

Transport of Metabolites across Isolated Envelope Membranes of Spinach Chloroplasts

Received for publication November 7, 1974 and in revised form January 14, 1975

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ABSTRACT

Isolated envelope membranes of spinach chloroplasts (*Spinacia oleracea* L. var. Viroflay) exhibited selective permeability. Metabolites such as 3-phosphoglycerate, bicarbonate, glyoxylate, and acetate were transported rapidly; 6-phosphogluconate, glycolate, glycine, L-malate, and succinate were intermediate; whereas glucose 6-phosphate, fructose 1,6-diphosphate, and sucrose were hardly transported. Transport rates, metabolite accumulations within the membrane vesicles, and the internal water volume of isolated and *in situ* envelope membranes were compared and found to show similar trends.

Metabolite exchange between the chloroplast and cytoplasm is controlled by the inner membrane of the membrane pair comprising the chloroplast envelope (10). Since photosynthetic CO₂ fixation is regulated by the concentration of various metabolites (13, 14, 21), and the cytoplasm in turn is provided with photosynthetically produced substrates released from the chloroplast (2, 9), this control of permeability undoubtedly regulates many processes. The exchange of a number of substrates across this membrane using intact chloroplasts has been examined in the past by several techniques, and the results obtained have been reviewed recently (7, 22).

A useful tool for further studies of permeability has become a possibility with the availability of isolated pure envelope membranes from spinach chloroplasts (5, 18). The characteristics of the transport of bicarbonate into the isolated envelope membranes were previously studied in this laboratory (19). Further refinements in the isolation procedure resulted in the isolation of complete envelope membranes (at least 75% with a double membrane structure) possessing a high ATPase activity (20). This paper describes differences in the permeability of the complete envelope membrane towards various substrates of physiological significance and compares these properties with those obtained by other workers with intact chloroplasts.

MATERIALS AND METHODS

Plant Material. Spinach (*Spinacia oleracea* L. var. Viroflay, Asgrow Seed Co.) was grown outdoors in sand trays to which nutrient was added twice each week.

Radioactive Sources. The following labeled sources were obtained from New England Nuclear: ¹⁴C-bicarbonate, ¹⁴C-acetate, 1,4-¹⁴C-succinate, ¹⁴C-6-P-gluconate, ¹⁴C-glucose-6-P, U-¹⁴C-fructose-1,6-diP, and ¹⁴C-glycine. L-U-¹⁴C-malate and ¹⁴C-glycolate were purchased from Amersham/Searle. 3-

Phosphoglycerate was a gift from the laboratory of M. Gibbs and ¹⁴C-glyoxylate was provided by I. Zelitch. The latter was synthesized from ¹⁴C-glycolate in the presence of glycolate oxidase and catalase and purified on a column of Dowex-1-acetate X8 (25). This resin was also used to purify ¹⁴C-6-P-gluconate, which was found to contain some 3-P-glycerate. The fraction preceding 3-P-glycerate contained the 6-P-gluconate.

Chloroplast Envelope Membranes. Complete envelope membranes were removed from intact spinach chloroplasts and purified on a three phase discontinuous sucrose gradient, as described previously (20). A slight modification of the volume and centrifugation time was necessary, since a larger swinging bucket rotor (Beckman Spinco SW 25.1) was used. The centrifuge tube volume of the gradient, buffered at pH 7.6 with 50 mM Tricine, was composed of a top portion of 16 ml of osmotically shocked chloroplasts suspended in 350 mM sucrose, a middle layer of 6 ml of 672 mM sucrose, and a bottom layer of 9 ml of 876 mM sucrose. The centrifugation time was 90 min at 75,000g. After purification, the complete envelope membrane layer was removed (about 3–4 ml in 780 mM sucrose) and diluted with 50mM Tricine buffer (pH 7.6), to decrease the sucrose concentration to 330 mM.

Protein. The procedure of Lowry *et al.* (15) was used to determine protein.

Transport. A sample of the envelope membranes maintained at about 4 C in an ice bath, was added to a test tube (10 × 75 mm) containing the reaction medium shown below and maintained at 30 C in a water bath. After a 30-sec incubation to allow temperature equilibration, the desired radioactive permeant was added. This constituted zero time. The final volume in each tube was 0.5 ml, and the reaction mixture contained the following: 50 mM Tricine (pH 7.6), 330 mM sorbitol, 3 mM Mn²⁺, 3 mM Mg²⁺ (chloride salts), 2 to 4 μg of envelope membrane protein, and 1 mM radioactive permeant (0.1 to 2.0 × 10⁶ cpm/100 μl). Each addition of envelope membranes and labeled permeant was performed with automatic pipettes and followed by vigorous shaking on a Vortex mechanical mixer. Transport experiments were terminated by rapid removal of the sample with a 1-ml syringe and subsequent vacuum filtration on a Millipore filter (25 mm diameter, GS 0.22 μm). A stainless steel microsyringe filter holder, mounted on a vacuum flask and attached directly to the syringe, housed the filter. Completion of filtration constituted the end time. The filter was washed by syringe injection with 10 ml of Tricine buffer containing 0.33 M sorbitol at 4 C and pH 7.6. After a 15-sec wait, to ensure completion of filtration, the filter was removed. Radioactivity remaining on the filter was determined with a scintillation counter. Loss in counting efficiency caused by the presence of the Millipore filter was compensated for by a correction factor determined with a known ¹⁴C standard.

Volume of Exchangeable Water in Envelope Membranes. The water volume of envelope membrane vesicles was deter-

mined with $^3\text{H}_2\text{O}$. The transport and subsequent filtration were carried out as described above. Incubation time was 2 min (12).

RESULTS

During the spring, transport of bicarbonate into the complete envelope membranes was comparable to that reported previously (19). However, as the weather became hot and dry, a general deterioration of the transport properties of the isolated envelope membranes became evident. Earlier studies by Jacobson and Stumpf (12) indicated that Mg^{2+} and Mn^{2+} improved the uptake of acetate by chloroplasts. Permeability of the deteriorated envelope membranes was restored to higher levels upon incubation with Mn^{2+} and Mg^{2+} . An equimolar concentration of 3 mM was optimal. During the fall, when spinach growth improved again, envelope membranes again showed transport rates comparable to those in the spring without the addition of Mn^{2+} and Mg^{2+} . Their inclusion did not decrease the rate. Therefore Mn^{2+} and Mg^{2+} were added routinely to the incubation medium. This need for Mg^{2+} and Mn^{2+} may be related to the Mg^{2+} -dependent ATPase present in the envelope membrane (20).

The activity of the complete envelope membranes used in this study were similar to those prepared by the earlier method (19), since their transport properties deteriorated with storage at 4 C. Consequently, envelope membranes were used immediately after isolation, and experiments were completed within 45 min after isolation. During each experiment, the transport rate of bicarbonate was determined and compared with previous rates (19) to ensure that the membranes were not damaged or deteriorated.

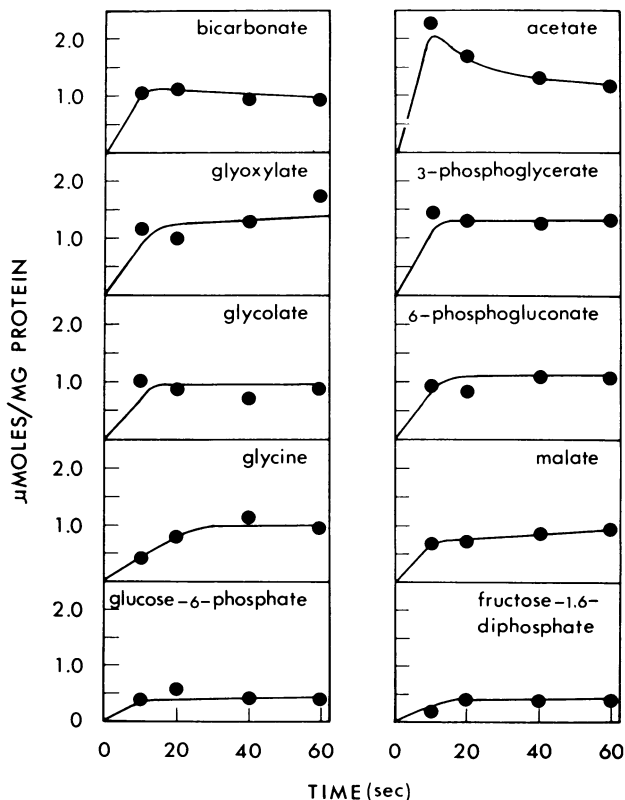


FIG. 1. Effect of time upon metabolite transport by isolated envelope membranes. A final volume of 0.5 ml at 30 C contained 50 mM Tricine at pH 7.6, 0.33 M sorbitol, 3 mM Mg^{2+} , 3 mM Mn^{2+} , 2 to 4 μg of envelope membrane protein, and 1 mM labeled metabolite.

Table I. Comparisons of Maximal Rates of Transport and Accumulation of Various Metabolites in Isolated and in Situ Envelope Membranes of Spinach Chloroplasts

Metabolite	Initial Rate of Transport ¹	Internal Conc ²
	$\mu\text{moles/mg protein}\cdot\text{hr}$	mM
3-Phosphoglycerate	515 (360) ³	11.9 (10) ⁴
Bicarbonate	389 (380) ⁵	9.3 (6) ⁵
Acetate	886 (88) ⁶	12.5 (2) ⁶
Glycolate	378 (70-700) ⁷	8.0
Glyoxylate	428 (70-700) ⁷	8.7
Glycine	151 (70-700) ⁷	7.8
Malate	245 (80) ⁸	7.5 (7) ⁴
Succinate	385 (80) ⁸	9.0
6-Phosphogluconate	335 (0.7-70) ⁷	9.6
Fructose 1,6-diphosphate	90 (0.7-7) ⁷	3.3
Glucose 6-phosphate	170 (7-70) ⁷	4.7
Sucrose		1.6

¹ Calculated from values obtained at 10 sec; average value of three replicates for at least three separate preparations of isolated envelope membranes. The values in parentheses are for membranes from intact chloroplasts taken from the literature.

² Determined at 20 sec; average value for at least three separate preparations of isolated envelope membranes. External metabolite concentrations were 1 mM. The values in parentheses are for membranes from intact chloroplasts taken from the literature.

³ See ref. 24.

⁴ See ref. 10.

⁵ See ref. 23.

⁶ See ref. 12.

⁷ See ref. 22.

⁸ See ref. 11.

The exchangeable water volume of the envelope membrane vesicles, as determined with $^3\text{H}_2\text{O}$, was 120 $\mu\text{l}/\text{mg}$ envelope membrane protein. Since the μmoles of labeled permeant transported per mg envelope protein at a given time were experimentally determined from their specific radioactivities, the actual concentration of permeant within the envelope membrane vesicles could be calculated.

This exchangeable water volume is exclusive of the intramembranous space, because the volume of the wash solution is sufficient to remove the tritium between the inner and outer membranes comprising the envelope membrane (19). Since only the inner membrane is selectively permeable, only the volume bounded by it is of any consequence. Optical planimetry of 40 electron micrographs (see ref. 20 for representative micrographs) showed about 11% of the total area of the complete envelope membrane is occupied by the intramembranous space.

In Figure 1 the transport of various permeants into the envelope membrane vesicles is shown as a function of time. Maximal transport usually occurred within 20 sec; a comparison of the initial transport rates attained within the first 10 sec, and the maximal internal concentration of various permeants attained in 20 sec are shown in Table I. In Figure 2 the effect of permeant concentration upon permeant transport is shown. The selected permeants were representative of substances having moderate or rapid rates of transport.

To compare absolute values, as opposed to relative values, one must know the relationship between the mg Chl and mg envelope membrane protein content of chloroplasts. Previously, this ratio was determined to be about 10 (19). However, with more complete envelope membranes, and hence more protein,

the ratio as determined from the amounts of protein in those preparations with the best yield is about 7. This value can be used to recalculate the data of other workers who used intact chloroplasts and to present it on an equivalent basis of envelope membrane protein. The present results can thus be compared with data determined by other investigators (see Table I).

Slight differences in the values obtained for transport rates and internal concentrations of permeant were observed among different preparations of envelope membranes. Similar observations were reported by Werdan and Heldt (24) for the same types of data determined with *in situ* envelope membranes. The values in Table I are, for the most part, obtained with the same preparations of envelope membranes, as opposed to separate preparations for the results shown in Figures 1 and 2.

DISCUSSION

The terms "transport" and "uptake" are used here according to the definition of Ghei and Kay (6). "Transport" means passage through the membrane without any further metabolism, whereas "uptake" implies both passage and subsequent internal metabolism. By these definitions isolated envelope membranes could only perform transport phenomena.

Throughout this paper the radioactive metabolites associated with the envelope membrane are designated as "transported". However, the possibility of some binding cannot be excluded, whether this be transitory binding during membrane passage or actual adsorption. Since methods to distinguish between the alternatives of transport or either type of binding are all subject to uncertainty (16), this question is not easily resolved. However, the results of permeability studies involving the release into the cytoplasm or suspending medium of various metabolites produced during photosynthesis are in good agreement with experiments on metabolite transport into the chloroplast (7, 22). Assuming the membrane permeability properties are the same in both directions, this would argue in favor of transport or at best transitory binding rather than adsorption. The agreement between results obtained with the attached and isolated envelope membrane would also suggest a transport mechanism for the isolated envelope membranes.

Previously, I have investigated the transport of bicarbonate in the envelope membranes (19). Complete envelope membranes in the present study, isolated by an improved procedure (20), gave similar results for bicarbonate transport as those previously examined (19). Since the two methods produce envelope membranes that differ mainly in the extent of their completeness, *i.e.* the percentage possessing both membranes, this similarity in bicarbonate transport is not unexpected. An implication of this similarity is that the envelope membrane vesicles are right side out and not inside out. This follows from the observation that the outer membrane of the envelope membrane pair is nonspecifically permeable, while the inner one exhibits selective permeability (10). If they were right side out, the newer method of isolation would be expected to yield more membrane vesicles possessing the outer membrane and this would not affect transport. Conversely, if they were reversed, one might expect the transport properties to be altered with the exposure of more membranes showing selective permeability.

If isolated chloroplast envelope membranes are to provide an alternate method for studying metabolite transport, they must show permeability characteristics consistent with envelope membranes of intact chloroplasts. A similarity between bicarbonate transport for the isolated and *in situ* envelope membrane has already been demonstrated (19).

From the data shown in Figure 1 and Table I, it is apparent

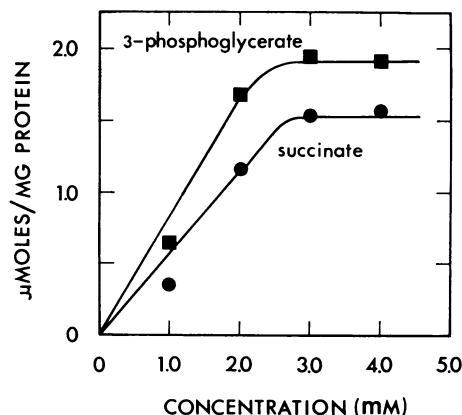


FIG. 2. Effect of metabolite concentration upon transport by isolated envelope membranes. A final volume of 0.5 ml at 30 C contained 50 mM Tricine at pH 7.6, 0.33 M sorbitol, 3 mM Mg^{2+} , 3 mM Mn^{2+} , 2 to 4 μg of envelope membrane protein, and labeled metabolites at the indicated concentrations terminated at 20 sec.

that isolated envelope membranes possess selective permeability. Metabolites such as 3-P-glycerate, bicarbonate, glyoxylate, and acetate are transported rapidly; 6-P-gluconate, glycolate, glycine, malate, and succinate are intermediate; whereas glucose-6-P, fructose-1,6-diP, and sucrose are hardly transported.

In terms of selective permeability there is good agreement between the present data (Fig. 1, Table I) and those obtained by others (9-12, 17, 22-24) with intact chloroplasts. The isolated envelope membrane, like the chloroplast, possesses high permeability toward malate, succinate, glycolate, glyoxylate, and glycine; and low permeability toward glucose-6-P and sucrose.

Likewise, absolute values for transport rates on a mg protein basis and permeant accumulation on an internal volume basis are also in agreement for isolated and *in situ* envelope membranes, as shown in Table I. The temperatures used were 30 C compared with the more usual 4 C, and not all concentrations of permeant were similar; nevertheless agreement in rates by the two kinds of preparations is good, especially for permeants with high rates of transport. However, differing temperatures (30 C versus 4 C) may result in the larger differences observed with permeants showing lower rates of transport.

Another similarity between isolated and *in situ* envelope membranes was that the internal volume of intact chloroplasts, as determined with 3H_2O (10, 12), expressed per mg envelope membrane protein, would be about 140 μl which compares favorably to the value of 120 μl cited here for isolated envelope membranes. This similarity indicates that the osmotic rupture and subsequent annealing of the envelope membranes during the isolation process does not effect the internal volume bounded by the chloroplast envelope membranes.

Similarities in the effect of permeant concentration upon its rate of transport are also apparent. In either the isolated (Fig. 2) or *in situ* (10) envelope membranes, transport rates become maximal at a concentration of 2 mM for dicarboxylic acids or 3-P-glycerate.

Some disagreement exists in the results for three metabolites: acetate, fructose-1,6-diP, and 6-P-gluconate (Fig. 1, Table I). However, this apparent discrepancy with respect to the permeability of acetate and fructose-1,6-diP has also been questioned with the intact chloroplast. Thus Heldt and Rapley (9) have described a low permeability of chloroplasts toward acetate, while Jacobson and Stumpf (12) found a higher permeability. My results support the latter observation. Interestingly, Jacobson and Stumpf (12) also observed an overshoot of acetate at

10 sec as I did (Fig. 1). Earlier evidence indicated a high permeability of the chloroplast toward fructose-1,6-diP (1, 2, 8), but later evidence indicates low permeability toward this metabolite (9, 11). The present results agree with the latter conclusion.

Earlier reports of fructose-1,6-diP being transported into and out of the chloroplast were later attributed to the action of adherent and inherent aldolase (7). The triosephosphates produced are highly permeable, and this would explain the apparent previous discrepancy. Chloroplast envelope membranes were not assayed for aldolase activity. However, the short duration of the present transport experiments (less than 20 sec), and the demonstrated absence of other chloroplastic enzymes (20) make it unlikely that such enzymic conversions occur with isolated envelope membranes.

The degree of permeability of 6-P-gluconate is of importance, especially because of its known regulatory effect upon ribulose-diP carboxylase whereby at saturating concentrations of bicarbonate and Mg^{2+} it is inhibitory and with limiting concentrations of bicarbonate and Mg^{2+} it enhances activity (3, 4). Heldt and Rapley (9) indicate a low permeability of the intact chloroplast toward 6-P-gluconate in contrast to the moderate permeability shown here (Fig. 1, Table I). It is not inconceivable that slight damage could occur during the isolation of the envelope membrane, resulting in altered permeability toward 6-P-gluconate. Also conformation changes might be induced, which would expose binding sites for that metabolite, so that the 6-P-gluconate is bound and not actually transported. This seems to be unlikely, in view of all the permeability similarities cited above. Since the presence of 6-P-gluconate may have a strong regulatory effect upon ribulose diphosphate carboxylase (3, 4), rapid rates of transport would facilitate the changes of internal concentration of 6-P-gluconate necessary for a regulatory function.

Thus, similarities in transport properties between the isolated and *in situ* envelope membranes suggest the usefulness of the isolated envelope membrane as an additional means to study transport of chloroplastic metabolites and to study the possible regulatory effect of membrane structure on transport.

Acknowledgments—I thank Dr. Israel Zelitch for his helpful discussions, Renée Shapiro for excellent technical assistance, George Smith for growing the spinach, and Dr. J. Michael Robinson for his efforts in supplying labeled 3-phosphoglycerate.

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