Direct and Indirect Transfer of ATP and ADP across the Chloroplast Envelope

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1. In intact leaves of *Elodea densa* illumination resulted in a large increase in the levels of chloroplastic and cytoplasmic ATP and a decrease in chloroplastic and cytoplasmic ADP. Reverse changes were observed on darkening. The kinetics of the fluctuations were similar in chloroplasts and cytoplasm suggesting effective transfer of ATP and ADP between chloroplasts and cytoplasm. Ratios of ATP to ADP were significantly lower in the chloroplasts than in the cytoplasm in the dark and in the light. This may indicate different phosphate potentials in chloroplasts and cytoplasm. Transfer of ATP across the chloroplast envelope as calculated from the light-dependent cytoplasmic ATP increase was 7 to 9 μmoles/mg chlorophyll per hour. Actual transfer rates are probably higher.

2. The determination of the rate of adenylate transfer across the envelope of intact isolated chloroplasts requires information on the composition of chloroplast preparations. Intact chloroplasts were quantitatively separated from envelope-free chloroplasts by density gradient centrifugation on L u d o x gradients. The percentage of envelope-free chloroplasts in preparations of intact chloroplasts was also determined from measurements of light-dependent ferricyanide reduction.

3. During isolation in sorbitol buffer ca. 50% of the adenylates were lost from intact chloroplasts which were still capable of high rates of phosphoglycerate reduction and photosynthesis.

4. Adenylate transfer across the envelope of isolated chloroplasts as measured by the light-dependent phosphorylation of added ADP in the absence of cofactors was slow and occured at a rate of 0 to 4 μmoles/mg chloroplyll per hour. In the dark chloroplastic adenylate kinase reacted only very slowly with added AMP and ATP to form ADP. Breakage of the chloroplast envelope stimulated reaction rates.

5. Indirect transfer of ATP and ADP across the chloroplast envelope occurred via a shuttle transfer of phosphoglycerate and dihydroxyacetone phosphate. 3-phosphoglyceraldehyde is not involved as a transport metabolite. Maximum transfer rates of phosphoglycerate and dihydroxyacetone phosphate across the chloroplast envelope were higher than maximum rates of photosynthesis and reached 300 μmoles/mg chlorophyll per hour. Indirect transfer of ATP was somewhat slower than rates of phosphoglycerate reduction by isolated chloroplasts. In vivo transfer of phosphate energy by this transport metabolite system is under control of the redox state of pyridine nucleotides and of the phosphate potential.

In heterotrophic cells of higher organism a considerable part of the total energy production takes place in the mitochondria. From these organells ATP is exported to other parts of the cell [1–3]. A back flow of ADP maintains phosphorylation. In autotrophic chloroplast-containing cells a very similar situation is likely to exist in the dark. In the light, however, ATP production in the chloroplasts by far exceeds that in the mitochondria. In fact, in these cells mitochondrial metabolism appears to be suppressed by light [4]. The question arises whether chloroplastic ATP is used exclusively inside the chloroplasts to drive photosynthetic reactions or whether it can also be made available for metabolic reactions in other parts of the cell.

From measurements of the distribution between chloroplasts and cytoplasm of ATP and ADP labelled in in vivo-experiments with ^{32}P in the dark and in the light [5, 6] and from determinations of fluctuations in intracellular ATP levels [7] it has been inferred that a considerable traffic of adenylates takes place across the chloroplast envelope. Alternatively, phosphate energy could be transferred across the chloroplast envelope indirectly via transport metabolite

* With the technical assistance of E. SEDLICK and R. ROTHES.

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[7] K. A. SANTARIUS u. U. HEBER, Biochim. biophysica Acta [Amsterdam] **102**, 39 [1965].

systems [8] analogous to those known for the transfer of hydrogen across biological membranes. Recent measurements of STROTMANN and HELDT [9] and HELDT [10] indicated that direct transfer of adenylates across the chloroplast envelope does occur, but is slow. In the present report attempts are described to measure rates of transfer of phosphate energy across the chloroplast envelope both in vivo and in vitro.

Material and methods

Shoots of *Elodea densa* were illuminated and/or darkened for different times and rapidly frozen in light petrol ether which was precooled to approximately $-110\,^{\circ}C$. After freezedrying chloroplasts were separated from the remainder of the cells in a nonaqueous medium (petrol ether/carbon tetrachloride) [11]. Yields of chloroplasts varied from 45 to 55 per cent. ATP and ADP were determined in the fractions as described previously [7]. Using the chlorophyll content of the remainder results were recalculated [11] to give the adenylate content of the nonchloroplastic part of the cells (= cytoplasm). Cytoplasmic contamination of the chloroplast fraction as measured by the pyruvate kinase test [12] was lower than 5 per cent.

For the isolation of chloroplasts in aqueous media leaves of field-grown spinach were used. Chloroplasts were isolated from 75 gr of spinach leaves by grinding them in a Waring Type blender for 7 to 9 seconds in 150 ml of Jensen & Bassham's buffer A [13] containing in addition 0,004 M cysteine. After filtration through 8 layers of cheese cloth the resulting suspension was either centrifuged for 12 min at 200 *g* (= slow chloroplasts) or for 40 sec at 2000 *g* (= fast chloroplasts). The supernatant and a semi-fluid layer of chloroplasts were carefully discarded, the sediment resuspended in 30 ml of Jensen & Bassham's buffer B [13], but without ascorbate, and centrifuged for 1 min at 170 *g*. The supernatant was then centrifuged either for 12 min at 200 *g* or for 25 to 30 sec at 2000 *g*. The sediment was again carefully separated from the supernatant and semi-fluid chloroplast material and resuspended in a small volume of Jensen & Bassham's buffer C [13], but without ascorbate and pyrophosphate. It contained between 60 and 97% intact chloroplasts contaminated by envelope-free chloroplasts. Preparations of "slow" chloroplasts did not photoreduce CO_2 at significant rates. "Fast" chloroplasts exhibited usually, but not always high rates of photosynthesis in the presence of bicarbonate (up to 140 μmoles per mg chlorophyll per hour). Added phosphoglycerate was photo-reduced at rates ranging from 30 μmoles up to occasionally 300 μmoles/mg chlorophyll per hour as measured polarographically with a Clark type electrode. Such measurements were performed routinely parallel to photophosphorylation experiments. Since in the absence of bicarbonate phosphate uptake of "slow" and "fast" chloroplasts was not different in photophosphorylation experiments, no special mention is made which type of chloroplasts was used in the experiments.

Photophosphorylation and Hill reaction rates were measured of "intact" and osmotically shocked preparations. "Intact" chloroplasts were suspended in a 1/1 mixture of freshly destilled water and double strength buffer C of Jensen & Bassham, which contained in addition $3 \cdot 10^{-3}$ M phosphate, $3{,}6 \cdot 10^{-3}$ M ADP and $6{,}6 \cdot 10^{-3}$ M $MgCl_2$ and in a number of experiments was made CO_2-free by bubbling with CO_2-free air. To obtain osmotically shocked material chloroplasts were added to an at least 10 fold greater volume of water and double strength buffer C as above was added to adjust osmolarity to that of the intact preparation. Brief exposure to hypotonic conditions was sufficient to rupture chloroplast envelopes. Chlorophyll concentrations (usually 100 μg chlorophyll per 1.4 ml reaction mixture) in the samples containing intact and shocked chloroplasts were identical. The reaction was terminated after 5 to 12 minutes illumination by turning off the light and adding trichloroacetic acid to a concentration of 4 per cent.

Phosphate was determined according to FISKE and SUBBA ROW [14] and phosphate consumption calculated from the differences between the illuminated sample and a control which was kept in the dark.

In a number of experiments with "fast" chloroplasts enzymes (from Boehringer, Mannheim, freed partially or completely from ammonium sulfate by centrifugation and dialysis against buffer C) and substrates were added to the reaction mixture used for photophosphorylation. Special additions, if any, are listed in the legends to the tables.

Rates of ferricyanide reduction (Hill reaction) were measured simultaneously with or shortly after the photophosphorylation experiments. They were recorded continuously by measuring the voltage drop across a load resistor produced by a photocurrent which was generated in a photomultiplier by a low intensity measuring beam of 400 nm after passing through the sample (light path 0.5 cm). Sensitivity was increased by zero suppression. The photomultiplier was shielded from exciting red light (produced by an RG 630 cutoff filter of Schott & Gen., Mainz, 60 000 ergs/cm^2/sec)

[8] U. HEBER, in: Proc. of the Internatl. Sympos. on Transport of Higher Plants, Reinhardsbrunn, 1968, in the press.
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[13] R. G. JENSEN u. J. A. BASSHAM, Proc. nat. Acad. Sci. USA **56**, 1095 [1966].
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by two Corning filters 9782 and 5030. The reaction mixture for the Hill reaction was identical with that used for photophosphorylation. Intact and shocked chloroplasts with 8 to 10 μg of chlorophyll and 0.6 μmoles ferricyanide were added to 1.4 ml reaction mixture, and the sample was illuminated after recording a dark trace. From the difference in the slope of the trace the reaction rate was calculated. Results were highly reproducible and served to determine the accurate percentage of envelope-free chloroplasts contaminating intact preparations as intact chloroplasts did not or only slowly photoreduce ferricyanide.

ADP formation from ATP and AMP by adenylate kinase was determined by adding a suspension of intact or shocked chloroplasts with 20 μg chlorophyll to 2 ml isotonic buffer C which contained 1,6 μmoles ATP, 2,5 μmoles phosphoenolpyruvate, 40 μg pyruvate kinase, 25 μg lactic dehydrogenase and 0,15 μmoles NADH. After measuring ADP production due to an ATPase reaction by recording the optical density at 340 nm the adenylate kinase reaction was started by adding 2 μmoles AMP. Adenylate kinase activity was calculated from the difference in slope before and after addition of AMP. Reaction rates of intact and shocked chloroplasts were corrected for adenylate kinase activity not associated with chloroplastic material. The data were processed as described previously [12] in similar studies involving other enzyme systems. Parallel to these experiments the percentage of intact chloroplasts in the preparations was determined.

Proper controls (measurements of ferricyanide reduction) ascertained that no significant membrane breakage occurred during handling (pipetting) of intact chloroplasts.

Intact chloroplasts were separated quantitatively from envelope-free chloroplasts by density gradient centrifugation. Following a suggestion of Prof. Willenbrink a Ludox-gradient was used and made up as follows: 4 volumes Ludox (DuPont, 40% solution), 1 volume water and 5 volumes of buffer C as used by Jensen & Bassham, but double strength and without ascorbate and pyrophosphate, were mixed and the pH was readjusted to 7.8. A density gradient was established by centrifuging 4.5 ml portions in tubes of 1,2 cm diameter in a swing out rotor for 30 to 40 min at 60 000 *g*. Mixtures of intact and broken chloroplasts containing ca. 150 μg of chlorophyll were layered on top of the gradient and separated by centrifugation for 30 min at 60 000 *g*. Bands containing intact and broken chloroplasts respectively were sharp (less than 2 mm wide) and almost 2 cm apart.

Results

I. Transfer of phosphate energy across the chloroplast envelope as indicated by in vivo experiments

On illumination of leaves of *Elodea densa* the level of chloroplastic ATP increased rapidly, reached a maximum after ca. 15 sec and then decreased to a steady state (fig. 1). Darkening produced a rapid fall in chloroplastic ATP. Very similar kinetics have been observed before in *Elodea* and other species of higher plants [7]. As should be expected, changes

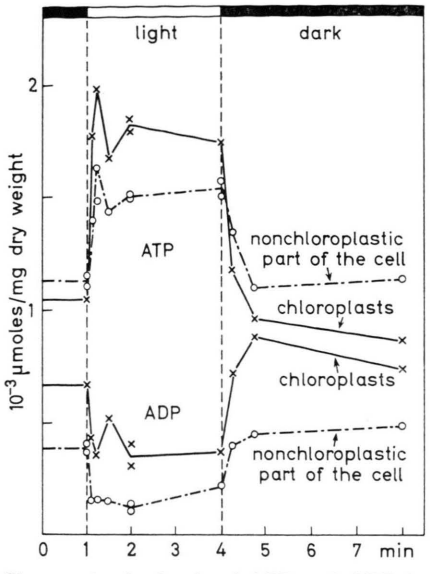

Fig. 1. Changes in the levels of ATP and ADP in chloroplasts and cytoplasm of leaf cells of *Elodea densa* caused by illumination and darkening.

in chloroplastic ADP were antiparallel to those of ATP, but, owing to the presence of adenylate kinase, not as large. AMP was not determined. Cytoplasmic changes in ATP and ADP were very similar to the chloroplastic changes. No significant lag behind the chloroplastic fluctuations was seen. The data show as other data before [5–7] that changes in chloroplastic ATP and ADP, which are produced by photosynthetic reactions, communicate to the cytoplasm. They indicate a rapid transfer of phosphate energy across the chloroplast envelope. Neglecting turnover of adenylates in the cytoplasm, an approximate and very probably minimal figure for the rate of export of phosphate energy into the cytoplasm can be obtained from the rate of increase of cytoplasmic ATP on illumination. Similarly, the rate of decrease in cytoplasmic ATP produced on darkening may serve to indicate a minimum rate of back transfer of phosphate energy especially as cytoplasmic metabolism after darkening is geared to an excess of ATP production rather than consumption. This ATP production which is a consequence of glycolytic triose phosphate oxidation [15], would tend to slow the de-

[15] O. Kandler u. J. Haberer-Liesenkötter, Z. Naturforsch. **18 b**, 718 [1963].

crease in cytoplasmic ATP levels down. The rate of export of phosphate energy as calculated from the increase in cytoplasmic ATP on illumination was 7 to 9 μmoles ATP/mg chlorophyll per hour, the rate of import as calculated from the decrease in cytoplasmic ATP about 4 μmoles ATP/mg chlorophyll per hour. Actual rates of transfer of phosphate energy may be considerably higher.

As noticed also by KEYS and WHITTINGHAM [16] for leaves of tobacco, the percentage of ADP found in the chloroplasts is higher than that of ATP. In *Elodea* 23 to 35% of the total ADP of the cells was located in the chloroplasts. Its distribution between chloroplasts and cytoplasm was similar to that of protein. In contrast, only 14 to 20% of the total ATP was found in the chloroplasts. In consequence, ATP/ADP ratios were distinctly lower in the chloroplasts than in the cytoplasm. This holds true not only in the dark, but surprisingly also in the light even though there is rapid ATP formation in the chloroplasts in the light (fig. 2). It is unknown whether the level of

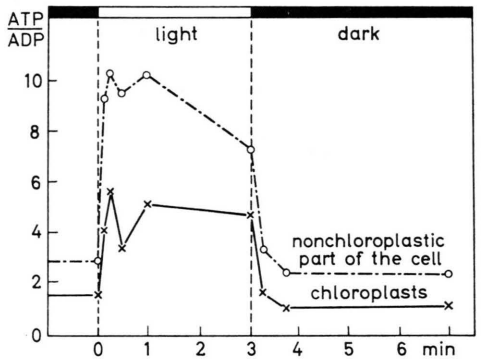

Fig. 2. The ratio of ATP/ADP in chloroplasts and cytoplasm of leaf cells of *Elodea densa* in the light and in the dark.

orthophosphate in chloroplasts and cytoplasm is similar. If it is, the different ratios of ATP/ADP in chloroplasts and cytoplasm indicate different phosphate potentials in these compartments. In in vitro-experiments it has been shown by KLINGENBERG et al. [17] that phosphate potentials inside respiring mitochondria were lower than in the surrounding medium.

II. Phosphorylation in the light of ADP added externally to intact chloroplasts

The rate of phosphorylation of externally added ADP to ATP by washed intact chloroplasts can be taken as an indication of the rate of transfer of phosphate energy across the chloroplast envelope as photophosphorylation is known to take place inside the chloroplasts and reactants have to enter and leave the chloroplasts to permit the reaction to proceed. However, preparations of "intact" chloroplasts as isolated by the usual methods are invariably contaminated by chloroplasts stripped of their outer membranes. These "broken" chloroplasts exhibit high rates of photophosphorylation as entry of ADP and phosphate is not hindered by a membrane barrier. They interfere with the determination of transfer rates. Rates of photophosphorylation can only be used to measure transfer if chloroplast preparations are either uncontaminated by broken chloroplasts or if the percentage of broken chloroplasts is accurately known to permit proper corrections.

Separation of intact and broken chloroplasts is usually performed in sucrose density gradients. However, "intact" chloroplasts from sucrose gradients which were brought back slowly and carefully to isotonic conditions did not evolve oxygen in the light on addition of CO_2 or PGA. Furthermore, added pyridine nucleotides could be oxydized or reduced by enzymic constituents of these chloroplasts although the envelope of intact chloroplasts is impermeable for pyridine nucleotides [33] (see also l. c. [18]). Obviously part or all of the chloroplasts isolated from the sucrose density gradients had damaged outer membranes.

Centrifugation of chloroplasts in a density gradient made by Ludox yields completely separated bands of intact and broken chloroplasts (fig. 3). Only the former evolved oxygen in the light on addition of PGA, though at reduced rates. Photosynthetic CO_2-reduction has not been observed. Since it was found difficult to remove intact chloroplasts from the gradients without breaking some membranes and since Ludox inhibits enzyme reactions to various degrees chloroplasts from Ludox gradients

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[17] M. KLINGENBERG, H. W. HELDT u. E. PFAFF, in: The Energy Level and Metabolic Control in Mitochondria, S. PAPA, J. M. TAGER, E. QUAGLIARIELLO u. E. C. SLATER eds., Adriatica Editriche, Bari 1969.

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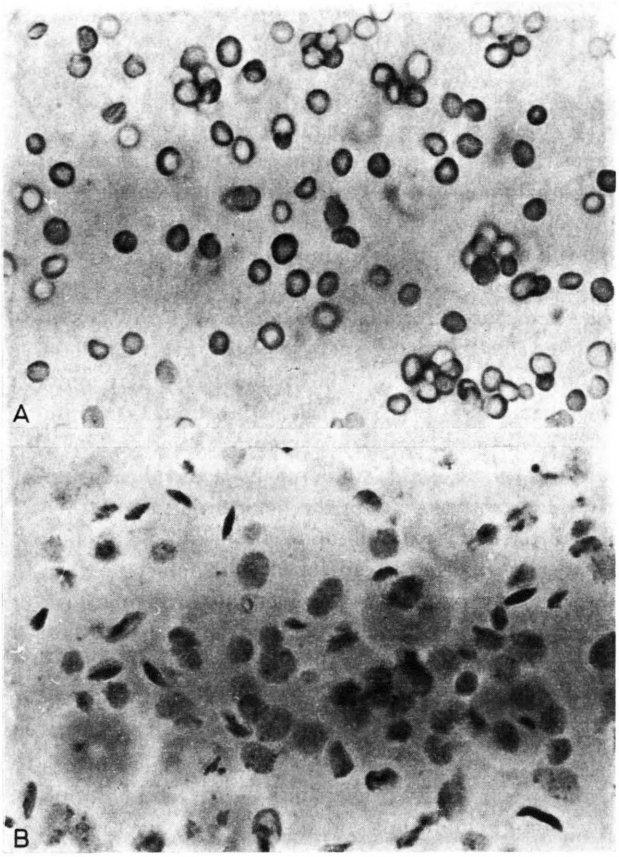

Fig. 3. A. Intact chloroplasts from the upper band of a Ludox density gradient after separation of intact and envelope-free chloroplasts by centrifugation. B. Envelope-free chloroplasts from the lower band.

were not used for photophosphorylation experiments.

To determine the percentage of broken chloroplasts contaminating a suspension of "intact" chloroplasts a Hill-reaction with ferricyanide as electron acceptor has been used. Using the separation of intact and broken chloroplasts on Ludox gradients as a reference it was found that, contrary to broken chloroplasts, intact chloroplasts do not or only very slowly photoreduce added ferricyanide, obviously because ferricyanide is unable to penetrate the chloroplast envelope. The ratio of the Hill-activity of the "intact" preparation to that of the same preparation after brief osmotic shock to rupture chloroplast envelopes (measured under identical conditions) served as a direct measure of the percentage of broken chloroplasts in the preparations. Results of relevant experiments are shown in fig. 4. Table 1 compares percentages of broken chloroplasts in "in-

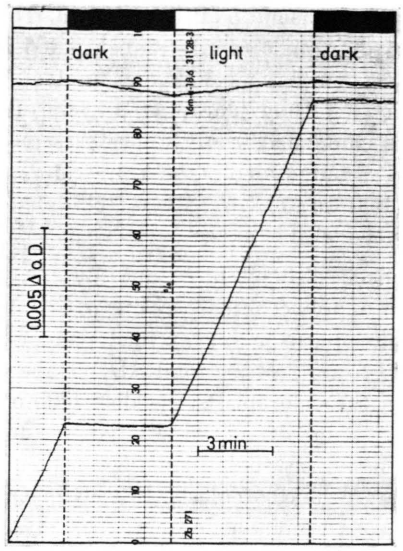

Fig. 4. Light-dependent reduction of ferricyanide by a chloroplast preparation before and after osmotic shock as revealed by changes in the optical density at 400 nm. Upper trace: Time course of optical density in the dark and during illumination with red light; chloroplast preparation with 6% envelope-free chloroplasts (7 μg chlorophyll per ml). Lower trace: Same chloroplast preparation as above, after brief osmotic shock.

exp. no.	% intact chloroplasts in the preparation as determined by	
	Hill-reaction measurements	chlorophyll distribution
1	72	67.4
2	92	92
3	35	29.7
4	70.7	73.2
5	90	92.9
6	75.1	83.4

Table 1. The percentage of intact chloroplasts in preparations containing also envelope-free chloroplasts as determined by measuring the light-dependent reduction of ferricyanide before and after osmotic shock and by measuring the chlorophyll distribution in density gradients made of Ludox after physical separation of the chloroplasts. Reproducibility of Hill-reaction measurements was better than that of chlorophyll determinations.

tact" preparations as measured by the chlorophyll distribution in Ludox density gradients and by reduction rates of ferricyanide. With rare exceptions, similar values were obtained.

Phosphorylation of ADP added externally to intact chloroplasts was investigated in the absence of cofactors. As shown in table 2, ADP is phosphorylated in the light by envelope-free shocked chloroplasts under these conditions at a rate of ca. 20 μmoles/mg chlorophyll per hour. As is made

exp. no.	μmoles phosphate uptake/mg chlorophyll per hour	
	envelope-free chloroplasts	intact* chloroplasts
1	17.8	1.6
2	20.6	4.3
3	23.6	3.6
4	24.5	1.7
5	21.5	0
6	19.4	3.4

Table 2. Light-dependent phosphorylation of ADP by intact * and envelope-free chloroplasts. No cofactors added to the system. * Values corrected for 5 to 20% envelope-free chloroplasts contaminating suspensions of intact chloroplasts.

evident by sensitivity to 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea and similar rates of oxygen uptake in the presence of cyanide (to poison catalase activity) ATP synthesis is supported in this system by pseudocyclic electron flow [19]. Preparations of intact chloroplasts display a much lower rate of phosphate uptake in the light. If rates are corrected for broken chloroplasts contaminating the preparations, intact chloroplasts phosphorylate ADP in the absence of bicarbonate at rates ranging from close to zero to more than 4 μmoles/mg chlorophyll per hour. Side reactions do not contribute significantly to the rates. As there is reason to assume that phosphate transfer into the chloroplasts is not rate limiting, the lowered rate of phosphorylation by intact chloroplasts as compared with that of shocked chloroplasts may indicate slow transfer of ADP across the chloroplast envelope. The results are in general agreement with direct measurements of ADP/ATP exchange [10].

To test whether the rate of transfer is linked with the rate of ATP turnover, phosphate uptake was measured in the absence of added cofactors, but with $2{,}1 \cdot 10^{-3}$ M phosphoglycerate present, which is reduced by intact but not by broken chloroplasts. Phosphate uptake was not increased as compared with controls which did not contain phosphoglycerate. Atractyloside, which is known to inhibit adenylate transfer across the mitochondrial membrane [20], had no significant effect on transfer in the chloroplast system.

III. Reaction of adenylate kinase present in intact chloroplasts

Chloroplasts contain a soluble adenylate kinase in their stroma [7] which catalyzes after ultrasonic or osmotic breakage of the chloroplast envelope the conversion of added AMP and ATP at rates of 50 to 120 μmoles/mg chlorophyll per hour. The slow appearance of ADP outside of intact chloroplasts can be used as a measure of penetration of the "slowest" participant of the reaction which probably is AMP [10, 21]. Unexpectedly high rates of ADP formation by preparations of intact chloroplasts could be explained by the fact that broken chloroplasts retain inspite of careful washing some adenylate kinase even though this is a soluble enzyme which can quantitatively be removed from broken chloroplasts by density gradient centrifugation. When the rate of ADP formation from AMP and ATP was plotted against the percentage of intact chloroplasts in different preparations from the same leaf material extrapolation of the resulting lines to 100% intact chloroplasts indicated that these chloroplasts formed less than 3 and possibly close to zero μmoles ADP/mg chlorophyll per hour from added AMP and ATP (fig. 5).

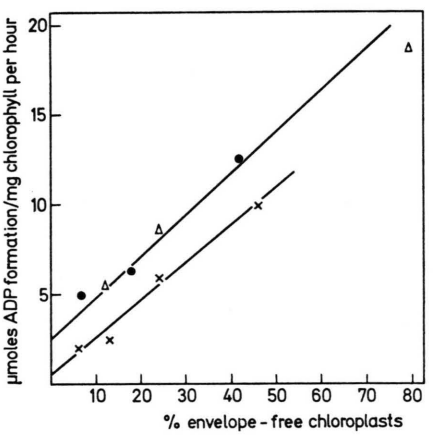

Fig. 5. ADP-formation from AMP and ATP by washed preparations of intact chloroplasts containing different percentages of envelope-free chloroplasts. Chloroplasts were isolated in 3 experiments from 3 spinach batches by differential centrifugation at 200, 300, 400 and, in one experiment, 500 *g*.

The rates are too low to account for the light-dependent cytoplasmic adenylate fluctuations in vivo. The measurements were performed in the dark in contrast to the photophosphorylation experiments described above which indicate penetration in the light.

IV. Adenylate content in isolated chloroplasts

Intact spinach chloroplasts washed in isotonic buffer contained between 22 and 30 nanomoles adeny-

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[20] H. W. HELDT, H. JACOBS, and M. KLINGENBERG, Biochem. biophysic. Res. Commun. **18**, 174 [1965].
[21] A. J. KEYS, Biochem. J. **108**, 1 [1968].

lates/mg chlorophyll (values corrected for broken chloroplasts, some values shown in table 3). The ATP content generally was very low. The level of adenylates in chloroplasts in situ is higher than that of aqueously isolated chloroplasts as shown by nonaqueous chloroplast isolation from the same leaf material. Between 60 and 100 nanomoles/mg chlorophyll were found in nonaqueous chloroplasts (some of the values shown in table 3). Although the leaves were kept in the dark, the ATP level was high and the AMP level low. More than 50% of the adenylates were ATP and less than 10% AMP.

exp. no.		nanomoles/mg chlorophyll in aqueously isolated chloroplasts*	nonaqueously isolated chloroplasts
		ATP	
1	expanded	2.8	54.7
2	leaves	3.6	55.2
3	young	4.5	29
4	leaves	4.9	39.5
		ADP	
1		15.2	25.2
2		14.6	22
3		15.1	24.6
4		14.9	28.5
		AMP	
1		22.2	3.2
2		18.8	3.2
3		19.4	4.8
4		13.5	6
		total adenylates	
1		40.2	83.1
2		37	80.4
3		39	58.4
4		33.3	74

Table 3. Content of ATP, ADP and AMP in chloroplasts after aqueous and nonaqueous isolation from the same leaf material *(Spinacia oleracea)*. Leaves were kept in the dark. * Corrected for a low percentage of envelope-free chloroplasts which had lost their adenylates.

The differences in the adenylate content of aqueously and nonaqueously isolated chloroplasts from the same source show that about 50% of the adenylates are lost during isolation. Still the adenylate content is not rate limiting in metabolic reactions of the isolated chloroplasts as shown by their photosynthetic activity. The large differences in the phosphorylation state of the adenylates may be considered as another indication that in vivo chloroplastic adenylate pools communicate with cytoplasmic pools. Even in the dark a high ATP level is maintained in the chloroplasts in vivo and only after separation from the cytoplasm does the ATP level drop and AMP accumulate owing to the action of ATPase and adenylate kinase.

V. Transfer of phosphate energy by transport metabolites

Obviously phosphorylation of added ADP by intact chloroplasts requires either transfer of ADP to the phosphorylation sites inside the chloroplasts and back transfer of ATP or a transphosphorylation system in the chloroplast envelope or transport metabolites capable of transferring phosphate energy from the chloroplasts to the surrounding medium[8]. A transport metabolite capable of passing largely unrestricted into and out of chloroplasts is phosphoglycerate[6, 22–26]. Addition of phosphoglycerate in the light to intact chloroplasts resulted in the immediate evolution of oxygen owing to its phosphorylation to 1,3-diphosphoglycerate and subsequent reduction to triosephosphate. Reduction rates by good chloroplast preparations as measured polarographically approached 300 μmoles/ml chlorophyll per hour. Chloroplasts from spinach grown in the spring or in the fall had considerably higher activity than winter material, which reduced added phosphoglycerate at rates as low as 30 μmoles/mg chlorophyll per hour.

An indirect transfer of ATP across the chloroplast envelope could be brought about by a shuttle involving 3-phosphoglycerate and 1,3-diphosphoglycerate. It is unknown whether 1,3-diphosphoglycerate can penetrate the chloroplast envelope. If this were the case, 1,3-diphosphoglycerate formed from 3-phosphoglycerate in the chloroplasts could be transferred to the cytoplasm and phosphorylate there ADP by phosphoglycerate kinase. The resulting 3-phosphoglycerate could then reenter the chloroplast. Net re-

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[24] J. A. BASSHAM u. R. G. JENSEN, in: Harvesting the Sun, A. SAN PIETRO, F. A. GREER u. T. I. ARMY eds., p. 79, Academic Press, London 1967.
[25] J. M. ROBINSON u. C. R. STOCKING, Plant Physiol. **43**, 1597 [1968].
[26] U. HEBER, in: Proc. of the Internatl. Sympos. „Productivity of Photosynthetic Systems", Moscow 1969, in the press.

sult would be the transfer of ATP without involving movements of adenylates. If this system functions in vivo, isolated washed chloroplasts should phosphorylate ADP in the light faster in the presence than in the absence of added phosphoglycerate kinase. Table 4 shows that this was not the case indicating that this possible transport metabolite system is inoperative in vivo.

exp. no.	μmoles phosphate uptake / mg chlorophyll per hour in the presence of phosphoglycerate kinase	without added phosphoglycerate kinase
1	2	0
2	1,6	1,2
3	0	0
4	0,2	—
5	3,0	2,0

Table 4. Light-dependent phosphate uptake of intact * chloroplasts in the presence of $2{,}1 \cdot 10^{-3}$ M phosphoglycerate as influenced by addition of phosphoglycerate kinase to the system. * Corrected for phosphate uptake of 12 to 40% envelope-free chloroplasts contaminating the preparations of intact chloroplasts.

Indirect transfer of ATP could also be mediated by the triosephosphate oxidation system of chloroplasts and cytoplasm [8]. Phosphoglycerate is reduced in the chloroplasts in the light by ATP and reduced pyridine nucleotide to glyceraldehyde-3-phosphate, which is in quasi-equilibrium with dihydroxyacetone phosphate. The latter is capable of penetrating the chloroplast envelope [23, 27]. Its oxydation in the cytoplasm would yield ATP and reduced pyridine nucleotide. Transfer of ATP should be expected to be considerable if accumulation of reduced pyridine nucleotide is prevented by a suitable oxidation system. Table 5 demonstrates that the phosphorylation of added ADP by intact chloroplasts is indeed greatly stimulated in the presence of added glyceraldehyde phosphate dehydrogenase, phosphoglycerate kinase, triosephosphate isomerase and a system capable of oxidizing NADH. These experiments were made with winter material. No attempt has been made to optimize rates and it is clear that transfer rates much higher than the $40 - 50$ μmoles/mg chlorophyll per hour listed for the complete system in table 5 can be observed when chloroplasts with a higher capacity to reduce phosphoglycerate are used. ATP added to the system reduced the rate of phosphorylation demonstrating that a control step of indirect transfer

	phosphate uptake, μmoles/mg chlorophyll · hour A	B
complete system**	40	47
plus $1.5 \cdot 10^{-3}$ M(A) or $4.5 \cdot 10^{-3}$ M(B) ATP	31.4	18.6
minus phosphoglycerate	0.4	5.4
complete system minus lactate dehydrogenase	8.4	10.7
minus triosephosphate isomerase	2.5	0.0
minus glyceraldehyde phosphate dehydrogenase	1.6	0.0
minus glyceraldehyde phosphate dehydrogenase and phosphoglycerate kinase	1.1	—
rate of phosphoglycerate reduction*** as measured by light- and phospho glycerate-dependent oxygen evolution in the complete system in μmoles/mg chlorophyll per hour	64.0	90.0

Table 5. Indirect transfer of ATP across the envelope of intact chloroplasts * via a shuttle transfer of phosphoglycerate and dihydroxyacetone phosphate. * Values corrected for envelope-free chloroplasts contaminating the preparations. ** 1.6 ml isotonic reaction mixture as used for photophosphorylation experiments containing in addition $1.5 \cdot 10^{-3}$ M glutathione, $1.5 \cdot 10^{-3}$ M phosphoglycerate, $1.9 \cdot 10^{-3}$ M pyruvate, $3.75 \cdot 10^{-4}$ M NAD, 100 μg glyceraldehyde phosphate dehydrogenase, 50 μg phosphoglycerate kinase, 50 μg triosephosphate isomerase and 50 μg lactatdehydrogenase. *** Rate not considerably influenced by omissions as indicated above except omission of phosphoglycerate.

is the phosphate potential outside the chloroplasts. Control is exerted also by the redox state of NAD as made evident by the decrease of indirect transfer in the absence of lactate dehydrogenase which served to keep NAD oxidized. The almost complete dependence of the system on the presence of triosephosphate isomerase outside the chloroplasts (which, however, can be seen only when carefully washed chloroplasts are used) demonstrates that 3-phosphoglyceraldehyde is not a transport metabolite and is retained inside the chloroplasts. This agrees with previously published evidence [12].

As glyceraldehyde phosphate dehydrogenase, phosphoglycerate kinase and triosephosphate isomerase in vivo reside both in chloroplasts and cytoplasm in high activities it is clear that in principle the shuttle phosphoglycerate/dihydroxyacetone phosphate is capable of transferring ATP indirectly from the chloroplasts into the cytoplasm in the light and vice versa in the dark. Limitations of this transfer system will be considered in the discussion.

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Discussion

The almost simultaneous fluctuations of ATP and ADP in chloroplasts and cytoplasm of leaf cells as shown in fig. 1 demonstrate in agreement with previous work [5–7] that chloroplastic and cytoplasmic adenylate pools are linked. No mechanism is known which could explain by light-stimulated metabolic events in the cytoplasm which are independent of chloroplasts the increase of cytoplasmic ATP and decrease of ADP seen on illumination. Rather ATP supplying and ADP consuming cytoplasmic reactions appear to be suppressed in the light [4, 37]. Therefore the observed cytoplasmic changes must be coupled by an effective mechanism to the similar changes caused by photophosphorylation in the chloroplasts. Similar considerations apply for reversed changes seen on darkening. Effectiveness of coupling can be expressed by the rate of light-dependent changes in cytoplasmic adenylate levels. This rate, which is close to 10 μmoles/mg chlorophyll per hour, reflects the minimum rate of transfer of phosphate energy across the chloroplast envelope. Actual transfer rates may be higher. Any transfer rate observed in vitro with isolated chloroplasts must be in this order of magnitude or higher to explain the in vivo observations.

Qualitatively, transfer of phosphate energy across the chloroplast envelope has also been observed in vitro. In experiments of BASSHAM isolated chloroplasts photosynthesizing in the presence of $H^{32}PO_4^{2\ominus}$ excreted labelled ATP. A fast decrease of external $AT^{32}P$ on darkening was explained by consumption inside the chloroplasts. The addition of bicarbonate to illuminated chloroplasts resulted in a concomitant decrease of internal and external ATP presumably by photosynthetic reactions inside the chloroplasts [28]. Addition of ATP to chloroplasts in the dark increased the level of endogenous ribulose diphosphate indicating that it reached the sites of synthesis in the stroma [29]. A minimum rate of transfer of ATP of ca. 2,5 μmoles/mg chlorophyll per hour was indicated by the observation that hexokinase reacted in intact chloroplasts with glucose and added ATP at a rate of 2,5 μmoles/mg chlorophyll per hour to form glucose-6-phosphate [12]. The enzyme reaction, not transfer, was rate limiting.

There are different possibilities to explain linkage of chloroplastic and cytoplasmic adenylate pools.

1. Pools may be linked by unspecific diffusion of adenylates across the chloroplast envelope.
2. Linkage may occur through a specific exchange-translocation mechanism within the chloroplast envelope for the transport of adenylates such as known for mitochondria [1–3].
3. Transfer of phosphate energy may occur in the absence of transfer of adenylates by a transphosphorylation mechanism in the chloroplast envelope.
4. Transfer of phosphate energy may, again in the absence of adenylate transfer, be mediated by a cyclic system of transport metabolites which are capable of traversing the chloroplast envelope.

Of these possibilities transfer by passive diffusion is unlikely. Although it appears that part of the endogenous adenylates is fairly readily lost from the chloroplasts during isolation (table 3), another part, which is obviously sufficient for photosynthetic reactions, is retained in spite of washing the chloroplasts in isotonic buffer. Even after separation on a Ludox gradient the adenylate content of the chloroplasts appears largely unaltered as compared with freshly isolated chloroplasts. Rates of leakage of adenylates from isolated chloroplasts by diffusion are low. In vivo ATP/ADP ratios in chloroplasts and cytoplasm are markedly different. Passive diffusion at rates indicated by the in vivo experiments should be expected to lead to equal ratios.

With the methods used in this work exchange-translocation of adenylates and transphosphorylation mechanisms in the membrane not involving direct transfer of adenylates cannot readily be distinguished. However, there are no specific data indicating that transphosphorylation mechanisms do exist. In most photophosphorylation experiments and in the adenylate kinase tests transfer of phosphate energy by transport metabolites cannot contribute to the observed reaction rates as external enzymes involved in transport metabolite systems were removed by washing.

A specific exchange translocation mechanism sensitive to atractyloside [20] is known to facilitate exchange of adenylates across mitochondrial membranes at rates comparable to those of oxidative phos-

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phorylation. A similar mechanism has been shown by STROTMANN and HELDT [9], STROTMANN and BERGER [30] and HELDT [10] to exist in chloroplasts. However, rates of exchange as measured by these authors are quite insufficient to explain transfer of phosphate energy as indicated by the in vivo experiments.

In our experiments phosphorylation of added ADP by intact chloroplasts in the light in the absence of added cofactors ranged in different experiments from zero to about 4 μmoles/mg chlorophyll per hour. An average rate of 2 μmoles is higher than the exchange rate with added ADP as reported by HELDT [10], but again insufficient to explain in vivo transfer of phosphate energy. However, it appears possible that in vivo exchange translocation across the chloroplast envelope is faster than in vitro experiments indicate. It has been shown for mitochondria that transfer of slowly penetrating metabolites can be greatly enhanced in the presence of other compounds [31]. STOCKING and ROBINSON observed that, even though additions of ATP in the light to chloroplasts actively photoreducing added phosphoglycerate does not increase the rate of reduction, additions in the dark immediately prior to illumination stimulate phosphoglycerate reduction in the subsequent light period [25]. This observation, which has been confirmed in our laboratory, may suggest that in vivo direct transfer of adenylates is a controlled process.

In addition to the direct penetration of the chloroplast envelope by adenylates there is the possibility of a link of chloroplastic and cytoplasmic adenylate levels by suitable transport metabolite systems.

An obvious possibility of linkage is through the phosphoglycerate/dihydroxyacetone phosphate system [8]. Both compounds are transport metabolites as discussed more fully elsewhere [8, 23, 26]. Transfer rates for phosphoglycerate are usually around 100 to 150 μmoles/mg chlorophyll per hour, sometimes even up to 300 μmoles/mg chlorophyll per hour. Transfer of dihydroxyacetone phosphate is about as fast as that of phosphoglycerate. These transport metabolites link chloroplastic and cytoplasmic metabolism at the glycolytic and the sugar level. In an unidirectional flow their capacity of carbon transfer is higher than 400 μatoms/mg chlorophyll per hour and thus higher than maximum rates of photosynthesis. In a cyclic system a transfer of phosphate energy and of reducing equivalents is possible. Phosphoglycerate is phosphorylated in the chloroplasts by ATP and reduced to glyceraldehyde-3-phosphate, which is in quasi-equilibrium with dihydroxyacetone phosphate. This can leave the chloroplasts. Its oxydation in the cytoplasm yields ATP and NADH. The resulting phosphoglycerate can return to the chloroplasts and thus establish a shuttle for the transfer of ATP and reduced pyridine nucleotide. In fact, transfer of reducing equivalents in this system across the chloroplast envelope has under suitable in vitro-conditions been observed by STOCKING [32] and an efficient transfer of ATP is demonstrated in table 5.

Although there is little doubt that the system functions also in vivo, its efficiency is probably not high for the following reasons: Transfer of ATP is coupled stoichiometrically to the transfer of reducing equivalents. The level of cytoplasmic NADH is lower than the level of cytoplasmic ATP by a factor of approximately 10 [7, 33]. A considerable transfer of ATP would thus have to be accompanied by either a large increase in or a fast oxidation of cytoplasmic NADH. However, a considerable increase of NADH would stop the oxidation of dihydroxyacetone phosphate and thereby the transfer of ATP for equilibrium reasons. In addition, no significant increase could be observed [33]. A considerable transfer of ATP into the cytoplasm by this transport metabolite system can therefore only be brought about if cytoplasmic NADH is rapidly oxidized. Such an oxidation could by itself contribute to a rise in cytoplasmic ATP. However, an accelerated oxidation of cytoplasmic NADH even though it may occur during transients is unlikely in the steady state in view of the control of respiratory events in the cytoplasm by the ATP/ADP ratio which is indicated by a large volume of experimental evidence [34–36]. It is also

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contradicted by the observation that oxygen uptake presumably of mitochondria is inhibited by light [4, 37].

A mode of transfer of ATP simpler than that by the system phosphoglycerate/dihydroxyacetone phosphate and not subject to its limitations is transfer via a shuttle system involving phosphoglycerate and 1,3-diphosphoglycerate. The concentration of the latter in the cell is very low. Any considerable transfer of ATP by this system should express itself as an increase in the phosphorylation of added ADP by intact chloroplasts which photoreduce added phosphoglycerate and are supplied externally with phosphoglycerate kinase. Such an increase could not be observed. Obviously this system does not operate, presumably because the chloroplast envelope is impermeable for 1,3-diphosphoglycerate.

From the results and the above considerations it is concluded that in vivo transfer of phosphate energy across the chloroplast envelope takes place directly via a probably controlled penetration of adenylates and in addition indirectly also via transport metabolite systems. It has previously been emphasized that this transfer has important metabolic consequences [7]. These are briefly summarized: In the dark the components of the triosephosphatedehydrogenase/phosphoglycerate kinase system of leaf cells are not far from thermodynamic equilibrium. This is shown by the fact that illumination by simply raising the level of ATP and NAD(P)H and lowering that of ADP reverses the direction of the reaction in the chloroplasts. A reversal is not possible in the cytoplasm because chloroplastic and cytoplasmic pyridine nucleotide pools are separated. However, the light-dependent increase in cytoplasmic ATP and the decrease in ADP as demonstrated in fig. 1 control oxidation of the increased levels [27] of cytoplasmic fructose-1,6-diphosphate and of triosephosphate (table 5). Inhibition of glycolysis has in fact been shown to occur [15].

Similarly, mitochondrial respiration is assumed to be under control of the phosphate potential ATP/ADP·P_i, a lowered potential inducing oxidation of substrates, an increased potential inhibiting it. The increase in the cytoplasmic phosphate potential in the light (cf. fig. 2) should be expected to communicate to the mitochondrial phosphate potential through the mitochondrial exchange translocation mechanism for adenylates producing an inhibition of respiration. Such an inhibition is in fact known for a long time although it can be obscured by other effects of light on oxygen uptake [4, 38].

It is likely that the suppression of mitochondrial oxidation in the light, the flooding of the cytoplasm with phosphoglycerate, triose phosphate and hexose diphosphate and the increase in the phosphate potential will also result in other shifts of cytoplasmic metabolism.

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