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Slow Passive Diffusion of Orthophosphate between Intact Isolated Chloroplasts and Suspending Medium[1]

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ABSTRACT

Isolated spinach chloroplasts purified by isopycnic centrifugation in density gradients of Percoll were found to be highly intact, to be devoid of extrachloroplastic contaminations, and to retain a high rate of CO_2-dependent O_2 evolution.

When suspended in a medium which avoided rupture of the envelope, intact purified chloroplasts progressively lost their phosphate content by passive diffusion. This led to a slow decrease in the uptake of labeled 3-phosphoglyceric acid or orthophosphate (Pi) and in the rate of CO_2-dependent O_2 evolution by isolated chloroplasts. Under these conditions, there was a good correlation between the rate of CO_2-dependent O_2 evolution and the concentration of Pi in the stroma space. Addition of Pi to the suspending medium at a final concentration of 10 millimolar, which counterpoised the slow efflux of Pi from the chloroplasts, slowed considerably the decrease in the rate of CO_2-dependent O_2 evolution.

It is well-established since the pioneering work of Heldt and Rapley (9) that there is a specific carrier situated in the inner membrane of the chloroplast envelope, named "phosphate translocator," which catalyzes a counter exchange of Pi^{2-}, PGA^{2-},[2] glyceraldehyde $phosphate^{2-}$, and dihydroxyacetone $phosphate^{2-}$. For each molecule entering the chloroplast, 1 molecule leaves the chloroplast, and vice versa. In this way, the total pool of Pi and phosphorylated compounds in the stroma space is kept constant.

In this paper we demonstrate that Pi alone effused slowly from intact isolated chloroplasts when suspended in a medium which avoids rupture of the two membranes of the envelope.

MATERIALS AND METHODS

Isolation of Chloroplasts. Young spinach (*Spinacia oleracea* L.) leaves (3 weeks old) were cut into small pieces directly into ice-cold chloroplast extraction medium [330 mM mannitol, 30 mM Mops-NaOH (pH 7.8), 2 mM EDTA and 0.15% BSA) with a tissue:medium volume ratio of 1:3.3. Leaves were homogenized three times for 2 s each with a Waring Blendor. The brei was filtered through eight layers of muslin (Ruby, Voiron, France) and intact chloroplasts (washed chloroplasts) were prepared as fast as possible according to the method of Nakatani and Barber (22). Chloroplasts thus obtained were purified by isopycnic centrifugation in a nontoxic silica sol (Percoll TM, Sigma) gradient which maintained isoosmotic conditions throughout the isolation procedure (20, 23, 25). Thirty-four ml Percoll medium [50% Percoll, 330 mM mannitol, 50 mM Mops-NaOH (pH 7.8), 2 mM EDTA, and 0.15% BSA)] were pipetted into each of two centrifuge tubes. The tubes were placed in a precooled Sorvall SS 90 vertical rotor and centrifuged (at 3 C) at 10,500 rpm (10,000*g*) for 100 min. At the conclusion of this step, a continuous gradient of Percoll was obtained in each tube. Aliquots (2 ml sample, 4 to 6 mg Chl) of the crude chloroplast suspension then were layered on the linear Percoll gradients. The tubes were centrifuged for 10 min at 5,000*g* (at 3 C) in the Sorvall SS 90 rotor. Intact chloroplasts were recovered as a broad band near the bottom of the tube, and stripped chloroplasts + extrachloroplastic membrane systems formed a band at the sample-gradient interphase (Fig. 1). The intact purified chloroplasts were diluted with extraction medium (extraction medium:chloroplast suspension, 10:1) and recovered as a pellet after centrifugation (3,500*g* or 90 s at 3 C, SS 34 rotor, Sorvall). The supernatant was removed by aspiration and the pellet of purified chloroplasts was gently resuspended in medium (suspending medium) containing 330 mM mannitol, 50 mM Mops-NaOH (pH 7.8), and 0.15% BSA; final Chl concentration was 7 to 8 mg ml^{-1}. The entire isolation procedure could be accomplished in less than 25 min. At this point in the procedure, the chloroplast suspension was stored in an ice bath under dark conditions. At various times, aliquots were taken for the measurement of CO_2-dependent O_2 evolution rates, per cent intactness as determined by ferricyanide reduction, uptake of [^{32}]Pi or [^{14}C]PGA as determined by silicone oil centrifugal filtration, and Pi content of the stroma space.

Photosynthesis Assay. O_2 evolution was followed polarographically at 25 C using a Clark-type electrode system (3) purchased from Hansatech Ltd, Hardwick, Industrial Estate, Kings Lynn, Norfolk. The reaction medium contained, in a total volume of 1 ml: 330 mM mannitol, 50 mM Mops-NaOH (pH 7.8), 2 mM EDTA, 5 mM $NaHCO_3$, 100 μM Na_2HPO_4, and chloroplasts equivalent to 70 to 80 μg Chl. The reaction medium (electrode medium) was gassed with argon before the addition of chloroplasts and equilibrated with the O_2 electrode at 25 C. The light was provided by a 150-w xenon arc lamp source (Oriel Corporation) giving an irradiance of 1,300 w m^{-2} at the surface of the vessel.

Chloroplast Intactness. This was determined by ferricyanide-dependent O_2 evolution in the presence of 2 mM NH_4Cl before and after an osmotic shock (8, 17), and under reduced irradiance (700 w m^{-2}).

Silicone Layer Filtering Centrifugation. The uptake of PGA or Pi by intact purified chloroplasts measured at 25 C was initiated by adding 10 μl chloroplast suspension (70 to 80 μg Chl) to 200

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[2] Abbreviations: PGA, 3-phosphoglyceric acid; Mops, 3-(*N*-morpholino)propanesulfonic acid.

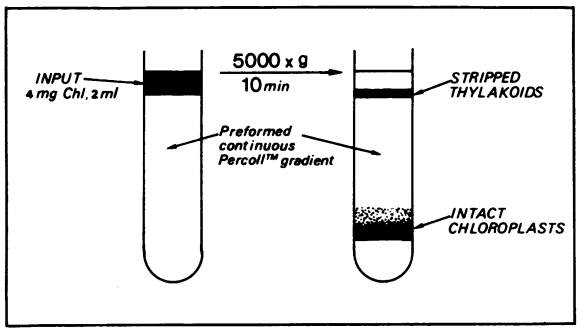

FIG. 1. Purification of isolated chloroplasts. Schematic representation of the Percoll gradient fractionation of the chloroplast preparation to yield intact chloroplasts and stripped thylakoids; the rotor used is a vertical rotor (SS 90, Sorvall).

μl electrode medium containing either [U-^{14}C]PGA (1 mM, 50 MBq mmol^{-1}), ^{32}Pi (mM, ≃ 37 MBq mmol^{-1}), [1-^{14}C]mannitol (250 kBq mmol^{-1}), or $^{3}H_2O$ (167 kBq ml^{-1}) in a 400-μl capacity polypropylene microtube. The uptake was stopped after 2 min by rapid centrifugation (Beckman, Microfuge B, 20 s, 10,000*g*) of the chloroplasts through a layer of silicone oil (70 μl Versilube F 50, General Electric) into 20 μl 10% $HClO_4$. The top and bottom layers then were separated by cutting the microtubes at the middle of the silicone layer. For details on the filtering centrifugation technique and on the evaluation of the uptake into the mannitol impermeable space, which is the space surrounded by the inner membrane of the chloroplast envelope, see Heldt and Sauer (10).

Pi Determination. An aliquot (50-μl; 350 to 400 μg Chl) of the chloroplast suspension was suspended in 5 ml O_2 electrode medium. The tube was centrifuged at 3 C for 1 min, at 1,500*g* (Sorvall SM 24 rotor). The supernatant was removed by aspiration and the pellet was resuspended in a low-osmolarity medium [10 mM Mes (pH 6), 2 mM EDTA, final volume, 1.1 ml]. One hundred μl of this suspension were used for Chl determination. The rest of the suspension was centrifuged 10 min at 7,000*g* (Sorvall SM 24 rotor) after addition of 200 μl 20% trichloroacetic acid. A fraction of the clear supernatant (1 ml) devoid of mannitol, which interferes with phosphomolybdate complex formation (11), was used for the colorimetric determination of Pi after isobutanol extraction according to the method of Martin and Doty (19) by Yanagita (28).

Enzymes and Other Assays. Catalase (H_2O_2:H_2O_2 oxidoreductase, EC 1.11.1.6) was assayed (26) followed by nitrate reductase (EC 1.6.6.1) (7), glycolate oxidase (glycolate:O_2 oxidoreductase, EC 1.1.3.1) (4), and fumarase (fumarate hydratase, EC 4.2.1.2) (24). Total protein was determined by the Folin-Ciolcalteu phenol reagent (18). Chl was extracted in 80% acetone (v/v) and measured (according to ref. 12).

RESULTS

In good agreement with Takabe *et al.* (25) and Mills and Joy (20), chloroplasts purified in Percoll showed markedly higher rates of CO_2-dependent O_2 evolution than did unpurified chloroplasts (20 to 40% higher) (Table I). We have observed that, upon illumination, the extension of time which elapsed (lag phase or induction period; see ref. 27) before attaining the maximum rate of photosynthesis was considerably shortened in the case of Percoll-purified chloroplasts. Estimation of the marker enzymes nitrate reductase, fumarase, catalase, and glycolate oxidase showed that sedimentation through a continuous Percoll gradient completely eliminates cytoplasmic, mitochondrial, and peroxisomal contamination in chloroplast pellets (Table I). The form of contamination depicted by Albertsson and Larsson (1) and Larsson *et al.* (14) in which individual chloroplasts are surrounded by a jacket of cytoplasm containing various cell organelles was excluded in the purified preparation. In addition, the rate of ferricyanide-dependent O_2 evolution was 0.5 to 2% of that recorded for osmotically shocked chloroplasts, giving an apparent percentage of intactness of 98 to 99.5. Finally, we found an average yield of 16 ± 1 g protein/g Chl for purified chloroplasts. This value is close to the theoretical average yield of 18.4 g protein/g Chl for 100% intact chloroplasts calculated by Lilley *et al.* (17).

Table I. *Percentage Intactness, Photosynthetic Capacity, and Distribution of Activity of Marker Enzymes in Crude Chloroplast Preparation and Chloroplasts Purified by Percoll Gradients*

Chloroplast	Intactness[a]	CO_2-dependent O_2 Evolution	Catalase	Glycollate Oxidase	Fumarase	Nitrate Reductase
	%	μmol O_2 mg^{-1}Chl h^{-1}	μmol mg^{-1} Chl h^{-1}			
Crude	78	76	82	0.2	12	Trace
Purified	98	108	n.d.[b]	n.d.	n.d.	n.d.

[a] Measured by ferricyanide-dependent O_2 evolution.
[b] n.d., not detectable.

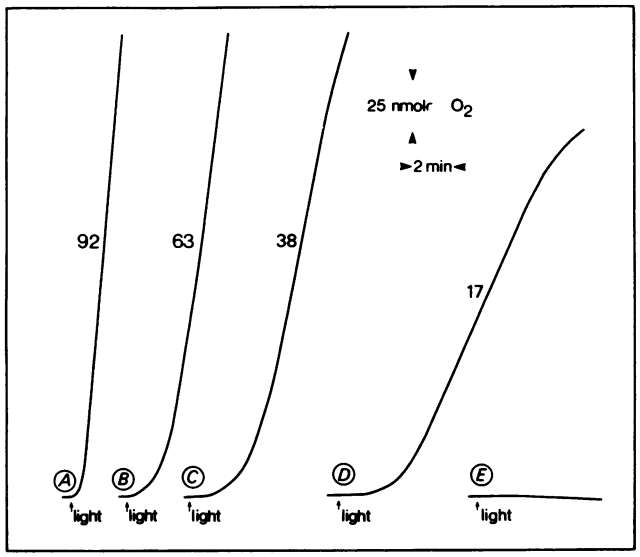

FIG. 2. Rates of CO_2-dependent O_2 evolution during aging of purified chloroplasts. A, 10 min after isolation; B, +10 h; C, +20 h; D, +30 h; E, +40 h. The suspending medium, from which Pi was omitted, and electrode medium are described in the text. For each time, the percentages of chloroplast intactness were: A, 98%; B, 96%; C, 93%; D, 91%; and E, 88%. The numbers along the traces indicate the rate of photosynthesis (in μmol O_2 mg^{-1} Chl h^{-1}). Note the lengthening of lag phase (or induction period; see ref. 27) and the slow decline of CO_2-dependent O_2 evolution during chloroplast aging.

Photosynthetic capacity of purified chloroplasts was made at intervals from 0.2 to 40 h after isolation (Fig. 2). We observed, with time, a slow decline in the rate of CO_2-dependent O_2 evolution which was accompanied by an increase of the induction period. In addition, during chloroplast aging *in vitro*, the final rate of photosynthesis was more and more sensitive to further additions of Pi in the electrode medium (at time zero, optimum Pi is 0.25 mM; at 20 h, it is 0.05 mM). Forty h after their isolation, chloroplasts could no longer evolve O_2 in the presence of CO_2. However, we have observed that the decline in the rates of CO_2-dependent O_2 evolution is less and less rapid as the ratio of chloroplast volume

to suspending medium increases, and vice versa (results not shown).

It is possible that the progressive loss of photosynthetic capacity of purified chloroplasts during their aging *in vitro* could be entirely attributable to a progressive loss of envelope membrane with a concomitant release of stroma material into the suspending medium. However, this hypothesis is most unlikely in the case of purified chloroplasts because, 24 and 90 h after their isolation, the apparent percentages of intact chloroplasts (as measured by ferricyanide-dependent O_2 evolution procedure) present in the suspending medium were 95 and 80% respectively.

It is interesting to note that unpurified chloroplasts were more fragile than Percoll-purified chloroplasts when maintained in the suspending medium. In this case, envelope membranes proved to be particularly more labile. In fact, we have shown that every crude preparation of intact chloroplasts was more or less contaminated by soluble vacuolar hydrolases, such as proteases or lipolytic acid hydrolases (6), which slowly disintegrate envelope membranes during chloroplast survival *in vitro*. In marked contrast, extrachloroplastic hydrolases were totally absent in Percoll-purified suspensions (results not shown).

These observations suggest that, once isolated, purified chloroplasts loose progressively one or several critical metabolites in the suspending medium. This simple hypothesis is reinforced by the fact that concentrated chloroplast suspensions maintained higher rates of photosynthesis when compared to dilute suspensions. In fact, substances lost from isolated chloroplasts will not accumulate in a large volume medium very rapidly: the medium acts as a buffer volume. As chloroplasts maintain a sufficiently large internal Pi pool both to satisfy the requirement of phosphorylations and to maintain sugar phosphate pools for photosynthesis (2, 16), it was therefore necessary to measure levels of phosphate inside the chloroplasts during their aging *in vitro*. The concentration of phosphate found in the stroma of freshly prepared chloroplasts was 11 ± 2 mM. Values between 4 and 14 mM have been reported (13, 16). Figure 3 shows that, with time, Pi concentration in isolated purified chloroplasts decreased steadily. This was confirmed by the fact that, with time, the accumulation of labeled PGA or Pi in the chloroplast stroma, measured by silicone layer-filtering centrifugation (Fig. 3), decreased also progressively (the uptake of PGA or Pi proceeds by a counter exchange with internal Pi + PGA, dihydroxyacetone phosphate). With ^{32}Pi-loaded chloroplasts, we have verified that the Pi lost from the chloroplasts appears in the suspension medium (unpublished data). In addition, there was an excellent correlation between the rate of CO_2-dependent O_2 evolution and the concentration of Pi in the stroma space (Fig. 3).

As the slow leakage of Pi was not attributable to a progressive rupture of chloroplast envelope, it would be interesting to see if addition of Pi to the suspending medium at a final concentration which counterpoised the internal Pi concentration would prevent the slow decrease in the rate of CO_2-dependent O_2 evolution during isolated chloroplast aging. The stabilizing properties of the Pi added in the suspending medium on isolated purified chloroplasts is demonstrated in Figure 4. Thus, after 30 h aging, the chloroplasts, maintained in a suspending medium containing 10 mM Pi, at a final concentration of 7 to 8 mg Chl ml^{-1}, lost only about 20% of their activity, against 80% in suspending medium devoid of Pi. Furthermore, under these conditions, Pi concentration in the stroma space is kept constant (Figs. 4 and 5).

DISCUSSION

The results presented here confirm that chloroplasts purified by isopycnic centrifugation in density gradients of modified silica (Percoll) are highly intact, retain a high rate of CO_2-dependent O_2 evolution, and have practically no contamination by mitochondria, peroxisomes, and chloroplast fragments (21).

These results strongly suggest that Pi effluxes from intact iso-

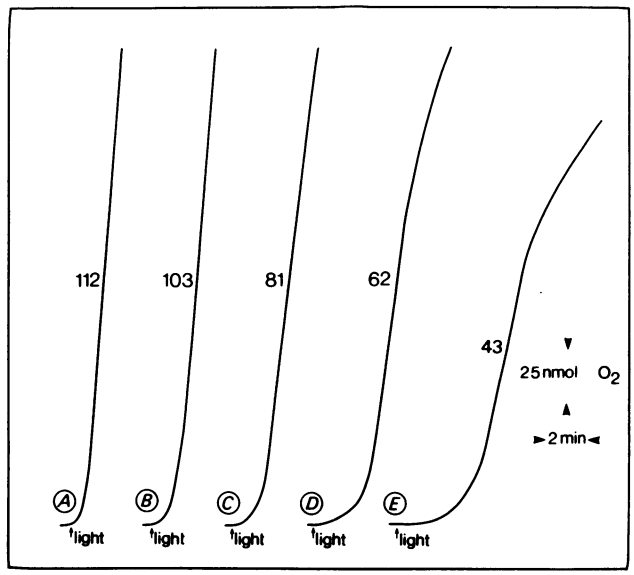

FIG. 4. Rates of CO_2-dependent O_2 evolution during aging of purified chloroplasts maintained in a suspending medium containing 10 mM Pi. A, 10 min after isolation; B, +20 h; C, +40 h; D, +60 h; E, +80 h. The suspending medium, which contains 10 mM Pi, and the electrode medium are described in the text. For each time, the percentage of chloroplast intactness were: A, 98%; B, 94%; C, 90%; D, 87%; and D, 83%. The numbers along the traces indicate the rate of photosynthesis (in μmol O_2 mg^{-1} Chl h^{-1}). Note the very low decline of CO_2-dependent O_2 evolution when the suspending medium contains 10 mM Pi.

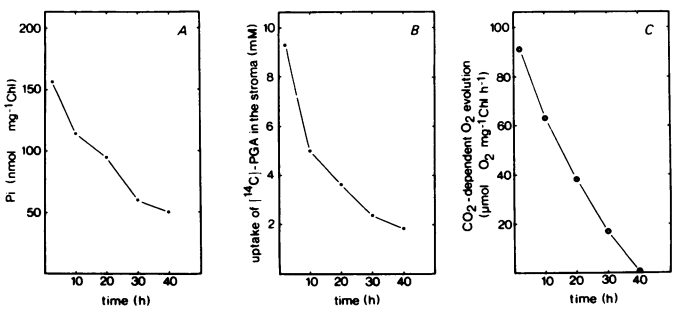

FIG. 3. Evolution of Pi content of the chloroplasts (A), uptake of labeled PGA in the stroma (B), and CO_2-dependent O_2 evolution rates (C) during aging of purified chloroplasts. Note the tight correlation between rates of photosynthesis and Pi concentration in the stroma.

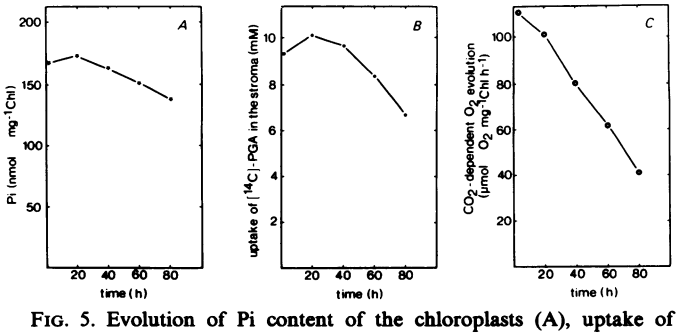

FIG. 5. Evolution of Pi content of the chloroplasts (A), uptake of labeled PGA in the stroma space (B), and CO_2-dependent O_2 evolution (C) during aging of chloroplasts maintained in a suspending medium containing 10 mM Pi. Note that the time scale is different from that of Figure 3.

lated chloroplasts maintained in a medium which avoids rupture of the two membranes of the envelope. The arguments in favor of this passive diffusion are numerous.

(*a*) The decrease in the rate of photosynthesis during aging of isolated chloroplasts is not attributable to a progressive rupture of envelope membranes. (*b*) The concentration of Pi in the chloroplast stroma decreases progressively during aging of chloroplasts. This, in turn, leads to a slow decrease in the uptake of labeled PGA or Pi by isolated chloroplasts. (*c*) Addition of Pi to the suspending medium at a final concentration of 10 mM, which counterpoises internal Pi concentration, prevents the slow leakage of Pi from the chloroplasts and slows down considerably the decrease observed in the rate of CO_2-dependent O_2 evolution. This result confirms the fact that the rate of photosynthesis is strongly dependent on the Pi concentration in the stroma space (2).

From Figure 3, it is clear that the rate of passive diffusion of Pi out of the chloroplasts is extremely low (*i.e.* 5 to 7 nmol mg^{-1} Chl h^{-1} during the first 10 h aging). However, this slow rate is sufficient to empty the chloroplast of its Pi content in 30 to 40 h. This tendency is more marked as the ratio of suspending medium to chloroplast volume increases. This slow rate of passive diffusion is considerably lower than the V_{max} of uptake of Pi catalyzed by the phosphate translocator and which was found to be in the range of 25 to 30 μmol mg^{-1} Chl h^{-1} at 4 C (5). It is possible that the passage of Pi through the inner membrane of the envelope by passive diffusion occurs either through the Pi translocator or directly through the membrane lipid bilayer. The later hypothesis, however, is most unlikely because the passage of ionic or polar molecules through the hydrophobic core of the membrane is thermodynamically a highly unfavorable process. The same thing can be true for some other small anions, such as dicarboxylates (15).

Finally, the data reported here will be helpful for scientists involved in chloroplast survival *in vitro* or in proteins, nucleic acids, pigments, and lipid synthesis. It is obvious that the physiological integrity of isolated chloroplasts can be maintained at least during 3 days if two conditions are fulfilled. (*a*) The suspending medium must be devoid of various hydrolases, such as proteases, lipolytic acid hydrolases, etc., capable of hydrolyzing the chemical constituents of envelope membranes during aging. To this end, chloroplasts must be purified by centrifugation in gradients of silica. (*b*) The suspending medium must prevent the slow leakage of critical metabolites from intact isolated chloroplasts.

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