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Photophosphorylation During Chloroplast Development in Red Kidney Bean. I. Characterization of the Mature System and the Effect of BSA and Sulfhydryl Reagents[1, 2]

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Abstract. Optimal conditions were determined for photophosphorylation and reduction in mature chloroplasts from *Phaseolus vulgaris* var. Red Kidney. Bovine serum albumin (BSA) at 1 mg/ml and various sulfhydryl reagents (0.1–0.5 mM) greatly enhanced cyclic and noncyclic phosphorylation, but had little effect on photoreduction. BSA and reduced glutathione also stimulated cyclic phosphorylation in spinach chloroplasts. BSA was needed in the reaction from the start to provide high rates of phosphorylation. BSA also protected against atebrin uncoupling but not against uncoupling by ammonium ions or inhibition by 3,-(3,4-dichlorophenyl)-1,1-dimethylurea. Similarly, BSA and glutathione protected against atebrin inhibition of cyclic phosphorylation. Chloroplasts incubated at 0° rapidly lost the ability to catalyze phosphorylation and BSA did not protect against inactivation.

A definitive study of coupled phosphorylation during chloroplast development has not as yet been undertaken. Dodge and Whittingham (13) followed the onset of cyclic phosphorylation along with photoreduction during chloroplast formation in flax, but noncyclic phosphorylation was not investigated. A study of this type requires a plant in which chloroplast development can be controlled and the plastids readily isolated. Although experiments involving photophosphorylation are usually performed with chloroplasts from spinach and Swiss chard, these plants are not suitable for developmental studies. Therefore, Red Kidney bean was selected as the experimental plant since beans could be grown with relative ease in the laboratory, the structural development of the bean chloroplast had been detailed and described (9, 21, 22), and the rates of phosphorylation obtained in initial experiments with mature chloroplasts appeared quite adequate for a developmental study. However, prior to examining photophosphorylation during development, the mature system was characterized.

In a preliminary communication (19) we noted the stimulatory effect of bovine serum albumin on the rates of photophosphorylation in isolated chloroplasts from Red Kidney bean. We also indicated that the effect of BSA[4] could be largely replaced by various sulfyhdryl reagents. Independently, Friedlander and Neumann (15) have also shown that serum albumin stimulated various photoreactions in isolated bean and lettuce chloroplasts and they suggested that it acted primarily by binding free unsaturated fatty acids. Other workers (34) have shown that BSA was effective in preserving photochemical activity in isolated spinach chloroplasts. In this paper, we describe conditions for optimal rates of photophosphorylation in isolated Red Kidney bean chloroplasts and further characterize the BSA and sulfhydryl effects.

Materials and Methods

Primary leaves of *Phaseolus vulgaris* var. Red Kidney (Agway Incorporated, Syracuse, New York) were obtained from plants grown in darkness for 8 days and subsequently exposed to light for 3 to 8 days. Germination in the dark was carried out at room temperature in sterilized wooden flats of vermiculite kept moist with distilled water. After 8 days the flats were transferred to a Percival growth chamber maintained at 23° and illuminated with 300 ft-c of light produced by cool white fluorescent lamps augmented with incandescent bulbs. Chloro-

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[2] The data are taken from a dissertation submitted by C. D. Howes to the graduate faculty of the University of Massachusetts in partial fulfillment of the requirements for the Ph.D. degree.

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[4] Abbreviations used: BSA, bovine serum albumin; DCMU, 3-(3,4-dichlorophenyl)-1, 1-dimethylurea; PCMB, *p*-chloromercuribenzoic acid; PHMB, *p*-phydroxymercuribenzoate; PMS, phenazine methosulfate.

plasts were prepared by grinding 5 to 10 g of primary leaves at 0 to 4° in a Waring Blendor for 25 sec at top speed in a medium containing 0.4 M sucrose, 0.05 M tris, and 0.01 M NaCl (pH 8.0) (STN). The homogenate was squeezed through a double layer of cheesecloth and was subjected to differential centrifugation at 0 to 2° as follows: after an initial centrifugation of 90 sec at 600*g*, the supernatant was centrifuged for 5 min at 1000*g*; the pellet was resuspended in STN and resedimented at 1000*g* for 5 min; finally, the washed chloroplast pellet was resuspended in 3 to 5 ml STN at a concentration of 50 to 150 μg chlorophyll/ml.

Light reactions were carried out in a stainless steel glass-sided aquarium (42 × 16 × 12 cm) similar to the one described by Avron (3) which was illuminated on each side by 2 Westinghouse 300w reflector spot lamps. Temperature was maintained at 24° with a thermostatically controlled circulating water pump. The reaction usually was allowed to proceed for 2 min in the light and was terminated by turning the light off and adding 0.3 ml of 30% (w/v) trichloroacetic acid. After centrifugation, aliquots of the supernatants were assayed for radioactive phosphate incorporation and photoreduction.

The standard reaction mixture contained in μmoles: tris (pH 8.0), 45; $MgCl_2$, 8; ADP, 4; Na, K phosphate (pH 8.0), 24; BSA where included, 0.045 (3 mg); PMS, 0.06 or $K_3Fe(CN)_6$, 1.5; sufficient $^{32}PO_4^{3-}$ (1–4 × 10^6 cpm); chloroplasts containing 24 to 45 μg chlorophyll, and water to a total volume of 3 ml.

For time course studies, the contents of the standard reaction mixture were increased 4-fold and at the specified times, 1 ml aliquots were removed with a long-nosed calibrated syringe and injected into tubes containing 0.1 ml 30% (w/v) trichloroacetic acid. After centrifugation, 0.7 ml of the supernatant was analyzed for radioactive phosphate incorporation.

The incorporation of ^{32}P into organic phosphate was measured by the method of Avron (3) and the reduction of ferricyanide by the colorimetric procedure of Avron and Shavit (6). Rates are expressed as net incorporation or reduction, *i.e.*, light minus dark controls. Chlorophyll was determined by the method of Arnon (2).

Bovine serum albumin, ADP, PMS, PCMB, and PHMB were purchased from Sigma Chemical Company, St. Louis, Missouri. Atebrin (quinacrine dihyrochloride) was obtained from Mann Research Laboratories, New York, New York. ^{32}P was purchased from New England Nuclear Corporation, Boston, Massachusetts. DCMU was a generous gift from Drs. P. B. Sweetser and R. W. Luckenbaugh, du Pont de Nemours, Incorporated, Wilmington, Delaware.

Results and Discussion

Requirement for Phosphate, ADP, and $MgCl_2$. No significant activity was detected in the absence of phosphate, ADP or $MgCl_2$. Optimal concentrations determined for each were, 8 mM phosphate; 1 mM ADP, and 1 mM $MgCl_2$. Sodium chloride was not added to the reaction mixture since the medium used to prepare the plastids (STN) contained sufficient salt to result in a final concentration of approximately 0.3 mM NaCl on adding chloroplast aliquots to the reaction tubes. Adding additional salt (up to 50 mM NaCl) had no effect on activity. The inclusion of $NaHCO_3$ (up to 100 mM) in the reaction mixture also had no effect on activity.

Stimulation of Photophosphorylation by BSA. The presence of BSA (1 mg/ml) in the reaction mixture markedly stimulated cyclic phosphorylation with PMS and noncyclic phosphorylation with ferricyanide as the electron acceptor (Fig. 1). Although a 3-fold enhancement of both types of phosphorylation was noted, BSA had little effect on the rates of photoreduction of ferricyanide (Fig. 1).

A time course study of cyclic phosphorylation indicated that within the shortest time tested (2 min), BSA stimulated the initial rate of phosphate incorporation. However, the stimulated rate was only maintained for several min more in the presence of the protein since the reaction leveled off after

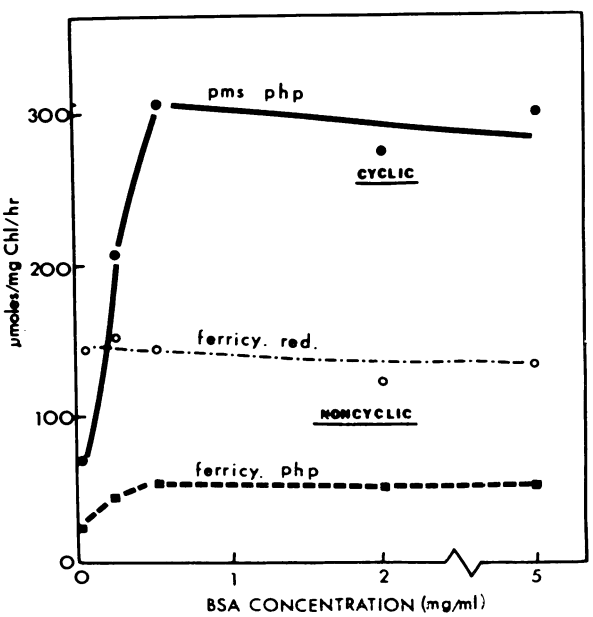

FIG. 1. The effect of BSA on photophosphorylation and photoreduction. Standard reaction conditions as described in Methods except that the reaction time with ferricyanide was 5 min rather than 2 min. Control values in the absence of BSA were: 64 (PMS phosphorylation); 147 (ferricyanide reduction), and 14 (ferricyanide phosphorylation). At 0.5 mg/ml BSA the P/e_2 was 0.76.

5 min without BSA and after 8 min with BSA. For this reason, reaction times of between 2 and 5 min were routinely employed. Ascorbate (4–100 mM) could not replace BSA in enhancing phosphorylation in similar experiments. In fact, ascorbate was found to be inhibitory which is in contrast to the stimulation of cyclic phosphorylation by ascorbate observed in spinach (31, 36) and Swiss chard (3). Also, adding 20 mM ascorbate to the isolating medium (STN) resulted in no increase in photochemical activity.

The effect of adding BSA to the reaction tubes before and after illumination is illustrated in Fig. 2. BSA was required from the start of the reaction, since it no longer promoted phosphorylation when added after 15 sec of light exposure. Incubating the chloroplasts with BSA for up to 4 min in the dark prior to light exposure, resulted in only a slight additional stimulation in activity. For example in a typical experiment, in the absence of BSA a specific activity of 134 was obtained; introducing BSA at zero time increased the specific activity to 571, while preincubating with BSA in the dark for 4 min resulted in a specific activity of 602. However, no additional increase in activity was observed when BSA (0.5 mg/ml) was also included in the preparation medium (STN). Higher concentrations

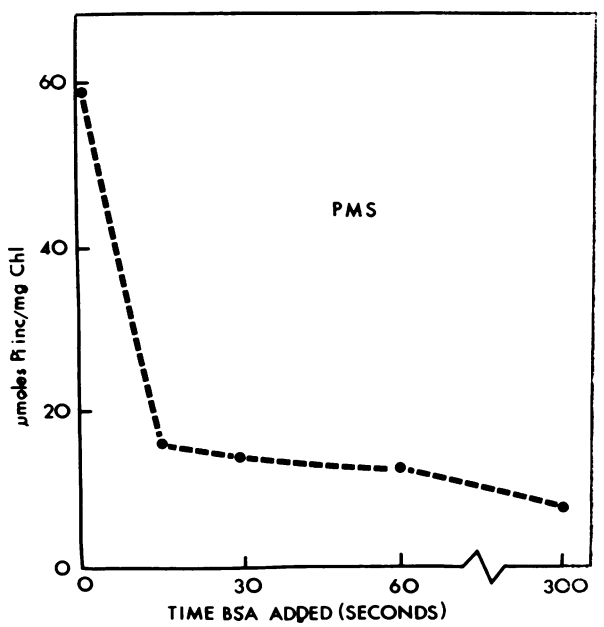

FIG. 2. The effect of adding BSA to the reaction mixture at various times in the light. Several tubes containing the standard reaction mixture for measuring cyclic phosphorylation were exposed to light to initiate the reaction and BSA (1 mg/ml) was then added to the various tubes at the times indicated. All tubes received a total of 10 min (600 sec) of light before they were assayed for activity; otherwise, standard reaction conditions.

of BSA (10 mg/ml) when added to the preparation medium inhibited phosphorylation. It was also found that boiling BSA at 100° for 5 to 10 min did not increase its ability to promote phosphorylation (table I).

These experiments suggest that BSA may be preventing the light inactivation or may be inhibiting the rate of decay of a factor(s) required for photophosphorylation. Friedlander and Neumann (15) also reported that BSA was only effective when added to their chloroplast preparations prior to illumination and concluded from their experiments that BSA was inhibiting the rate of decay of a high energy intermediate produced in the light. Even if this were so, the high energy intermediate should be continually regenerated in the light and BSA would be expected to affect its decay rate whenever it was introduced into the reaction. Since it was observed that activity was irreversibly lost in the initial absence of BSA (Fig. 2), it is unlikely that this is the sole explanation for the BSA promotion of phosphorylation.

The effect of BSA on chloroplast swelling was examined since BSA was found to reduce the rate of spontaneous swelling of animal and plant mitochondria and the associated loss of membrane integrity (14, 35, 37), and it could also affect the conformational changes observed in chloroplasts in the presence of oleate (30). Two experiments were performed in which the length and width of several hundred Red Kidney bean chloroplasts were measured with an optical micrometer at 1000 X and no difference was noted in the degree of swelling in the light in the presence or absence of BSA. However, the final size of the plastids with BSA was 10 % smaller than controls incubated without the protein due to initial shrinkage in the dark of the plastids with BSA. Since BSA did not inhibit the light-induced swelling in these experiments, we do not believe that the swelling phenomenon is directly related to the rapid loss in activity observed.

Temperature and pH. In their study with Brittlewax bean chloroplasts, Friedlander and Neumann (15) reported a difference in optimal temperature for cyclic phosphorylation in the presence

Table I. *Effect of Boiled BSA on Cyclic Phosphorylation*

A BSA solution (20 mg/ml) was boiled for 5 min at 100° and an aliquote of the resulting suspension was added where indicated. Final BSA concentrations were 1 mg/ml. Standard reaction conditions as described in Methods.

Reaction condition	Rate of phosphorylation
	μmoles P_i/mg chl·hr
Control - BSA	112
Control + BSA	448
Control + boiled BSA	465

and absence of serum albumin. In the presence of the protein, the temperature optimum was between 20° and 25° for this activity, while in the absence of the protein, the optimal temperature dropped to 15°. With Red Kidney bean chloroplasts, we did not observe the lower temperature optimum in the absence of BSA (Fig. 3).

A pH of 8 was found to be optimal for cyclic phosphorylation (PMS) and noncyclic phosphorylation and reduction (ferricyanide) in the presence or absence of BSA.

Light Intensity and Chlorophyll Concentration. Maximal rates of photophosphorylation were obtained in the presence of BSA at the highest light intensity our apparatus could provide (16,000 ft-c) with chlorophyll concentrations between 0 and 20 μg/ml. However, BSA was also effective at lower light intensities (table II). Although activity was still increasing at 16,000 ft-c in the presence of BSA, this intensity appeared to be close to light saturation. Also at high light intensities, no significant pigment bleaching was observed in the chloroplast preparations in the presence or absence of BSA until after 30 min of light exposure.

Cofactors and Nucleotide Specificity. Concentrations of 0.02 mM PMS and 0.5 mM ferricyanide were found to be optimal for cyclic phosphorylation and for noncyclic phosphorylation and reduction respectively. The presence of BSA did not alter the optimal concentrations for these cofactors although it stimulated phosphorylation. The highest rates of cyclic phosphorylation (PMS) obtained were over 1100 μmoles phosphate incorporated/mg chlorophyll • hr; noncyclic reduction, over 300 μmoles ferricyanide reduced/mg chlorophyll • hr, and phosphorylation (ferricyanide), 120 μmoles phosphate incorporated/mg chlorophyll • hr (P/e_2 was generally close to unity, 0.8).

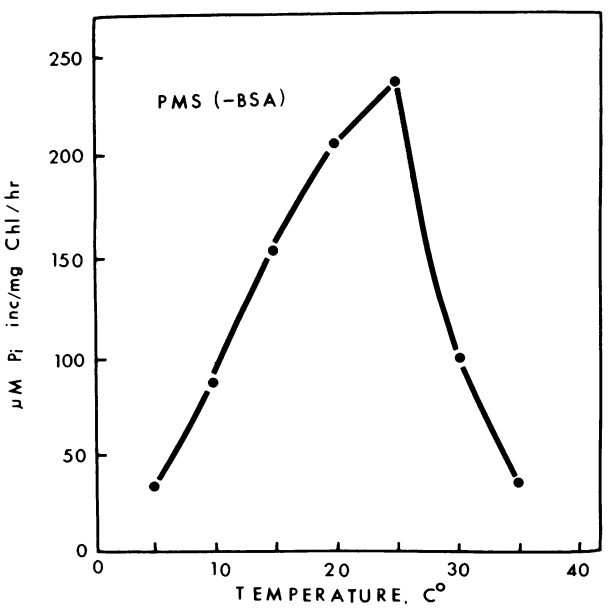

FIG. 3. The effect of temperature on cyclic phosphorylation. Standard reaction conditions except for the variation in temperature and the absence of BSA.

Table II. *Effect of Light Intensity on Cyclic Phosphorylation*

Standard reaction conditions except for the variation in light intensity. Different chloroplast preparations were used in Experiments A and B. Chlorophyll concentration in A, 9.5 μg/ml; B, 15.5 μg/ml. BSA concentration in A, 0.5 mg/ml; B, 1 mg/ml.

	Light intensity	Rate of phosphorylation — BSA	Rate of phosphorylation + BSA
	ft-c	*μmoles P_i/mg chl•hr*	
Experiment A	500	116	166
	1000	186	255
	4000	210	340
	8000	246	400
Experiment B	2000	74	196
	6000	512	635
	16000	538	858

Various nucleotide diphosphates (CDP, UDP, GDP) were substituted for ADP in the reaction mixture. Significant rates of phosphorylation were only observed in the presence of ADP or GDP and these were equally effective. Again, the presence of BSA did not alter the specificity for the nucleotide diphosphate acceptor, although activity was greatly stimulated.

The rates of photophosphorylation and reduction reported above are comparable to those previously found for bean (15, 26, 27) and other plants such as spinach (20, 23) and Swiss chard (3). Conditions for optimal activity are also similar with regard to temperature (4, 15), pH (3, 5), light intensity (3), chlorophyll concentration (3), and nucleotide specificity (3, 8). GDP was also found to substitute for ADP as a phosphate acceptor in Swiss chard (3, 8) where the evidence suggested that it was directly esterified during photophosphorylation (8).

The Effect of Sulfhydryl Reagents. Since boiling BSA did not diminish its ability to promote phosphorylation (table I) and since it has been reported that the number of active sulfhydryl groups (0.3–0.75 per BSA molecule) did not change on denaturation (12), the possibility that BSA was acting as a sulfhydryl reagent in this system was examined. It was found that GSH, cysteine and mercaptoethanol could almost completely replace BSA in stimulating phosphorylation in bean chloroplasts (table III, A and B). Optimal concentrations for the various reagents was between 0.1 and 0.5 mM. A similar effect was noted for both BSA and GSH on phosphorylation catalyzed by spinach chloroplasts (table III, C) and the BSA and sulfhydryl effects were not additive in either system (bean or spinach).

Table III. *Effect of Sulfhydryl Reagents on Cyclic Phosphorylation*

Standard reaction conditions as described in Methods. Experiments A and B were performed with bean chloroplasts while Experiment C, utilized spinach chloroplasts isolated in an identical manner. GSH and cysteine concentrations Experiment A, 0.5 mM. Mercaptoethanol experiment B, 0.1 mM. GSH experiment C, 0.1 mM. BSA where included, 1 mg/ml.

Experiment	Added compounds (s)	Rate of phosphorylation
		μmoles P_i/mg chl·hr
A	None	483
	GSH	843
	Cysteine	840
	BSA	946
	BSA + GSH	723
	BSA+ cysteine	872
B	None	112
	2 - Mercaptoethanol	420
	BSA	448
C	None	207
	GSH	361
	BSA	480
	BSA + GSH	419

Other amino acids (alpha- or beta-alanine, asparagine, glutamate, glutamine, histidine, lysine, and proline) at concentrations up to 1 mM failed to promote phosphorylation. Only a slight stimulation in the rates of phosphorylation was noted when 0.4 M and 0.8 M sucrose was substituted for BSA in the reaction mixture. The effects of various thiol alkylating or mercaptide-forming reagents such as iodoacetate, PCMB and PHMB were also examined. Only the latter 2 exhibited inhibition at concentrations higher than 10 μM, and these appeared to act non-specifically, inhibiting phosphorylation in both the presence and absence of BSA.

The stimulation of phosphorylation by BSA cannot be directly attributed to osmotic phenomena since its concentration in the reaction tubes was quite low, 0.0145 mM [assuming a molecular weight of 69,000 (33)]. Also, sulfhydryl reagents at relatively low concentrations (0.5 mM) could replace BSA (table III) and sucrose at high molarities had little effect on activity. The fact that BSA appears to be at least 40 times more effective than thiol reagents used in this study [on the basis of SH-groups available per molecule, 0.3–0.75 (12)] indicates that the protein nature and/or size of the molecule may be important factors contributing to its action.

Previously, it has been postulated that BSA affected the activity of isolated chloroplasts by binding endogenously released fatty acids (15). This explanation was based upon the findings that BSA could bind unsaturated fatty acids (10) which inhibited phosphorylation and electron transport when added to chloroplasts (16, 24, 28). Also, a pH-dependent release of fatty acids had been observed during the aging of spinach chloroplasts which was correlated with a loss in Hill activity (11, 28). However, this loss in activity could be largely prevented by the addition of BSA (11). Similarly, serum albumin was shown to prevent the loss of Hill activity due to heating the chloroplasts or by the addition of fatty acids which were released during heating (29). The fact that various sulfhydryl reagents could substitute for BSA in promoting phosphorylation in the present study suggests that there may be an additional site of action of BSA and that it may also behave as an SH-reagent in chloroplasts.

Inhibitors and Uncouplers. Typical patterns of inhibition and uncoupling of photophosphorylation were observed with DCMU and ammonium chloride (table IV). At 0.5 μM DCMU, noncyclic phosphorylation and reduction (ferricyanide) were inhibited 84 % and 72 % respectively while cyclic phosphorylation (PMS) remained unaffected. Concentrations higher than 1 μM DCMU were required for inhibition of cyclic activity. For unexplained reasons, ammonium chloride uncoupled Red Kidney bean chloroplasts at lower concentrations (0.1 mM) than those previously reported with other plant systems (around 1.0 mM) (7, 25). At 0.1 mM ammonium chloride, ferricyanide reduction was stimulated over 200 % whereas phosphorylation was inhibited 50 %. Cyclic phosphorylation was also greatly inhibited at this concentration of NH_4Cl. The absence of BSA (results not shown) did not change the patterns of inhibition and of uncoupling observed with either inhibitor although the rates of phosphorylation were lower.

Table IV. *The Effect of DCMU and NH_4Cl on Photophosphorylation and Photoreduction*

Standard reaction conditions with the inclusion of 1 mg/ml BSA. Activities are expressed in μmoles/mg chlorophyll·hr. Different chloroplast preparations were employed in the two experiments.

	Ferricyanide		PMS
DCMU concn	Reduction	ATP formation	ATP formation
μM			
0	326	118	572
0.05	331	122	582
0.10	242	94	556
0.30	140	38	590
0.50	91	19	588
1.00	52	7	490
NH_4Cl concn			
μM			
0	193	46	455
1	204	53	458
5	199	50	454
10	240	50	401
50	308	37	247
100	417	23	87

However, BSA markedly affected the pattern of uncoupling found in the presence of atebrin (Fig. 4 A & B). When BSA was omitted from the reaction, atebrin at concentrations higher than 1 μM showed typical uncoupling of photophosphorylation (Fig. 4B). For example, 10 μM atebrin stimulated reduction 200 % while completely inhibiting phosphorylation. On the other hand, when BSA was included, the same concentration of atebrin had no effect on reduction, and phosphorylation was only inhibited 40 % (Fig. 4B). Completely inhibiting phosphorylation by adding higher atebrin concentrations (50 μM) still had no effect on reduction when BSA was included in the reaction mixture. Atebrin stimulation of reduction is lowered by BSA in the absence of phosphorylation (Fig. 4A) although maximum resistance to atebrin uncoupling was noted only when a phosphate acceptor system and BSA were both included in the reaction mixture (Fig. 4B).

BSA also protected against inhibition of cyclic phosphorylation by atebrin (Fig. 5A). In the presence of BSA, a 10-fold higher concentration of atebrin (50 μM) was required to completely inhibit cyclic phosphorylation. Since it was found that various thiol reagents could replace BSA in stimulating photophosphorylation, GSH was tested to determine whether it could also substitute for BSA in protecting against atebrin inhibition of cyclic phosphorylation (Fig. 5B). Indeed, it was found that GSH could provide comparable protection against atebrin inhibition. For example, at 10 μM atebrin in the BSA experiment (Fig. 5A), the control was completely inhibited while the tubes with BSA still exhibited 55 % activity; at the same atebrin concentration in the GSH experiment (Fig. 5B), the control was inhibited 75 % while the tubes with GSH also exhibited 55 % activity. However, the concentration at which atebrin completely inhibited phosphorylation was not extended by GSH, reflecting the higher resistance of the controls to atebrin in this experiment as compared to the BSA experiment.

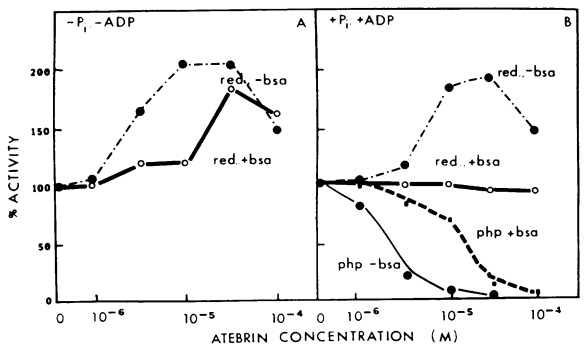

FIG. 4. Atebrin uncoupling in the presence and absence of photophosphorylation and BSA. A) Standard reaction conditions except P_i and ADP were omitted from the reaction mixture. BSA where included, 1 mg/ml. B) Standard reaction conditions. BSA where included, 1 mg/ml. Noncyclic activities with ferricyanide are shown as the percentage of the activity observed in controls conducted in the absence of atebrin under identical conditions. The specific activities of the controls in Experiment A were, 82 (—BSA) and 81 (+BSA). In Experiment B, the control specific activities were as follows: 115 (reduction, —BSA); 156 (reduction, +BSA); 76 (phosphorylation, —BSA), and 145 (phosphorylation, +BSA). All values were determined with aliquots from the same chloroplast preparation.

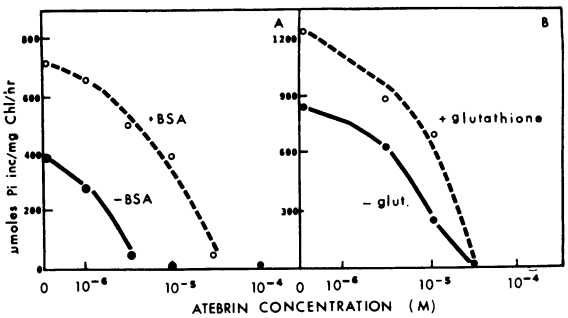

FIG. 5. Resistance to atebrin inhibition of cyclic phosphorylation in the presence of BSA and glutathione. A) Standard reaction conditions. BSA where included, 1 mg/ml. B) Standard reaction conditions. Glutathione where included, 2.5 mM. Different chloroplast preparations were used in A and B.

To check the possibility that BSA and GSH were not inactivating or removing atebrin by complexing it, the spectrum of atebrin was examined under a variety of conditions. In water, atebrin exhibited a major broad peak in the ultraviolet at about 270 nm and minor peaks in the near-ultraviolet and visible at 340, 357, 420, and 444 nm (the ratio of 270:420 nm was approximately 6.5). When atebrin (10 μM) was introduced into the standard reaction mixture, in which it was shown to be effective as an inhibitor (Fig. 4b and 5), a shift in the ultraviolet portion of the spectrum was noted. The broad band at 270 nm was converted to a much sharper peak with a lower extinction at 283 nm. However, there were no changes in the minor peaks in the near-ultraviolet and visible regions of the spectrum and the ratio of 283:420 nm was approximately 4.5. No additional changes in the spectrum were noted when BSA (1 mg/ml) or GSH (2.5 mM) were added in the presence or absence of chloroplasts (9–16 μg/ml) and/or light (2 min, 16,000 ft-c). The spectral shift was apparently caused by ADP in the reaction mixture since no change was observed in the absence of ADP, while a shift could be immediately induced by adding the nucleotide diphosphate. In similar experiments performed in the absence of ADP in the reaction mixture, under conditions in which atebrin was shown to stimulate photoreduction (Fig. 4A), no spectral changes were observed with or without chloroplasts when adding BSA or GSH in the presence or absence of light.

Although the spectral data revealed an unexpected interaction between ADP and atebrin, they did not indicate a similar interaction between atebrin and BSA or GSH, at least under the conditions tested. Apparently, the interaction between atebrin and ADP did not impair atebrin uncoupling or inhibition or photophosphorylation (Figs. 4B and 5). Atebrin may be uncoupling photophosphorylation in Red Kidney bean chloroplasts by affecting a specific thiol-sensitive site and the protection noted in the presence of BSA and GSH could be explained as competition for this site.

The fact that BSA had no effect on uncoupling by ammonium chloride (table IV), suggests that atebrin and ammonium ions are acting at different sites. This conclusion is also supported by the finding of others that although both uncouplers increase the rate of decay of a high energy intermediate formed in the light (18), ammonium ions inhibited 2 stage photophosphorylation when added either in the light or dark, while atebrin primarily affected a dark stage in this process (17). However in the latter study, a possible light-driven inactivation or complexing of atebrin was not taken into consideration (18). It has also been observed that chloroplasts uncoupled with ammonium ions show a decrease in light scattering due to swelling, while chloroplasts uncoupled with atebrin exhibit an increase in scattering due to shrinking when exposed to light (5, 18).

Dark Decay of Phosphorylation. It became apparent early in this study that chloroplasts did not retain photochemical activity when incubated in the dark at 0° for extended periods (table V). Within 2 hr, the chloroplast preparations no longer exhibited significant rates of phosphorylation (table V, 105 min). The presence of BSA in the preparation medium (0.5 mg/ml), the reaction tubes (1 mg/ml) or both, did not prevent the loss of activity in the dark at 0° (table V). Higher concentrations of BSA in the preparation medium (10 mg/ml) were inhibitory. Also, adding BSA to the preparation medium as well as to the reaction mixture, resulted in no further stimulation in phosphorylation (table V, 0 min).

Table V. *Dark Decay of Cyclic Phosphorylation at 0°*

Chloroplasts were isolated in the usual manner except for the inclusion of 0.5 mg/ml BSA in the preparation medium (STN) where indicated. The chloroplasts were incubated in the dark at 0° and assayed for activity at the times shown. Standard reaction conditions as described in Methods. Final BSA concentrations where included in the reaction mixture, 1 mg/ml.

Preparation medium	Reaction mixture	Incubation time 0	30 min	105 min
		μmoles P_i/mg chl·hr		
—BSA	—BSA	287	215	5
+BSA	—BSA	305	223	9
+BSA	+BSA	380	318	30
—BSA	+BSA	402	352	14

A similar loss of Hill activity in bean chloroplasts has been observed by Anderson and Boardman (1). The inactivation may be due to inhibitors released during the isolation of the chloroplasts (26, 28) such as fatty acids, since bean leaves are known to contain enzymes which hydrolyze galactolipids (28, 32). However, the failure of BSA to preserve activity as reported in spinach (34) (presumably by binding free fatty acids although the authors found no evidence for such a mechanism) suggests additional unknown factors may be involved.

Perhaps the most significant observation in this study was the promotion of photophosphorylation noted in the presence of BSA and sulfhydryl reagents. Although Friedlander and Neumann (15) reported a similar effect by BSA on various photoreactions catalyzed by Brittle wax bean and lettuce chloroplasts, they did not include thiol reagents in their experiments. Our findings also differ from theirs in several other important respects. For example, we did not observe a stimulation by BSA on photoreduction which they reported (Fig. 1). Also, we did not find a change in the optimal temperature for activity in the absence of BSA (Fig. 3); no requirement was observed for BSA in the homogenizing medium as well as in the reaction mixture for optimal rates of phosphorylation (table V), and we failed to observe a stimulation of photoreduction by atebrin in the presence of BSA (Fig. 4). However, it should be pointed out that we employed another variety of bean and subjected our plants to different growth conditions and light regimes. Our plants were routinely germinated and maintained in the dark prior to light exposure. While their plants were germinated in the light. Also, for chloroplast development, our plants were subjected to continuous illumination, while they employed a 12 hr photoperiod. It is conceivable that these differences may have affected the response of the chloroplasts to BSA.

Having characterized photophosphorylation in the mature Red Kidney bean chloroplast and having determined the optimum conditions for the cyclic and noncyclic processes we can now proceed to examine these activities during chloroplast development.

Literature Cited

1. Anderson, J. M. and N. K. Boardman. 1964. Studies on the greening of dark-grown bean plants. II. Development of photochemical activity. Australian J. Biol. Sci. 17: 93–101.
2. Arnon, D. I. 1949. Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. Plant Physiol. 24: 1–15.
3. Avron, M. 1960. Photophosphorylation by Swiss-

chard chloroplasts. Biochim. Biophys. Acta 40: 257–72.

4. AVRON, M. 1962. Light-dependent adenosine triphosphatase in chloroplasts. J. Biol. Chem. 237: 2011–17.
5. AVRON, M. AND J. NEUMANN. 1968. Photophosphorylation in chloroplasts. Ann. Rev. Plant Physiol. 19: 137–66.
6. AVRON, M. AND N. SHAVIT. 1963. A sensitive and simple method for determination of ferrocyanide. Anal. Biochem. 6: 549–54.
7. AVRON, M. AND N. SHAVIT. 1963. On the coupling of photophosphorylation to electron transport. In: Photosynthetic Mechanisms in Green Plants. Natl. Acad. Sci. Natl. Res. Council, Publ. 1145, p 611.
8. BENNUN, A. AND M. AVRON. 1965. The relation of the light-dependent and light-triggered adenosine triphosphatases to photophosphorylation. Biochim. Biophys. Acta 109: 117–27.
9. BOARDMAN, N. K. AND J. M. ANDERSON. 1964. Studies on the greening of dark-grown bean plants. I. Formation of chloroplasts from proplastids. Australian J. Biol. Sci. 17: 86–92.
10. BOYER, P. D., G. A. BALLOU, AND J. M. LUCK. 1947. The combination of fatty acids and related substances with serum albumin. III. The nature and extent of the combination. J. Biol. Chem. 167: 407–24.
11. CONSTANTOPOULOS, G. AND C. N. KENYON. 1968. Release of free fatty acids and loss of Hill activity by aging spinach chloroplasts. Plant Physiol. 43: 531–36.
12. DIEZ, M. J. F., D. T. OSUGA, AND R. E. FEENEY. 1964. The sulfhydryls of avian ovalbumins, bovine β-lactoglobulin, and bovine serum albumin. Arch. Biochem. Biophys. 107: 449–58.
13. DODGE, A. D. AND C. P. WHITTINGHAM. 1966. Photochemical activity of chloroplasts isolated from etiolated plants. Ann. Botany 30: 711–19.
14. EARNSHAW, M. J. AND B. TRUELOVE. 1968. Swelling and contraction of *Phaseolus* hypocotyl mitochondria. Plant Physiol. 43: 121–92.
15. FRIEDLANDER, M. AND J. NEUMANN. 1968. Stimulation of photoreactions of isolated chloroplasts by serum albumin. Plant Physiol. 43: 1249–54.
16. FRIEND, J. AND D. M. HAWCROFT. 1967. Carotenoids and fatty acids as uncouplers of photophosphorylation in isolated chloroplasts. Biochem. J. 104: 60 p.
17. GROMET-ELHANAN, Z. AND M. AVRON. 1965. Effect of inhibitors and uncouplers on the separate light and dark reactions in photophosphorylation. Plant Physiol. 40: 1053–59.
18. HIND, G. AND A. T. JAGENDORF. 1965. Effect of uncouplers on the conformational and high energy states of chloroplasts. J. Biol. Chem. 240: 3202–09.
19. HOWES, C. D. AND A. I. STERN. 1967. Photophosphorylation in mature and developing chloroplasts of Red Kidney bean. J. Cell. Biol. 35: 60a.
20. JAGENDORF, A. T. AND M. AVRON. 1958. Cofactors and rates of photosynthetic phosphorylation by spinach chloroplasts. J. Biol. Chem. 231: 277–90.
21. KLEIN, S., G. BRYAN, AND L. BOGORAD. 1964. Early stages in the development of plastid fine structure in red and far-red light. J. Cell. Biol. 22: 433–42.
22. KLEIN, S. AND J. NEUMANN. 1966. The greening of etiolated bean leaves and the development of chloroplast fine structure in absence of photosynthesis. Plant Cell Physiol. 7: 115–23.
23. KROGMANN, D. W. AND A T. JAGENDORF. 1959. Comparison of ferricyanide and 2,3'6-trichlorophenol indophenol as Hill reaction oxidants. Plant Physiol. 34: 277-82.
24. KROGMANN, D. W. AND A. T. JAGENDORF. 1959. Inhibition of the Hill reaction by fatty acids and metal chelating agents. Arch. Biochem. Biophys. 80: 421-30.
25. KROGMANN, D. W., A. T. JAGENDORF, AND M. AVRON. 1959. Uncouplers of spinach chloroplast photosynthetic phosphorylation. Plant Physiol. 34: 272–77.
26. MARGULIES, M. M. 1966. Concerning the preparation of chloroplasts active in Hill and photosynthetic phosphorylation activities from leaves of *Phaseolus vulgaris*. Plant Physiol. 41: 1320–22.
27. MARGULIES, M. M. AND A. T. JAGENDORF. 1960. Effect of cold storage of bean leaves on photosynthetic reactions of isolated chloroplasts. Arch. Biochem. Biophys. 90: 176–83.
28. MCCARTY, R. E. AND A. T. JAGENDORF. 1965. Chloroplast damage due to enzymatic hydrolysis of endogenous lipids. Plant Physiol. 40: 725-35.
29. MOLOTKOVSKY, Y. G. AND I. M. ZHESTKOVA. 1965. The influence of heating on the morphology and photochemical activity of isolated chloroplasts. Biochem. Biophys. Res. Commun. 20: 411–15.
30. MOLOTKOVSKY, Y. G. AND I. M. ZHESTKOVA. 1966. Morphological and functional changes in isolated chloroplasts under the influence of oleate. Biochim. Biophys. Acta 112: 170–72.
31. OHMURA, T. 1958. Photophosphorylation by chloroplasts. J. Biochem. (Tokyo) 45: 319–31.
32. SASTRY, P. S. AND M. KATES. 1964. Hydrolysis of monogalactosyl and digalactosyl diglycerides by specific enzymes in runner bean leaves. Biochemistry 9: 1280–87.
33. SCATCHARD, G., A. L. BATCHELDER, AND A. BROWN. 1946. Preparation and properties of serum and plasma proteins. VI. Osmotic equilibria in solutions of serum albumin. J. Am. Chem. Soc. 68: 2320–29.
34. WASSERMAN, A. R. AND S. FLEISCHER. 1968. The stabilization of chloroplast function. Biochim. Biophys. Acta 153: 154–69.
35. WEINBACH, E. C., H. SHEFFIELD, AND J. GARBUS. 1963. Restoration of oxidative phosphorylation and morphological integrity to swollen uncoupled rat liver mitochondria. Proc. Natl. Acad. Sci. 50: 561–68.
36. WESSELS, J. S. C. 1958. Studies on photosynthetic phosphorylation. II. Photosynthetic phosphorylation under aerobic conditions. Biochim. Biophys. Acta 29: 113–23.
37. WOJTCZAK, L. AND A. L. LEHNINGER. 1691. Formation and disappearance of an endogenous uncoupling factor during swelling and contraction of mitochondria. Biochim. Biophys. Acta 51: 442–56.