sufficient phosphate acceptors and by the rate of oxygen diffusion.

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PHOTOSYNTHESIS BY ISOLATED CHLOROPLASTS IX. PHOTOSYNTHETIC PHOSPHORYLATION AND CO_2 ASSIMILATION IN DIFFERENT SPECIES[1]

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Understanding the role of chloroplasts in photosynthesis has been greatly increased during the past five years by experiments with chloroplasts isolated from the leaves of one species, spinach (*Spinacia oleracea* Linn.). Previously, the only experimentally documented photochemical activity of chloroplasts isolated from several species, including spinach, was the Hill reaction (18) in which illuminated chloroplasts evolve oxygen in accordance with equation I, where

$$A + H_2O \xrightarrow{\text{Light}} AH_2 + 1/2\ O_2 \qquad \text{I}$$

A represents a non-physiological electron or hydrogen acceptor such as ferricyanide or benzoquinone (27).

The new work with spinach chloroplasts has provided direct experimental evidence for the capacity of isolated chloroplasts to assimilate CO_2 photosynthetically to the level of starch and sugars (4, 5, 1, 17, 25). Previously, the view that photosynthetic CO_2 assimilation in green plants is, like oxygen evolution, localized in chloroplasts, was at first asserted without the support of critical experimental evidence (22, 23) and was later abandoned because of evidence to the contrary (18, 15, 14, 21, 6).

By fractionating spinach chloroplasts, CO_2 assimilation proper was shown to be a dark process (24), but one dependent on assimilatory power, i.e. $TPNH_2$ and ATP[4], formed by two light reactions (11, 13), cyclic (equation II) and non-cyclic photophosphorylation (equation III).

$$n\ ADP + n\ H_3PO_4 \xrightarrow{\text{Light}} n\ ATP \qquad \text{II}$$

$$2TPN + 2H_2O + 2ADP + 2H_3PO_4 \xrightarrow{\text{Light}} 2TPNH_2 + O_2 + 2ATP \qquad \text{III}$$

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[4] The following abbreviations will be used: TPN, $TPNH_2$, oxidized and reduced forms of triphosphopyridine nucleotide; ATP, adenosine triphosphate; ADP, adenosine diphosphate; P, *ortho*phosphate; FMN, riboflavin phosphate; Tris, Tris(hydroxymethyl)-aminomethane buffer, neutralized with HCl.

The assimilatory power generated by reactions II and III is used for CO_2 assimilation in accordance with reaction IV.

$$CO_2 + 2TPNH_2 + n\,ATP \longrightarrow (CH_2O) + 2TPN + n\,ADP + n\,H_3PO_4 \quad IV$$

The sum of reactions II, III, and IV gives the overall equation for photosynthesis:

$$CO_2 + 2H_2\overset{*}{O} \xrightarrow{\text{Light}} (CH_2O) + \overset{*}{O}_2 + H_2O \quad V$$

A non-physiological variant of reaction III is reaction VI in which TPN is replaced by ferricyanide

$$4Fe^{3+} + 2H_2O + 2ADP + 2H_3PO_4 \xrightarrow{\text{Light}} 4Fe^{2+} + O_2 + 2ATP + 4H^+ \quad VI$$

(represented here by Fe^{+++}). The Hill reaction (equation I) was shown to be an uncoupled photophosphorylation (11, 13, 19), i.e., that fragment of reaction VI which represents the electron transport when it is not accompanied by ATP formation.

It was desirable to demonstrate the newly found photosynthetic capacity of isolated chloroplasts in other species besides spinach, especially since the work with spinach was used in formulating a concept of photosynthesis (7) no longer based on the photolysis of water as the common denominator of photosynthesis in green plants and photosynthetic bacteria (26). This article presents evidence that chloroplasts isolated from the leaves of sugar beet *Beta vulgaris* Linn. (Chenopodiaceae), tobacco *Nicotiana tabacum,* Linn. (Solanaceae), pokeweed[5] *Phytolacca americana,* Linn. (Phytolaccaceae), sunflower *Helianthus annuus,* Linn. (Compositae) and New Zealand spinach, *Tetragonia expansa* Murr. (Aizoaceae) can carry out the same three processes which were recently discovered in spinach chloroplasts: cyclic photophosphorylation, non-cyclic photophosphorylation and assimilation of CO_2 to the level of carbohydrates.

Preliminary reports of some of these findings have been made previously (6, 31).

Methods

The plants were all grown in the greenhouse in water culture using the nutrient solution technique described elsewhere (3, 8). Mature leaves were used in preparing the chloroplasts. Broken chloroplast particles (P_{1s} or C_{1s}) and chloroplast extract (CE) were made by the methods previously described (32). The only modification was that 0.5 molar NaCl was used instead of 0.35 molar NaCl for grinding the leaves and washing the chloroplasts in the case of sugar beet and tobacco. Chloroplasts from the other species were made in 0.35 molar NaCl. Measurements of chlorophyll (3), phosphorylation (10), TPN reduction (12), and CO_2 fixation (1) were made as described previously.

Results

Cyclic Photophosphorylation: Cyclic photophosphorylation (equation II) is a photochemical reaction of chloroplasts in which all the trapped light energy is converted into ATP. Cyclic photophosphorylation is not accompanied by oxygen evolution (or absorption), and is independent of CO_2 assimilation. No reductant for CO_2 assimilation is formed; the sole product is ATP, which is formed by esterification of inorganic phosphate (cf. review, 6).

Work with spinach chloroplasts has shown that there are two kinds of cyclic photophosphorylation, one catalyzed by FMN[4] and the other by vitamin K compounds (30, 28, 32). The FMN and vitamin K pathways, (or systems) are similar in many respects, but may be distinguished in that the FMN system is more sensitive to inhibition by *o*-phenanthroline and dinitrophenol than the vitamin K system (32). Moreover, the FMN pathway differs from the vitamin K pathway in its requirement for TPN (32) and chloride ions (7) (J. Bové, C. Bové, F. R. Whatley, and D. I. Arnon. 1959. Manuscript in preparation.)

Table I shows the two pathways of cyclic photophosphorylation in isolated chloroplasts from the different species. A dark control is shown in the FMN system, to demonstrate the strict light dependence of the process, which was also observed in experiments with the vitamin K system.

Non-cyclic Photophosphorylation: In non-cyclic photophosphorylation (equation III) ATP formation is coupled with the reduction of TPN and evolution of oxygen; the generation of the energy-rich

Table I

Cyclic Photophosphorylation by Chloroplasts from Different Plants

Plant	P esterified (μmoles/30 min/0.1 mg chl)		
	FMN system		Vit K_3 system
	Light	Dark	Light
Spinach	7.6	0.3	7.9
Sugar beet	8.4	0.2	7.8
Pokeweed	6.6	0.3	7.7
Tobacco	4.3	0.3	5.1
Sunflower	4.2	0.2	3.9
Tetragonia	8.3	...	7.6

The reaction mixture (3 ml final volume) contained, in micromoles: tris[4], pH 8.3, 80; KCl, 20; $MgCl_2$, 5; ADP, 10; $K_2HP^{32}O_4$, 10; Na ascorbate, 10; and chloroplast fragments (C_{1s}), containing 0.1 mg chlorophyll. In addition the FMN system contained, in micromoles: TPN, 0.3; and FMN, 0.1; in the vitamin K_3 system the TPN and FMN were omitted and 0.3 μmole vitamin K_3 was added. The reaction was run under N_2 at 15° C for 30 min and the esterification of phosphate was measured as described previously (10).

[5] Drs. C. S. French and Helen M. Habermann suggested the use of this species and kindly supplied the seed.

TABLE II

NON-CYCLIC PHOTOPHOSPHORYLATION BY CHLOROPLASTS FROM DIFFERENT PLANTS

PLANT	$TPNH_2$ FORMED (μMOLES)	OXYGEN EVOLVED (μATOMS)	P ESTERIFIED (μMOLES)
Spinach	3.3	4.0	6.5
Sugar Beet	3.3	3.4	6.4
Pokeweed	1.8	1.6	3.7
Tobacco	2.6	2.0	4.4
Sunflower	2.7	1.2	3.0

The reaction mixture contained (3 ml final volume), in micromoles: Tris, pH 8.3, 80; KCl, 20; TPN, 4; $MgCl_2$, 5; ADP, 10; $K_2HP^{32}O_4$, 10; sodium ascorbate, 10; chlorophyll-free chloroplast extract from chloroplasts containing 2 mg chlorophyll; and chloroplast fragments (P_{1s}) containing 0.25 mg chlorophyll. Experiments were run in the light at 15° C under nitrogen for 30 min. $TPNH_2$ was measured by its absorption at 340 mμ (12), oxygen was measured manometrically (KOH in center well), and esterification of phosphate was measured as described previously (10).

pyrophosphate bonds of ATP accounts for only a portion of the light energy captured by the chloroplasts. Another portion is used for forming $TPNH_2$. Non-cyclic photophosphorylation provides the three products of the light phase of photosynthesis in green plants: molecular oxygen, and the two components of assimilatory power (11) required for converting CO_2 into sugars, namely, $TPNH_2$ and ATP. Table II illustrates the ability of chloroplasts from different species to carry out non-cyclic photophosphorylation.

The salient features of non-cyclic photophosphorylation are that ATP formation is accompanied by TPN reduction and oxygen evolution. As shown in table II, this was found with all species investigated. The stoichiometric relation, however, expressed by equation III, between the pairs $TPNH_2$ and oxygen, and ATP and $TPNH_2$ was reasonably good for the first pair (except in sunflower) but not for the second. More ATP was formed than $TPNH_2$, suggesting that some cyclic photophosphorylation also occurred in the reaction mixture.

It was previously reported (12) that the P: 2e ratio of one between ATP formed and TPN reduced was observed only under special experimental conditions. In the case of spinach (12) a P: 2e ratio of one was obtained by omitting ascorbate, washing chloroplasts and dialyzing the TPN-reducing factor, supplied by the chloroplast extract (CE). These special experimental conditions, which were considered to be beyond the scope of this investigation, were necessary to eliminate the additional ATP formation by the cyclic photophosphorylation pathway which is operative, albeit at a lower rate, even without added FMN or vitamin K (4, 9). The degree of suppression of this residual cyclic photophosphorylation may vary from species to species and also in different chloroplast preparations of the same species, depending upon the carryover of the cofactors of cyclic photophosphorylation that are present in the intact leaf.

ATP FORMATION COUPLED WITH FERRICYANIDE REDUCTION: A non-physiological variant of Reaction III is Reaction VI, in which TPN is replaced by ferricyanide. Reaction VI, first found with spinach chloroplasts, has led to an interpretation of the Hill reaction as an uncoupled photophosphorylation, i.e. as a measure of photochemical electron transport which is proceeding without an associated phosphorylation reaction (11, 12, 13, 19, 20). This conclusion is based upon the observation that, with spinach chloroplasts, the rate of oxygen evolution in the Hill reaction, when it is coupled with phosphorylation, is higher than in the conventional Hill reaction without phosphorylation (equation I). As shown in table III, photophosphorylation coupled with reduction of ferricyanide and oxygen evolution, was observed with chloroplasts of several different species. In general, as in spinach chloroplasts, the rate of oxygen evolution accompanying ferricyanide reduction was increased by coupling the system with photophosphorylation (11, 13, 19).

CO_2 ASSIMILATION: Photosynthetic CO_2 assimilation for spinach was first demonstrated with whole chloroplasts (4, 1, 17, 25), and subsequently with broken chloroplasts supplemented with chloroplast extract and a number of cofactors (29). In experiments carried out with other species the ability to

TABLE III

HILL REACTION, WITH AND WITHOUT COUPLED PHOSPHORYLATION, BY CHLOROPLASTS FROM DIFFERENT PLANTS

PLANT	HILL REACTION	HILL REACTION COUPLED WITH PHOSPHORYLATION	
	OXYGEN EVOLVED (μATOMS)	OXYGEN EVOLVED (μATOMS)	P ESTERIFIED (μMOLES)
Spinach	4.8	6.2	5.8
Sugar Beet	5.1	7.5	6.6
Pokeweed	5.8	6.0	4.9
Tobacco	3.8	4.7	1.8
Sunflower	2.4	2.1	1.2
Tetragonia	...	5.2	6.3

The Hill reaction mixture contained (3 ml final volume), in micromoles: tris, pH 8.3, 80; KCl, 20; $K_3Fe(CN)_6$, 15; and chloroplast fragments (P_{1s}) containing 0.1 mg chlorophyll, prepared as described previously (10). The light reaction was carried out at 15° C under nitrogen for 30 min, and the oxygen evolution measured manometrically (with KOH in the center well). For the experiments with coupled phosphorylation the reaction mixture contained, in addition, in micromoles: $MgCl_2$, 5; ADP, 10; $K_2HP^{32}O_4$, 10. The oxygen evolution was measured manometrically and the phosphate esterified as described previously (10).

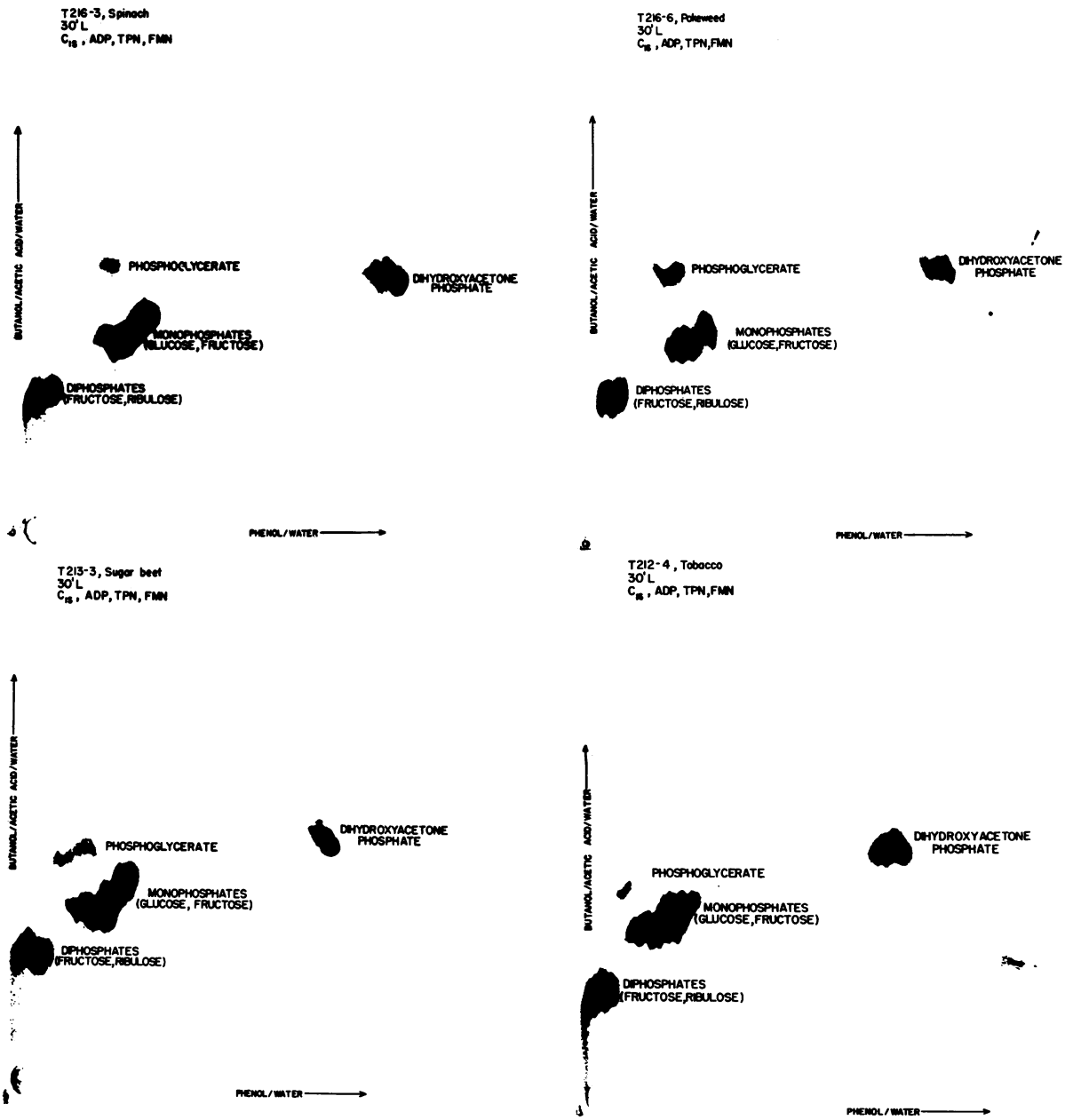

FIG. 1 (*upper left*). Products of CO_2 assimilation by illuminated chloroplasts isolated from spinach. Experimental conditions are described in the legend to table IV.

FIG. 2 (*lower left*). Products of CO_2 assimilation by illuminated chloroplasts isolated from sugar beet. Experimental conditions are described in the legend to table IV.

FIG. 3 (*upper right*). Products of CO_2 assimilation by illuminated chloroplasts isolated from pokeweed. Experimental conditions are described in the legend to table IV.

FIG. 4 (*lower right*). Products of CO_2 assimilation by illuminated chloroplasts isolated from tobacco. Experimental conditions are described in the legend to table IV.

TABLE IV

CO_2 FIXATION BY CHLOROPLASTS FROM DIFFERENT PLANTS

PLANT	CO_2 FIXED (μMOLES/MG CHL/HR)
Spinach	2.2
Sugar Beet	3.5
Pokeweed	2.2
Tobacco	1.7
Sunflower	1.4
Tetragonia	2.0

The reaction mixture contained, in micromoles: Tris, pH 7.4, 80; $MgCl_2$, 5; $MnCl_2$, 2; Na ascorbate, 10; TPN, 0.3; ADP, 0.5; K phosphate, pH 7.4, 5; FMN, 0.0001; glucose-1-phosphate, 0.3; $NaHC^{14}O_3$, 10; chlorophyll-free chloroplast extract made from chloroplasts containing 2 mg chlorophyll; and chloroplast fragments (C_{1s}) containing 0.5 mg chlorophyll. The reaction was run at 15° C under N_2 for 30 min in the light. The CO_2 fixation was measured as described previously (1).

carry out extracellular CO_2 assimilation was tested with broken chloroplast preparations. The results are summarized in table IV.

CO_2 assimilation in all cases has progressed to the level of sugar phosphate (table IV). Individual compounds were identified by radioautography and paper chromatography (1). The pattern of compounds formed in light from CO_2 is shown in figures 1 to 4. The close similarity of products formed by chloroplasts from different species suggests that the same basic mechanisms for CO_2 assimilation are involved.

DISCUSSION

Although extracellular photosynthesis by chloroplasts cannot be equated in all respects with photosynthesis in intact cells, it retains the principal features of that process, and permits their investigation and isolation from concurrent cellular activities. Extracellular photosynthesis by chloroplasts is similar to that in whole cells in that carbon dioxide reduction by visible light to the level of carbohydrates is accompanied by oxygen evolution. Extracellular photosynthesis by chloroplasts differs from that process in whole cells, notably in that it proceeds independently of concurrent cellular activities (such as respiration, which cannot be divorced from photosynthesis in intact cells (16)).

Separation of photosynthesis by chloroplasts from the structural and functional complexity of the whole cells has opened a way for investigating the central problem of photosynthesis, the conversion of light into chemical energy, in a manner which has already forced a re-examination of certain established concepts in photosynthesis (7). It was therefore deemed especially important to base the new conclusions about the mechanism of photosynthesis on evidence that the three key photosynthetic processes recently identified in spinach chloroplasts, CO_2 assimilation, cyclic, and non-cyclic photophosphorylation, also occur in chloroplasts isolated from other species. The aim of this investigation was to establish this point in principle, leaving for subsequent research the determination for each species of those experimental conditions, which result in highest rates of CO_2 fixation and photosynthetic phosphorylation and which, in the case of non-cyclic photophosphorylation, give the closest agreement with the stoichiometry of reaction III.

SUMMARY

Isolated chloroplasts from leaves of spinach, sugar beet, pokeweed, sunflower, and *Tetragonia expansa*, when prepared by techniques developed for spinach, had the ability to carry out three basic reactions found in spinach chloroplasts: cyclic photophosphorylation of either the flavin mononucleotide (FMN) or the vitamin K type, non-cyclic photophosphorylation, and assimilation of carbon dioxide to the level of carbohydrates.

Chloroplasts from all the species tested have also shown a capacity for photosynthetic phosphorylation coupled with the reduction of ferricyanide.

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