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Cold Storage of Isolated Class C Chloroplasts

OPTIMAL CONDITIONS FOR STABILIZATION OF PHOTOSYNTHETIC ACTIVITIES

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ABSTRACT

Preservation of photosynthetic activities (photophosphorylation, electron transport, fluorescence induction, 0.3-second delayed light emission) of isolated broken (class C) chloroplasts by low temperature storage was investigated under a wide range of conditions in order to optimize long time activity retention.

The more labile functions (photophosphorylation and electron transport) required very low temperatures (below −79 C) and relatively high (above 20%, v/v) concentrations of cryoprotectives for satisfactory stabilization. Fluorescence induction and delayed light emission were less sensitive, especially during the 1st month of storage.

Taking into account the effect of cryoprotectives on absolute activities prior to freezing, optimum activity retention was observed with a medium containing ethylene glycol (30%, v/v) and a storage temperature of −100 C or below. In this case, given fast thawing and high chloroplast concentration, practically 100% preservation of all of the photosynthetic activities investigated was obtained for at least 10 months, even with very simple freezing and storage procedures.

The same optimal medium at somewhat higher temperatures (−79 C and to a lesser extent at −41 C) caused a dramatic uncoupling effect: photophosphorylation was inhibited in a few hours, while electron transport increased 3- to 5-fold. The enhanced electron transport was stable for almost a month and then declined sharply. This uncoupling effect was specific only to ethylene glycol.

Isolated broken chloroplasts from higher plants rapidly lose their photosynthetic activities when stored in the usual preparation medium (10, 13, 22, 23) even at low temperatures (11, 22). The most successful method for stabilizing activities has been a storage at low temperatures (−20 C and below), using laborious procedures for rapid freezing and thawing (7) and special protective mixtures (13, 17), the favored cryoprotective being glycerol (2, 4, 19, 23). We felt that there is little systematic information (2, 13, 19, 23) to yield conclusions reaching beyond the limits of a particular case. The randomness of the data makes it difficult to compare various reports, since several important parameters differed simultaneously (*e.g.* source and preparation of chloroplasts, freezing and thawing velocities, assays, etc.).

The purpose of this research was to design a simple method for optimal storage, by comparing systematically a number of photosynthetic activities in a wide range of conditions. Since photophosphorylation is the most demanding in terms of structural integrity, this activity was used as the main indicator of preservation, retention of the other activities being usually equal or better, according to the conditions.

MATERIALS AND METHODS

Class C (9) chloroplasts were prepared from greenhouse-grown tobacco (*Nicotiana tabacum*, var. Xanthi) and market lettuce (*Lactuca sativa*, var. romaine) essentially as described by Avron (3). Chl concentrations were determined according to Arnon (1). Immediately after preparation chloroplasts were resuspended (at 0 C) in aqueous mixtures containing usually a certain amount of cryoprotective (0, 10, 20, 30, 40, or 50% [v/v] glycerol, DMSO,[1] or ethylene glycol), 20 mM sucrose, 20 mM Tris-HCl buffer (pH 7.8), and 125 mM KCl, at final concentrations corresponding to 0.6 to 5.0 mg Chl/ml. In certain experiments, some of the secondary components were changed (*e.g.* 60, 110 mM sucrose, 25, 50 mM KCl, NaCl instead of KCl, Tricine instead of Tris) or supplemented (*e.g.* by 10 mg/ml BSA). Aliquots of 0.3 to 1.0 ml were placed in thin walled screw-cap plastic vials and brought to the various final storage temperatures by alternatively placing them into crushed ice (0 C), a freezing chamber (−20 C), a cooled ethanol bath (−41 C), a container with dry ice (−79 C), or a Dewar with liquid N_2, above (−100 C) or immersed in (−196 C) the liquid. Thawing occurred by either transfer to a 4 C bath, dipping in a 25 C bath, a 35 C bath or continuous heating by hand.

All assays were carried out at 25 C, in a total volume of 2.0 ml at an initial pH of 7.8, and a chloroplast concentration equivalent to 25 μg Chl/ml. Photophosphorylation was assayed by the pH change method (see 14), and ATP formation was calculated according to Lilley and Walker (14). The reaction mixture contained 30 mM KCl, 5 mM $MgCl_2$, 0.5 mM ADP, 1.3 mM Pi. These were supplemented with 0.025 mM PMS (for cyclic photophosphorylation) or 1 mM diquat and 1.5 mM Na azide (for noncyclic photophosphorylation). Proton uptake ability was measured in the same medium, with no ADP and Pi added. Incandescent illumination, provided from a slide projector, was passed through a water filter and a Corning 3-66 (>540 nm) cut-off filter and had an intensity of about 0.7 $\mu E/cm^2 \cdot s$ between 540 and 700 nm. The pH change was measured with a Radiometer Copenhagen PHM 64 Research pH meter, connected to a Houston Instrument Omniscribe recorder.

Electron transport was assayed by measuring the rate of O_2 evolution (with 2 mM FeCy) or uptake (with 1 mM diquat and 1.5 mM Na azide). The reaction mixture also contained 50 mM NaCl and 20 mM Tris-HCl. The O_2 electrode used was a Clark type (Yellow Springs-Ohio or Rank Bros-Bottnisham, U.K.). Signal recording, chloroplast concentrations, and illumination were the same as for photophosphorylation.

Fluorescence induction was measured as previously described (21); the symbols used are those given in reference 16. The quantity $(F_\infty - F_0)/F_\infty$ is considered to be an indicator of the quantum yield of Q reduction, under certain conditions (16). Delayed luminescence at 300 ms was measured as before (15).

The chemicals used were of the highest degree of purity commercially available. All of the data presented are average values of at least two separate determinations.

[1] Abbreviations: DMSO: dimethylsulfoxide; FeCy: (potassium) ferricyanide; PMS: phenazine methosulfate; DCCD: *N,N'*-dicyclohexylcarbodiimide.

RESULTS AND DISCUSSION

EFFECTS OF CRYOPROTECTIVES ON THE ABSOLUTE (INITIAL) ACTIVITIES

Preliminary experiments indicated that a reasonable degree of protection of the photosynthetic activities is obtainable for all three cryoprotectives used (glycerol, DMSO, and ethylene glycol). Quantitative differences were apparent in the control activity prior to freezing and storage. Suspension of freshly prepared chloroplasts in the protective media, followed by the assay of photosynthetic activities (by dilution of 5–50 × 10^{-3} ml of the stock in 2 ml reaction mixture) revealed some remarkable changes in activities (Figs. 1 and 2). Photophosphorylation was only insignificantly affected by increasing concentrations of ethylene glycol (Fig. 1). Glycerol, and to lesser extent also DMSO, caused a considerable decrease of photophosphorylation as their concentration varied from 10 to 50%. Electron transport was affected in a more complicated way (Fig. 2). With glycerol it decayed monotonically *versus* concentration. With DMSO there was a phase of increased activity after a phase of drop in activity which could be due to uncoupling effects. The noticeable drop of the ferricyanide reduction activity as ethylene glycol concentration increased above 30% was noted also by Inoue and Nishimura (12). In concentrations up to 30% ethylene glycol by itself affected the activity in a marginal way. The effect of the cryoprotectives on fluorescence and delayed luminescence was limited and practically concentration-independent.

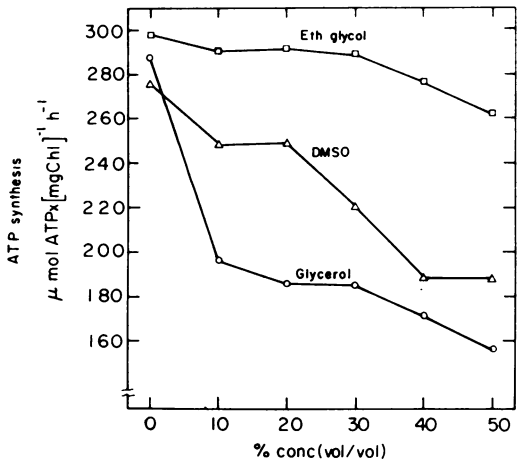

FIG. 1. Initial cyclic photophosphorylation (+PMS) as a function of cryoprotective concentration in the storage medium. Chloroplast concentrations for storage were equivalent to 3.60 to 4.22 mg Chl/ml (this small variation had no detectable effect on activity retention).

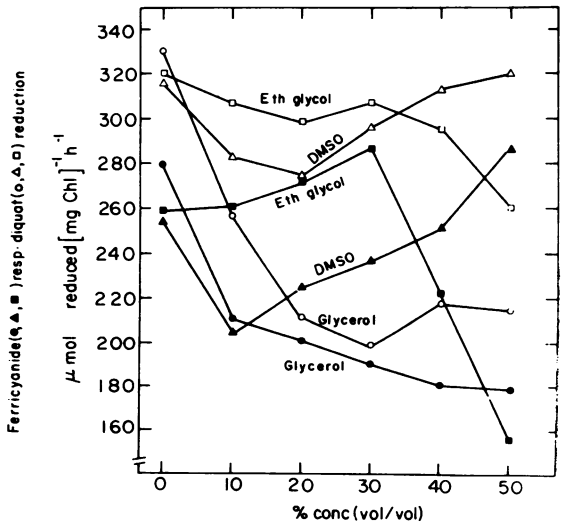

FIG. 2. Initial photosynthetic electron transport as a function of cryoprotective concentration in the storage medium. Chloroplast concentrations for storage were the same as for Figure 1. (○, ●): Glycerol; (△, ▲): DMSO; (□, ■): ethylene glycol. Tobacco chloroplasts.

STABILIZATION OF PHOTOSYNTHETIC ACTIVITIES AS A FUNCTION OF CRYOPROTECTIVE CONCENTRATION, STORAGE TIME AND TEMPERATURE

Assays for all temperatures and protective media were performed after storage times ranging from minutes to months, in order to define optimum chloroplast preservation.

Cryoprotective Type and Concentration. A characteristic set of results is given in Figure 3, for the absolute rate of cyclic photophosphorylation (after 10 days of storage), as a function of the cryoprotective concentration. A reasonable protection was achieved even at relatively low concentration (*e.g.* 10% ethylene glycol for storage at −196 C). Activity retention decayed somewhat at the highest concentrations used, the optimal concentration depending on the storage temperature and cryoprotective activity. Activity retention with ethylene glycol was far above that with the other cryoprotectives, reflecting in part the medium effect *per se* (without storage) as also depicted in Figure 1. The over-all optimum is obtained for 30% (v/v) ethylene glycol at −196 C, when practically 100% activity retention was achieved. Similar results were obtained for different storage times. The other functions assayed behaved similarly, but the optimal points were less sharply defined due to the interference of uncoupling effects (and enhancement of electron transport) or to the lesser sensitivity to damage. Ethylene glycol (30%, v/v) at −100 C and below remained nevertheless the best choice for all activities (*cf.* below).

Storage Time and Temperature. Figure 4 demonstrates the dependence on temperature and storage time for all of the activities tested, for the optimal preserving medium: ethylene glycol at 30% (v/v). Figure 5 gives the same data for the corresponding—in terms of molarity—concentration of glycerol. Data for DMSO were also collected but are not shown as they are similar to those for glycerol. Generally the stabilization improved as the temperature decreased. An exception to the above was the preservation of phosphorylation in ethylene glycol which behaved in a more complex way. It was rapidly lost at −79 C while relatively better preserved at −20 C (Fig. 4). This seemingly strange behavior is due to a specific effect (*cf.* later). At 0 C all activities were rapidly lost; −20 C provided already considerable protection, phosphorylation decaying with a half-time of about 6 days, other activities decaying much slower; −79 C was better for protection (except for phosphorylation in ethylene glycol), but only below −100 C was there preservation of all activities for a period of at least 10 months. In all of the other cryoprotectives, except ethylene glycol, there was an initial phase of drop of electron transport and phosphorylation activity for storage even at the lowest temperatures, which was finally stabilized at about 60 to 80% of the initial activity after storage. The temperature of −100 C used for storage in ethylene glycol was as efficient as −196 C for preserving activities, the results having a better reproducibility at −100 C possibly due to avoiding the penetration of liquid N_2 into the storage vials.

The functions assayed exhibited different degrees of sensitivity to low temperature storage, the order being approximately photophosphorylation–electron transport–delayed luminescence–fluorescence, in concordance with their respective structural requirements and in agreement with earlier reports (2, 10, 19, 23). Thus, in 26 days at −20 C cyclic phosphorylation was almost completely lost, while electron transport decayed to 40 to 60% of its initial value. Delayed light and fluorescence usually changed very little at −20 C (less than 20%) in the first 20 to 30 days of storage, and only after a few months an important decrease began to appear (but still little change is detectable for lower temperatures). This

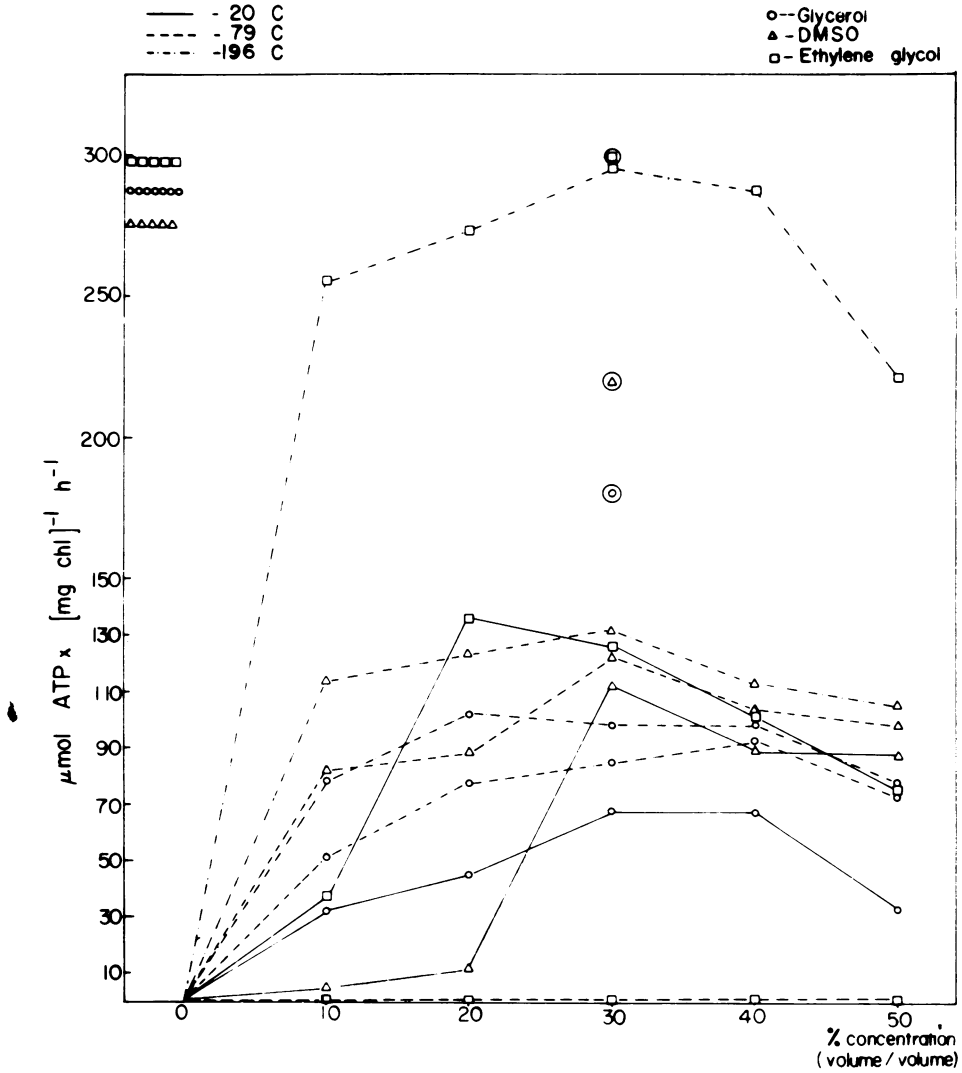

FIG. 3. Cyclic photophosphorylation (+PMS) assayed after 10 days of storage of tobacco chloroplasts. Symbols in circles (◎, Δ, □) represent rates of photophosphorylation of the same chloroplasts stored at −196 C in glycerol, DMSO, or ethylene glycol, in presence of 200 mM sucrose instead of the usual 20 mM. Series of symbols at the upper left side (○○, ΔΔ, □□) represent initial absolute rates, in the buffer, with no cryoprotective added, for three sets of experiments. Chloroplast concentrations during storage were the same as for Figure 1.

long term change might be due to a slow disorganization of the pigment arrays.

OTHER FACTORS

Freezing and Thawing Rates. As already stated, freezing and thawing procedures were as simple as possible, the initial rates being higher the lower the final storage temperatures. Usually, for −20 C storage there was an initial fast drop in activity, which later slowed down (Figs. 4 and 5). This could be due to the effect of freezing and thawing. Such effect was minimal or nonobservable for storage at lower temperature. We found repeatedly that freezing to −20 C and immediate thawing induces a 15 to 30% decrease in photophosphorylation (depending on cryoprotective concentration), whereas a similar freeze-thaw cycle to −196 C only marginally affected photophosphorylation (on the average by 5%). These differences were probably due to the differences in the initial rate of freezing (being higher for cooling to −196 C). For assessing the effect of thawing we utilized four different thawing rates, and although differences were again small (up to 24%), we constantly found that the higher the rates, the better the activities that were obtained. These results are consistent with earlier reports of higher freezing and thawing rates being more favorable for damage prevention in chloroplast suspensions (*e.g.* ref. 7), unlike for plant cells, algae, and other biological systems (see ref. 17).

Chloroplast Concentrations. For storage at −196 C, activity retention was unchanged for chloroplast concentrations corresponding to 1.0 to 4.2 mg Chl/ml. Concentrations below 1.0 (0.6) yielded poorer results by up to 27% in agreement with earlier reports (23).

Other Components of Storage Medium. The standard storage mixture contained Tris, KCl, and sucrose at low concentration. For the most optimal conditions for storage (*i.e.* with 30% ethylene glycol at temperature below −100 C) we did not find any further improvement by additives generally considered beneficial for activity retention as the following examples show:

BSA (10 mg/ml) was an efficient protective for short storage times at 0 C (13, 23) and even superior to the cryoprotectives used here. At −20 C it became less effective and at very low temperatures it did not afford any protection (data not shown). When used concomitantly with the cryoprotectives it gave no improvement on low temperature activity preservation. These findings are consistent with most of the data in the literature (13, 23) and with albumin's supposed mechanism of protection, by binding of unsaturated fatty acids released endogenously especially during isolation and aging at 0 C.

Sucrose, used as an effective cryoprotective with special procedures (7) or for relatively short times (8, 10), was usually present in our experiments in a concentration of 20 mM which by itself

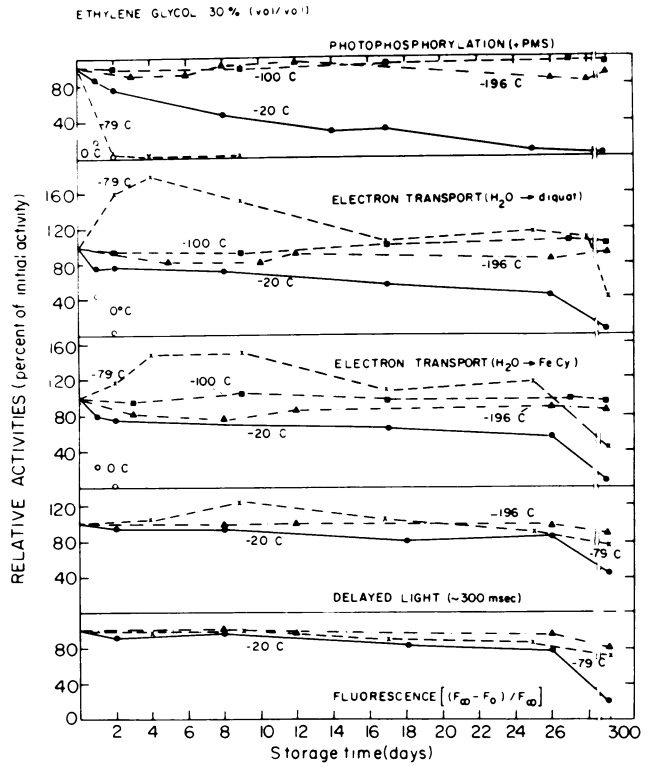

FIG. 4. Effect of storage time and temperature in 30% (v/v) ethylene glycol on the photosynthetic activities. Abscissa: storage time, in days; ordinate: per cent photosynthetic activities, relative to the initial activities (*i.e.* after addition of cryoprotective and before freezing). Absolute values for photophosphorylation and electron transport are as in Figures 1 and 2. Symbols used for storage temperatures: (○····○): 0 C; (●——●): −20 C; (×– – –×): −79 C; (■– –■): −100 C; (▲·–·–·▲): −196 C. Tobacco chloroplasts. Chloroplast concentration in the storage medium was equivalent to 4.20 mg Chl/ml.

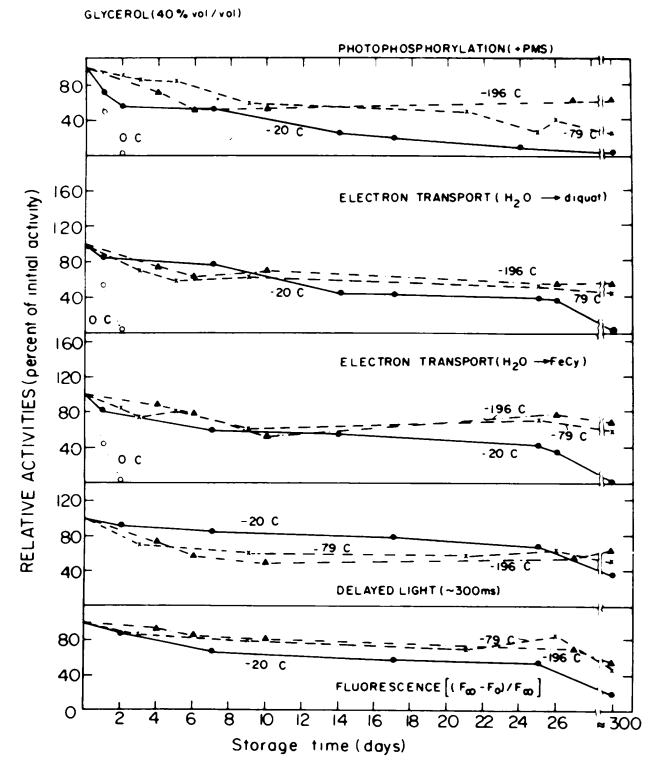

FIG. 5. Same as Figure 4, for storage in glycerol 40% (v/v). Chloroplast concentration in the storage medium was equivalent to 4.05 mg Chl/ml.

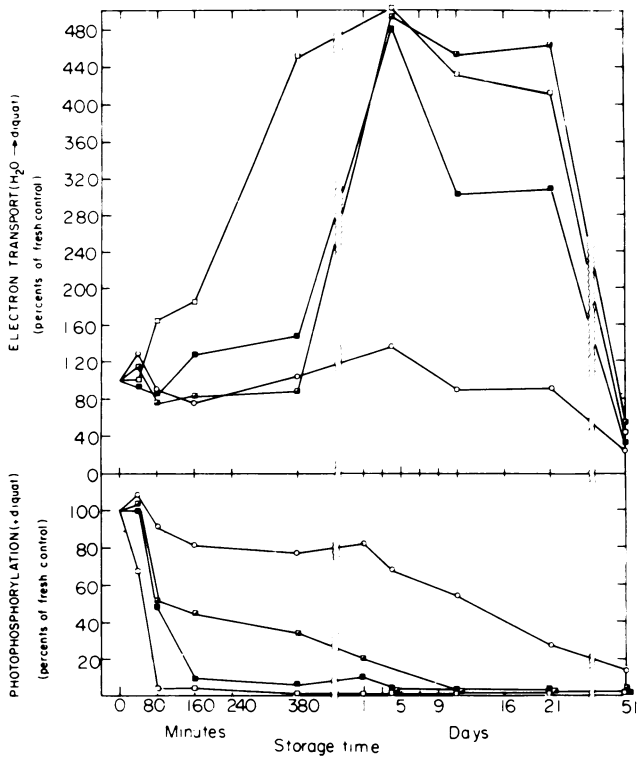

FIG. 6. Noncyclic phosphorylation and electron transport (H_2O → diquat), measured simultaneously, for storage at −79 C. Tobacco chloroplasts. Initial absolute activities (with no cryoprotectives added) were: 142 μmol ATP/mg Chl·h for phosphorylation (PHP) and 321 μmol/mg Chl·h (of diquat reduced) for electron transport (ET). Symbols used for the storage media and the respective initial activities (in the same units as above) are: (○): 40% (v/v) glycerol, 20 mM sucrose, 125 mM KCl; PHP: 95, ET: 282. (□): 30% (v/v) ethylene glycol, 20 mM sucrose, 125 mM KCl; PHP: 104, ET: 306. (■) 30% (v/v) ethylene glycol, 60 mM sucrose, 125 mM KCl; PHP: 120, ET: 311. (◪): 30% (v/v) ethylene glycol, 110 mM sucrose, 125 mM KCl; PHP; 122, ET: 308. Chloroplast concentration in the storage media corresponded to 3.82 to 4.01 mg Chl/ml.

does not afford protection (8). At 200 mM it gave considerable improvement in stabilization, when combined with glycerol and DMSO (symbols in circles in Fig. 3). This synergism is probably due to the fact that sucrose is a nonpenetrating agent while the others are penetrating ones; hence their mechanism of action is different.[2] With ethylene glycol, however, there was no effect of added sucrose, since the preservation was about 100% already.

UNCOUPLING IN PRESENCE OF ETHYLENE GLYCOL

The information gathered so far showed that chloroplast storage is most efficient in 30% (v/v) ethylene glycol at −100 C or below. At somewhat higher temperatures we encountered an unsuspected phenomenon: chloroplasts stored at −79 C in ethylene glycol lost photophosphorylation quite rapidly (within hours), while electron transport increased considerably (Fig. 4, the three upper parts). This effect looked like a true uncoupling, and was found to be specific to ethylene glycol at all concentrations used (*i.e.* 10–50%), but only at a storage temperature around −79 C. Storage at −20 C, −100 C, and −196 C, or in glycerol (and DMSO) at any temperature did not entail this effect (Figs. 4 and 5). Looking for the reasons for such specificity, the possibility of an artifact due to impurities was ruled out by redistillation of the ethylene glycol used. The possibility that freezing and thawing rates are important was eliminated by prior freezing to −196 C and then transfer to

[2] Penetrating cryoprotectives increase viscosity inside and prevent dehydration, whereas nonpenetrating ones are supposed to form an outside glass (see *e.g.* 17).

Table I. *Light-induced Proton Uptake (under Nonphosphorylating Conditions)*

Absolute initial activities are expressed in neq/mg Chl. All of the other activities in the table are expressed as per cents of these controls. Initial activity of the fresh chloroplasts (with no cryoprotective added) was 266 neq/mg Chl.

Protective Substance (v/v)	Sucrose	Absolute Initial Activity	Storage Temperature and Time									
			−20 C		−41 C			−79 C			−100 C	
			6 h	24 h	6 h	24 h	48 h	6 h	24 h	48 h	6 h	24 h
	mM											
30% Ethylene glycol	20	237	107	96	34	21	3	36	20	0	97	86
30% Ethylene glycol	60	232	95	91	31	17	2	45	33	15	89	91
30% Ethylene glycol	110	238	99	101	61	36	35	58	48	42	99	92
40% Glycerol	20	246	89	90	78	72	70	95	86	86	93	89

−79 C, followed by thawing, or the opposite (−79 C to −196 C, then thawing). In all of these cases the strong uncoupling was still present.

In order to investigate the nature and kinetics of the process as a function of temperature and of other elements of the storage medium, we measured both cyclic and noncyclic photophosphorylation—the latter simultaneously with the corresponding electron transport ($H_2O \rightarrow$ diquat)—as well as proton uptake under nonphosphorylating conditions. Figure 6 shows that in ethylene glycol noncyclic photophosphorylation was lost after very short storage times at −79 C under the most unfavorable conditions (high salt and low sucrose). This was followed by a remarkable enhancement (up to 5-fold) of electron transport persisting for about 20 days of storage and steeply declining afterward. The presence of sucrose (up to 110 mM) slowed down the process of uncoupling but could not prevent it (Fig. 6). Salt, on the other hand, accelerated the decay of phosphorylation (results not shown). In contrast to ethylene glycol, glycerol, used for the control, stabilized the activities of the same chloroplast preparation and only very weak signs of uncoupling can be noticed if at all. (*cf.* also ref. 6).

A strong uncoupling also appeared after storage in ethylene glycol at −41 C so that there seems to be a temperature range rather than a specific temperature for the effect. At −20 C this effect was absent.

The uncoupling effect was not unique to the plant species used (*i.e.* tobacco), as it was manifested also in lettuce chloroplasts (results not shown).

Loss of photophosphorylation was paralleled by the loss of proton uptake ability (Table I), in accordance with the Mitchell hypothesis, strongly suggesting leakiness induced by structural damage of the thylakoid membrane as the source of the uncoupling. This damage could be alleviated by certain additives, such as sucrose (Fig. 6 and Table I), but no restoration of activity seemed possible although DCCD used under certain conditions induced a short lived improvement. Preliminary experiments using SDS-gel electrophoresis (in collaboration with Dr. Varda Shoshan, Ben Gurion University) revealed the presence of a considerably increased amount of coupling factor subunits (mainly the α and β subunits) in the suspension medium of the uncoupled chloroplasts as compared to controls stored in either glycerol at −79 C or ethylene glycol at −196 C, so that release of the coupling factor to the medium is probably involved.

An interesting property of this uncoupling effect is that the kinetic behavior of photophosphorylation loss and electron transfer enhancement was not matched (Fig. 6). In a typical case the photophosphorylation decay was completed in about 80 min, while at this time only a slight change in the electron transfer activity occurred. The enhancement in electron transfer was completed several hours later. It is probable that the coupling factor is released in two steps at least. For example, one could think that the "head" part is presumably released faster, without any concomitant changes in the electron transport. Sometime later the proton channel part of the coupling factor is released, exposing free channels in the membrane through which protons may leak. At that time the enhancement of electron transfer takes place. This interpretation is supported by the kinetics of loss of proton uptake activity (Table I). This uncoupling effect of ethylene glycol could perhaps be utilized intentionally, as it appears to be an easy and delicate way (probably quite specific too) to affect coupling. Uncoupled chloroplasts could then be stored at less than −100 C for long periods and compared with coupled chloroplasts of the same batch.

The phenomenon of fast uncoupling in presence of ethylene glycol by storage at −41 C and −79 C is puzzling, especially because of its specificity and intensity. One could think in terms of eutectic crystallization as in (20), a phase transition (18), or conformational changes, like those reported for enzymes in 50% ethylene glycol which are temperature-dependent (5).

CONCLUSIONS

We found that ethylene glycol is a superior preserving medium as: (*a*) it does not affect control rates achieved without the protective substance, while the other cryoprotectives tried decreased the control rates; (*b*) it preserves the control rates of all activities to an extent of 100% at temperatures below −100 C. In the other cryoprotectives there was a further decrease of activities during the storage.

We tentatively recommend the following method in order to preserve chloroplast activity for long periods. After isolation of the chloroplasts the final pellet should be suspended in a preservation medium containing 30% ethylene glycol (v/v) as well as 20 mM Tris at pH 7.8, 20 to 200 mM sucrose, and 125 mM KCl (the concentrations of the secondary components are not critical in this case). Chl concentration should be within 1 to 5 mg/ml. The best storage is in a Dewar (above the liquid phase of the nitrogen, so as to avoid its penetration into the storage vials) at temperatures at −100 C or below. All pertinent activities will probably be kept close to the control level for very long periods.

A convenient method to prepare uncoupled chloroplasts would be to keep them for a short period (about 1 day) at dry ice temperature and then transfer them to the usual lower storage temperature.

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