PHOTOSYNTHESIS BY ISOLATED CHLOROPLASTS

VI. RATES OF CONVERSION OF LIGHT INTO CHEMICAL ENERGY IN PHOTOSYNTHETIC PHOSPHORYLATION

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It has previously been shown that illuminated spinach chloroplasts esterify orthophosphate into the energy-rich pyrophosphate bonds of ATP** under conditions which exclude CO_2 fixation[1-5]. This process, which has been named photosynthetic phosphorylation, thus affords an independent measure of the transformation of light into chemical bond energy, by a technique employing neither the evolution of oxygen nor the assimilation of CO_2[6,3].

The determination of maximal rates of photosynthetic phosphorylation is of interest not only in assessing the efficacy of ATP formation at the expense of light by a cell-free system but also in appraising the possible quantitative importance to the overall photosynthetic process of this special mechanism for generating ATP independently of respiration[6,3].

Prior to the identification of the cofactors[7,8] of photosynthetic phosphorylation and of improved experimental conditions, it was deemed premature[9] to embark on the investigation of rates of photosynthetic phosphorylation which is now being reported. As with most newly-discovered cell-free reactions, the early rates were low[1,6,9]. This paper describes the experimental conditions under which we obtained rates of photosynthetic phosphorylation up to 170 times higher than those first described[1]. These new rates of photosynthetic phosphorylation are comparable with the maximum rates of conversion of light into chemical energy during photosynthesis by whole leaves with optimal light and CO_2 supply[10].

METHODS

Whole chloroplasts were prepared from spinach leaves as previously described[3] (suspension P_1), except that the leaves were ground in 0.35 *M* NaCl containing 0.02 *M* Tris buffer, pH 8. The whole chloroplasts (P_1) were then disrupted by suspending in ice-cold dilute NaCl (0.035 *M*). The resultant chloroplast fragments, which were used in al the experiments described here, are similar to those prepared in earlier experiments by disrupting whole chloroplasts in distilled water[4], but have the advantage that they lend themselves better to repeated washing and centrifugation.

Chloroplast extract (CE) was prepared as previously described[4] except that dilute NaCl (0.035 *M*) was substituted for water as the extractant. Chlorophyll determinations and measurements of photosynthetic phosphorylation were carried out by methods previously reported[3].

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** The following abbreviations will be used: AMP, adenosine-5-phosphate; ADP, ATP, adenosine di- and triphosphate; P_i, orthophosphate; PGA, 3 phosphoglyceric acid; FMN, flavin mononucleotide; tris, (hydroxymethyl) aminomethane.

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The reaction was carried out at 20° C. Unless otherwise specified, the reaction mixture (final volume 3 ml) contained chloroplast fragments and, in micromoles: Tris** buffer, pH 8.3, 80; $K_2H^{32}PO_4$, 20 or 10; AMP, 20 or ADP, 10; sodium ascorbate, 10; $MgCl_2$, 10; FMN, 0.1; and vitamin K_5 (2-methyl-4-amino-1-naphthol hydrochloride) 0.3.

RESULTS

Light saturation

Since the object of our experiments was to measure the rate of photosynthetic phosphorylation in saturating light it was first necessary to establish the range of chloroplast concentration (expressed in mg of chlorophyll) for which light saturation could be provided with the particular light source used in our experiments (cerise fluorescent tubes[11] submerged in the constant temperature bath). Fig. 1 shows that at constant light, photosynthetic phosphorylation was proportional to the concentration of chlorophyll up to 0.3 mg per 3 ml of reaction mixture. At higher concentrations of chloroplast material light was obviously limiting photosynthetic phosphorylation.

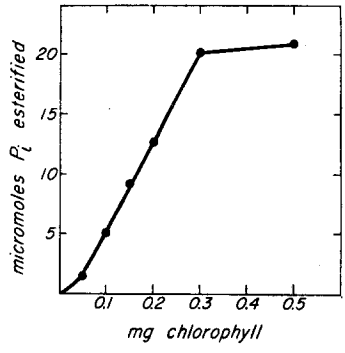

Fig. 1. Photosynthetic phosphorylation as a function of chorophyll concentration. Experimental conditions as described under METHODS. The reaction was carried out for 30 minutes.

Based on these results, the concentration of chloroplast material used in all subsequent measurements of rates of photosynthetic phosphorylation was reduced to a level well below the limit of light saturation; it was never in excess of 0.2 mg chlorophyll per 3.0 ml of reaction mixture.

Phosphate acceptor

In our earlier experiments[1,2] ADP and AMP were found to be equally effective as phosphate acceptors in the phosphorylation reaction. The question which of these two is the primary phosphate acceptor was left open. It seemed likely that in photosynthetic phosphorylation by chloroplasts, as in respiratory phosphorylation by mitochondria[12], ADP was the primary phosphate acceptor and that the equal effectiveness of AMP was made possible by the activity of adenylic kinase (myokinase), the enzyme which catalyzes the reversible reaction:

$$ATP + AMP \rightleftarrows 2ADP$$

This enzyme was indeed shown later to be concentrated in spinach chloroplasts[13]. The catalytic amounts of ATP required for its activity would be expected to be normally present in the chloroplast system.

On washing chloroplast fragments in distilled water or dilute salt (0.035 *M* NaCl) it was found, in agreement with the results of FRENKEL on bacterial preparations[14],

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that photosynthetic phosphorylation was almost completely abolished when AMP was used as the phosphate acceptor (Table I). Phosphorylation was restored, however, by substituting ADP as phosphate acceptor or by adding to the washed chloroplast fragments either purified rabbit myokinase[15] or chloroplast extract (CE), a preparation containing the water-soluble constituents of chloroplasts removable by washing. Adenylic kinase thus appears to be easily removed by washing whole chloroplasts (*cf.*[13,16]).

TABLE I

EFFECT OF MYOKINASE ON PHOTOSYNTHETIC PHOSPHORYLATION BY WASHED CHLOROPLASTS

Additions	*μM P_i esterified*
AMP	1.3
AMP + CE	8.7
AMP + myokinase	11.9
AMP	2.2
ADP	12.8

Chloroplast fragments containing 0.2 mg chlorophyll were used. Other components are described under "METHODS". Phosphorylation was carried out for 15 minutes (top) or 20 minutes (bottom).

In subsequent experiments on rate measurements ADP rather than AMP was used as the phosphate acceptor. This modification of experimental procedure was based on observations that at low concentrations of chloroplast material the supply of adenylic kinase limited the overall rate of phosphorylation, even in unwashed preparations, when AMP was added as the phosphate acceptor. Unwashed fragments of chloroplasts were used in all subsequent experiments discussed in this paper.

The chloroplast extract (CE) was shown in earlier experiments[17,4] to be important as a source of soluble enzymes in CO_2 fixation. It now appears that it also contains soluble factors involved in phosphorylation. Among these we can identify not only adenylic kinase but also the TPN-reducing factor described elsewhere[18,19] and the TPN-diaphorase isolated by AVRON AND JAGENDORF[20]. Since TPN diaphorase has been found[20] to catalyze the transfer of electrons between TPNH (but not DPNH) and known cofactors of photosynthetic phosphorylation (FMN and vitamin K), its role as a component of the electron transport chain in this process was rendered likely by the recent finding that TPN (but not DPN) is a catalyst in photosynthetic phosphorylation[18].

pH dependence

The replacement of AMP by ADP as the phosphate acceptor has led to a reexamination of the pH optimum of the phosphorylating system, independent of the activity of adenylic kinase. Previously published phosphorylation experiments[1–4], were run at pH 7.2–7.4. This pH was chosen since it was compatible with good activity of all three photochemical reactions of chloroplasts—photolysis, phosphorylation and carbon assimilation. With AMP as the phosphate acceptor the phosphorylation reaction had a broad pH optimum; the rate at pH 7.2–7.4 was still high (Fig. 2).

In contrast to phosphorylation with AMP, the reaction with ADP was found

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to have a rather sharp pH optimum at 8.3 (Fig. 2). Accordingly, this pH was adopted for all subsequent experiments on photosynthetic phosphorylation.

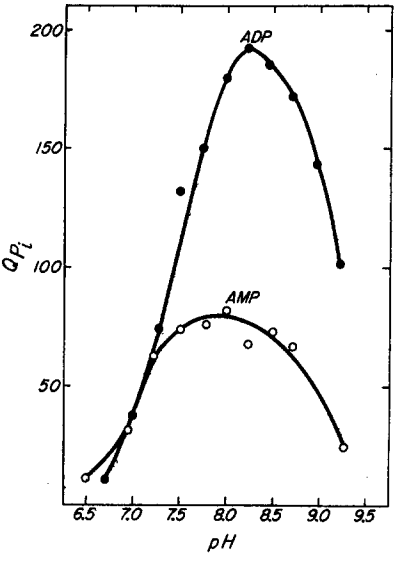

Fig. 2. Effect of pH on photosynthetic phosphorylation. The reaction mixture (final volume 3 ml) in the AMP series contained: chloroplast fragments equivalent to 0.5 mg chlorophyll, and the following in micromoles: tris, 80; AMP, 20; $^{32}P_i$, 20; Na ascorbate, 10; FMN, 0.1; vitamin K_5, 0.3; $MgCl_2$, 10. In the ADP series the reaction mixture (final volume 3 ml) contained: chloroplast fragments equivalent to 0.2 mg chlorophyll; and the following in micromoles: tris, 80; ADP, 10; $^{32}P_i$, 10; $MgCl_2$, 5; FMN, 0.03; vitamin K_5, 0.1; Na ascorbate, 10. The pH was adjusted by titrating individually to the desired value the following components of the reaction mixture: Tris and K_2HPO_4 with HCl; AMP and ADP with NaOH. Other conditions as described under METHODS.

Rates of photosynthetic phosphorylation

Table II illustrates the rates of photosynthetic phosphorylation which have been obtained under the changed experimental conditions when: (a) ADP was the phosphate acceptor, (b) the pH was 8.3, and (c) the concentration of the chloroplast material in the reaction mixture was adjusted to correspond to a chlorophyll concentration not in excess of 0.1 mg per 3 ml. Under these conditions, and in the presence of FMN, vitamin K_5, and ascorbate, chloroplast fragments esterified anaerobically into ATP 360 to 510 micromoles of orthophosphate per mg chlorophyll per hour. Expressed on the basis of the nitrogen content of chloroplast fragments these rates correspond to 554–785 micromoles of orthophosphate per mg nitrogen (Table II).

TABLE II

RATES OF PHOTOSYNTHETIC PHOSPHORYLATION

Exp. No.	$Q^{Chl.}_{P_i}$	$Q^{N}_{P_i}$
209	405	624
210	360	554
211	510	785
212	450	693
212	420	647

Chloroplast fragments containing 0.05 or 0.1 mg chlorophyll were used. Other conditions as described under "METHODS". $Q^{Chl.}_{P_i}$ = micromoles P_i esterified, per hour, per mg chlorophyll. $Q^{N}_{P_i}$ = micromoles P_i esterified per hour, per mg nitrogen. These rates were computed on the basis of the quantity of P_i esterified during the first 3 minutes of the reaction. The rate decreased with time but this falling off was prevented in other experiments by the addition of TPN[18].

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JAGENDORF AND AVRON[21] have recently reported rates for photosynthetic phosphorylation for spinach chloroplasts of 200 micromoles of orthophosphate esterified per hour per mg of chlorophyll. Higher rates were also obtained by these authors when several cofactors were replaced by artifical compounds.

DISCUSSION

Rates of carbon assimilation and photosynthetic phosphorylation

The maximum capacity of a chlorophyll-bearing plant to convert light energy into chemical energy is often expressed as the "assimilation number" of WILLSTÄTTER AND STOLL[22]. The "assimilation number", defined as the maximum quantity of carbon dioxide that can be reduced in saturating light by a unit quantity of chlorophyll in a unit time, is different for various plants. The "assimilation number" for spinach leaves is not known but we can assume that it does not exceed 4000 mm^3 CO_2 (*ca.* 180 micromoles) per mg chlorophyll per hour, cited by RABINOWITCH[10] as the maximum assimilation number for green leaves of land plants.

A comparison of this maximum rate of carbon assimilation for *intact* leaves (180 micromoles CO_2/h/mg chlorophyll) with the observed rate (Table II) for photosynthetic phosphorylation by spinach chloroplast fragments *outside* the living cell (500 micromoles P_i esterified/h/mg chlorophyll), leads to the conclusion that the capacity of chloroplasts to convert light energy into ATP is significant. When CO_2 fixation is excluded, the photosynthetic apparatus localized in chloroplasts is capable of using light energy to esterify inorganic phosphate into ATP, at a rate equal to producing several high-energy pyrophosphate bonds for each molecule of CO_2 that would have been assimilated by intact leaves at maximum photosynthesis. This high capacity for converting light into pyrophosphate bond energy is demonstrable by direct measurement with present experimental techniques, which may still be suboptimal, without making allowances for the probable damage that accompanies the disruption of the cell and the isolation and fractionation of chloroplasts.

Stoichiometric ATP: CO_2 ratio

The high rates of photosynthetic phosphorylation invite consideration of recent proposal of a stoichiometric ratio between ATP and CO_2 in photosynthesis. The two most recent ones differ widely. BASSHAM AND CALVIN[23] arrived at a ratio of 3 moles ATP per mole CO_2 assimilated whereas KANDLER[24] suggested a ratio of 1/6.

The wide divergence in the estimation of the ATP requirement in photosynthesis stems from uncertainty about the path of carbon assimilation. CALVIN's scheme[25] is based on the key premise that phosphoglyceric acid is the first stable product of photosynthesis. This view is not shared by WARBURG[26], KANDLER[24] and KANDLER AND GIBBS[27,28]. WARBURG regards PGA, not as an intermediate in carbohydrate synthesis, but as a sugar degradation product* of induced respiration. KANDLER AND GIBBS question some of CALVIN's kinetic data on which the phosphoglyceric

* In this connection it may be recalled that an investigation of the glycolytic enzymes in photosynthetic tissues yielded evidence for the emergence, with the onset of photosynthesis[34], of a TPN-specific enzyme that catalyzes the oxidation of glyceraldehyde-3-phosphate without the addition of either inorganic phosphate or arsenate[35,36]. This new enzyme differs from the other two triose-phosphate dehydrogenases of green plants in having only a degradative action; it oxidizes triose phosphate to PGA irreversibly[36].

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acid theory was founded (compare also UTTER AND WOOD[29]) and cite their new degradation experiments which fail to support one of the main postulates of CALVIN's scheme, *i.e.* that the hexose molecule is formed in photosynthesis by a condensation of two identically labelled trioses[25]. It seems clear that precise formulations of ATP requirements in carbon assimilation will remain premature until the intermediate steps in the conversion of CO_2 to sugar are known with greater certainty.

With isolated chloroplasts the study of the intermediates in carbon assimilation is also not sufficiently advanced as yet to justify specific conclusions about pathways[9]. What is known is that isolated chloroplasts contain the necessary enzymic equipment to accomplish the complete photosynthetic transformation of CO_2 to sugar and starch[11,3,4,30]. Photosynthesis by chloroplasts might therefore be expected to provide, in time, an evaluation of the ATP requirement in carbon assimilation as well as of the role of photosynthetic phosphorylation as a source of ATP in a system free from oxidative phosphorylation by mitochondria. The evidence so far indicates that a capacity for phosphorylation is a prerequisite to CO_2 assimilation since only those chloroplast preparations which can phosphorylate are able to fix CO_2[1,11,6,30].

Rates of phosphorylation by chloroplasts and mitochondria

KANDLER, in his proposed carbon assimilation scheme[24], bases the low ATP requirement on postulations that ATP is needed neither in the reduction of a carboxyl to a carbonyl group, nor, basically, in the regeneration of the CO_2 acceptor, but principally in the polymerization of hexose units into oligo- and polysaccharides. The evaluation of the validity of these postulations must, of course, await their experimental substantiation. What can be disputed now is KANDLER's view that the current evidence favors the assignment of a subordinate role to photosynthetic phosphorylation as a source of ATP in photosynthesis.

KANDLER has concluded from a survey of the literature (*cf.* review[31]) and his own recent work[24] that the rate of photosynthetic phosphorylation is of the same magnitude as that of respiratory (oxidative) phosphorylation and as such is too low to play an important role in carbon assimilation. The high rates of photosynthetic phosphorylation by isolated chloroplasts which are now known were not before him. His appraisal of the potentialities of this process was mainly based on experiments with whole cells. In these the estimation of the rate of photosynthetic phosphorylation can only be made indirectly since it is difficult, if not impossible, to isolate with certainty from the over-all phosphorus metabolism of the intact cell those phases which are solely linked to photosynthetic events. Unlike isolated chloroplasts, whole cells do not accumulate appreciable quantities of ATP either in the presence or absence of CO_2. In the course of normal photosynthesis in intact cells the rate of ATP formation by chloroplasts is probably geared to the rate of CO_2 fixation. ATP is very likely consumed rapidly in the assimilation of CO_2 without diffusing to the external cytoplasm.

A more direct comparison between rates of photosynthetic and respiratory phosphorylation is afforded by studying the two processes, outside the living cell, with the aid of the specific cytoplasmic structures to which they are confined[6,3,30]: chloroplasts and mitochondria. In the cell-free systems both phosphorylating processes are isolated from their associated cellular reactions which consume ATP; ATP is thus allowed to accumulate.

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Chloroplasts, freed of mitochondria, do not respire (statements to the contrary in the literature as, for example, on p. 33, ref.[23] are not supported by experimental evidence) and do not carry on respiratory (oxidative) phosphorylation either in the light or in the dark[3,6,30,31]. Photosynthetic phosphorylation, which is strictly dependent on chlorophyll and light, can thus be readily distinguished from the respiratory phosphorylation carried on by mitochondria.

Table III summarizes rates of oxidative phosphorylation by mitochondrial preparations from plant and animal sources. A comparison, on a nitrogen basis, of these rates with those in Table II shows that the rate of ATP formation by chloroplasts, anaerobically, with light as the "substrate", is several times greater than that observed in oxidative phosphorylation by mitochondria at the expense of various organic substrates and oxygen.

TABLE III

RATES OF OXIDATIVE PHOSPHORYLATION BY MITOCHONDRIAL FRAGMENTS (F) AND INTACT MITOCHONDRIA (M) FROM PLANT AND ANIMAL SOURCES

(Q_{P_i} = μmoles P_i ester/mg N/h)

Reference	*Source*	Q_{P_i}
GREEN *et al.*[37]	heart (F)	39–117
COOPER AND LEHNINGER[38]	liver (F)	22–33
GREEN *et al*[37]	heart (M)	173
COPENHAVER AND LARDY[39]	liver (M)	51–54
CONN AND YOUNG[40]	lupine (M)	38–75
LIEBERMAN AND BIALE[41]	sweet potatoes (M)	145–200
LATIES[42]	cauliflower (M)	30–50
BONNER AND MILLERD[43]	mung beans (M)	16–23

This comparison between chloroplasts and mitochondria must not be pressed too far in our present state of knowledge. For example, a computation from the present data of ratios between photosynthetic and oxidative phosphorylation would be of limited value. To be more meaningful such a ratio should be computed from a comparison of the phosphorylating activity of chloroplasts and of mitochondria isolated from the same tissue. Because of the experimental difficulties involved[30] reliable evidence of this sort is as yet unavailable.

A comparison of the importance of chloroplasts and mitochondria as sources of ATP in leaves cannot be limited to rates of phosphorylation but should also include the relative abundance of these cytoplasmic structures in the cell. The mesophyll of leaves, which contains most of the chloroplasts, is a tissue remarkably specialized for photosynthesis[32]. Within the mesophyll cells, especially in the palisade parenchyma, chloroplasts are the dominant cytoplasmic bodies; mitochondria are relatively few. On this basis alone the contribution of mitochondria to the total ATP requirement in photosynthesis would not be expected to be large[30,33]. It seems therefore probable that, whatever the stoichiometric ratio between ATP and CO_2 may turn out to be, the main source of ATP in carbon assimilation is photosynthetic phosphorylation by chloroplasts rather than oxidative phosphorylation by mitochondria.

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SUMMARY

Under improved experimental conditions, rates of photosynthetic phosphorylation up to 500 micromoles of orthophosphate esterified per hour per mg of chlorophyll were obtained with isolated chloroplast fragments. Expressed on a nitrogen basis these rates correspond to about 780 micromoles of orthophosphate esterified per hour per mg N.

These rates are similar to maximum rates of photosynthesis in intact leaves of land plants with optimal light and CO_2 supply (about 180 micromoles of CO_2 fixed per hour per mg of chlorophyll) and are several times higher than rates of oxidative phosphorylation from mitochondria from various plant and animal sources.

The significance of these rates in the evaluation of the role of photosynthetic phosphorylation as a source of ATP in photosynthesis is discussed.

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