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THE STABILIZATION OF CHLOROPLAST FUNCTION

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SUMMARY

Preparations of chloroplasts as described in the literature have very limited stability. This study is concerned with factors which affect the stability of chloroplast function.

1. Storage of suspensions of chloroplast fragments (2 mg chlorophyll per ml) for several days at 0° can be achieved simply by supplementation with bovine serum albumin (9 mg/ml). Hill reaction activity, non-cyclic photophosphorylation and ADP control of the Hill reaction are all largely retained. Dilution of the chloroplast fragment concentration leads to instability; supplementation with bovine serum albumin enhances stability.

2. Chloroplast function can be preserved in the frozen state for extended periods (at least 6 weeks) by the simple and convenient procedure of freezing the preparations at ordinary freezer temperature (−20°) in the presence of bovine serum albumin (1 %) and glycerol (10 %). Glycerol is required to prevent clumping, to enable refreezing and to retain photophosphorylation.

INTRODUCTION

Chloroplast fragments capable of the Hill reaction and photophosphorylation are readily prepared from spinach[1] but have limited storage stability even at 0°. DUANE AND KROGMANN[2] retained moderate Hill and photophosphorylation activity by freezing their preparations at liquid N_2 temperature. This method required relatively complex and exacting conditions for freezing and thawing small amounts of material.

The present study has found it possible to stabilize the activities of chloroplast fragments both in liquid suspension at 0° as well as in the frozen state at ordinary freezer temperature (−20°). Chloroplast fragments could be stabilized in liquid suspension at pH 8 for several days by the addition of bovine serum albumin. Hill activity, non-cyclic photophosphorylation, and enhancement of Hill activity by ADP were largely stabilized by this procedure. These activities were also largely preserved after freezing and storage at −20° by simply supplementing the suspensions with glycerol (10 %, v/v) and bovine serum albumin (9 mg/ml). The preservation of chloro-

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plast function for extended periods in liquid suspension or in the frozen state can thus be achieved both simply and effectively.

MATERIALS AND METHODS

Ovalbumin (twice crystallized) was the product of Nutritional Biochemicals. Human γ-globulin ("Gamastan") was from Cutter Laboratories. Bovine serum albumin was the recrystallized product of Armour Pharmaceutical, and Tris ("Trizma") base was the recrystallized product of Sigma Chemical. All other chemicals were reagent grade.

Chlorophyll was assayed by the method of ARNON[3]. Particulate protein was determined by the LOWRY–FOLIN method[4] with crystalline bovine serum albumin as a standard.

Preparation of chloroplast fragments

Washed chloroplast fragments were prepared from commercial spinach leaves essentially according to the procedure of WHATLEY AND ARNON[1]. This method consisted of isolating the chloroplasts, disrupting them in hypotonic medium, and resedimenting the fragments to remove soluble components. The following modifications were introduced: (1) Depetiolated spinach leaf blades were sliced and then fragmented in a Waring blendor at 2 to 3 sec bursts for a total of 10 sec at full speed rather than by grinding the leaves in a mortar and pestle with sand. The blendor was more convenient for producing larger quantities of particles whose Hill activity was as high as that from preparations ground in sand. (2) All resuspensions were performed with a Brendler (Potter–Elvehjem type) homogenizer equipped with a teflon pestle. This was more effective in dispersing particles uniformly than stirring with a glass rod and cotton or glass wool. (3) The particles were resuspended finally in media containing Tris chloride (or acetate) buffer (pH 8) rather than in unbuffered salt alone to ensure constant pH. All unfrozen suspensions were stored in glass-stoppered tubes in the dark in a crushed-ice bath.

Washed chloroplast fragments were used rather than intact chloroplasts to maximize permeability of added reagents and to minimize the influence of soluble components. Two types of preparations of chloroplast fragments were made[1] with the following modified preparative media: I. Acetate–ascorbate preparation: (1) The leaves were blended and the chloroplasts washed in a solution containing 0.5 M sucrose, 0.05 M Tris acetate (pH 8), 0.05 M sodium acetate, 0.01 M ascorbate, 0.001 M EDTA. (2) The chloroplasts were broken hypotonically and the fragments resuspended, after centrifugation, in the same medium but without sucrose. II. Chloride preparation: (1) The leaves were blended in 0.35 M NaCl–0.02 M TrisCl (pH 8) and the chloroplasts washed in 0.35 M NaCl. (2) The chloroplasts were shocked hypotonically in 0.035 M NaCl and resuspended in 0.035 M NaCl–0.02 M TrisCl (pH 8). Washed chloroplast fragments (chloride preparation) contained 7.2 ($\pm$ 0.2) mg of particulate protein per mg of chlorophyll.

Assay of enzyme activities

(*A*) *Hill reaction with $K_3Fe(CN)_6$ as electron acceptor.* Elements of the procedure are based on that described by STILLER AND VENNESLAND[5]. Washed chloroplast

fragments were assayed aerobically with $K_3Fe(CN)_6$ as Hill electron acceptor. White light (intensity 4000–5000 ft-candles) was provided by three 150-W reflector flood lamps. Particles containing about 0.090 mg chlorophyll in a total volume of 0.03 to 0.08 ml were pipetted into dry 25-ml erlenmeyer flasks held in ice in a Dubnoff shaker rack. The tray was then wrapped in aluminum foil, the room lights were turned off, and 3 ml of the Hill reaction medium, pre-equilibrated to temperature (usually 25°) were injected into each flask through the aluminum foil with a calibrated syringe. The tray of flasks was then uncovered in the dark, placed in a Dubnoff shaker already pre-equilibrated to temperature, and the reaction was initiated by turning on the light. Generally no more than 30 sec elapsed between the time the first flask was injected and the onset of illumination. Bath temperature during the 3-min assay period was maintained within $\pm 0.5°$ by the addition of ice. The reaction was terminated by delivering 0.24 ml of 50% trichloroacetic acid to each flask with a 1-ml syringe to bring the final acid concentration to 4% and by turning off the lights. All the flasks were acidified within 15 sec after the 3-min assay period, a time variation of at most 8%.

The final assay medium contained 1 mM $K_3Fe(CN)_6$, 33 mM Tris chloride (pH 8), 3 mM $MgCl_2$, 10 mM potassium phosphate (pH 8), 25 mM KCl. Total volume was 3.00 ml *plus* 0.03 to 0.08 ml of chloroplast fragments. Duplicates of each suspension were spaced in the shaker in 'staggered' fashion to minimize variability[5]. Dark control suspensions of identical composition were particularly necessary when the suspensions contained bovine serum albumin because a small decrease in ferricyanide concentration was produced by bovine serum albumin alone, with or without light. The acidified suspensions were centrifuged 2 min in a clinical centrifuge, and their ferricyanide concentration was assayed at 420 mμ in a Beckman DU-2 spectrophotometer.

(*B*) *Measurement of O_2 evolution and enhancement of Hill activity in the presence of ADP with the oxygen electrode.* The signal from a Clark oxygen electrode (Yellow Springs Instrument Co.) was amplified and recorded with an instrument designed by Dr. ROBERT RIKMENSPOEL. Light for the reaction, white light of about 5000 ft-candles, had been cooled by passing it through 8 cm of water. Additionally, the reaction cell was water-jacketted and thermostated at 23–25°. The assay medium (3 mM $MgCl_2$, 10 mM K_2HPO_4 (pH 8), 25 mM KCl, 33 mM TrisCl (pH 8), $\pm$ 10 mg bovine serum albumin per ml) was flushed (before any bovine serum albumin addition) with N_2 to achieve an O_2 concn. of 30 to 40% satn. at 23–25°. Chloroplast fragments (0.04 to 0.09 mg chlorophyll) were added and equilibrated for about 1 min in the light. No O_2 evolution was observed until the Hill reaction was initiated in the light by the addition of the electron acceptor. The initial $K_3Fe(CN)_6$ concn. was 1 mM. Rates of O_2 evolution were based on a 1-min interval, although their linearity usually persisted for 3 min with little change in slope. In experiments measuring the effect of 2 mM ADP on O_2 evolution, 0.04 ml of 0.1 M ADP (pH 7.4) was injected after 1 min and a second 1-min interval was recorded.

The presence of 10 mg bovine serum albumin per ml of assay medium helped to stabilize the rates of some aged particle preparations but usually had little or no effect on O_2 rates of fresh or partly inactive particles.

(*C*) *Measurement of non-cyclic photophosphorylation with ferricyanide as electron acceptor.* The reaction conditions in Part A were used except that the phosphate concn. was 3 mM rather than 10 mM to facilitate subsequent phosphate analysis,

and 2 mM ADP and $^{32}P_i$ were present. After the 3-min light period, the assay mixtures were acidified and centrifuged, and the centrifuge tubes were cooled in ice to minimize acid hydrolysis of ATP. 1 ml of clear supernatant was withdrawn and the amount of esterified [^{32}P]phosphate determined[6]. Rates of phosphate esterification are expressed as net photophosphorylation, *i.e.* light *minus* dark. Phosphorylation rates in the dark with chloroplast fragments were negligible, being identical usually with rates of the dark reaction of a reagent blank without particles. The remaining clear, acidified supernate was assayed for ferricyanide concentration as in Part A to determine the rate of photoreduction of ferricyanide by fragments in the presence of ADP.

Freezing and storage of chloroplast fragments at —20°

Suspensions of chloroplast fragments at 0° were brought to a desired glycerol concentration by adding glycerol solutions (40 %, v/v, or less) containing the same components as the chloroplast fragment suspending medium. The suspensions were swirled gently for about 30 sec as the glycerol solution was added. Slower addition was not necessary. The suspensions were frozen in the dark by merely placing them in a freezer at —20°. Suspensions varying in any one parameter *e.g.* glycerol concentration, were identical in all other parameters, *e.g.* fragment concentration, volume and type of tube. Suspensions were thawed by swirling them gently, alternately in cold water and in ice to keep the suspension temperature near 0°. Suspensions containing 10 or 20 % glycerol thawed within seconds, those with 5 % glycerol in about 2 min, those without glycerol thawed in about 5 min. Thawed suspensions were kept at 0° in the dark until aliquots were withdrawn for assay; they were then put back at —20°, if refreezing was desired, 10 min after thawing.

RESULTS

General considerations on assay conditions

The Hill assay medium contained sufficient magnesium and phosphate (*cf.* METHODS) to elicit the enhanced Hill-electron-transport activity associated with these reagents in phosphorylating particles[7] but contained no ADP unless specified. Short assay times (3 min for ferricyanide reduction, 1 min for O_2) were chosen to minimize particle instability during assay and thus to better reflect instability during storage. The pattern of results reported here also was observed with assays using other media, reaction times and temperatures.

Hill reaction activity was linearly proportional to chlorophyll concentration in the amounts used and to reaction time. Because the Hill reaction of these particles showed a Q_{10} of about 2 between 15° and 25° temperature control to $\pm 0.5°$ was required to keep rate errors due to temperature to less than 5 %.

Correspondence of Hill rates as measured by ferricyanide reduction and by O_2 evolution

On the basis of electron balance the rate of O_2 evolved, when expressed as μmoles e^- transferred per (mg chlorophyll) (h) *i.e.* μmoles $O_2 \times 4$/(mg chlorophyll) (h) should equal the rate of ferricyanide reduction, expressed as μmoles ferricyanide reduced/(mg chlorophyll) (h). Although the two assays were conducted with the same final medium and at comparable particle concentrations using the same source of particle suspensions, some differences in procedure existed: Ferricyanide reduction

measurements were conducted aerobically and measurements of O_2 evolution with the oxygen electrode were necessarily made at low O_2 tensions (approx. 40% times 270 μM O_2 or an initial O_2 concn. of about 108 μM). Experimental errors due to timing (8% in stopping the ferricyanide assay) and temperature (generally 1° lower in the oxygen-electrode assay) would together raise the rates with ferricyanide about 18% higher than those for O_2. Considering these errors and differences in assay conditions, the two rates obtained with the separate assay procedures (*cf.* Figs. 3, 4) were in surprisingly good agreement. The evidence presented indicates that both fresh and aged suspensions of spinach chloroplast fragments evolved O_2 as fast as they reduced ferricyanide and that both capacities were preserved or lost to the same extent during storage of the particles. Studies using Warburg manometry also showed this stoichiometry between ferricyanide reduction and O_2 evolution.

Individual values for Hill rates were generally reliable to 5 to 10% as were values for phosphorylation efficiency ($P/2e^-$) because aliquots of the same acidified suspension were used to measure [^{32}P]phosphate esterification and ferricyanide reduction. Values for ADP control of the Hill reaction were intrinsically more variable —particularly when measured by ferricyanide reduction, which required dark–control subtractions. Values of ADP control were more reliable when measured with the oxygen electrode because in the same assay particles could be assayed in the presence or absence of ADP.

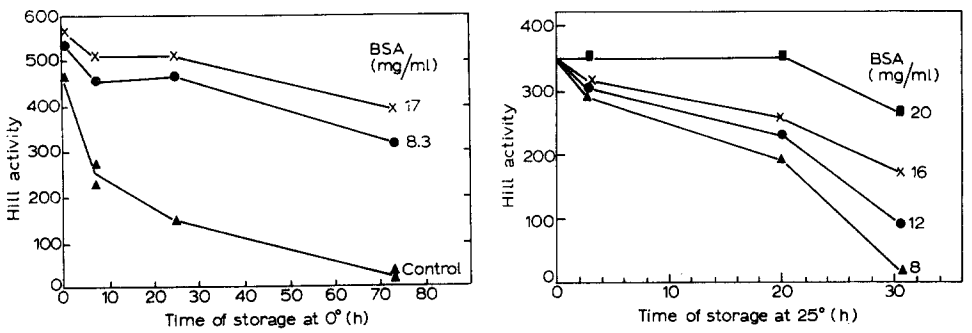

Fig. 1. Stability of Hill ferricyanide activity of a chloride preparation of chloroplast fragments (*cf.* METHODS) stored at 0° as a function of bovine serum albumin (BSA) added to the suspensions, or no addition. Hill activity is in units of μmoles ferricyanide reduced per mg chlorophyll per h. Fragment concentration during storage was 1.26 mg chlorophyll per ml suspension. All suspensions contained 30 mM NaCl–20 mM TrisCl (pH 8) during storage, with supplements of bovine serum albumin as indicated. 0.088 mg chlorophyll was used per assay.

Fig. 2. Stabilization by bovine serum albumin (BSA) of the Hill activity of particles stored at 25°. Hill activity is expressed in units of μmoles ferricyanide reduced per mg chlorophyll per h. The storage medium contained: 35 mM NaCl, 50 mM TrisCl (pH 8), 10 mM EDTA, 6 mM $MgCl_2$, and bovine serum albumin in the concentrations indicated in the figure. The fragments used in this experiment had been frozen in acetate–ascorbate medium containing 20% glycerol and 8 mg bovine serum albumin per ml. Removal of glycerol, and other additives as well as the transfer to the storage medium was accomplished by washing the fragments at 0° in 10 volumes of storage medium containing 8 mg bovine serum albumin per ml, centrifuging them at 25000 × *g* for 10 min, and resuspending the pellet to the original chloroplast concentration with storage medium supplemented with bovine serum albumin in the concentrations indicated in the figure. Aerobic storage in sealed containers was performed in the dark. Aliquots were withdrawn at the indicated times. When particles were washed at 0° without bovine serum albumin, their activity dropped to half its original value, and subsequent storage at 25° eliminated the remaining activity within 1 h.

I. Stabilization of Hill activity with bovine serum albumin

Supplementation of chloroplast fragments with bovine serum albumin during storage resulted in a dramatic stabilization of their Hill activity (Fig. 1). $MgCl_2$ and EDTA (singly, together, and in combination with bovine serum albumin) when tested as preserving agents during storage could not replace bovine serum albumin nor could they augment particle stability in the presence of bovine serum albumin (Table I). Mg^{2+} (2.5 or 5 mM) was totally ineffective as a stabilizing agent; EDTA (10 mM) was partially effective, but less so than bovine serum albumin.

TABLE I

EFFECT OF $MgCl_2$, EDTA, AND BSA ON THE STABILITY OF HILL ACTIVITY OF FRAGMENTS STORED AT 0° IN NaCl–TrisCl (pH 8)

All suspensions were stored in the dark at 0° in stoppered tubes. Suspensions contained 28 mM NaCl, 16 mM TrisCl (pH 8) and 2.28 mg chlorophyll/ml. The final bovine serum albumin (BSA) concentration, where added, was 8 mg/ml suspension. Other supplements to the storage medium are indicated in the table headings. An aliquot of 0.04 ml of suspension containing 0.091 mg chlorophyll was used in each assay. (*cf.* METHODS for assay medium and conditions.) Comparable results were obtained when 5 mM $MgCl_2$ was used instead of 2.5 mM $MgCl_2$, and 5 mM $MgCl_2$ *plus* 10 mM EDTA instead of 2.5 mM $MgCl_2$ *plus* 10 mM EDTA.

Time of storage (h)	*Hill activity [μmoles ferricyanide reduced/(mg chlorophyll) (h)]*							
	Control		*10 mM EDTA*		*2.5 mM $MgCl_2$*		*10 mM EDTA + 2.5 mM $MgCl_2$*	
	—BSA	*+BSA*	*—BSA*	*+BSA*	*—BSA*	*+BSA*	*—BSA*	*+BSA*
0	524	600	386	469	509	622	488	534
8	309	580	357	413	314	492	374	420
28	183	437	332	405	212	382	312	337
70	103	390	272	290	170	346	224	246

Stabilization with bovine serum albumin of Hill reaction capability of particles stored at 25°. Particle suspensions stored at 25° lost their Hill activity much more rapidly than did cold suspensions, and supplementation with bovine serum albumin was consequently even more imperative at 25°. Unsupplemented suspensions were totally inactive within 1 h of storage at 25° (*cf.* legend of Fig. 2) and the stability of supplemented suspensions was a direct function of relatively higher concentrations of bovine serum albumin (Fig. 2). Although this experiment was performed at pH 8 using TrisCl–NaCl, bovine serum albumin also protected suspensions at 25° in phosphate buffer (pH 7) for at least 30 min with no detectable loss in Hill activity.

Stabilizing effect of bovine serum albumin during assay. The Hill activity of freshly prepared particles or particles freshly washed with bovine serum albumin–TrisCl (pH 8)–NaCl medium was unaffected when bovine serum albumin (5 or 10 mg/ml) was present in the assay medium. Neither the linearity nor magnitude of Hill rates (ferricyanide reduction or O_2 evolution) was affected during a 6-min period. However, with aged particles the presence of bovine serum albumin helped maintain linear rates during assay and thus stabilized but did not activate these particles.

Effect of washing the particles with bovine serum albumin. Washing fresh or partly aged particles with bovine serum albumin–TrisCl–NaCl medium before assay could not significantly increase their activity (Fig. 3). However, such washing in

media containing bovine serum albumin produced no great loss in activity as was usually encountered when dilution or washing of particles was performed in the absence of bovine serum albumin. Homogenizing the fresh particles with bovine serum albumin conferred no greater stability upon them than did simply adding the bovine serum albumin by mixing (Fig. 3).

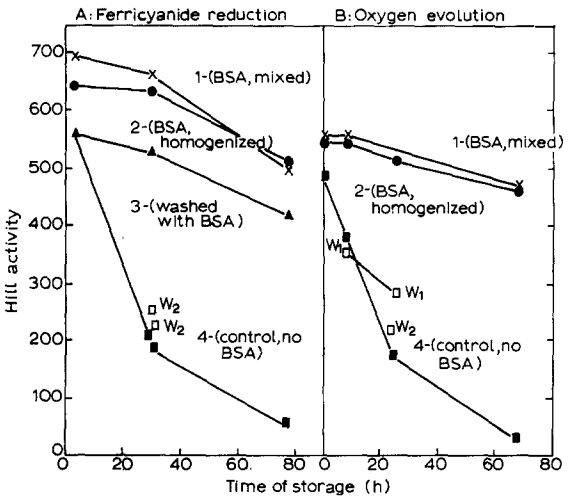

Fig. 3. Stabilization of Hill reaction as a function of the type of mixing of the particles with bovine serum albumin (BSA) and the effect of bovine serum albumin washings. In 3A, the Hill reaction was measured by ferricyanide reduction (μmoles ferricyanide reduced/(mg chlorophyll) (h)], and in 3B by O_2 evolution with the oxygen electrode (μmoles e^- transferred/(mg chlorophyll) (h)]. The suspensions were supplemented or treated as follows: (1) 9 mg bovine serum albumin per ml suspension (mixed by swirling); (2) 9 mg bovine serum albumin per ml suspension (mixed with Potter–Elvehjem homogenizer); (3) as in (2) but then washed immediately with 10 vol. of 10 mg bovine serum albumin per ml, 20 mM TrisCl (pH 8), 35 mM NaCl and resuspended in 9 mg bovine serum albumin per ml, 20 mM TrisCl (pH 8), 35 mM NaCl; (4) control, no addition of BSA; the Point W_1 represents an aliquot of control washed at the 8-h point with 10 vol. of wash, as in (3) and resuspended, as in (3); the Point W_2 represents an aliquot of control washed at the 25-h point with 10 vol. of wash as in (3), and resuspended as in (3). All suspensions were stored in the dark at 0° in 20 mM TrisCl (pH 8)–35 mM NaCl, *plus* or *minus* 9 mg bovine serum albumin per ml suspension, as indicated. The particle concentration during storage was (mg chlorophyll per ml): (1) 1.73; (2) 1.73; (3) 1.73; (4) 1.69; W_1, 1.33; W_2, 1.38. The assays were carried out without bovine serum albumin in the assay medium.

II. Stability of Hill reaction (±ADP) and photophosphorylation capability of particles during storage as a function of particle concentration and of the presence of bovine serum albumin

We were led to a more careful examination of the effect of fragment concentration on stability because, over the course of several experiments, more concentrated preparations appeared to be more stable. Using the same preparation of chloroplast fragments for all suspensions in this experimental series (Figs. 4 and 5), we examined two factors—the concentration of chloroplast fragments (1, 2, and 4 mg chlorophyll per ml) and the presence or absence of bovine serum albumin (9 mg/ml)—to test their importance and interrelation in preserving the function of chloroplast fragments during storage at 0°. The following particle activities were assayed at various times during storage and used as measures of particle stability: (1) the Hill reaction (O_2 evolved and ferricyanide reduced) assayed with ADP in the reaction

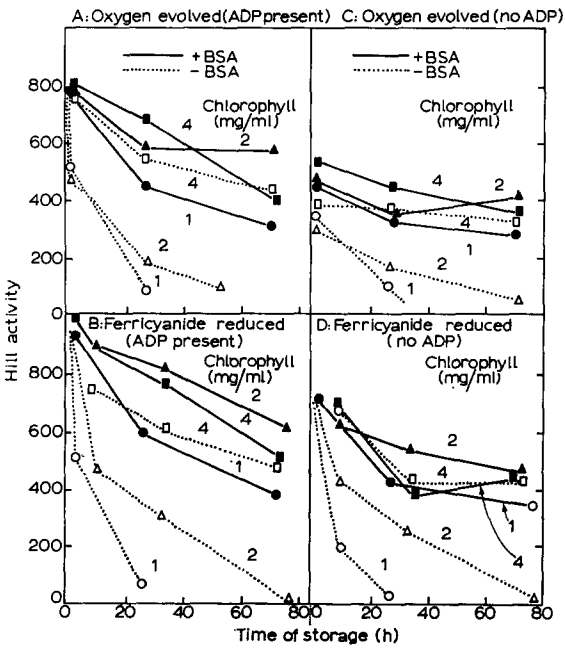

Fig. 4. Decay of Hill reaction during storage of fragments as a function of particle concentration and of the presence or absence of bovine serum albumin (BSA) (9 mg/ml) in the suspensions. In 4A and B, ADP was present during assay; in 4C and D, no ADP was present during assay. O_2 evolution is expressed as μmoles e^- transferred/(mg chlorophyll) (h) and ferricyanide reduction as μmoles ferricyanide reduced/(mg chlorophyll) (h). During storage all suspensions of chloroplast fragments contained 35 mM NaCl–20 mM TrisCl (pH 8) and were kept at 0° in the dark. At the designated times, aliquots were withdrawn for assay of the rates of O_2 evolution or ferricyanide reduction (*cf.* METHODS). The suspension of chloroplast fragments used was a chloride preparation (*cf.* METHODS) which was subsequently diluted either with TrisCl–NaCl or with TrisCl–NaCl containing 9 mg bovine serum albumin per ml to achieve the particle concentrations indicated in the figure.

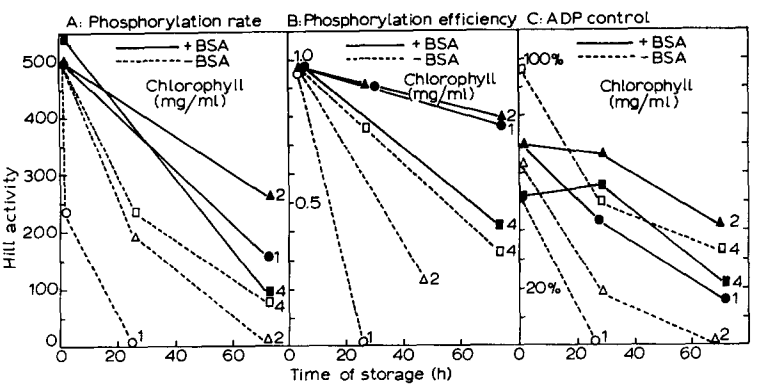

Fig. 5. Decay of non-cyclic phosphorylation and ADP control of the Hill reaction as a function of fragment concentration and of the presence or absence of bovine serum albumin (BSA) (9 mg/ml) in the suspensions during storage. 5A shows rates of non-cyclic phosphorylation, [μmoles phosphate esterified/(mg chlorophyll) (h)]; 5B presents values of phosphorylation efficiency (P/2e$^-$), (μmoles phosphate esterified/2 μmoles ferricyanide reduced); and 5C shows % ADP control of the Hill reaction (O_2 evolution). % ADP control is defined as $[(\text{Rate})_{+\text{ADP}} - (\text{Rate})_{-\text{ADP}}]/(\text{Rate})_{-\text{ADP}}$. For assay conditions *cf.* METHODS. The particles used as well as other experimental conditions are described in the legend of Fig. 4.

medium (Figs. 4A, B) or without ADP (Figs. 4C, D); (2) the rate of non-cyclic phosphorylation (Fig. 5A) and the efficiency of non-cyclic phosphorylation ($P/2e^-$), *i.e.* μmoles phosphate esterified/2 μmoles ferricyanide reduced (Fig. 5B); and (3) ADP control of the Hill reaction (Fig. 5C), expressed as the percentage

$$\frac{(\text{Rate})_{+\text{ADP}} - (\text{Rate})_{-\text{ADP}}}{(\text{Rate})_{-\text{ADP}}}$$

(Typical oxygen-electrode traces of O_2 evolved showed that the addition of ADP by injection promptly produced an increase in the rate of O_2 evolution.)

Both higher fragment concentration and bovine serum albumin were important interrelated factors in retaining all functional activities during storage (Figs. 4, 5)— the presence of bovine serum albumin was particularly beneficial with dilute suspensions of fragments, and higher fragment concentration was increasingly required in the absence of bovine serum albumin. Phosphorylation (Figs. 5A, B, C) appeared to be more labile than electron transport (Fig. 4) and the beneficial effect of bovine serum albumin is most clearly indicated in its stabilization of phosphorylation efficiency (Fig. 5B). Storage of suspensions at a chloroplast fragment concentration of 2 mg chlorophyll per ml with 9 mg bovine serum albumin per ml was the most effective and practical condition.

Stability of chloroplast function in the presence of other proteins. Of the three proteins tested, bovine serum albumin was the best stabilizer of the Hill reaction ($\pm$ADP), ovalbumin was moderately effective, and γ-globulin was practically ineffective (Table II).

III. Preservation of chloroplast function by storage in the frozen state with glycerol and bovine serum albumin

Chloroplast fragments could be readily stored in the frozen state with retention of function if both glycerol and bovine serum albumin were added to the TrisCl–NaCl medium before freezing. Glycerol and bovine serum albumin protected about 85 % of the Hill activity of chloroplast fragments during the process of freezing and thawing (Table III). About 50 % was preserved in the presence of glycerol alone, and about 25 % with bovine serum albumin alone.

Glycerol protected the particles against damage by freezing and thawing but had no marked effect on the stability of unfrozen particles during storage (Table IV). (The variability in the values shown in the table (as well as in similar studies not shown) was about $\pm$ 20 %.)

Hill activity was largely preserved in the presence of glycerol and bovine serum albumin even after multiple cycles of freezing and thawing (Fig. 6). Suspensions without glycerol progressively lost activity with each cycle. The high activity after one cycle for the control without glycerol occurred rarely; a loss of 30 to 50 % or more after one freeze-and-thaw cycle was more common.

Fragments frozen without glycerol, with or without bovine serum albumin, clumped badly after freezing and thawing, without exception; 10 or 20 % glycerol always prevented this aggregation, 5 % was partially effective. Homogenization of particles aggregated by freezing had little effect in restoring their activity. The loss after freezing is thus not attributable merely to an incomplete exposure of active particles to assay because of aggregation. The relation of clumping to loss of Hill

TABLE II

STABILITY OF HILL ACTIVITY OF SUSPENSIONS DURING STORAGE *vs.* TYPE OF PROTEIN ADDED

Storage conditions: 20 mM TrisCl (pH 8)–35 mM NaCl; 0°, with storage in the dark. The concentration of chloroplast fragments was 1 mg chlorophyll per ml in all suspensions. Hill activity was measured with the oxygen electrode (*cf.* METHODS). Percentage ADP control is defined as:

$[(\text{Rate}_{+\text{ADP}} - (\text{Rate})_{-\text{ADP}}] / (\text{Rate})_{-\text{ADP}}$

Protein added (*10 mg/ml*)	*Time of storage*	O_2 *evolved* [*μmoles electrons transferred/(mg chlorophyll) (h)*]								
		2 h			*28 h*			*72 h*		
		+ADP	*−ADP*	*% ADP control*	*+ADP*	*−ADP*	*% ADP control*	*+ADP*	*−ADP*	*% ADP control*
Bovine serum albumin		770	455	69	456	314	45	313	266	18
No addition		533	345	54	94	94	0	0	0	0
γ-Globulin		471	283	66	79	79	0	7	7	0
Ovalbumin		534	377	42	299	251	19	219	219	0

TABLE III

PROTECTION BY GLYCEROL AND BOVINE SERUM ALBUMIN AGAINST FREEZING DAMAGE TO CHLOROPLAST FRAGMENTS

The suspensions were aliquots of a fresh chloride preparation (*cf.* METHODS). The final concentration was in all cases 1.11 mg chlorophyll per ml of suspension. 0.089 mg of chlorophyll was used in each Hill assay. Values of Hill activity are expressed in units of μmoles ferricyanide reduced/(mg chlorophyll) (h) and represent the average of duplicate determinations.

	Controls (no freezing)			*Frozen and thawed*			
				No glycerol		*20 % glycerol*	
Bovine serum albumin, 10 mg/ml final concn.	+	+	−	−	+	+	−
Glycerol, 20 % final concn.	+	−	−	−	−	+	+
Hill activity	453	548	450	26	117	386	231
% original activity remaining after freezing (450 = 100 %)				6	26	86	52

TABLE IV

EFFECT OF GLYCEROL (10%, v/v) ON THE STABILITY OF CHLOROPLAST FRAGMENTS STORED AT 0° (NOT FROZEN)

During storage in the dark, all suspensions contained 10 mg bovine serum albumin per ml, 30 mM NaCl, and 20 mM TrisCl (pH 8). Aliquots were withdrawn for assay in the oxygen electrode at the times indicated.

Particle concentration [chlorophyll (mg/ml)]	*10% glycerol*	*Time of storage*	*O_2 evolved (μmoles e^- transferred/(mg chlorophyll) (h)]*								
			2 h			*28 h*			*72 h*		
			+ADP	*−ADP*	*% ADP control*	*+ADP*	*−ADP*	*% ADP control*	*+ADP*	*−ADP*	*% ADP control*
4	−		814	533	52	686	437	57	420	343	22
3	+		1045	756	39	756	503	50	498	375	33
1	−		770	455	70	456	314	45	313	266	18
1	+		756	409	85	628	408	54	500	359	39

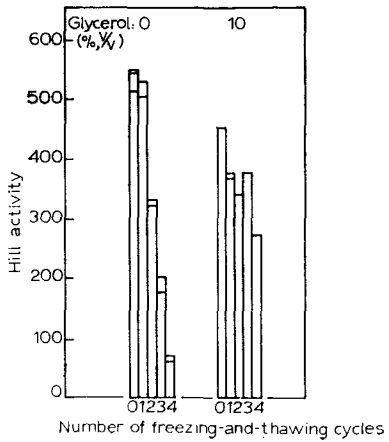

Fig. 6. Stability of the Hill reaction activity of chloroplast fragments prepared in chloride medium to multiple freezing and thawing cycles as a function of the presence or absence of 10% glycerol in the freezing medium. Results with 5 or 20% glycerol (not shown) were essentially the same as those for 10% glycerol. Hill activity is expressed as μmoles ferricyanide reduced/(mg chlorophyll) (h). All suspensions contained 8.3 mg bovine serum albumin per ml suspension, 30 mM NaCl–20 mM TrisCl (pH 8), 0.63 mg chlorophyll per ml suspension. 0.0882 mg of chlorophyll was used per assay. (Where more than one horizontal line appears within a vertical cycle-column it represents a duplicate determination.)

activity is not clear. Occasionally, as in the control of Fig. 6 after 1 cycle, extensive clumping of fragments after freezing produced little loss of Hill activity. More often, particles clumped by freezing were largely inactive. Thus glycerol served both to protect against loss of activity during freezing cycles and to prevent clumping.

The pattern of observations described for preparations frozen in chloride medium applied equally well to particles frozen in acetate–ascorbate medium.

Suspensions in glycerol and bovine serum albumin were very convenient to use—they thawed in seconds, were easy to pipette, and the particles remained well dispersed with no tendency to sediment on standing.

The protection by glycerol and bovine serum albumin against freezing-and-thawing damage to a number of chloroplast activities is summarized in Table V. This combination of reagents was effective in preserving Hill activity and non-cyclic phosphorylation as well as ADP control of the Hill reaction.

TABLE V

PROTECTION BY GLYCEROL AND BOVINE SERUM ALBUMIN AGAINST FREEZING DAMAGE TO THE HILL AND PHOSPHORYLATION SYSTEMS

Final concentrations of additions, where added, were: bovine serum albumin, 10 mg/ml final suspension; glycerol 10% (v/v, final suspension). All suspensions contained 35 mM NaCl, 20 mM TrisCl (pH 8). The chloroplast fragments were aliquots of a chloride preparation (*cf.* METHODS). All suspensions were kept frozen for 4 days at −20° in the dark. O_2 evolution, assayed with or without ADP, is expressed in units of μmoles e^- transferred/(mg chlorophyll) (h). Rates of non-cyclic photophosphorylation with ferricyanide as electron acceptor are expressed in units of μmoles phosphate esterified/(mg chlorophyll) (h) (*cf.* METHODS for assay conditions). Phosphorylation efficiency ($P/2e^-$) was calculated from twice the phosphorylation rate divided by the rate of ferricyanide reduction in the presence of ADP. In this study, the assay medium (*cf.* METHODS) was supplemented with 10 mg bovine serum albumin per ml. For the rates designated with an asterisk, the rates of O_2 evolution were unstable and declined rapidly, with or without ADP. Although the data presented are for O_2 evolution, the same pattern of results was obtained when ferricyanide reduction was followed.

Particle concentration [chlorophyll (mg/ml)]	*Additions to suspensions*	*O_2 evolved*		*% ADP (control)*	*Phosphorylation*	
		+ADP	*−ADP*		*Rate*	*Efficiency ($P/2e^-$)*
4	No addition	31	31	0	—	—
4	+ BSA	156*	265*	0	0	0
3	+ BSA + glycerol	466	226	106	282 ± 8	0.85 ± 0.01
1	No addition	0	0	—	—	—
1	+ BSA	187*	249*	0	0	0
1	+ BSA + glycerol	467	358	31	234 ± 2	0.82 ± 0.05

Abbreviation: BSA, bovine serum albumin.

The preservation of these activities in preparations frozen over long periods of time is observed in Table VI. It is noteworthy that preparations stored in the frozen state for periods as long as 6 weeks still retained much of their Hill activity and photophosphorylation activity as well as ADP control of the Hill reaction.

TABLE VI

ACTIVITY OF CHLOROPLAST FRAGMENT PREPARATIONS STORED IN THE FROZEN STATE FOR EXTENDED TIME PERIODS

Hill rate by ferricyanide reduction is expressed as μmoles ferricyanide reduced/(mg chlorophyll) (h); phosphorylation efficiency ($P/2e^-$) is in units of μmoles phosphate esterified for each 2 μmoles of ferricyanide reduced; Hill rate as measured by the oxygen electrode is in units of μmoles e^- transferred/(mg chlorophyll) (h). All preparations contained 9 mg bovine serum albumin per ml, 20 mM TrisCl (pH 8), 35 mM NaCl, and 20% glycerol (v/v) for I and II, 10% glycerol for III. Storage was at −20° in the dark. The preparations shown were typical. The original rates and stability in suspension at 0° (unfrozen) of Prep. III are shown in Figs. 4 and 5.

Preparation	*Chlorophyll concentration (mg/ml)*	*Total time stored frozen (−20°)*	*Hill rate (ferricyanide reduction)*				*Hill rate (O_2 evolution)*		
			+ADP	*−ADP*	*%ADP control*	*$P/2e^-$*	*+ADP*	*−ADP*	*%ADP control*
I	1.16	6 weeks	443	300	48	0.65	352	312	13
II	0.81	3.5 weeks	450	384	17	0.60	450	360	25
III Thawed once	1.00	4 days	580	415	40	0.82	467	358	31
Thawed once	1.00	16 days	—	—	—		269	249	19
Thawed twice	1.00	16 days	—	—	—		312	234	33
III Thawed once	3.00	4 days				0.85	466	226	106
Thawed once	3.00	16 days					497	339	47
Thawed twice	3.00	16 days					518	331	56

DISCUSSION

These studies show that at pH 8 chloroplast fragments are more stable when stored with bovine serum albumin and at high fragment concentration. The activities stabilized by bovine serum albumin represent high, coupled rates rather than low residual rates of aged fragments. (The maximum rates of photosynthetic electron transport when expressed on a protein basis, 0.9 μatom O_2 evolved at 25° per min per mg protein, are comparable to rapid mammalian respiratory rates (*cf.* BLAIR *et al.*[8]).)

Bovine serum albumin has been demonstrated in these studies to be a stabilizing agent but not an activator for it has not been possible to reverse inactivation once it occurred. Bovine serum albumin has previously been found effective in maintaining the activity of animal mitochondria[9] where its effect is probably due to its binding of long-chain fatty acids and a consequent reversal of fatty acid inhibition[10–13] (*cf.* BORST *et al.*[13]). Similarly, FLEISCHER, CASU AND FLEISCHER[14,15] used a bovine serum albumin washing procedure to remove fatty acids and lysophosphatides which were formed when mitochondria were digested with phospholipase A. We have no evidence that bovine serum albumin stabilizes chloroplast fragments at pH 8 by binding inhibitors produced by the breakdown of lipids since: (1) addition of bovine serum albumin to the assay medium did not increase the initial Hill activities of partly inactive fragments; (2) washing with bovine serum albumin did not reactivate the Hill activity of aged fragments; and (3) no change was detected in the pattern of polar lipids (as observed by thin-layer chromatography) in preparations at pH 8 which had lost most of their Hill activity. Furthermore, we could detect no alteration of lipids due to peroxidation as measured by the thiobarbituric acid reaction. (Although MCCARTY AND JAGENDORF[16] suggested that lipid breakdown might explain the inactivation of spinach chloroplasts during aging at pH 6, this phenomenon did not appear to occur or account for the inactivation of spinach chloroplasts aged at pH 8.) The mechanism both of chloroplast inactivation at pH 8 and of its prevention by bovine serum albumin remains unknown.

Glycerol, first used successfully[17] to freeze sperm, has now been shown to be highly effective at 10 % concn. in preserving photosynthetic function during freezing of chloroplast fragments at —20°. Glycerol need not be removed by washing after thawing because it does not interfere with assays of chloroplast function and does not affect fragment stability in cold liquid suspension. (If glycerol removal is desired after thawing, this can be accomplished with relative impunity by washing suspensions with a medium containing bovine serum albumin–TrisCl (pH 8)–NaCl.) The procedure for freezing chloroplast fragments is not only effective but easy to perform (*cf.* METHODS) and the suspensions are easily manipulated after thawing. Freezing separate aliquots of chloroplast suspensions in 10 % glycerol and 9 mg bovine serum albumin per ml at —20° now affords a convenient source of suspensions of identical chemical composition and reasonably high, reproducible activity.

During the preparation of this manuscript, it has been reported[18] that unwashed spinach chloroplasts stored at —20° in 50 % glycerol retain their capacity for cyclic and non-cyclic phosphorylation for several weeks. Several comments might be made: (1) the stated rate of phosphorylation for chloroplasts preserved in 50 % glycerol was at best (time zero) only 50 μmoles phosphate esterified per mg chloro-

phyll per h (phenazine methosulfate-supported phosphorylation), a rate which is almost negligible compared to phosphorylation rates for fresh particles; (2) rates of electron transport were reported as percentages of an unstated control rate; (3) suspensions of 50 % glycerol at −20° were not stated to have frozen, and, in our experience, do not freeze. This appears then to have been storage in the liquid state with 50 % glycerol.

In the present studies both glycerol (10 %) and bovine serum albumin (9 mg/ml) were required for freezing suspensions of chloroplast fragments to preserve maximal activity. The presence of glycerol (10 %) was the major factor in (1) protecting all photosynthetic activities during freezing and thawing at −20° especially photophosphorylation and ADP control of the Hill reaction, (2) protecting function during additional freezing and thawing cycles, and (3) preventing the fragments from 'clumping' due to freezing and thawing. Although loss of function especially of photophosphorylation was correlated with 'clumping' of fragments which lacked glycerol during freezing and thawing, the mechanism of this relation is unclear. The preserving action of glycerol is probably due to physical or physicochemical factors in freezing at −20° rather than to a direct chemical role because glycerol did not affect the stability of fragments in liquid suspension. Bovine serum albumin was an important but lesser factor in freezing whose function need not be related to the freezing process itself but rather might be the same as its action (not understood) in preserving activity of fragments in liquid suspension. Although 60 % of the high initial chloroplast activity could be preserved for 1 to 2 months by freezing at −20°, additional factors need to be investigated to allow greater recovery of function—particularly of photophosphorylation and ADP control—after brief freezing and to prevent further gradual decline of function during prolonged (months) storage at −20°.

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REFERENCES

1 F. R. WHATLEY AND D. I. ARNON, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. VI, Academic Press, New York, 1963, p. 308.
2 W. C. DUANE AND D. W. KROGMANN, *Biochim. Biophys. Acta*, 71 (1963) 195.
3 D. I. ARNON, *Plant Physiol.*, 24 (1949) 1.
4 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.

5 M. STILLER AND B. VENNESLAND, *Biochim. Biophys. Acta*, 60 (1962) 562.
6 O. LINDBERG AND L. ERNSTER, in D. GLICK, *Methods of Biochemical Analysis*, Vol. III, Academic Press, New York, 1956, p. 1.
7 M. AVRON AND A. T. JAGENDORF, *J. Biol. Chem.*, 234 (1959) 967.
8 P. V. BLAIR, T. ODA, D. E. GREEN AND H. FERNÁNDEZ-MORÁN, *Biochemistry*, 2 (1963) 756.
9 B. SACKTOR, J. J. O'NEILL AND D. G. COCHRAN, *J. Biol. Chem.*, 233 (1958) 1233.
10 B. C. PRESSMAN AND H. A. LARDY, *Biochim. Biophys. Acta*, 21 (1956) 458.
11 B. D. POLIS AND H. W. SHMUKLER, *J. Biol. Chem.*, 227 (1957) 419.
12 M. E. PULLMAN AND E. RACKER, *Science*, 123 (1956) 1105.
13 P. BORST, J. A. LOOS, E. J. CHRIST AND E. C. SLATER, *Biochim. Biophys. Acta*, 62 (1962) 509.
14 S. FLEISCHER, A. CASU AND B. FLEISCHER, *Federation Proc.*, 23 (1964) 486.
15 B. FLEISCHER, A. CASU AND S. FLEISCHER, *Biochem. Biophys. Res. Commun.*, 24 (1966) 189.
16 R. E. MCCARTY AND A. T. JAGENDORF, *Plant Physiol.*, 40 (1965) 725.
17 C. POLGE, A. SMITH AND A. S. PARKES, *Nature*, 164 (1949) 666.
18 L. PACKER AND A. C. BARNARD, *Biochim. Biophys. Acta*, 126 (1966) 443.