PHOTOPHOSPHORYLATION BY SWISS-CHARD CHLOROPLASTS

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SUMMARY

1. The preparation of chloroplasts and chloroplast fragments from swiss-chard, which were active in photophosphorylation is described. A chloroplast preparation stable for 24–48 h could be obtained.

2. A sensitive assay for the ATP formed was adopted. It allowed the measurement of very small amounts of ATP formed, and avoided several difficulties encountered in other assays.

3. The requirements in the homogenizing medium and in the reaction mixture were studied. Factors studied included: ascorbate, gas phase, pH, cofactors, magnesium, phosphate, nucleotide specificity and light intensity.

4. The chloroplasts could phosphorylate ADP, GDP, or IDP at high rates, UDP at low rates, and CDP not at all. Nucleoside diphosphokinase(s) in the preparations was demonstrated.

5. No adenyl kinase activity could be found in washed or fragmented swiss-chard chloroplasts.

6. Rates of 2000–2500 μmoles ATP formed/mg chlorophyll/h were obtained under optimal conditions, which included low chloroplast concentrations, short reaction times, appropriate ascorbate concentration, and very high light intensities (approx. 150,000 lux).

7. Confirmation was presented for the ineffectiveness of 2,4-dinitrophenol and effectiveness of ammonium ions as uncoupling agents, and the lack of exchange between inorganic phosphate and ATP in swiss-chard chloroplasts.

8. No exchange between ADP and ATP could be demonstrated.

INTRODUCTION

Photophosphorylation catalyzed by particulates isolated from higher plants was first demonstrated by ARNON *et al.*[1], and reviewed by him[2]. It is believed to be represented by the equations:

$$H_2O \xrightarrow{\text{light, chloroplasts}} (H) + (OH) \qquad (1)$$

Abbreviations to be used include: Pi, inorganic phosphate; AMP, adenosine monophosphate; ADP, adenosine diphosphate; ATP, adenosine triphosphate; GDP, guanosine diphosphate; IDP, inosine diphosphate; UDP, uridine diphosphate; CDP, cytidine diphosphate; Tris, tris-(hydroxymethyl)aminomethane; PMS, phenazine metho-sulfate; FMN, flavin mono-nucleotide; TCA, trichloroacetic acid.

$$(H) + (OH) + ADP + Pi \xrightarrow{Mg,\ cofactor} H_2O + ATP + OH^- \qquad (2)$$
$$\text{Total: } ADP + Pi \longrightarrow ATP + OH^-$$

Almost all the work conducted in the elucidation of the requirements, potentialities, mechanism, and function of the system utilized spinach particulates[1-10].

Several considerations prompted the present investigation with chloroplasts and chloroplast fragments from swiss-chard. It was thought desirable to make a thorough study of the photophosphorylative capacity of chloroplasts from a different plant so as to emphasize the similarities and differences which one may find in a reaction which is considered to be a basic one, common to all plants[11]. In addition, since spinach is a long-day plant[12], it will flower and terminate leaf production as soon as the critical day length is reached. This makes its growing in a greenhouse for leaf material difficult during a substantial part of the year. Swiss-chard on the other hand, is quite insensitive to day-length and can continuously supply leaves for very long periods of time. Thirdly, chloroplasts isolated from spinach possess certain disadvantages, which it was hoped, might be overcome by the use of chloroplasts isolated from a different plant. Two such disadvantages—the fast decrease of activity with storage time at 0°, and the adherence of the adenyl-kinase activity to the chloroplasts—were indeed overcome by the use of these chloroplasts.

METHODS

Preparation of chloroplasts

Chloroplasts are generally prepared by grinding the leaves by hand in a mortar and pestle, followed by differential centrifugation[1-10]. It was thought desirable to have a more standardized grinding procedure and thus several alternative methods were tried. The Virtis "45" homogenizer (Virtis Co., 160 Ashburton Ave., Yonkers, New York), under optimal conditions, was found to provide chloroplasts which were just as active as those prepared by mortar and pestle grinding, and was consequently adopted in the standard preparatory technique.

In addition, it was thought desirable to sediment the chloroplasts within the smallest range of gravity forces practicable. The usual range of 200–1000 $\times$ *g* was therefore narrowed down to 500–1000 $\times$ *g* with only a minor loss of chlorophyll containing material.

The standard procedure adopted consisted of the following stages: 5 g of a leaf, whose midrib had been removed, was cut into small pieces (approx. 1 $\times$ 1 cm in size) and put within a 100 ml capacity Virtis cup, placed in the ice water bath of the instrument. 40 ml of chilled homogenizing medium (0.4 *M* sucrose, 0.05 *M* Tris, 0.01 *M* NaCl, 0.02 *M* sodium ascorbate, pH 7.8) were added and the homogenizer run for 5 sec at 50 on its variac, followed by 25 sec with the variac set on 20. The slurry was squeezed through a double layer of cheese cloth and centrifuged at 0–2° for 90 sec at 500 $\times$ *g*. The upper, light colored, layer was sucked off and the supernatant centrifuged for 7 min at 1000 $\times$ *g*. The pellet from this centrifugation was sometimes resuspended in a small volume (2–3 ml) of homogenizing medium and used directly (unwashed chloroplasts). Usually it was washed by resuspension in 40 ml of the homogenizing medium and recentrifugation at 500 $\times$ *g* for 90 sec, followed by transfer of the supernatant and its centrifugation at 2000 $\times$ *g* for 7 min. The pellet was resuspended in 2–3 ml of homogenizing medium (once washed chloroplasts).

For the preparation of chloroplast fragments the latter pellet was resuspended in 40 ml of 10^{-2} *M* Tris, pH 7.8, put within the Virtis homogenizer and homogenized for 5 min with the variac set at 0, at 0–2°. The resulting suspension was centrifuged for 2 min at 2000 × *g*, the supernatant transferred and recentrifuged at 10,000 × *g* for 7 min. The pellet of the latter centrifugation was resuspended in a small volume of homogenizing medium and used (chloroplast fragments).

Reaction conditions

The standard reaction mixture contained in μmoles: Tris, pH 7.8 – 45; NaCl, 60; $MgCl_2$, 12; Na ascorbate, 30; Na, K phosphate, pH 7.8 – 12; adequate amounts of ^{32}P ($5 \cdot 10^4$–$2 \cdot 10^5$ counts/min); ADP, 12; phenazine methosulphate, 0.09, chloroplasts or chloroplast fragments containing less than 30 μg chlorophyll, and water to a total volume of 3.0 ml. The reaction was started by turning the light on, and terminated by turning the light off and adding 0.3 ml of 20 % trichloroacetic acid to the reaction mixture. The flask contents were transferred to small centrifuge tubes, and after centrifugation a sample of the supernatant was analyzed for $AT^{32}P$ as described below.

The reaction was carried out in one of two ways: (a) In an illuminated Aminco-Warburg apparatus, fitted with two rows of 150 W Mazda spot lights (4 to a row). The reaction mixture was placed in 50-ml Erlenmeyer flasks, mounted on a metal rod connected to the Warburg shaker, and shaken at 100 rev./min with an amplitude of 4 cm. For anaerobic runs, the flasks were closed with rubber stoppers fitted with glass tubing and nitrogen gas was continuously run through the flasks. The flask bottoms were approximately 17 cm from the light. Maximum light intensity at flask level in this setup was 45,000 lux. (b) For higher light intensities an aquarium was constructed (14 × 16 × 8 cm) with an inlet and an outlet for a continuous flow of water. Two of the sides were made of glass, and the aquarium was placed between two 150 W Mazda lamps, 10 cm from each other. Water flowing through it from the Warburg bath maintained the temperature throughout the experiments at 16–17°. 4 spectrophotometer cuvettes (3 ml capacity) were placed within a holder especially contructed to allow full illumination of the two optical surfaces. The holder was placed within the aquarium, and reaction started by turning the light on. Maximum light intensity in the cuvette under these conditions was 154,000 lux.

For time course studies, samples of 0.5 ml were removed at specified times and placed into test tubes containing 0.05 ml of 20 % TCA. After centrifugation, 0.3 ml of the supernatant was analysed for its $AT^{32}P$ content as described below.

Assay of ATP formation

The progress of photophosphorylation is generally followed by either measuring the change in the inorganic phosphate content of the reaction[4–7,10], or by measuring the radioactivity incorporated from inorganic ^{32}P into the organic phosphate fraction[3,8]. The first and most commonly used method possesses several disadvantages. The main ones are: (a) The initial phosphate concentration is the highest measured and thus one is starting with a high reading which decreases with time. This lowers the sensitivity and makes it very difficult to measure small amounts of Pi uptake. In addition, one cannot measure the reaction in the presence of high phosphate concentrations. (b) The addition of reducing agents, such as ascorbic acid, interfered greatly in the measurements. (c) Arsenate behaves similarly to phosphate in the color reactions

involved. The method adapted overcomes all these difficulties and is still rather simple and rapid.

The assay is based on the procedure of NIELSEN AND LEHNINGER[13]: an appropriate sample of the supernatant from the TCA denatured reaction-mixture was placed within a 18 × 150 mm O.D. tube, 1.2 ml acetone added, contents mixed and allowed to stand for 10 min. The water content of the tube was made up to 2.5 ml with water saturated with a 1/1 mixture of isobutanol and benzene. 7.0 ml of a 1/1 mixture of isobutanol and benzene saturated with water was added, and the tube contents mixed. After phase separation, 0.8 ml of molybdate reagent (made by dissolving 5 g ammonium molybdate in 40 ml of 10 N H_2SO_4 and making it up to 100 ml with water) were added to the side of the tube, the water layer gently mixed, and allowed to stand for 5 min. Each tube was now vigorously mixed for 30 sec. After phase separation the upper layer was sucked off into a trap. 0.02 ml of 0.02 M KH_2PO_4 were added to the aqueous layer, followed by 7.0 ml of the isobutanol–benzene and the tube contents vigorously mixed for 30 sec. After phase separation the upper layer was again sucked off and 1.0 ml of the water phase placed in a stainless-steel planchet and counted directly.

All mixings throughout the procedure were made with a glass rod thickened at one end to form a small ball whose diameter was slightly smaller than the diameter of the glass tube. Moving this rod up and down the tube provided very good mixing of phases and avoided the necessity of using separatory funnels.

For each reaction two extra samples were counted. One was a zero-time control for the reaction mixture. This value was substracted from all counts/min to give the corrected counts/min. The second was a sample of the supernatant from the TCA treated reaction mixture, diluted to 1.0 ml with water saturated with isobutanol–benzene, and counted directly to give the total counts/min introduced. The μmoles of ATP formed can be calculated from the following equations:

$$\mu\text{moles ATP formed} = (\text{corr. counts/min in planchet}) \times 3.3 \times \frac{(\text{volume in flask after TCA addition})}{(\text{volume used for assay})} \times \frac{(\mu\text{moles Pi in flask})}{(\text{total counts/min in flask})}$$

It was found initially that significant amounts of counts could be recovered in the zero-time sample (0.1–0.2 % of the total introduced). This could be completely overcome by first treating the ^{32}Pi as it was received in 1N HCl at 100° for 1 h, followed by the addition of acid washed charcoal and filtration. After such treatment zero-time values decreased to 0.01–0.02 % which was completely negligible in the standard assay.

Chlorophyll was determined by the modification of ARNON[16].

Materials

Phenazine methosulfate was synthesized by the method of DICKENS AND MCILLWAIN[14] according to directions kindly supplied by Dr. T. P. SINGER. All other chemicals were commercial preparations.

Swiss-chard (*Beta vulgaris*) was grown in pots in a greenhouse. 10–20 plants supplied sufficient leaves for several months of work.

RESULTS

Preparatory requirements

It was noticed early in the preparation of chloroplasts from swiss-chard that when the leaves were homogenized in the sucrose–Tris–NaCl mixture used for spinach[10] the homogenate would rapidly darken. In addition the swiss-chard chloroplasts lost their activity very fast upon standing at 0°. Both of these effects could be overcome by the inclusion of ascorbate in the homogenizing medium. Table I illustrates the stability with time, shown by chloroplasts prepared in this manner. Although not all preparations maintained activity for such long periods, they will usually maintain it for 24–48 h. 0.02 *M* ascorbate seemed optimal and was thus adopted in the standard preparative technique.

Since spinach chloroplasts never showed such stability, a comparative experiment was run using spinach and swiss-chard chloroplasts prepared in the identical manner and tested together for their activity. Table II shows that the effect was indeed, much more marked in swiss-chard chloroplasts. In the absence of ascorbate spinach chloroplasts lost activity slowly, as shown earlier[10], while chloroplasts prepared from swiss-chard lost it rapidly. The addition of ascorbate to the homogenizing medium had a marked effect in the swiss-chard chloroplasts but small to no effect in spinach.

TABLE I

EFFECT OF ASCORBATE IN HOMOGENIZING MEDIUM

Reaction conditions as described under METHODS. Reaction time, 15 min. Light intensity approximately, 40,000 lux. Chloroplasts once washed. Chlorophyll 12–18 μg/ml. Temperature, 25° gas phase–air. Chloroplasts kept at 0° in the dark between runs. All reaction mixtures contained a total of 10 μmoles ascorbate/ml.

Ascorbate in medium	*Time after homogenization at which activity was tested*					
	Hours					
M	*1*	*23*	*46*	*70*	*94*	*214*
	μmoles ATP formed/mg chlorophyll/h					
0	230	64	0	20	0	0
0.01	368	362	336	356	274	128
0.02	496	508	468	464	386	164
0.04	432	396	370	386	302	178

TABLE II

EFFECT OF ASCORBATE IN HOMOGENIZING MEDIUM-SPINACH *vs.* SWISS-CHARD

Reaction conditions as described under METHODS. Reaction time, 10 min. Light intensity approximately, 40,000 lux. Chloroplasts once washed. Chlorophyll: Spinach, 14–15 μg/ml, swiss-chard-6–7 μg/ml. Temperature, 25°. Gas phase-air. Chloroplasts kept at 0° in the dark between runs. All reaction mixtures contained a total of 10 μmoles ascorbate/ml.

Source of chloroplasts	*Ascorbate in medium*	*Hours after homogenization at which activity was tested*		
	M	*0.5*	*23.5*	*56*
		μmoles ATP formed/mg chlorophyll/h		
Swiss-chard	0	272	14	2
Swiss-chard	0.02	356	342	165
Spinach	0	297	165	6
Spinach	0.02	218	117	35

Occasionally spinach chloroplasts showed some response to ascorbate in the homogenizing and storage medium, but the effect was always small and the activity continuously decreased in all cases.

The stability of swiss-chard chloroplasts prepared in the sucrose–Tris–NaCl–ascorbate medium was also emphasized by the maintainance of activity during washing and subfragmentation. Table III shows that 3 washes or subfragmentation did not change the activity per unit chlorophyll of the preparation.

TABLE III

EFFECT OF WASHING AND SUBFRAGMENTATION

Reaction conditions as described under METHODS. Reaction time: Expt. 1, 30 min, Expt. 2, 15 min. Light intensity approximately 40,000 lux. Chlorophyll: Expt. 1, 7–11 μg/ml; Expt. 2, 10–12 μg/ml. Temperature, 25°. Gas phase–air.

Experiment	*Treatment*	*μmoles ATP formed/mg chlorophyll/h*
1	No wash	392
1	One wash	380
1	Three washes	372
2	One wash	456
2	Subfragmented	460

It could be mentioned at this point that microscopically the chloroplast preparation can be seen to be composed mainly of whole chloroplasts. The fragmented chloroplasts have the appearance of small oval shaped dark particles of a rather definite size. The latter preparation contains, however, a few whole chloroplasts. Both preparations contain a small amount of cell fragments of indefinite shape, other than chloroplasts or their fragments.

Requirements in medium

Ascorbate: In addition to the requirement for ascorbate in the homogenizing medium, it soon became apparent that its addition to the reaction mixture markedly increased the rates of phosphorylation. Closer examination of the latter effect revealed that it was strongly time-dependent. Fig. 1 illustrates a time course study in the presence of increasing concentrations of ascorbate. It can be seen that at very short time intervals the addition of ascorbate had no significant effect. However, if the reaction was allowed to proceed for longer time periods the effect became very marked. Ascorbate seemed to act in maintaining the activity of the chloroplasts for longer time periods. It should be noted that the activity of ascorbate is rather specific. It is not at all replaced by other reducing agents such as glutathione or cysteine. In addition, the requirement for ascorbate is just as pronounced with fragmented chloroplasts as it is with whole ones (see Fig. 3), indicating that whatever it acts on is very closely associated with the enzymic complex responsible for photophosphorylation.

The time-dependent requirement for ascorbic acid in the medium is just as apparent, in our experience, with spinach or swiss-chard chloroplasts.

Gas phase: Fig. 2 illustrates a time-course study of the reaction under nitrogen or air, in the presence or absence of ascorbate. It is clear that nitrogen and ascorbate act in a similar manner, prolonging the maintainance of activity in the chloroplasts. Under nitrogen ascorbate has little to no effect.

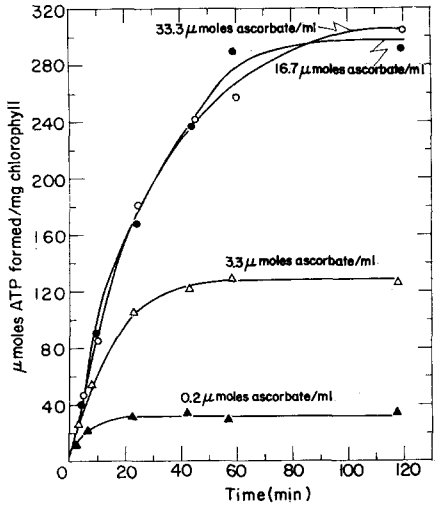

Fig. 1. Effect of ascorbate in the reaction mixture. Reaction conditions as described under METHODS. Light intensity approximately 40,000 lux. Chloroplasts once washed. Chlorophyll, 11 μg/ml. Temperature 25°. Gas phase–air.

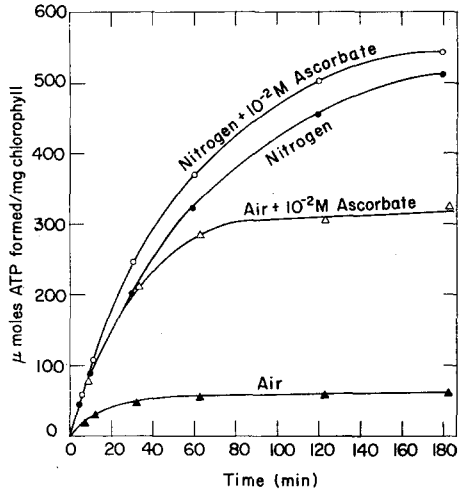

Fig. 2. Effect of ascorbate in the reaction mixture; Nitrogen *vs.* Air. Reaction conditions as described under METHODS. Light intensity, 45,000 lux. Chloroplast fragments. Chlorophyll, 2.9 μg/ml. Temperature, 15°.

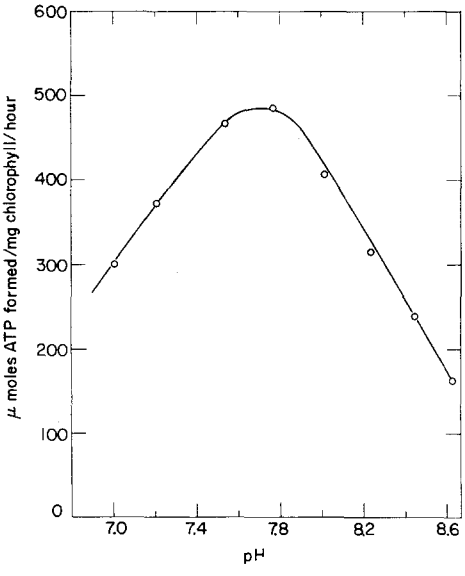

Fig. 3. pH curve. Reaction conditions as described under METHODS. Reaction time, 10 min. Light intensity, 40,000 lux. Chloroplasts, once washed. Chlorophyll, 11 μg/ml. Temperature, 25°. Gas phase–air. pH changed by varying the pH of the Tris added. The pH of the reaction mixture was measured at the end of the reaction.

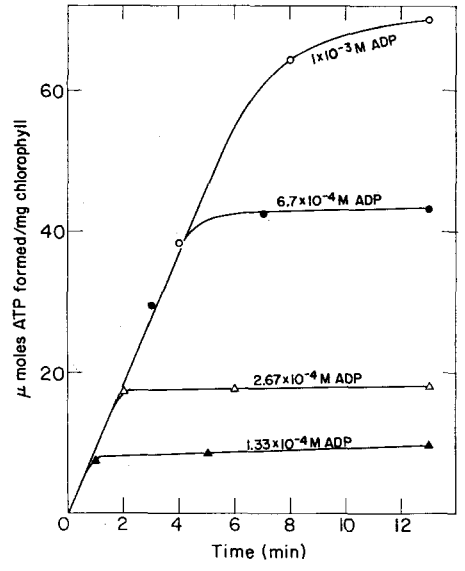

Fig. 4. Requirement for ADP. Reaction conditions as described under METHODS. Light intensity, approximately 40,000 lux. Chloroplasts, once washed. Chlorophyll, 11 μg/ml. Temperature, 25°. Gas phase-air.

pH: As previously shown for spinach[15], swiss-chard chloroplasts also possess a rather sharp pH optimum around 7.6–7.9 (Fig. 3). We were not able to observe the peak at pH 7.0 reported by WALKER AND HILL[9] in several of these experiments.

Cofactor: Swiss-chard chloroplasts show the same lack of specificity for a cofactor as do spinach chloroplasts[10]. Table IV illustrates the activity of the chloroplasts with several different cofactors. It is evident that phenazine methosulfate permits by far the highest rates of phosphorylation. As previously shown, the concentration range for maximal activity is rather narrow. FMN, menadione and indigo carmine all catalyse the reaction at about half to a third the rate allowed by phenazine methosulfate. With the sensitive assay used it is possible to detect some phosphorylation in the absence of any cofactor. Its rate is about 10 μmoles ATP formed/mg chlorophyll/h, but it is definitely present.

TABLE IV

ACTIVITY WITH SEVERAL COFACTORS

Reaction conditions as described under METHODS. Reaction time, 10 min. Light intensity, 45,000 lux. Chloroplasts, one washed. Chlorophyll, 15 μg/ml. Temperature, 25° Gas phase–air.

Cofactor	*Concentration (M)*				
	$1 \cdot 10^{-5}$	$3.3 \cdot 10^{-5}$	$1 \cdot 10^{-4}$	$3.3 \cdot 10^{-4}$	$1 \cdot 10^{-3}$
	μmoles ATP formed/mg chlorophyll/h				
PMS	428	712	366	340	34
FMN	194	272	358	346	310
Menadione	186	304	290	246	120
Indigo carmine	194	226	216	210	140

TABLE V

REQUIREMENT FOR MAGNESIUM

Reactions conditions as described under METHODS. Reaction time, 10 min. Light intensity, 45,000 lux. Chloroplasts once washed. Chlorophyll, 7 μg/ml. Temperature, 25° Gas phase–air.

Magnesium concentration	*μmoles ATP formed/mg chlorophyll/h*
0	70
$5.3 \cdot 10^{-4}$ *M*	520
$4 \cdot 10^{-3}$ *M*	640
$2 \cdot 10^{-2}$ *M*	520

Magnesium: An optimal amount of $4 \cdot 10^{-3}$ *M* magnesium is required (Table V). significant activity is present in the absence of any added Mg.

Phosphate: It was previously noted[15,10] that for maximal activity much more phosphate was required than the amount necessary for ATP production. With the assay employed this could be retested with greater ease. Table VI illustrates the continuously increasing rates obtained with increasing phosphate concentrations. Higher phosphate concentrations could not be conveniently used at present because of assay difficulties. In all cases the uptake of phosphate was not greater than 10 % of the total phosphate present in the reaction mixture. The high rates shown were due to the high light intensity and the short reaction time (see below).

Nucleotide: In contrast with phosphate, the ADP concentration required for saturation is very low. Fig 4 illustrates a time course study in the presence of increasing concentrations of ADP. It is clear that, for the concentrations used, the initial rate of the reaction is independent of the ADP concentration. The reaction seems to proceed at the maximal rate until almost all the ADP is utilized. In view of the absence of adenyl-kinase and ATPase in these preparations (see below) the reaction could serve as a very sensitive assay for small quantities of ADP, in the presence of AMP and/or ATP.

TABLE VI

REQUIREMENT FOR PHOSPHATE

Reaction conditions as described under METHODS. Reaction time, 2 min. Light intensity approximately 100,000 lux. Chloroplasts, once washed. Chlorophyll, 8.6 μg/ml. Temperature, 16°. Gas phase–air.

Phospahte concentration	*μmoles ATP formed/mg chlorophyll/h*
$1.4 \cdot 10^{-3}$ *M*	900
$4.2 \cdot 10^{-3}$ *M*	1004
$8.4 \cdot 10^{-3}$ *M*	1210
$1.7 \cdot 10^{-2}$ *M*	1290

TABLE VII

NUCLEOTIDE SPECIFICITY

Reaction conditions as described under METHODS. Reaction time, 10 min. Light intensity, 45,000 lux. Chloroplasts, Expt. 1, once washed, Expt. 2, washed 3 times. Chlorophyll: 19 μg/ml. Temperature, 25°. Gas phase–air.

Experiment	*Nucleotide*	*Concentration μmoles/ml*	*μmoles ATP formed/mg chlorophyll/h*
1	AMP	6	0
1	ADP	6	420
1	ATP	0.7	0
1	AMP + ATP	6 + 0.7	0
2	ADP	1.6	796
2	IDP	1.6	640
2	GDP	1.6	514
2	UDP	1.6	196
2	CDP	1.6	2

It is now accepted by all workers in the field that ADP, rather than AMP, is the actual acceptor in the photophosphorylation by chloroplasts[3,6,10]. This can be very beautifully demonstrated with swiss-chard chloroplasts since they are virtually free of adenyl kinase activity. Table VII, experiment 1 shows that even with once washed whole chloroplasts, activity is seen only in the presence of ADP. This is in striking contrast with spinach chloroplasts, where only after exhaustive washings can one achieve a relatively adenyl-kinase free preparation[3,10,15].

The specifity of the reaction for nucleosides diphosphate other than ADP, has not been thoroughly tested. KRALL *et al.*[6] reported that UDP, GDP, IDP and CDP can be phosphorylated by spinach chloroplasts, but at lower rates than ADP.

Table VII, experiment 2, illustrates the activity of swiss-chard chloroplasts with these phosphate acceptors. It is evident that thrice washed chloroplasts were capable of phosphorylating IDP and GDP at almost as fast a rate as ADP. UDP was utilized at a lower rate, while CDP was inactive. The same results were obtained with fragmented chloroplasts.

The question arose whether the results were a reflection of the specificity of the enzyme combining with ADP. It could also be consequence of the esterification of small amounts of contaminating ADP in the preparations of the other nucleosides diphosphate, followed by nucleoside diphospho-kinase activity. The latter possibility was tested in the following manner: chloroplasts were incubated in the usual reaction mixture and allowed to synthesize $AT^{32}P$ in the light. After practically all of the ADP was phosphorylated, the light was turned off and another nucleoside diphosphate added to the reaction mixture. The reaction was allowed to proceed for another 30 min in the dark, after which denaturation and paper chromatography was carried out. The radioactivity distribution between the adenine nucleotide spot and the other nucleotide in question at the end of the reaction is recorded in Table VIII. If no nucleoside diphosphokinase activity was present all the radioactivity should have been recovered in the $AT^{32}P$ spot. However, it is seen that in the case of IDP, GDP, and UDP a substantial part of the radioactivity has been transferred to their triphosphates. In the case of CDP, however, only a small percentage has been transferred. This percentage might even be accounted for by other contaminating nucleotides in the CDP preparation. The relative inactivity of the nucleoside diphosphokinase for CDP is in agreement with the inability of the chloroplasts to phosphorylate CDP.

TABLE VIII

NUCLEOSIDE DIPHOSPHOKINASES IN CHLOROPLASTS

Reaction conditions as described under METHODS. Reaction time: light, 10 min. Dark, 30 min. Light intensity, 40,000 lux. Chloroplasts, once washed. Chlorophyll, 25 μg/ml. Temperature, 25°. Gas phase–air. See text for details. One dimensional paper chromatography according to the method of BERGKVIST AND DEUTSCH[20], with saturated ammonium sulfate, water, isopropanol (79 : 19 : 2; v/v) as the developing agent.

Nucleotides in dark reaction	*Radioactivity distribution % of total in*	
	ATP	*Other nucleotide*
$1.3 \cdot 10^{-3}$ *M* $AT^{32}P$	100	0
$1.3 \cdot 10^{-3}$ *M* $AT^{32}P$ + $1.3 \cdot 10^{-3}$ *M* IDP	63	37
$1.3 \cdot 10^{-3}$ *M* $AT^{32}P$ + $1.3 \cdot 10^{-3}$ *M* GDP	61	39
$1.3 \cdot 10^{-3}$ *M* $AT^{32}P$ + $1.3 \cdot 10^{-3}$ *M* UDP	59	41
$1.3 \cdot 10^{-3}$ *M* $AT^{32}P$ + $1.3 \cdot 10^{-3}$ *M* CDP	88	12

These data do not allow a definite conclusion as to the specificity of the nucleotide combining enzyme of photophosphorylation. They seem to indicate, however, that the activity observed with nucleotides other than ADP may be accounted for by the nucleoside diphosphokinase(s) present in the chloroplasts. The preparation of chloroplast fragments, free of the latter enzyme, is being attempted. Such a preparation would provide means of testing the specificity of the nucleotide combining enzyme of photophosphorylation.

Light: As soon as light intensity studies were begun it became apparent that

the intensity of 45,000 lux used, on the basis of the requirements previously determined for spinach chloroplasts[10], was not sufficient. Fig. 5 illustrates the light intensity requirements as measured with the set up described under methods (b). It is evident that the reaction was not approaching saturation until at least 100,000 lux were supplied. Complete saturation does not seem to have been attained even at 154,000 lux, the limiting intensity with that apparatus. The obvious result of this finding was the extremely high rates of photophosphorylation recorded. Under 154,000 lux of light we have continuously observed rates of 1300–2000 μmoles phosphate esterified /mg chlorophyll/h.

Several other factors emerge as important in the attainment of these high rates. As can be seen in all time-course curves presented, the activity in the preparations is not linear with time. Even under the best conditions attained to date, the rate continuously decreased as time proceeds. It is thus important to measure the reaction at short intervals for maximal rates. A second factor can be seen in Fig. 5. Although it was previously found that the effect of ascorbate in the reaction mixture is apparent only at longer time intervals, it is clear that it is also dependent on the intensity of the light supplied. Thus in a 5-min reaction (Fig. 5), ascorbate has little to no effect up to about 40,000 lux, but the effect is increasingly marked thereafter. A third factor can be seen in Fig. 6. It illustrates chloroplast concentration curves measured at several light intensities. The statement is often made on the basis of one such curve that the concentration of chloroplasts used was below the limit of light saturation (see, for example, ref.[3]), meaning in the lowest portion of the curve illustrated. Although this is reasonably sound, Fig. 7 illustrates that even at the lowest portion of the curves, at chlorophyll concentration of 3 μg/ml, light saturation was certainly not reached until 154,000 lux were supplied. It is therefore important to run a light concentration curve of the type shown in Fig. 5 to determine the level of light saturation under the particular conditions in question.

At the lowest chlorophyll concentrations, highest light intensity, in a 2-minute reaction time, and with optimal ascorbate concentration the data of Fig. 6 represents a specific activity of 2500 μmoles ATP formed/mg chlorophyll/h.

Inhibitors

Arsenate: It has previously been shown that arsenate was a competitive inhibitor for phosphate in photophosphorylation by spinach chloroplasts[17]. Since the strictly competitive nature of the effect was valuable as a tool in several investigations concerning the mechanism of ATP formation[17,18], it was thought important to confirm it with chloroplasts isolated from swiss-chard. Fig. 7 represents a LINEWEAVER-BURK plot of the effect of arsenate on the reaction. It is evident that here, too, arsenate inhibits ATP formation only by virtue of its competition with phosphate.

Uncouplers: The classical uncoupler of phosphorylation from electron transport, 2,4-dinitrophenol, was shown to be ineffective in uncoupling the non-cyclic photophosphorylation of spinach chloroplasts[18], and effective in inhibiting its cyclic phosphorylation only at high concentrations[4,19]. As indicated in Table IX, swiss-chard chloroplasts were even less sensitive than spinach to dinitrophenol. Whereas spinach chloroplasts were inhibited 60–80 % by 10^{-3} *M* dinitrophenol[4,19], only 20 % inhibition is seen with the swiss-chard preparation.

By contrast, ammonium ions, which were previously shown to uncouple noncyclic

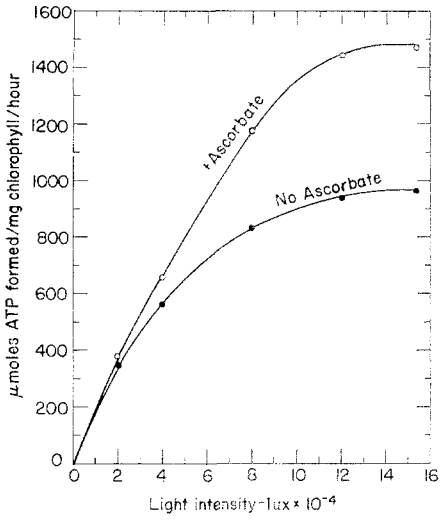

Fig. 5.

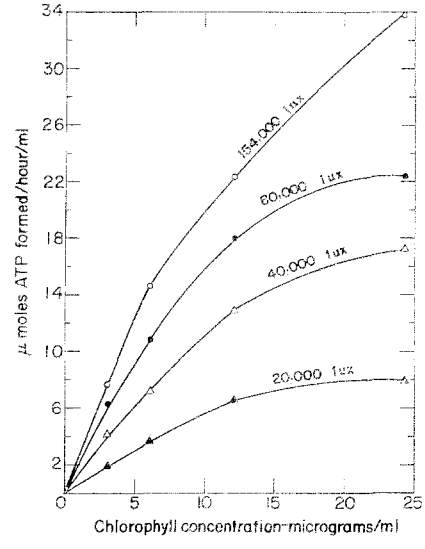

Fig. 6.

Fig. 5. Requirement for light intensity. Reaction conditions as described under METHODS. Reaction time, 5 min. Light intensity, as marked. Chloroplasts, once washed. Chlorophyll, 7 µg/ml. Temperature 16°. Gas phase-air.

Fig. 6. Effect of chloroplast concentration at several light intensities. Reaction conditions as described under METHODS. Reaction time, 2 min. Light intensity, as marked. Chloroplasts, once washed. Chlorophyll, as marked. Temperature 16°. Gas phase-air.

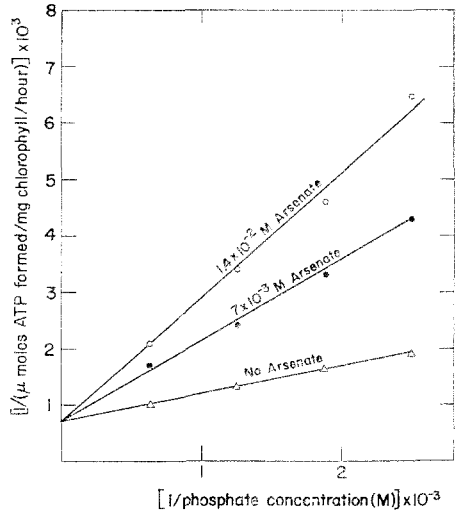

Fig. 7.

Fig. 7. Arsenate as a competitive inhibitor. Reaction conditions as described under METHODS. Reaction time, 5 min. Light intensity 40,000 lux. Chloroplasts, once washed. Chlorophyll, 6.6. µg/ml Temperature, 16°. Gas phase-air.

photophosphorylation[1,8] and inhibit cyclic phosphorylation[4] of spinach preparations, behave in the identical manner with swiss-chard chloroplasts. Table IX illustrates the strong inhibition produced by ammonium chloride. The concentration required for 50 % inhibition was about $6 \cdot 10^{-4}$ *M*, and the inhibition seemed to be directly proportional to the logarithm of the ammonium ion concentration.

Mechanism

Pi-ATP exchange and ATPase: The surprising lack of ATPase or Pi-ATP-exchange in spinach chloroplasts was previously shown[17]. It is here strongly confirmed with chloroplasts from swiss-chard. Table X illustrates the complete lack of any reaction

TABLE IX

EFFECT OF 2,4-DINITROPHENOL AND AMMONIUM CHLORIDE

Reaction conditions as described under METHODS. Reaction time, 5 min. Light intensity, 45,000 lux. Chloroplasts, once washed. Chlorophyll: dinitrophenol experiment, 4.8 μg/ml; NH_4Cl experiment, 6.6 μg/ml. Temperature, 25°. Gas phase–air.

Inhibitor	*Concentration M*	*μmoles ATP formed/mg chlorophyll/h*	*% of control*
2,4-dinitrophenol	0	625	(100)
2,4-dinitrophenol	$1.0 \cdot 10^{-5}$	630	101
2,4-dinitrophenol	$1.0 \cdot 10^{-4}$	700	112
2,4-dinitrophenol	$1.0 \cdot 10^{-3}$	500	80
NH_4Cl	—	630	(100)
NH_4Cl	$5.0 \cdot 10^{-4}$	375	60
NH_4Cl	$1.3 \cdot 10^{-3}$	186	30
NH_4Cl	$5.0 \cdot 10^{-3}$	15	2

TABLE X

LACK OF Pi-ATP EXCHANGE AND ATPASE

Reaction mixture contained the usual components (see METHODS), except for ADP and inorganic phosphate being replaced by the materials indicated under vessel contents. Reaction performed in test tubes in the dark, with once washed chloroplasts. Total volume, 1.0 ml. Chlorophyll, 25 μg/ml. Temperature, 25°. Gas phase–air. In the first and second experiments samples of 0.2 ml were taken out at the indicated times, and transferred into tubes containing 0.02 ml of 20% trichloroacetic acid. The samples were applied to Whatman No. 1 paper and chromatographed by the method of BURROWS *et al.*[21], using acetone, 15% trichloroacetic acid (65 : 35, v/v). Samples of the third experiment were analyzed as described under METHODS. The $AT^{32}P$ used was synthesized with swiss-chard chloroplasts, adsorbed on charcoal, and extracted from it with 50% ethanol, 0.2% NH_4OH. The extract was applied to Whatman No. 1 paper and developed by the method of KREBS AND HEMS[22], using isobutyric acid–concentrated ammonium hydroxide–water (66 : 1 : 33, v/v). After drying, the $AT^{32}P$ strip was cut out and eluted with water.

Vessel contents	*Reaction time (min)*	*% of total radioactivity in*	
		ATP	*Pi*
1 μmole $AT^{32}P$	0	81	19
	60	80	20
1 μmole $AT^{32}P$ + 2 μmoles Pi	0	82	18
	60	82	18
1 μmole ATP + 4 μmoles ^{32}Pi	0	0	100
	60	0	100

in 60 min, which was sufficient to phosphorylate, under the same conditions, many times the amount of ATP supplied.

The addition of ADP and changes in the reaction components did not alter the results.

ADP–ATP exchange: Since these chloroplasts are free of adenyl kinase, they provide excellent material for the study of the exchange between ADP and ATP. This exchange was found in mitochondria[23] and was related to the last step in oxidative phosphorylation. Its rate was several fold faster than that of the Pi–ATP exchange, which in itself is many fold faster than the phosphorylating reaction.

The data of Table XI illustrate the complete lack of such an exchange in photophosphorylation by swiss-chard chloroplasts. The absence of adenyl kinase is indicated

by the lack of any simultaneous increase in the AMP and ATP in any of the experiments. This was true even when the reaction was run with washed chloroplasts. The third experiment indicates that, in the light, the reaction conditions used were sufficient to completely esterify the ADP supplied in 10 min. However, in the dark, even 60 min of reaction did not show any measurable exchange between ADP and ATP under the identical conditions.

TABLE XI

LACK OF ADP–ATP EXCHANGE

Reaction mixture contained the usual components (see METHODS) except for ADP and inorganic phosphate being replaced by the materials indicated under vessel contents. In addition, all vessels contained $2 \cdot 10^{-3}$ *M* ATP. The reaction was carried out in small test tubes, with chloroplast fragments. Total volume, 0.3 ml. Chlorophyll, 29 μg/ml. Temperature, 25°. Gas phase–air. Samples of 0.05 ml were taken at the indicated times and transferred into tubes containing 0.005 ml of 20% trichloroacetic acid. The samples were applied to Whatman No. 1 paper, together with a marker for AMP, and chromatographed by the method of KREBS AND HEMS[22], using isobutyric acid–concentrated ammonium hydroxide–water (66 : 1 : 33, v/v). After thorough drying, the spots were marked under an u.v. lamp, cut out and their radioactivity determined in a gas flow counter.

Vessel contents	*Illumination (lux)*	*Reaction time (min)*	*% of total counts in*		
			AMP	*ADP*	*ATP*
$2 \cdot 10^{-3}$ *M* [^{14}C]ADP	None	0	9	82	9
		60	11	79	10
$2 \cdot 10^{-3}$ *M* [^{14}C]ADP + $5 \cdot 10^{-3}$ *M* Pi	None	0	9	82	9
		60	8	83	9
$2 \cdot 10^{-3}$ *M* [^{14}C]ADP + $5 \cdot 10^{-3}$ *M* Pi	45,000	0	10	81	9
		10	8	6	86

DISCUSSION

The effects of ascorbate in the photophosphorylation system have been a source of continuous and contradicting reports. It was first reported by ARNON and his group[1] that it was essential for maximal rates of photophosphorylation. AVRON, JAGENDORF AND EVANS[15] confirmed the requirement, but observed no such need when phenazine methosulfate was used as a cofactor[10]. ARNON *et al.*[24] reported that the inclusion of 0.01 *M* ascorbate in the preparatory medium increased the stability of spinach chloroplasts. OHMURA[4] confirmed both effects of ascorbate, stabilizing and increasing the rates of photophosphorylation. He further showed that the increase in rate was apparent only when the reaction was carried out for longer periods of time. WESSELS[5] also observed the ascorbate and time dependent increase of rate. In addition he reported that the effect was absent when the reaction was run under anaerobic conditions.

All of these observations are much more clearly seen with the swiss-chard chloroplasts as demonstrated under results. It would seem that where the effect of ascorbate was not observed, short time periods were used[10]. The increase in rate due to ascorbate was not, therefore, cofactor dependent, as it seemed at first[10]. It is, however, time dependent (Fig. 1), and therefore when a cofactor permitting higher rates was used, shorter time intervals were required and the response could not be seen. However, under very high light intensities, and consequently very high rates, a requirement for ascorbate was apparent even at short time intervals.

It is difficult to define the mode of action of ascorbic acid. Attempts to relate its effect to an inhibitory action of polyphenol oxidase. which can be demonstrated in these preparations, have not succeeded. Although sodium diethyl dithio carbamate, a potent inhibitor of the latter enzyme, causes an increase in the rate of photophosphorylation, it does so independent of the presence of ascorbate. One can only say that both actions of ascorbate can be attributed to a stabilizer, rather than a cofactor function. It is also clear that this stabilizing action is related to its ability to prevent or correct some deleterious oxidative effect, since no increase of rate was observed under anaerobic conditions (Fig. 2).

The requirements in the medium for swiss-chard chloroplasts were, in general, quite similar to the ones previously described for chloroplasts from spinach. The high concentration of phosphate needed for saturation was strongly confirmed with these chloroplasts. Although no explanation can, as yet, be given to this phenomenon, it is especially striking when compared to the very high affinity of the same chloroplasts for ADP.

The presence of nucleoside diphosphokinase(s) in the chloroplasts, makes it difficult to reach a conclusion regarding the final identification of the primary phosphate acceptor. The data presented, however, would place ADP or GDP as the only likely candidates.

Most workers in the field seem to employ relatively low intensity light for the reaction[3–5,8]. Although light is recognized by all to be an essential component, it was rarely given a thorough study, and its intensity is often expressed in non-comparable terms. It is clear, however, from the results presented herewith, and earlier[10], that under optimal conditions it often was the limiting factor in the reaction. The rates reported here of 2000 μmoles ATP formed per mg chlorophyll per h, could be obtained only at very high intensities around 150,000 lux. It might be worthwhile to call attention again to the inconclusiveness of chloroplast concentration curves, when interpreted as indicating saturation by light (see Fig. 7 and text under RESULTS).

The continuous increase in maximal rates of photophosphorylation as obtained by different workers in the field was quite remarkable. In units of μmoles ATP formed/mg chlorophyll/h, the initial observations[1] reported rates of 3, which were improved by the addition of FMN or menadione to 34. Close study of the requirements of the system resulted in another increase up to a rate of 200[15]. ALLEN *et al.*[3] upon further study, obtained rates of 360–510. Rates in the same order of magnitude were also reported by OHMURA[4]. The use of phenazine methosulfate as a cofactor[10], resulted in rates of 700–900, and in this report the rate has been boosted another 2–3 fold to 2000–2500. It was obtained as a result of a higher light intensity, higher phosphate concentration, lower chloroplast concentration, a very short reaction time, and a sensitive assay permitting the accurate measurement of the small amounts of ATP formed.

The demonstration of no exchange between ADP and ATP emphasizes again the difference between the mechanisms of oxidative and photophosphorylations[17]. It is in agreement with the mechanism previously proposed[17,25] and a consequence of the irreversibility of such a step as has been predicted from earlier, unrelated data[17].

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THE ROLE OF THE SULFHYDRYL GROUPS IN THE STABILISATION OF THE STRUCTURE OF THE D-GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE

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SUMMARY

The effect of the blocking of the SH groups on the spatial structure of the swine muscle D-glyceraldehyde-3-phosphate dehydrogenase was studied. The treatment with *p*-chloromercuribenzoate resulted in significant changes in the optical rotation and the intrinsic viscosity of the protein, the D-glyceraldehyde-3-phosphate dehydrogenase molecule being altered in the direction of denaturation. From these data it is assumed that the SH groups may play an important role in the stability of the secondary structure of the enzyme.

Abbreviations: PGAD = D-glyceraldehyde-3-phosphate dehydrogenase; DPN = diphosphopyridine nucleotide; PCMB = *p*-chlormercuribenzoate.