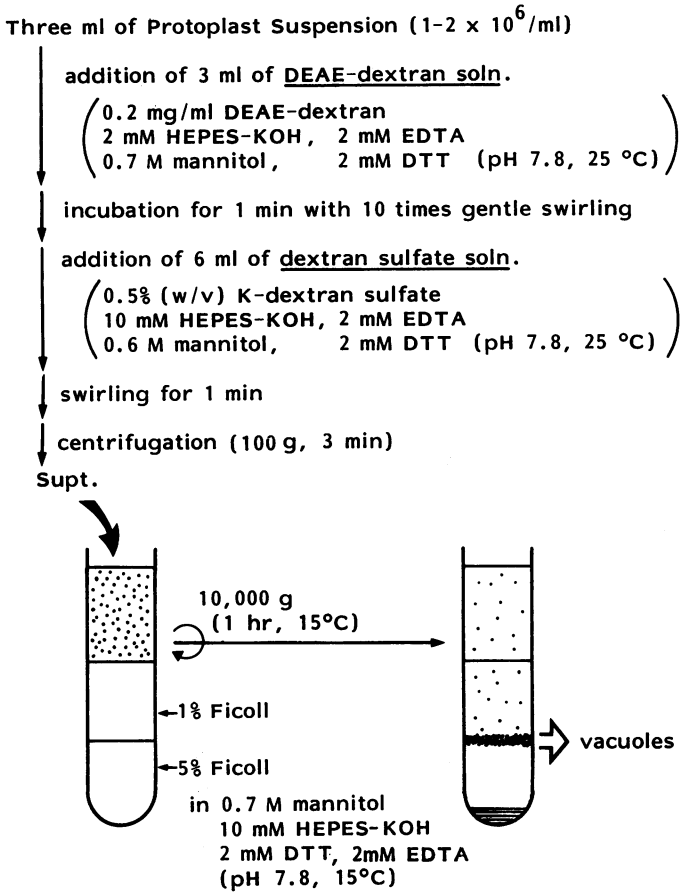

FIG. 1. Method for isolation of vacuoles from spinach leaf protoplasts.

fixation was measured by withdrawing 50 μl aliquots of the reaction mixture at the selected time intervals (*cf.* Fig. 3). Three-ml samples were then used to isolate vacuoles according to the method described above. Simultaneously, a 0.15-ml aliquot was used for the analysis of radioactive labeling of photosynthetic products in protoplasts. To determine what leaked from the protoplasts (Figs. 5 and 6), the extracellular fraction was analyzed using 2 ml of the supernatant fraction obtained by centrifuging the protoplast suspension (1000 *g*, 1 min). Each protoplast, vacuole, and extracellular fraction was treated with ethanol (final concentration, 80% v/v) to stop the reaction.

Analysis of ^{14}C-Labeled Photosynthates. Ethanol-treated samples were cooled to crystallize mannitol and Ficoll, which were then removed by filtration on a glass filter. The solution containing low mol wt labeled compounds was applied to a column (0.5 × 2.5 cm) of Dowex 50 (H^+ form), which was washed with 6 ml of H_2O to elute the neutral-acidic fraction. Amino acids were eluted from the column with 3 ml of 2 M NH_4OH solution. Each fraction was then dried in a flash evaporator and dissolved in 1 ml of H_2O, and quantitative analysis of each metabolite was performed as follows.

Five μl each of the neutral acidic fraction were applied to high voltage paper electrophoresis (20 × 20 cm, Toyo 51A) (10 mM acetic acid-ammonium acetate [pH 5.5], 1400 v, 45 min), and then subjected to ascending paper chromatography (*n*-butanol-propionic acid-H_2O = 94:44:62, 4 h) in the second dimension. Amino acids were separated by high voltage paper electrophoresis (acetic acid:formic acid:H_2O = 3:1:16, 1400 v, 2 h). In each case, the paper was then exposed to x-ray film, and the radioactive spots were identified by comparing with the location of nonradioactive authentic standards. Radioactivities of each identified spot were measured in a liquid scintillation spectrometer using a nonaqueous scintillator.

Quantitative Assay of Neutral Sugars, Malic Acid, and Amino Acids. The content of neutral sugars and malate was determined enzymically using each neutral-acidic fraction, sucrose and hexose by Jones *et al.* (8), and malate by Williamson and Corkey (26), respectively. Amino acid content was analyzed using a Hitachi automatic amino acid analyzer.

Enzyme Assays. All the enzyme assays were carried out using a 0.5-ml reaction mixture containing 0.1% Triton X-100. (a) Catalase was assayed by measuring the disappearance of H_2O_2 (240 nm) (13). (b) Fumarase activity was determined by measuring the decrease of A_{340} due to the formation of NADPH in a coupling reaction using NADP-malic enzyme (6). (c) Cyt *c* oxidase activity was determined by measuring the decrease of A_{550} due to the oxidation of reduced Cyt *c* (16). (d) RuBisCO activity was measured following the method of Nishimura *et al.* (21). (e) PEPCase activity as measured as described by Maruyama *et al.* (14).

Analytical Method. Chl content was determined by the method of Arnon (1). Protein content was determined according to the method of Lowry *et al.* (12) after the precipitation of proteins by the addition of TCA (final concentration, 10%). The number of protoplasts and vacuoles was counted with a Thoma haemacytometer.

Reagents. The following commercial products were used: Percoll, Ficoll, and DEAE-dextran from Pharmacia (Uppsala, Sweden); K-dextran sulfate (Meito-Sangyo, Nagoya).

RESULTS

We have tested several reported methods of isolating vacuoles from spinach leaf protoplasts (10, 11, 19), and the method using polycationic DEAE-dextran/dextran sulfate dissolved in osmoticum (0.6–0.7 M mannitol) (4) proved to be most satisfactory for the isolation of pure vacuolar fractions (Fig. 2). Since the density of spinach leaf vacuoles is light, it was thus essential to remove epidermal protoplasts from the mesophyll protoplasts at the beginning of experiments as described in "Materials and Methods". The vacuole fractions separated at the interface of the 1 to 5% Ficoll dissolved in 0.7 M mannitol solution were judged to be practically free from mitochondria (determined by the presence of fumarase, Cyt *c* oxidase), chloroplasts (Chl and RuBisCO), and peroxisomes (catalase) as shown in Table I. It is noteworthy that the absence of PEPCase activity (as a marker of cytosol) in vacuoles is indicative of the pure state of the preparations. In contrast, activities of acid phosphatase, phosphodiesterase, and α-mannosidase are shown to be high.

As presented in Table II, vacuoles are shown to contain a substantial amount of both neutral sugars (sucrose and hexoses)

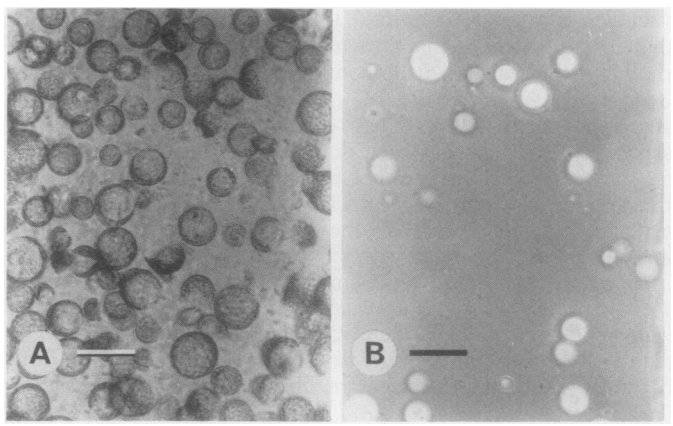

FIG. 2. Photomicrographs of: A, protoplast preparation; B, purified vacuole fraction from spinach leaf protoplasts. Bars, 100 μm.

Table I. *Enzymic Content of Vacuoles and Protoplasts from Spinach Leaves*

To obtain the protoplast and vacuolar extracts, the purified protoplasts and vacuoles fraction containing about 2×10^5 were mixed with 0.5 ml of homogenizing buffer (50 mM Tricine-KOH [pH 7.5], 15% sucrose, 0.1 mM EDTA) and ruptured by sonication for 10 s at 0°C. Enzymes were assayed in vacuole and protoplast extracts and gave the activities per 10^7 particles shown.

	Protoplasts	Vacuoles	V/P
	μmol/min/10^7 particles		%
Fumarase	0.210	<0.001	<0.1
Cyt *c* oxidase	1.57	0.01	0.6
Catalase	3459	17	0.5
RuBisCO	5.21	0.03	0.6
PEPCase	0.356	0.002	0.6
Acid phosphatase	1.01	1.23	122
Phosphodiesterase	0.059	0.080	136
α-Mannosidase	0.078	0.046	59
	mg/10^7 particles		
Chl	0.885	<0.001	<0.1
Protein	18.9	0.8	4.2

Table II. *Net Metabolite Contents in Vacuoles and Protoplasts from Spinach Leaves*

The purified protoplast and vacuole fraction containing about 2×10^5 were treated with ethanol (final, 80%). The ethanol-treated samples were cooled to crystalize mannitol, which were removed by filtration on a glass filter. The eluate was applied to automatic amino acid analyzer and enzymic analysis of sucrose, hexose, and malate.

	Exp. 1		Exp. 2	
	μmol/10^7 protoplasts (% in vacuoles)			
Sucrose	0.44	(48)	0.18	(53)
Hexose	0.57	(86)	0.38	(89)
Malate	1.03	(51)	1.00	(56)
Aspartate	0.40	(25)	0.41	(20)
Serine	2.47	(14)	4.11	(8)
Glutamate	1.10	(11)	1.02	(19)
Glycine	0.06	(267)	0.10	(110)
Alanine	1.73	(13)	1.97	(16)
Valine	0.65	(52)	0.77	(68)
Methionine	0.05	—	0.11	(91)
Isoleucine	0.44	(80)	0.52	(94)
Leucine	0.92	(78)	0.95	(93)
Tryptophan	0.38	(37)	0.53	(51)
Phenylalanine	0.73	(77)	0.91	(95)
Lysine	0.23	(30)	—	—
Arginine	0.42	(45)	—	—

and organic acid (malic acid). It can be seen that about 50% of sucrose, 90% of hexose, and 55% of malate initially applied to the analysis are present in the vacuoles. Among amino acids determined, the content of aspartate, glutamate, serine, and alanine in the vacuoles is relatively small, whereas the major portions of valine, methionine, leucine and isoleucine appear to be localized in the vacuoles.

Translocation of Photosynthates into Vacuoles. A time-dependent transport of ^{14}C-labeled photosynthetic products into vacuoles after photosynthetic CO_2 fixation by protoplasts was determined. As shown in Figure 3, there occurs a gradual increase in the accumulation of ^{14}C-labeled products in the vacuoles, but its proportion is small relative to protoplasts and is only about 5 to 6% in a 40-min incubation period.

The distribution of various ^{14}C-labeled compounds in the vacuoles is presented in Figure 4 A and B. Although sucrose comprises about 60% of the total photosynthetic products in

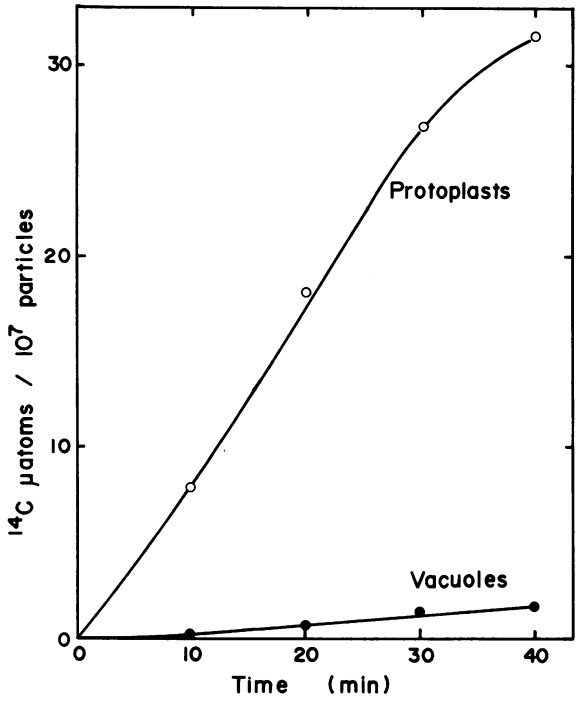

FIG. 3. Translocation of photosynthates into vacuoles. The light-dependent CO_2 fixation of spinach leaf protoplasts was carried out for various time periods under the conditions described in "Materials and Methods." Total $^{14}CO_2$ fixation was measured after drying a 50-μl aliquot withdrawn from the reaction mixture. The remaining 3-ml samples were used to isolate the vacuoles as described in "Materials and Methods." A 50-μl aliquot withdrawn from the purified vacuole fraction was used to measure radioactivity.

protoplasts, only approximately 4% of the [^{14}C]sucrose formed is tranlocated into vacuoles after the 40-min incubation. Since there is only a small increase in the net content of sucrose in the vacuoles in comparison with that observed in the protoplasts (compare data of Fig. 4A with Table II), it is unlikely that sucrose is transported extensively into vacuoles. A similar tendency was also observed with hexose (glucose and fructose). On the other hand, although the movement of malate into vacuoles is not significant in the initial 10 min, its content showed a prominent increase afterwards and the specific activities also clearly increased. Results of analysis with several other compounds are presented in Figure 4B, showing that none of the amino acids, *e.g.* glycine, alanine, and serine, moves extensively into vacuoles within a short incubation period. In spite of the fact that the net content of glycine in the vacuoles is relatively high, it does not appear to accumulate rapidly in the organelle. The accumulation of hexose-P in the vacuoles was not detectable, but organic acids, such as glycerate and citrate were shown to accumulate in vacuoles, although the rate of translocation was not as high as malate.

Extracellular Translocation of Photosynthates. Analysis indicates that both [^{14}C]hexose and sucrose do not accumulate extensively in the vacuoles, and the pertinent question arises where they are located? Since the 'protoplasts' signifies protoplasts *per se* plus suspending medium, there is a possibility that neutral sugars are extracellularly translocated after their production during photosynthesis. To examine this possibility, after the photosynthetic $^{14}CO_2$ fixation by protoplasts, the whole preparation was fractionated into intracellular and extracellular fractions following the procedures described in "Materials and Methods", and each of them was subjected to the analysis of individual components. Figure 5 shows that very little ^{14}C is present in the

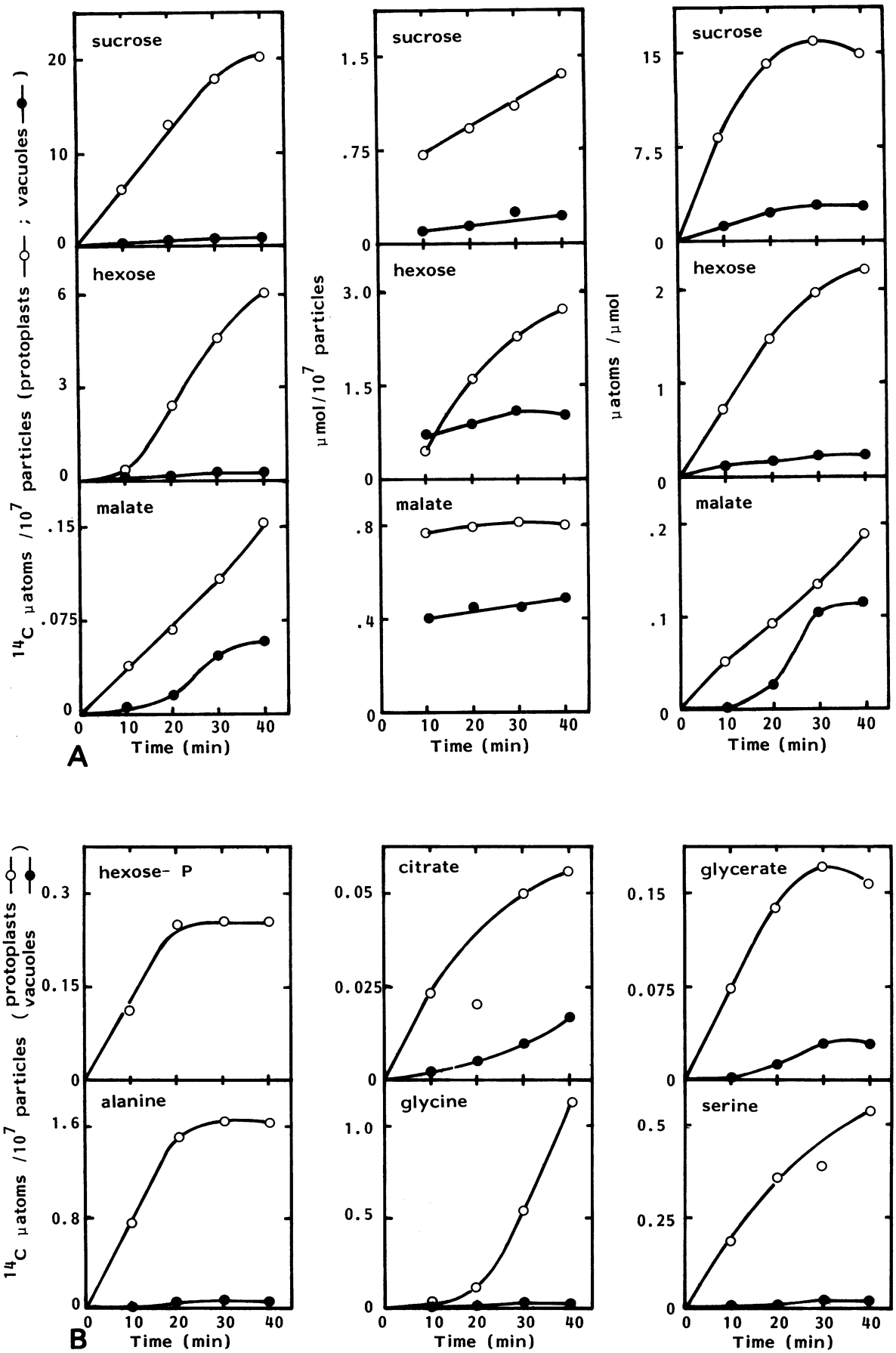

FIG. 4. Distribution of ^{14}C in various compounds in protoplasts and vacuoles. The various analytical methods employed are described in "Materials and Methods." Both protoplast and vacuole fractions were treated with hot ethanol (final concentration, 80%) and were then used for the analysis of the ^{14}C-labeled photosynthates. A, sucrose, hexose, malate; B, hexose-P, alanine, glycine, serine, citrate, glycerate.

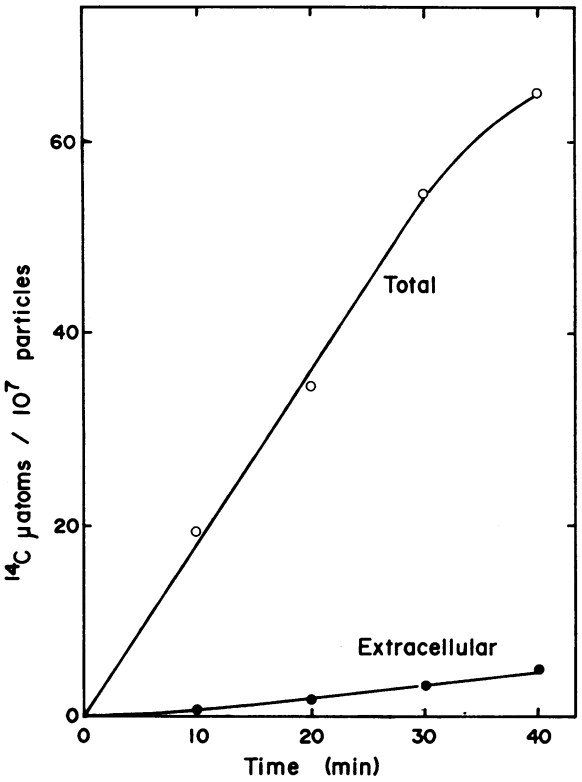

FIG. 5. $^{14}CO_2$ fixation by protoplasts and extracellular efflux of photosynthates. Photosynthetic $^{14}CO_2$ fixation by protoplasts was carried out under the conditions described in "Materials and Methods." A 0.15-ml aliquot was withdrawn for the analysis of photosynthetic $^{14}CO_2$ fixation by protoplasts. Remaining samples were centrifuged at 1,000*g* for 1 min. Two ml of the supernatant fraction was used to measure ^{14}C content and is designated as the extracellular fraction.

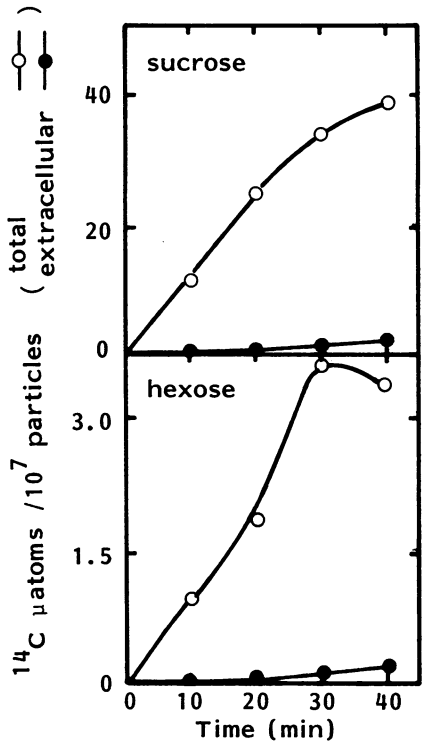

FIG. 6. Distribution of ^{14}C in sucrose and hexose in protoplasts and the extracellular fraction. Experimental conditions are the same as shown in Figure 5. Analytical methods are described in "Materials and Methods."

extracellular fraction. Figure 6 shows that only insignificant amounts of [^{14}C]sucrose and [^{14}C]hexose escape from the protoplasts. It is concluded that the major portion of [^{14}C]sucrose and [^{14}C]hexose remain in the cytosol or another intracellular fraction other than the vacuole.

DISCUSSION

Regardless of the origin, it is well known that various types of higher plant cells contain a well-developed central vacuole, sometimes comprising 90% or more of the entire cell space, where various metabolites accumulate. In some cases, the role of vacuoles as a metabolic reservoir has been verified (15), and the exclusive localization of hydrolytic enzymes in vacuoles appears to indicate a lysosomal function for them in higher plant cells (20). From the present study, it can be surmised that vacuoles of spinach leaf also have two such functions (Tables I and II). However, the mechanism of the transport of these metabolites into vacuoles has remained unknown. In answering this question, we have attempted to study the role of vacuoles in photosynthetic carbon assimilation. There is interaction of organelles, *e.g.* chloroplasts, mitochondria, with the cytosol during the flow of C and N compounds in photosynthesizing leaf cells (7). In addition, there is evidence indicating the crucial role of peroxisomes in the C-N flow in green leaf tissues placed under photorespiratory conditions. However, the role of vacuoles in photosynthetic carbon metabolism in C_3 plants are scanty and the translocation of photosynthetates has been characterized only in barley leaves (9). It is thus an intriguing question how rapidly some of the photosynthetic assimilates can move into the vacuolar system. The possibility has been considered that the time required to isolate the vacuoles from the protoplasts would invalidate the study of the transfer of photosynthetic assimilates into the vacuole. However, when the protoplasts are broken in order to prepare the vacuoles, there is an immediate dilution of the nonvacuolar components of the protoplast into the suspending medium which is centrifuged for 3 min (to remove cell debris, chloroplasts, etc.) before placing on the Ficoll gradient. The results are quite reproducible (Table II) and the level of metabolites in the vacuoles is quite high and similar to those found in other vacuoles (23). The fact that about 90% or more of several amino acids (methionine, isoleucine, leucine, phenylalanine, and glycine) and hexose are found in the isolated vacuoles indicates that, at least for these components, there is little leakage from the vacuoles during isolation. The situation must be left open for other metabolites; but, in any case, the vacuoles given in Figure 4, A and B, represent the minimum values for accumulation in the vacuole.

Our experiments show that, at least in protoplasts derived from spinach leaves, many of photosynthetic products do not appear in vacuoles in a short incubation period (10–40 min) (Table I), indicating that the vacuoles do not actively participate in photosynthetic carbon metabolism in this system. Among a variety of photosynthates, malate appears to be the only exception, which moves fairly rapidly into the vacuoles. Similar malate uptake has been observed in barley vacuole system (9). It has been demonstrated that, in CAM plants, malate is actively transported into the vacuoles (10), but our results cannot differentiate between active and passive transport systems. It appears that sucrose, one of the major photosynthates, does not accumulate intracellularly but conceivably is discharged extracellularly by the symplastic transport mechanism. However, in spinach leaf protoplasts it must be recognized that sucrose inevitably accumulates in the cytosol because of lack of plasmadesmata in the protoplast. Under the experimental conditions employed here,

the level of sucrose in the cytosol is calculated to be 0.7 M after 40 min incubation. It is worthwhile to note that, under such conditions, there is some inhibition of the photosynthetic activity of the spinach leaf protoplasts.

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