PHOTOSYNTHESIS BY ISOLATED CHLOROPLASTS

V. PHOSPHORYLATION AND CARBON DIOXIDE FIXATION BY BROKEN CHLOROPLASTS

by

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The attainment of complete extracellular photosynthesis by chloroplasts has made it possible to study photosynthesis as an isolated process divorced from the structural and metabolic complexity of a whole cell[1,2]. It has now been found that extracellular photosynthesis does not depend on the structural integrity of chloroplasts. This paper reports experiments in which whole chloroplasts were broken and active photosynthetic systems reconstituted by a recombination of chloroplast fractions and several cofactors. With proper additions, the photosynthetic activity of the reconstituted system, as measured by CO_2 fixation per unit of chlorophyll, was several times greater than that of the intact chloroplasts.

The fractionation of chloroplasts and the reconstitution of photosynthetically active systems with broken chloroplasts provides further experimental support for the previously discussed concept[1,2,3] that whole chloroplasts are complete cytoplasmic structures, specialized for photosynthesis, which contain three groups of enzymes, each group catalyzing an increasingly complex phase of the process: photolysis of water, photosynthetic phosphorylation, and CO_2 fixation.

METHODS

Preparation of broken chloroplasts (P_{1w}) *by treatment with water*

Whole chloroplasts (P_1) were prepared by grinding 100 g spinach leaves, freshly harvested from the greenhouse, in 0.35 M NaCl as described previously[1]. After removal of sand and large cell debris the whole chloroplasts were collected from the green suspension by centrifugation for 7 minutes at 1000 g at 0° C. The chloroplasts were washed once with 0.35 M NaCl and collected again by centrifugation. The washed whole chloroplasts were then suspended in about 50 ml of ice-cold water. The water treatment caused the chloroplasts to become swollen and irregular in shape; they could no longer be sedimented at 1000 g. The water-treated chloroplasts are referred to in this paper as "broken chloroplasts" (P_{1w}).

The chlorophyll content of each chloroplast suspension was determined[1] and an aliquot containing 0.5 mg chlorophyll was used in each reaction vessel.

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Preparation of chloroplast extract (CE)

Whole chloroplasts were prepared from 300 g spinach leaves as described above. The leaves were ground in 3 batches, each of 100 g and the washed chloroplasts from all three were suspended in water to give a total volume of 30 ml. A small aliquot of the suspension was taken for chlorophyll estimation[1] and the remaining suspension centrifuged at 20,000 *g* for 10 minutes at 0° C. The green sediment was discarded and the clear, straw-colored supernatant liquid was decanted and designated "chloroplast extract" (*CE*). It was found convenient to express the concentration of the chloroplast extract on the basis of the chlorophyll in the chloroplasts from which the extract originated.

In some cases the chloroplast extract was lyophilized and stored as a dry powder at —18° C. Aqueous solutions of this material were used. Here the quantity of the lyophilized powder used was calculated from the chlorophyll content of the chloroplasts from which it was derived. Even when stored at —18° C the lyophilized material lost about 50% of its activity in 7–14 days.

Measurement of photochemical reactions with broken chloroplasts

Photolysis, photosynthetic phosphorylation, and CO_2 fixation were measured as described previously except that the reaction mixtures were made to volume by the addition of glass-distilled water instead of the 0.35 *M* NaCl formerly used with whole chloroplasts[1].

RESULTS

Photosynthetic phosphorylation with broken chloroplasts

In our earlier experiments[1,3,4] active photosynthetic phosphorylation was obtained only with whole chloroplasts. It has now been found that on destroying the structure of the whole chloroplasts by treatment with water their capacity for photosynthetic phosphorylation was unimpaired when the requisite cofactors, Mg^{++}, FMN*, vitamin K_3 (2-methyl-1,4-naphthoquinone), and ascorbate, were added under anaerobic conditions. As shown in Table I, the response of broken chloroplasts to the addition of these cofactors was similar to that of whole chloroplasts[5,6].

Photosynthetic phosphorylation by broken chloroplasts depended on the addition of soluble cofactors only. The enzymes involved in photosynthetic phosphorylation were apparently unaffected by the disruption of chloroplast structure by the water treatment, and remained bound to the broken chloroplast particles.

TABLE I

COMPARISON OF EFFECTS OF COFACTORS ON PHOTOSYNTHETIC PHOSPHORYLATION BY WHOLE AND BROKEN CHLOROPLASTS

(See the preceding paper[1] for experimental details)

Additions (μmoles)			*P_i esterified (μmoles)*	
Vitamin K_3	*FMN*		*Whole chloroplasts*	*Broken chloroplasts*
0	0.1	Complete	7.0	10.5
0.003	0.1	Complete	6.8	9.5
0.03	0.01	Complete	11.7	11.5
0.03	0.1	Complete	14.3	17.2
0.3	0	Complete	16.0	18.2
0.03	0.1	Complete	12.6	20.0
0.03	0.1	Mg omitted	0.34	5.7
0.03	0.1	Ascorbate omitted	2.7	1.9

* The following abbreviations will be used in this paper: ADP, ATP, adenosine di- and triphosphate; FMN, flavin mononucleotide; DPN, TPN, di- and tri-phosphopyridine nucleotides.

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CO_2 fixation by broken chloroplasts

The ability of chloroplasts to fix CO_2[7] was almost completely lost when their structure was damaged by treatment with water. Their CO_2-fixing capacity was restored upon the addition of the clear, straw-colored water extract (*CE*) from whole chloroplasts (Table II). The addition of *CE* to the broken chloroplasts (P_{1w}) gave a small increase in CO_2 fixation in the dark; CO_2 fixation in the light was approximately 10 times as great as that in the dark, leaving little doubt that the addition of *CE* influenced principally the light-dependent CO_2 fixation.

TABLE II

EFFECT OF CHLOROPLAST EXTRACT (*CE*) ON PHOTOSYNTHETIC AND DARK CARBON DIOXIDE FIXATION BY BROKEN CHLOROPLASTS (P_{1w})

(See text and preceding paper[1] for experimental details)

	$^{14}CO_2$ *fixed (c.p.m.)*		
Expt. No.	P_{1w}	P_{1w} + *CE*	P_{1w} + *CE Dark fixation*
54	2700	108,300	14,700
56	1950	149,400	7,200
65	1900	276,800	24,400
67	950	137,300	—

Table III shows that the addition to the broken chloroplasts of DPN, TPN, or ATP, singly or in combination, failed to restore their capacity for CO_2 fixation in the absence of *CE*, but significantly increased photosynthetic CO_2 fixation in the presence of *CE*. Heating the *CE* almost completely abolished its effect on CO_2 fixation by broken chloroplasts.

TABLE III

EFFECT OF ADENOSINE TRIPHOSPHATE (ATP) AND DI- AND TRIPHOSPHOPYRIDINE NUCLEOTIDES (DPN AND TPN) ON THE FIXATION OF $^{14}CO_2$ BY BROKEN CHLOROPLASTS (P_{1w}) SUPPLEMENTED WITH A WATER EXTRACT OF CHLOROPLASTS (*CE*)

(See text and preceding paper[1] for experimental details)

Additions	$^{14}CO_2$ *fixed (c.p.m.)*		
	P_{1w}	P_{1w} + *CE*	P_{1w} + *heated CE*
None	950	137,300	19,750
ATP	1300	204,950	35,400
DPN	2250	347,700	20,250
TPN	1600	450,250	19,450
ATP + DPN	2000	411,950	35,550
ATP + TPN	2800	659,350	29,900

The stimulating effect of pyridine nucleotides on CO_2 fixation by broken chloroplasts supplemented with *CE* was of special interest. No such effect of pyridine nucleotides was observed on photosynthetic phosphorylation with either whole or broken chloroplasts, nor, as reported earlier[3], on CO_2 fixation by whole chloroplasts.

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Fig. 1 indicates that the effect of pyridine nucleotides on CO_2 fixation by broken chloroplasts varied with the concentration of *CE* in the reaction mixtures. At high concentrations of *CE* the effect of added pyridine nucleotides disappeared. It seems reasonable to conclude that pyridine nucleotides which appear to be required for CO_2 fixation, though not for photosynthetic phosphorylation, are present in adequate amounts in whole chloroplasts. Only when these cofactors are leached out by the water treatment of the whole chloroplasts could a dependence of CO_2 fixation on pyridine nucleotides be experimentally demonstrated. The effect of added TPN was, on an equimolar basis, somewhat greater than that of DPN. The significance of this observation must await future work.

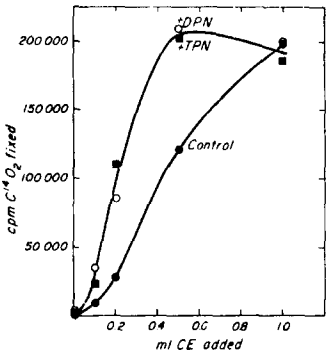

Fig. 1. Effect of diphosphopyridine nucleotide (DPN) and triphosphopyridine nucleotide (TPN) on CO_2 fixation by broken chloroplasts supplemented with varying amounts of chloroplast extract (*CE*). Experimental conditions as given in text.

The reconstituted broken chloroplast system reduced CO_2 to the level of carbohydrates. The products, shown in Fig. 2, were similar to those obtained with whole chloroplasts[7] and included phosphate esters of fructose, glucose, ribulose, dihydroxy acetone, and glyceric acid; glycine, alanine, glycolic acid, glucose and dihydroxyacetone. The reconstituted system differed from whole chloroplasts in that appreciable quantities of starch were not synthesized unless soluble starch was added.

The photosynthetic activity of the reconstituted broken chloroplast system indicates that treating whole chloroplasts with water removes both soluble enzymes and cofactors, such as ATP and pyridine nucleotides, which are required for CO_2 fixation. The identification of the enzymes contained in *CE* is now in progress; among those already identified are the glyceraldehyde phosphate dehydrogenases of green leaves[8], carboxydismutase[9], phosphoglyceryl kinase, phosphopentokinase, phosphoglucomutase, and phosphorylase. The role of these and other water-soluble enzymes of chloroplasts in CO_2 fixation is currently under investigation.

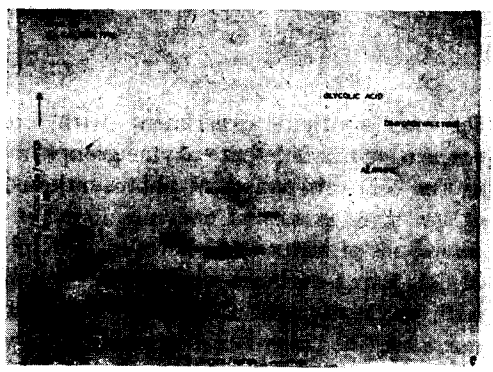

Fig. 2. Chromatogram showing products of photosynthetic CO_2 fixation by broken chloroplasts. Compound directly above area labeled "monophosphates" has been identified as phosphoglyceric acid. Experimental procedures as given in text.

Photosynthetic CO_2 fixation by broken chloroplasts supplemented with chloroplast extract, TPN, and ATP equalled or exceeded that obtained with the most active preparations of whole chloroplasts. A further several-fold increase in the rate of CO_2 fixation was obtained by the addition of any one of a number of compounds, principally phosphorylated sugars (Table IV). The products of CO_2 fixation were again similar to those obtained with whole chloroplasts. As seen from the diagrammatic

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summary in Fig. 3, the combined effect of the various addenda has resulted in a level of CO_2 fixation many times higher than that obtained with whole chloroplasts.

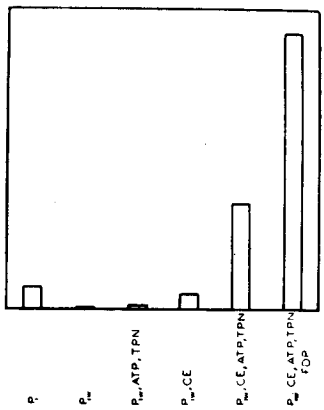

Fig. 3. Diagrammatic representation of the effects of various addenda on CO_2 fixation by broken chloroplasts. Experimental conditions as in text. Abbreviations: P_1, whole chloroplasts; P_{1w}, water-treated chloroplasts; *CE*, chloroplast extract; ATP, adenosine triphosphate; DPN, TPN, di- and triphosphopyridine nucleotides; FDP, fructose-1,6-diphosphate.

The stimulating effect of manganese on CO_2 fixation previously noted with whole chloroplasts[7] was much more pronounced with broken chloroplasts supplemented with *CE* and all the cofactors described. CO_2 fixation was reduced approximately eighty per cent when manganese was omitted.

TABLE IV

EFFECT OF PHOSPHORYLATED COMPOUNDS ON PHOTOSYNTHETIC CARBON DIOXIDE FIXATION BY BROKEN CHLOROPLASTS (10 MICROMOLES CO_2 ADDED)

Additions	*CO_2 fixed, µmoles*
None*	2.0
3-Phosphoglyceric acid	3.7
Ribose-5-phosphate	4.0
Glucose-1-phosphate	4.4
Glucose-6-phosphate	3.8
Fructose-6-phosphate	3.1
Fructose diphosphate	5.2

* P_{1w}, *CE*, ATP, TPN.

3 µmoles of each phosphorylated compound was added to the reaction mixture. Other experimental conditions as described in text and in the preceding paper[1].

DISCUSSION

The photosynthetic experiments with broken chloroplasts provide further evidence concerning the different enzyme groups in whole chloroplasts[1] and their participation in the different phases of photosynthesis. Broken chloroplasts contained only two of the three groups of enzymes found in whole chloroplasts: those controlling the photolysis of water and photosynthetic phosphorylation. At least some of the group of CO_2-fixing enzymes were leached out by treating whole chloroplasts with water, with the result that CO_2 fixation was completely abolished. The phosphorylating enzymes, like the enzymes of photolysis, were not water-soluble and remained bound to the particles.

A distinction should be made between broken chloroplasts (P_{1w}) described here and the chloroplast fragments (P_2) described earlier[1]. The P_{1w} preparation was obtained from whole chloroplasts first separated from other cytoplasmic particles and then broken, not by mechanical means, but by treatment with water. The P_2 preparation consisted of a mixture of chloroplasts, mechanically fragmented in the grinding of leaves, with other small cytoplasmic particles, which probably included mitochondria. The feeble capacity for photosynthetic phosphorylation of the P_2 preparation[1], as contrasted with the P_{1w}, may have resulted from damage during the mechanical breaking of the chloroplasts and also from possible interference by the non-chloroplast particles.

The technique of breaking whole chloroplasts by treating them with water has

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provided a direct experimental demonstration of the cofactors required for CO_2 fixation. The effect of ATP in restoring the capacity for CO_2 fixation to broken chloroplasts is in harmony with the conclusion[1,2] that ATP is essential for CO_2 fixation. As with whole chloroplasts, phosphorylated sugars, known to be formed with the aid of ATP, were identified among the products of CO_2 fixation by broken chloroplasts (Fig. 2).

It seems likely that pyridine nucleotides (PN^+) serve as electron carriers in the reduction of CO_2. The reduction of pyridine nucleotides, linked to photolysis of water, could be aided by ATP (compare[10–13]) as represented by Equation 1. The reduced pyridine nucleotide (PNH), again with the aid of ATP, would then reduce CO_2 in accordance with Equation 2:

$$\text{Light} + H_2O + PN^+ + ATP \longrightarrow PNH + H^+ + \tfrac{1}{2}O_2 + ADP + P_i \quad (1)$$

$$CO_2 + 2PNH + 2H^+ + n\text{ATP} \longrightarrow (CH_2O) + 2PN^+ + n\text{ADP} + nP_i \quad (2)$$

The inclusion of ATP in reaction 1 is attractive for it permits us to retain the concept discussed elsewhere[2] that the key reaction in photosynthesis, the photolysis of water, is accomplished by the energy of 1 quantum of red light (*ca.* 43 kcal). The pyrophosphate bonds of ATP could provide the supplementary energy for chloroplast reactions which require an input of more than 43 kcal, such as reduction of pyridine nucleotides. Taking the standard oxidation-reduction potential of TPN at pH 7 as —0.32 V[14], it would require approximately 50 kcal to give a TPN_{red}/TPN_{ox} ratio of 1.

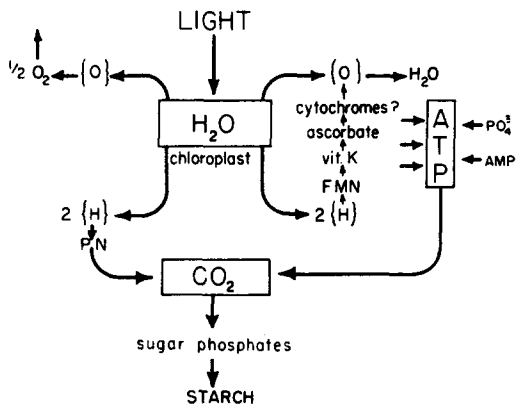

Fig. 4. Scheme for photosynthesis by isolated chloroplasts. Photolysis of water (center) leading either to ATP synthesis and the reconstitution of water (right) or to CO_2 reduction (below) linked to oxygen evolution (left).

The reduction of CO_2 to the level of carbohydrate (Equation 2) almost certainly involves a reduction of a carboxyl to a carbonyl group. The reducing potential of pyridine nucleotides is insufficient to accomplish this step without a lowering of the potential barrier, which again could be accomplished by phosphorylation of the carboxyl group by ATP[15].

The overall scheme for photosynthesis by chloroplasts[1] has now been modified (Fig. 4) to include pyridine nucleotides in the CO_2 reduction pathway.

SUMMARY

1. Photosynthetic esterification of inorganic phosphate into adenosine triphosphate, and reduction of CO_2 to the level of carbohydrate, hitherto found to occur only in whole chloroplasts, have now been observed with chloroplasts broken by treatment with water.

2. Broken chloroplasts retained only two of the three groups of enzymes contained in whole chloroplasts, namely, those controlling the photolysis of water and photosynthetic phosphorylation. At least some of the enzymes concerned in reduction of CO_2 were leached out by treating the chloroplasts with water, with the result that CO_2 fixation was completely abolished.

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3. On addition of the requisite cofactors, the capacity of broken chloroplasts for photosynthetic phosphorylation was the same as that of whole chloroplasts.

4. The restoration to broken chloroplasts of the full capacity for photosynthetic CO_2 fixation of whole chloroplasts required the addition of pyridine nucleotides, adenosine triphosphate, and the soluble enzymes removed by water treatment of whole chloroplasts.

5. An additional several-fold increase in the rate of CO_2 fixation by the reconstituted broken chloroplast system was obtained by the further addition of one of several compounds, principally phosphorylated sugars. This has resulted in a level of CO_2 fixation by broken chloroplasts which is much higher than that obtained with whole chloroplasts.

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