BBA 46097

THE INNER MEMBRANE OF THE CHLOROPLAST ENVELOPE AS THE SITE OF SPECIFIC METABOLITE TRANSPORT*

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(Received November 9th, 1970)

SUMMARY

The permeability of intact isolated chloroplasts for ^3H_2O and [^{14}C]sucrose has been measured. Part of the chloroplast ^3H_2O space is found to be unspecifically permeable to sucrose and other molecules of low molecular weight. The other part of the chloroplast ^3H_2O space which is impermeable for sucrose is accessible for specific transport of certain metabolites (*e.g.* 3-phosphoglycerate and malate).

From the comparison of these spaces with the morphological picture as obtained by electron microscopy, the sucrose-permeable space is attributed to the intermembrane space, situated between the inner and the outer membrane of the chloroplast envelope, and the sucrose-impermeable space to the stroma. It is therefore concluded that the outer membrane is unspecifically permeable to metabolites of low molecular weight, the inner membrane being the site of specific metabolite transport.

INTRODUCTION

Chloroplasts represent a metabolic compartment which is distinct from the cytosol. The main function of chloroplasts is to provide the plant cell with substrates generated by photosynthesis. It is therefore necessary to transport the products of photosynthesis from the chloroplast stroma to the cytosol of the cell. The envelope of the chloroplast, separating the stroma from the cytosol, consists of two membranes, the outer and the inner membrane (see ref. 2). The transport of metabolites across the chloroplast envelope is facilitated by specific translocators, catalysing a counter-exchange of anions. So far, three specific translocators have been characterized in spinach chloroplasts:

(a) The *phosphate translocator*[3] is specific for the transport of inorganic phosphate, 3-phosphoglycerate and dihydroxyacetone phosphate. The main products of photosynthesis in spinach, 3- phosphoglycerate and dihydroxyacetone phosphate, are thus transported from the chloroplast stroma into the cytosol in exchange for inorganic phosphate.

Abbreviation: HEPES, *N*-2-hydroxyethyl piperazine *N'*-2-ethane sulphonic acid.

* These data have been orally presented at the *8th Intern. Congr. Biochem., Symp. 4, Biological Oxidations and Bioenergetics, Lucerne*. Some of the data have been also included in a preliminary report[1].

(b) The *dicarboxylate translocator*[3] is specific for certain dicarboxylates, *e.g.* malate, and aspartate.

(c) The *ATP translocator*[4] has a high specificity for external ATP. The ATP translocator is not involved in the photophosphorylation of cytosolic ADP[4,5], but makes it possible to supply the chloroplasts with ATP during the night period.

The experiments described in the present paper were performed in order to decide whether these specific translocators are located in the inner or the outer membrane of the chloroplast envelope. It will be shown that the outer membrane is unspecifically permeable for the metabolites dealt with, the inner membrane being the site of specific metabolite transport.

METHODS

Preparation of chloroplasts

The spinach, obtained from a local gardener, was usually harvested a few hours before the experiment. The preparation of chlorplasts was carried out according to the method of COCKBURN *et al.*[6], with the following modifications: The filtrate obtained after homogenizing the spinach leaves was first centrifuged for 1 min at 4000 rev./min, and the supernatant discarded. The sediment was resuspended in a medium containing 0.33 M sorbitol, 20 mM *N*-2-hydroxyethylpiperazine *N'*-2-ethane sulphonic acid (HEPES), pH 7.6, 1 mM $MnCl_2$, 1 mM $MgCl_2$ and 2 mM EDTA, centrifuged for 1 min at 500 rev./min, the resultant sediment discarded and the supernatant centrifuged for 1 min at 2000 rev./min. The sediment was resuspended and centrifuged for 1 min at 2000 rev./min. Resuspension of this sediment yielded the chloroplast suspension employed in our experiments. The centrifugations were all carried out with a centrifuge "Sorvall RC 2b" using Rotor SS 34. From phase contrast microscopy[7] 70–90% of the chloroplasts appeared to have retained their outer membrane. CO_2 dependent oxygen evolution was 45–110 μmoles O_2 per mg chlorophyll per h. Chlorophyll was assayed according to the method of WHATLEY AND ARNON[8].

Conditions of incubation

The incubations were carried out at 4° in a medium containing 0.16, 0.33, or 0.66 M sorbitol, 20 mM HEPES, pH 7.6, 1 mM $MnCl_2$, 1 mM $MgCl_2$ and 2 mM EDTA, and chloroplasts equivalent to 0.1 mg chlorophyll per ml.

Filtering centrifugation

The filtering centrifugation[9] was carried out at 4° with a "Coleman" centrifuge using 0.5 ml plastic tubes. The tube contained 20 μl 10% $HClO_4$, 70 μl silicone oil (Wacker Chemie, München) and 200 μl chloroplast suspension. The density of the silicone was varied with the different media.

Sorbitol in medium (M)	*Silicone oil*
0.16	AR 50: AR 20 = 1:4
0.33	AR 50
0.66	AR 150

For counting of radioactivity, 50μl of the supernatant were taken. The content of the plastic tube was then frozen, the tip of the tube containing the $HClO_4$ fraction cut off with a knife and suspended in 300 μl water. After vigorous shaking and centrifugation of the protein precipitate, 200 μl supernatant of this sediment fraction was taken for counting.

Radioactive labelled substances used

The specific activities are given in parentheses: [U-^{14}C]sucrose (Boehringer, 100 μCi/mmole); [8-^{14}C]guanosine triphosphate (Amersham, 600 μCi/mmole); 3-Phospho[U-^{14}C]glycerate (Boehringer, 500 μCi/mmole); [U-^{14}C]malate (Amersham, 50 μCi/mmole).

$^{3}H_2O$ (Amersham, 3 μCi/ml); [^{14}C]dextrane (New England, 1.3 μCi/mg.) The dextrane was used (2 mg/ml) to correct for medium adherent to the outer surface of the chloroplasts.

The radioactivity measurements were carried out by liquid scintillation counting (Philips LSA) of ^{3}H–^{14}C double labelling.

Fixation of chloroplasts and electron microscopy

500 μl suspension of chloroplasts equivalent to 0.05 mg chlorophyll were added at 4° to 500 μl of 0.1 M glutaraldehyde containing 0.01 M HEPES, pH 7.6, and sorbitol (0.06, 0.23 or 0.56 M) according to the tonicity of the incubation medium). After 15 min, the suspension was centrifuged for 2 min at 1000 rev./min (Sorvall RC 2B with Rotor SS34). The pellet was washed 3 times with distilled water within 1 h, postfixed with osmium tetroxide and chromate according to the method of Dalton[10], dehydrated with isopropanol, embedded with Maraglas[11], cut with glass knives, stained with lead citrate and viewed in a Zeiss EM9A-microscope, aperture 60 μm. Initial magnification, 7000 ×.

RESULTS AND DISCUSSION

Permeability of chloroplasts

The permeability of chloroplasts for [^{14}C]sucrose and [^{14}C]GTP was investigated using silicone-layer centrifugation. GTP was selected as a nucleotide which is not transported into the stroma by the ATP translocator[4]. Sucrose was chosen as a compound presumably not freely permeating the osmotically active membranes of chloroplasts, since it can be used with chloroplasts to maintain the tonicity of the medium.

In the experiment shown in Fig. 1 the concentration of [^{14}C]GTP and of [^{14}C]-sucrose in the medium is varied. The H_2O space of the filtered chloroplasts is defined as the space accessible for $^{3}H_2O$ but inaccessible for [^{14}C]dextrane. The amount of sucrose or GTP assayed in the filtered chloroplasts as related to the H_2O space yields the apparent concentration of these substances within the chloroplasts. For sucrose and GTP there is a linear relatonship observed between the concentration in the medium and the apparent concentration in the chloroplasts, the latter amounting to about 40% of the concentration in the medium. It is concluded from these results that part of the H_2O space of chloroplasts is permeable for sucrose and GTP. The size of the permeable space is exactly the same for both compounds, it is about 40%

of the H_2O space when the medium contained 0.33 M sorbitol. This sucrose-permeable space does not derive from that portion of broken chloroplasts being present in the preparations[1].

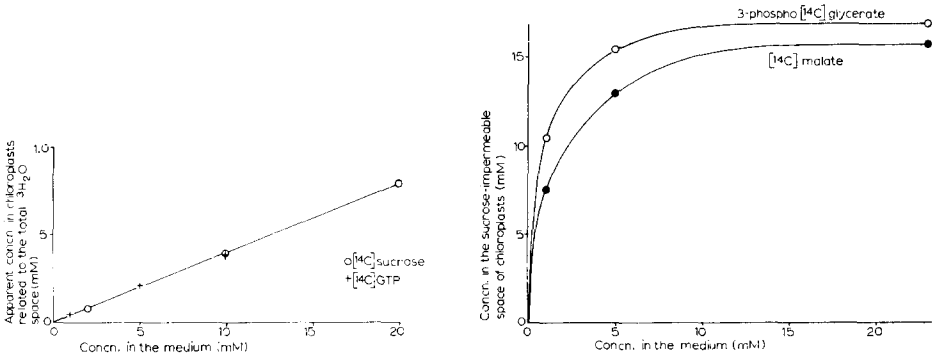

Fig. 1. Permeability for [^{14}C]sucrose and [^{14}C]GTP. Dependence of the apparent concentration in the chloroplasts on the concentration in the medium. Time of incubation, 2 min. Temp., 4°.

Fig. 2. Uptake of 3-phospho[^{14}C]glycerate and of [^{14}C]malate into the sucrose-impermeable space of chloroplasts, depending on the concentration in the medium. The time of incubation (2 min, at 4°) was long enough to measure the total uptake independent of the time. From the amount of [^{14}C]sucrose measured in a parallel experiment in the filtered chloroplasts, the amount of 3-phospho[^{14}C]glycerate or [^{14}C]malate adhering to the outer surface of the chloroplasts and present in the sucrose-permeable space is calculated and subtracted from the total amount of these substances assayed in the filtered chloroplasts. In this way the amount of these substances in the sucrose impermeable space is obtained. From the volume of the sucrose-impermeable space as measured with ^3H_2O the concentrations are calculated.

Subsequent measurements carried out as described above showed that the sucrose-permeable space of chloroplasts is permeable to all compounds of low molecular weight tested (AMP, ADP, ATP, GDP, inorganic phosphate, 3-phosphoglycerate, glucose 6-phosphate, fructose 6-phosphate, fructose 1,6-diphosphate, glucose, fructose, 6-phosphogluconate, malate, glutamate, aspartate, succinate, citrate, acetate).

The sucrose-impermeable space of chloroplasts is accesible to certain metabolites *e.g.* 3-phosphoglycerate and malate. The concentration dependence for this uptake is shown in Fig. 2. With low concentrations of 3-phosphoglycerate or malate in the medium, an accumulation of these compounds within the sucrose-impermeable space is observed. An increase of the concentration in the medium leads to a saturation of the uptake. This saturation curve for the specific uptake into the sucrose-impermeable space is very different from the linear concentration dependence of the unspecific permeation into the sucrose permeable space.

Besides this characteristic difference between the concentration dependence of the extent of metabolite uptake into the two spaces, there are also marked differences observed between the kinetics of uptake into those two spaces. In experiments not shown here, the unspecific permeation of sucrose and other metabolites into the sucrose permeable space was found to occur without any measurable time dependence at 4°. This suggests that there is a diffusion-limited permeation occurring faster than the resolution time of our experiment (5 sec). This is in contrast with the specific uptake of metabolites, *e.g.* 3-phosphoglycerate, into the sucrose-impermeable space, proceeding at 4° with a measurable time dependence[1].

In the experiment of Fig. 3 the size of the sucrose permeable and sucrose-impermeable spaces was measured when varying the tonicity of the medium. In hypertonic medium (0.66 M sorbitol) the H_2O space of the chloroplast is the smallest.

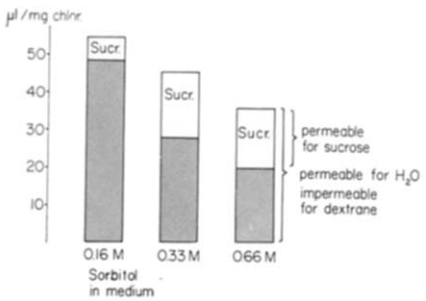

Fig. 3. Permeability of spinach chloroplasts for [^{14}C]sucrose depending on the tonicity of the medium.

Almost half of it is permeated by sucrose. With 0.33 M sorbitol the H_2O space is increased with only little change of the size of the sucrose permeable space. In hypotonic medium (0.16 M sorbitol) the H_2O space is increased furthermore, concurring with a decrease of the sucrose-permeable space. It appears that the osmotic swelling of the sucrose-impermeable space leads to a compression of the sucrose-permeable space.

It is concluded from the data discussed above that:

(a) Part of the chloroplast volume is accessible for diffusion-limited unspecific permeation by molecules of low molecular weight.

(b) Another part of the chloroplast volume, functionally defined as the sucrose-impermeable space, is accessible for carrier-mediated specific transport of certain

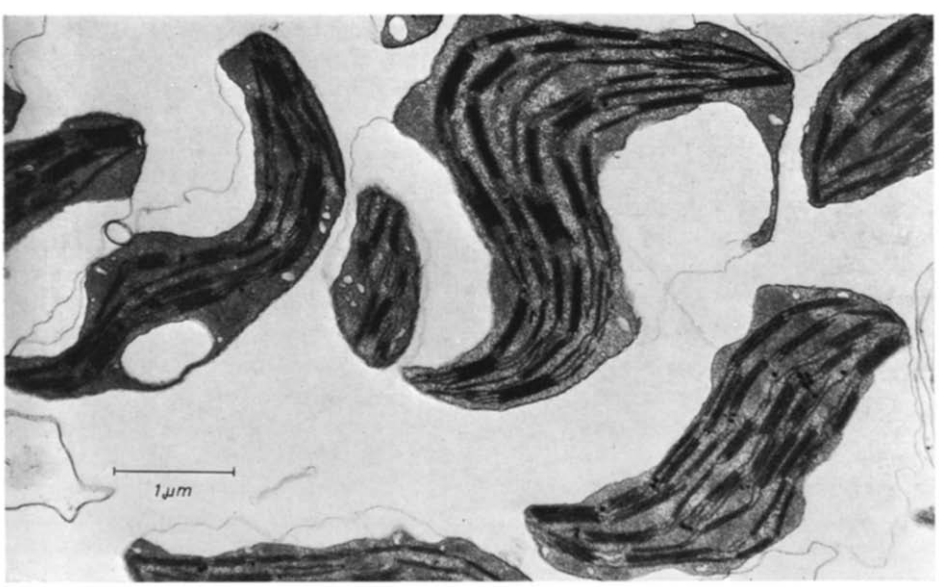

Fig. 4. Spinach chloroplasts in 0.66 M sorbitol medium.

metabolites. This compartment shows an osmotic response to variation of the tonicity of the medium.

Morphological studies

In order to correlate these findings with the morphology, the chloroplasts from the experiment in Fig. 3 suspended in different media were fixed in 0.5% glutaraldehyde and viewed through an electron microscope. Care was taken that the osmolarity of the fixing medium was the same as in the chloroplast suspension.

Fig. 4 shows a typical picture of chloroplasts in 0.66 M sorbitol. The stroma material gives a strong contrast. It appears to be packed along the grana, indicating extensive shrinkage of the stroma space. Part of the outer membrane is separated from the inner membrane, with large empty spaces between the two membranes. In 0.33 M sorbitol (Fig. 5) the stroma space is less condensed. There are again large empty spaces visible between the inner and outer membrane. In 0.16 M sorbitol (Fig. 6) the stroma space has a spherical shape, its size being larger and the stroma material showing less contrast than in 0.33 or 0.66 M sorbitol. The stroma space is highly swollen, the inner membrane coming into close contact with the outer membrane, leaving only little space between the two membranes. It may be noted that with the media of different tonicity there is no marked change of the size of the thylakoid spaces observed. In order to gain more quantitative information about these spaces, a number of micrographs obtained from the same preparation were evaluated by planimetry. The areas occupied by the empty spaces between the outer and the inner membrane are taken as a rough measure for the relative size of the intermembrane space (see ref. 12). The results of this estimation are summarized in Table I and are compared with the data of permeability measurements obtained from the experiment shown in Fig. 3.

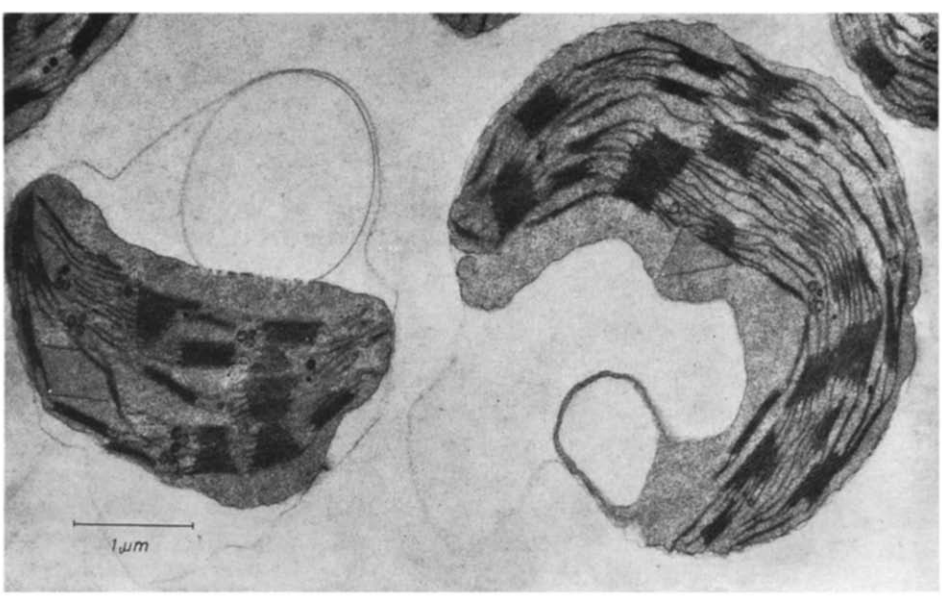

Fig. 5. Spinach chloroplasts in 0.33 M sorbitol medium.

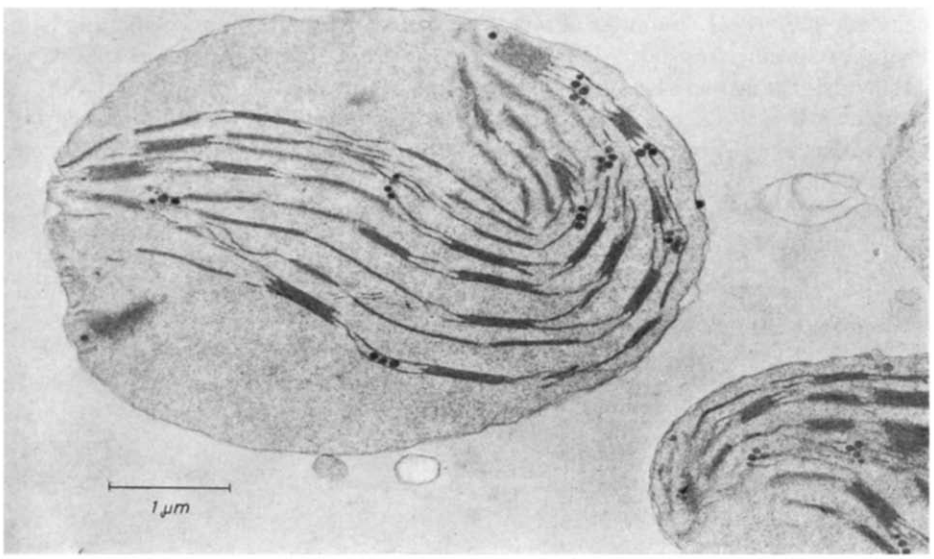

Fig. 6. Spinach chloroplasts in 0.16 M sorbitol medium.

TABLE I

ESTIMATION OF THE RELATIVE SIZES OF THE INTERMEMBRANE SPACES IN CHLOROPLASTS, AS OBTAINED BY PLANIMETRY OF ELECTRONMICROGRAPHS

Sorbitol in the medium (M)	*Area fraction of the section occupied by the intermembrane space related to the area occupied by the whole chloroplast (± S.D., n = number of sections)*		*Volume of the sucrose-permeable space related to the ^3H_2O space (data from Fig. 3)*
0.16	0.06 ± 0.04	$n = 19$	0.11
0.33	0.26 ± 0.10	$n = 21$	0.38
0.66	0.30 ± 0.07	$n = 20$	0.44

There is a fairly good agreement between the relative sizes of the intermembrane space and the stroma space (including the thylakoid space) as observed by electron-microscopy, and the sucrose-permeable and sucrose-impermeable spaces defined on functional terms. Methodical errors (*e.g.* partial rupture and shrinkage of the outer membrane occurring during the procedure of fixation and staining) may account for the differences between the sizes observed with both methods. It is concluded from these results that the sucrose-permeable space is identical with the intermembrane space. This implies that the outer membrane of chloroplasts is unspecifically permeable to sucrose and other molecules of similar size. Considering the generally accepted view that there is not direct communication between the intermembrane space and the thylakoid spaces, the inner membrane has to be regarded as the site of specific metabolite transport from the cytosol into the stroma or *vice versa*.

Essentially the same results as reported here have been obtained earlier with

mitochondria[13,14]. Also in mitochondria the outer membrane was found to be unspecifically permeable to solutes of low molecular weight, the inner membrane being the site for specific transport. It may be regarded as a minor difference that in the mitochondria the intracristae space is part of the intermembrane space, whereas in the chloroplasts the thylakoid space seems to be separated from the intermembrane space. It has been shown, however, that the thylakoids of chloroplasts are formed from invaginations of the inner membrane during development of the plastid (see ref. 2). Thus the thylakoid space in chloroplasts may be compared with the intracristae space of mitochondria (Fig. 7).

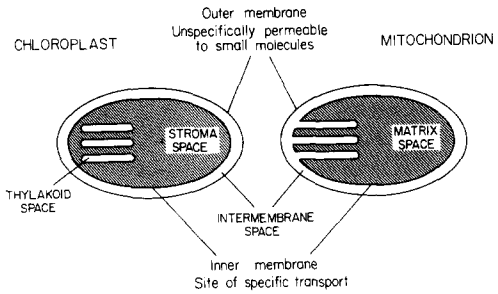

Fig. 7. Schematic diagram of the structure of chloroplasts and of mitochondria.

The great similarities between mitochondria and chloroplasts have been partially obscured due to semantics. Ion transport into mitochondria is generally understood as a transport from the intermembrane space across the inner membrane into the matrix space. The matrix is regarded as being inside the mitochondrion. For technical reasons ion transport with chloroplasts has been studied mainly with broken preparations, which have lost the envelope (see ref. 15). Ion uptake by these preparations involves a transport across the thylakoid membrane into the thylakoid space. This has often been referred to as a transport *into* the chloroplast. For the sake of clarity it may be advisable to look upon the stroma space, in parallel to the mitochondrial matrix space, as being *inside* the chloroplast. In this view a transport of substances into the thylakoid space may be regarded as a transport *out* of the chloroplast (stroma). The close relationship between mitochondria and chloroplasts (*e.g.* same direction of energy driven proton fluxes) may thus be visualized.

ACKNOWLEDGEMENTS

The authors thank Dr. A. D. Greenwood (London) and Prof. W. Menke (Cologne) for helpful discussions. The assistance of Miss Lynn Rapley and Miss Veronika Hilka is gratefully acknowledged. This research was supported by a grant from the Deutsche Forschungsgemeinschaft.

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