Photosynthesis by Isolated Chloroplasts

XI. CO₂ ASSIMILATION IN A RECONSTITUTED CHLOROPLAST SYSTEM

M. LOSADA, A. V. TREBST, AND DANIEL I. ARNON*

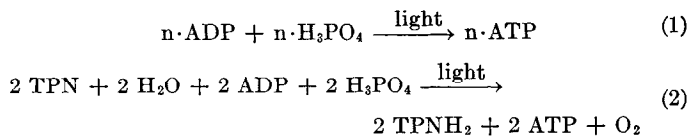*From the Laboratory of Plant Physiology, University of California, Berkeley, California*

In 1943, Ruben (1), elaborating on earlier suggestions by Thimann (2) and Lipmann (3), proposed that sugar formation in photosynthesis is a completely dark, chemosynthetic process which depends on only two products formed by light reactions: reduced pyridine nucleotide and adenosine triphosphate. Ruben's proposal distinguished two phases of sugar synthesis in the dark: a carboxylative phase, dependent on ATP only, in which CO₂ enters cellular metabolism by carboxylating an acceptor molecule, and a reductive phase, in which a carboxyl group is reduced by pyridine nucleotide with the aid of ATP.

In the ensuing 15 years Ruben's scheme received experimental support from several directions: (a) Calvin, Horecker, Ochoa, Racker and their associates (4–7) have identified the ATP-dependent carboxylative phase in CO₂ assimilation. They have shown that the entry of CO₂ into the metabolism of photosynthetic cells depends on the phosphorylation of ribulose monophosphate by ATP to ribulose diphosphate, which is then carboxylated by CO₂ and cleaved to give 2 molecules of 3-phosphoglyceric acid. (b) The kinetic studies of Calvin's group (8) suggested that the reductive phase of CO₂ assimilation is the reduction of 3-phosphoglyceric acid to triosephosphate by a reversal of the well known glycolytic reactions, a mechanism that requires reduced pyridine nucleotide and ATP. (c) In addition to a carboxylative and a reductive phase, the findings in photosynthetic tissues of components of the pentose cycle (*cf.* 9) afforded a mechanism for the regeneration of the CO₂ acceptor in photosynthesis, in what might be designated as a third, regenerative phase of CO₂ assimilation. (d) Racker (10) obtained in the *dark*, a synthesis, driven by DPNH₂[1] and exogenous ATP, of hexose phosphate from CO₂, in a model multi-enzyme system consisting of glycolytic enzymes from rabbit muscle and yeast[2] and pentose cycle enzymes from spinach leaves.

The carboxylative, reductive, and regenerative phases constitute a cyclic sequence of dark reactions which jointly might be termed a reductive carbohydrate cycle (*cf.* 7). Was this reduc-

tive carbohydrate cycle then, although comprising exclusively dark reactions, a "photosynthetic" cycle for sugar formation, peculiar to chlorophyllous cells? This question was answered in the negative when the most distinctive enzyme of the reductive carbohydrate cycle, ribulose-di-P carboxylase, was found in such heterotrophs as *Escherichia coli* (9, 11) and when the cycle in its entirety was found in the nonphotosynthetic sulfur bacterium *Thiobacillus denitrificans* (12, 13).

It thus became clear that CO₂ assimilation is removed from the domain of photosynthetic reactions proper in which light is converted into chemical energy. In green plants the first stable, chemically defined products of this energy conversion are not intermediates of CO₂ assimilation but are TPNH₂ and ATP (14). These two compounds are now known to be formed by two light reactions, cyclic and noncyclic photophosphorylation (14–16) which, in their over-all balance, were found to be independent of CO₂ assimilation (Equations 1 and 2).

$$n \cdot ADP + n \cdot H_3PO_4 \xrightarrow{\text{light}} n \cdot ATP \qquad (1)$$

$$2\,TPN + 2\,H_2O + 2\,ADP + 2\,H_3PO_4 \xrightarrow{\text{light}}$$
$$2\,TPNH_2 + 2\,ATP + O_2 \qquad (2)$$

Although the reductive carbohydrate cycle thus seemed to be the same in photosynthetic and nonphotosynthetic cells, this conclusion could not be drawn with finality without an investigation of the pathway of CO₂ assimilation at the particular cellular site where sugar synthesis occurs in green plants. This site is now known to be the chloroplast (17–22), a view that was once widely held without the support of critical experimental evidence (23, 24) and was later abandoned because of evidence to the contrary (25–29; *cf.* 30), only to be formulated anew on the basis of new experiments with tracer carbon and improved methods for isolating functional chloroplasts from leaves (17–22). Recent experiments have also shown that within the chloroplast itself, the enzymes for CO₂ assimilation are localized in the water-soluble portion and are capable of carrying out the synthesis of sugar in the dark, even when they are physically separated from the chlorophyll-containing particles (31).

The present investigation was concerned with the characterization of CO₂ assimilation in a reconstituted chloroplast system that was supplied, as is the case in intact cells, with only catalytic amounts of TPN and ADP. In this "catalytic" chloroplast system, CO₂ assimilation is possible only in light, since the dark reactions of sugar synthesis depend on TPNH₂ and ATP that are being continuously regenerated by Reactions 1 and 2 at the expense of absorbed light energy (32).

---

[1] The abbreviations used are: FMN, flavin mononucleotide; 3-PGA, 3-phosphoglyceric acid; 1,3-diPGA, 1,3-diphosphoglyceric acid; TPNH₂ and DPNH₂, reduced tri- and diphosphopyridine nucleotide (these abbreviations will be used, in preference to the more common TPNH and DPNH; *cf.* Dixon, M., and Webb, E. C., *Enzymes*, p. 395. Longmans, Green and Co., London, 1958). (At the request of the authors, the Editors have permitted the use of this terminology in this instance, although they strongly prefer the symbols DPNH and TPNH.)

[2] Racker, E., personal communication (1958).

Preliminary reports of this work have been published previously (30, 33).

METHODS

Broken chloroplasts from spinach were used in all the experiments described herein. The preparation of broken chloroplasts (C_{ls}) and of chloroplast extract was previously described (34). In certain experiments washed chloroplast particles and dialyzed chloroplast extract were used (34). With certain preparations of chloroplasts, CO_2 fixation was greatly increased by adding 5 μmoles of glutathione to the reaction mixture.

CO_2 fixation was carried out at 20° in rectangular Warburg manometer vessels, placed in a glass-bottomed constant temperature bath and flushed with argon gas before turning on the light. Illumination was from below by a bank of 300- and 150-watt reflector flood lamps, providing approximately 23,000 Lux at the level of the reaction vessels. Unless otherwise indicated, the period of illumination was 30 minutes. The reaction was stopped by adding to each vessel 0.1 ml of glacial acetic acid. Total CO_2 fixation was measured by pipetting aliquots from each treatment on stainless steel planchets, evaporating to dryness and counting C^{14} with a thin window Geiger-Müller counter. For the identification of the product of CO_2 fixation, the contents of the Warburg vessels were centrifuged and aliquots of the supernatant liquid were subjected to two-dimensional paper chromatography (on Whatman No. 41 paper) using as solvents (a) 80 phenol:20 water and (b) a mixture of 52 parts n-butanol, 14 parts glacial acetic acid, and 34 parts water.

The radioactivity in the individual compounds, located on the papers by radioautography, was determined by direct counting on the dried paper. The individual compounds were identified by elution, and cochromatography with samples of authentic compounds. Sugar phosphates were further identified by dephosphorylation with phosphatase (polidase) and rechromatography with the corresponding authentic sugars.

Preparation of Ribulose-di-P by Cyclic Photophosphorylation—Following a suggestion by E. Racker, ribulose-di-P was prepared with the aid of illuminated chloroplasts from ribose-5-P. The following procedure was used: ATP was formed by cyclic photophosphorylation from catalytic amounts of ADP and substrate amounts of orthophosphate. The reaction mixture (3 ml) contained chloroplast extract (34) equivalent to 2 mg of chlorophyll, and broken chloroplasts (C_{ls}) containing 0.5 mg of chlorophyll, and, in μmoles: ADP, 0.5; sodium phosphate, pH 8.0, 40; ribose-5-P, 30; vitamin K₃, 0.3; $MgCl_2$, 5; and Tris, pH 8.0, 80. The reaction was carried out in Warburg vessels, flushed with argon gas before turning on the light. The period of illumination was 1 hour. The reaction was stopped by adding 0.1 ml of 20% trichloroacetic acid. After centrifugation the pH of the supernatant fluid was adjusted to pH 6.5 and 60 μmoles of barium acetate and an equal volume of ethanol were added. The precipitate was washed with 80% ethanol, dried, and used without further purification as a source of ribulose-di-P.

The amount of ribulose-di-P in the preparation was estimated by measuring the $C^{14}O_2$ fixation by the carboxylation enzyme of chloroplasts, in the dark and in the absence of ATP. The reaction mixture (1.5 ml) contained in μmoles: Tris, pH 8.0, 80; cystein, 5; $MgCl_2$, 5; an aliquot of about 0.5 μmoles of ribulose-di-P; $NaHC^{14}O_3$, 10; and chloroplast extract equivalent to 2 mg chlorophyll. The reaction was stopped with acetic acid and the

Each vessel was illuminated and contained in a final volume of 2.5 ml, broken chloroplasts washed 3 times (C_{ls3}) and containing 0.5 mg of chlorophyll, chloroplast extract (dialyzed overnight against 2 liters of 0.01 M Tris buffer, pH 8.3, and 0.0001 M ethylenediamine tetraacetate) equivalent to 2 mg of chlorophyll, and the following in μmoles: Tris buffer pH 7.5, 80; $MnCl_2$, 2; $MgCl_2$, 5; Na ascorbate, 10; sodium phosphate pH 7.5, 5; TPN, 0.3; FMN, 0.001; $NaHC^{14}O_3$, 10 and, where indicated, 0.5 μmole of ADP and 1 μmole of either ribose-5-P or ribulose-di-P.

Treatment	Total $C^{14}O_2$ fixed
	c.p.m.
Ribose-5-P	10,600
Ribose-5-P, ADP	410,000
Ribulose-di-P	342,000
Ribulose-di-P, ADP	372,000

total $C^{14}O_2$ fixed measured. The moles of $C^{14}O_2$ fixed were taken as equivalent to the moles of ribulose-di-P present.

As for other chemicals used, ADP, TPN, and FMN were from Sigma Chemical Company; sugar phosphates from Nutritional Biochemicals Corporation, and Schwarz Laboratories. A "D,L-glyceraldehyde-1-bromide-3-phosphoric acid dioxane complex" was obtained from California Biochemical Research Corporation and soluble starch from Pfanstiehl Chemical Company. 2-PGA was a gift of Dr. C. E. Ballou.

Enzyme Assays—Aldolase, phosphoglycerate kinase and the triosephosphate dehydrogenases were assayed by measuring, at room temperature, the reduction of DPN, or the oxidation of $TPNH_2$ and $DPNH_2$, at 340 mμ, in cuvettes with a light path of 1 cm (Figs. 1 and 4). Fructose diphosphatase was assayed (Fig. 5) at 38°, by measuring the liberated inorganic phosphate according to Sumner (35).

RESULTS

I. Carboxylative Phase of CO₂ Assimilation

Photophosphorylation and CO_2 Fixation—CO_2 fixation was investigated in the presence of ribose-5-P and ribulose-di-P in an illuminated chloroplast system, the components of which were washed and dialyzed. Without added ADP, photosynthetic phosphorylation could not proceed and the system was unable to form ATP; significant CO_2 fixation occurred then only in the presence of ribulose-di-P (Table I). When ADP was included in the reaction mixture and photophosphorylation could proceed, CO_2 fixation occurred also in the presence of ribose-5-P (Table I).

These results suggest that in the absence of preformed ribulose-di-P, CO_2 fixation in the reconstituted chloroplast system was dependent on the photochemically generated ATP, the ATP being required for the phosphorylation step (Equation 3) in the conversion of ribulose-5-P to ribulose-di-P (36, 6, 7).

$$Ribulose\text{-}5\text{-}P + ATP \xrightarrow{Mg^{++}} ribulose\text{-}di\text{-}P + ADP \quad (3)$$
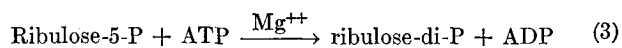
Effect of Time on Pattern of CO_2 Fixation—As shown in Table II, the early product of CO_2 fixation in the presence of ribulose-di-P was PGA; after 1-minute illumination PGA was the only
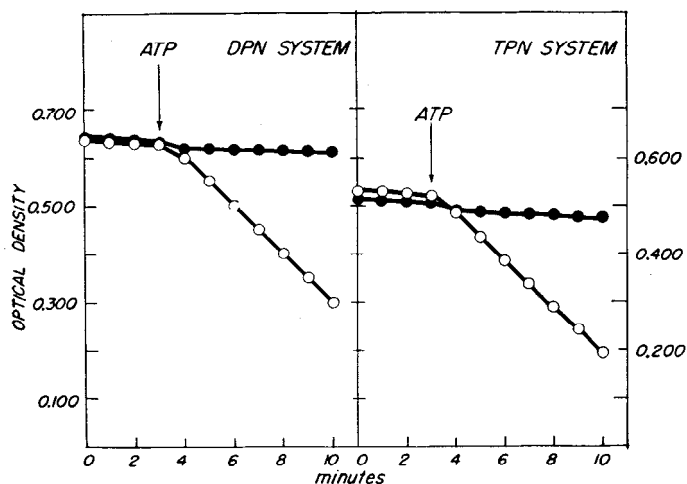
Fig. 1. Phosphoglyceric acid kinase and DPN- and TPN-dependent triosephosphate dehydrogenases in chloroplasts. The reaction mixture (3.0 ml final volume) contained dialyzed (see Table I) chloroplast extract equivalent to 0.6 mg of chlorophyll, and in micromoles: Tris buffer, pH 8.0, 120; cysteine, 12; phosphoglyceric acid, 1; and DPNH$_2$ or TPNH$_2$, 0.4. At the time indicated by the arrow, 2 μmoles of ATP were added. No phosphoglyceric acid was added to the controls.

TABLE II

Effect of time on pattern of CO_2 fixation in light by isolated chloroplasts

Each vessel contained in a final volume of 2.5 ml: broken chloroplasts (C$_{18}$) containing 0.5 mg of chlorophyll; chloroplast extract equivalent to 2 mg of chlorophyll and the following in μ-moles: Tris buffer, pH 7.5, 80; MnCl$_2$, 2; MgCl$_2$, 5; Na ascorbate, 10; sodium phosphate pH 7.5, 5; TPN, 0.3; ADP, 0.5; FMN, 0.001; reduced glutathione, 5; ribulose-di-P, 1; NaHC^{14}O$_3$, 10.

Illumination	Total C^{14}O$_2$ fixed	Total C^{14}O$_2$ fixed as	
		PGA	Sugar phosphates
min	*c.p.m.*	%	%
1	108,000	100	
2	196,000	75	24
5	450,000	54	38
10	600,000	40	57
20	750,000	13	86

measurable product. With continuing exposure to light and increasing total CO$_2$ fixation, the proportion of PGA diminished and that of sugar phosphates increased. After 20 minutes of illumination, sugar phosphates accounted for 86% of the photosynthetic products.

These findings are consistent with the view that the initial CO$_2$ fixation in chloroplasts is the reaction catalyzed by ribulose-di-P carboxylase (4–7), resulting in the cleavage of ribulose-di-P by CO$_2$ to 2 moles of PGA (Equation 4).

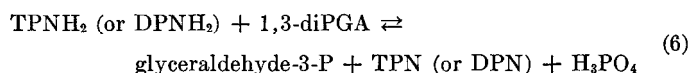$$\text{Ribulose-di-P} + CO_2 \rightarrow 2\ \text{PGA} \qquad (4)$$
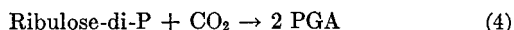
The PGA is then reduced to sugar phosphates, in a reversal of the glycolytic pathway of sugar breakdown, the TPNH$_2$ and ATP, required for the reduction of PGA (Equations 5 and 6), being formed by light. Evidence for this second phase of CO$_2$ assimilation in chloroplasts will now be presented.

II. Reductive Phase

The reduction of PGA to hexose phosphate by a reversal of the glycolytic pathway involves the participation of phosphoglycerate kinase, triosephosphate dehydrogenase, triosephosphate isomerase, aldolase, and fructose diphosphatase. These glycolytic enzymes were found to be present in the aqueous extract of chloroplasts, with the improved methods now used for the isolation of chloroplasts. Isolation methods used earlier (37, 38) failed to include the soluble portion of chloroplasts and the preparations obtained were chloroplast fragments rather than whole chloroplasts. Consequently, the glycolytic enzymes, because of their solubility, were usually lost in the older chloroplast preparations; for example, the triosephosphate dehydrogenases of green leaves were reported to be localized in the cytoplasmic fluid rather than in chloroplasts (37, 38). It must be emphasized, however, that even with the current improved methods for preparing chloroplasts the leakage of soluble enzymes cannot be completely prevented. This may account, at least in part, for the lower rate of CO$_2$ fixation in isolated chloroplasts than in intact leaves (Table VI).

Phosphoglycerate Kinase and Triosephosphate Dehydrogenases—The presence of these enzymes in an aqueous extract of chloroplasts is shown in Fig. 1. The oxidation of TPNH$_2$ and DPNH$_2$ by the triosephosphate dehydrogenases occurred only in the presence of ATP, indicating that PGA was phosphorylated by phosphoglycerate kinase to 1,3-diphosphoglycerate before its reduction to glyceraldehyde-3-P (Equations 5 and 6).

$$3\text{-PGA} + \text{ATP} \rightleftarrows 1,3\text{-diPGA} + \text{ADP} \qquad (5)$$

$$\text{TPNH}_2\ (\text{or DPNH}_2) + 1,3\text{-diPGA} \rightleftarrows$$
$$\text{glyceraldehyde-3-P} + \text{TPN (or DPN)} + H_3PO_4 \qquad (6)$$

Both the DPN- and the TPN-dependent triosephosphate dehydrogenases (37–39) were found to be present in the chloroplast extract, under the conditions of the test, in which exogenous DPNH$_2$ and TPNH$_2$ were supplied in the dark. However, as shown below, in a complete, illuminated chloroplast system only TPN was effective in reducing PGA.

Specificity of TPN in Photochemical Reduction of PGA—When, instead of supplying exogenous TPNH$_2$ and DPNH$_2$ to a chloroplast extract in the dark, the oxidized forms of pyridine nucleotides were supplied to a complete chloroplast system in the light, only TPN was effective in bringing about the reduction of PGA since only TPN was reduced in light by chloroplasts (14, 40). Table III shows that the addition of TPN, but not that of DPN, increased significantly total CO$_2$ fixation in the light. In earlier experiments with broken chloroplasts (41, 42), under somewhat different experimental conditions, TPN increased CO$_2$ fixation more than DPN, but DPN was also effective.

In the present investigation, the sole product of CO$_2$ fixation in the DPN system was phosphoglycerate (Fig. 2), whereas in the TPN system a reductive pattern of CO$_2$ assimilation, including the formation of sugar phosphates, was observed (Fig. 3).

Aldolase—Evidence for the presence in chloroplasts of aldolase, which catalyzes Reaction 7, is given in Fig. 4.

$$\text{Glyceraldehyde-3-P} + \text{dihydroxyacetone phosphate} \rightleftarrows$$
$$\text{fructose-1,6-di-P} \qquad (7)$$
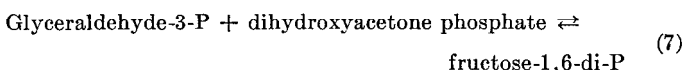
The presence of aldolase in chloroplasts was determined by coupling Reactions 7 and 6, and measuring the reduction of DPN

TABLE III

Effect of TPN and DPN on CO_2 fixation by isolated chloroplasts in light

Reaction mixture as in Table II, except that glutathione was omitted, 0.1 μmole of FMN was used, 0.3 μmole of glucose-1-P replaced ribulose-di-P, and the indicated additions of TPN or DPN replaced the uniform TPN addition.

TPN or DPN added	$C^{14}O_2$ fixed	
	TPN series	DPN series
μmoles	*c.p.m.*	*c.p.m.*
0	84,000	84,000
0.05	196,000	86,000
0.1	226,000	98,000
0.3	252,000	93,000
1.0	260,000	102,000

in Reaction 6 after the addition of fructose-1,6-di-P to the reaction mixture (Fig. 4).

Fructose Diphosphatase—Chloroplast extracts were found to have an active fructose diphosphatase (Fig. 5) which catalyzes the hydrolysis of the phosphate group linked to carbon 1 of fructose-1,6-di-P (Equation 8).

$$\text{Fructose-1,6-di-P} + H_2O \rightarrow \text{fructose-6-P} + H_3PO_4 \qquad (8)$$
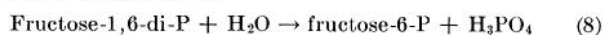
The occurrence of this enzyme in chloroplasts and its purification from spinach leaves have recently been described by Racker and Schroeder (43). The presence of fructose diphosphatase in
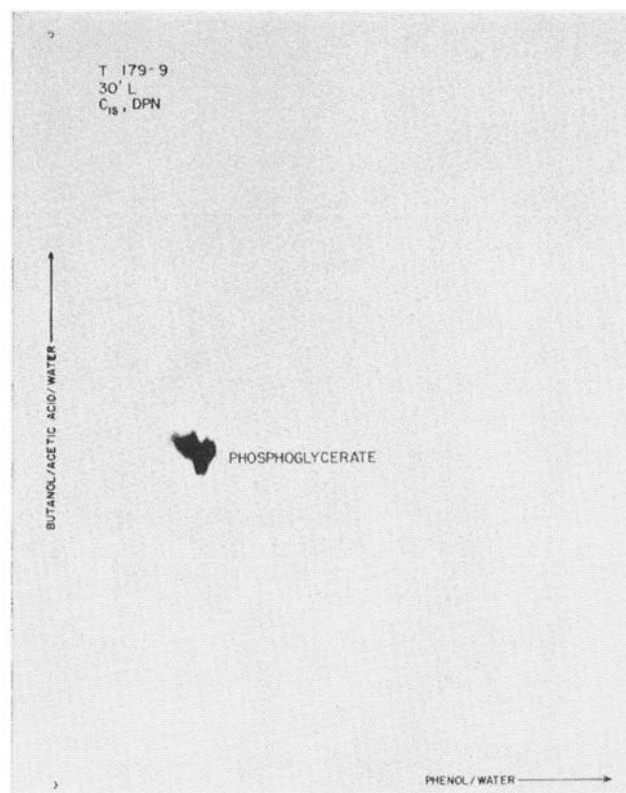

FIG. 3. Radioautograph of a chromatogram showing products of photosynthetic CO_2 assimilation by illuminated chloroplasts in the presence of 1 μmole of TPN. Other conditions as given in Table III.
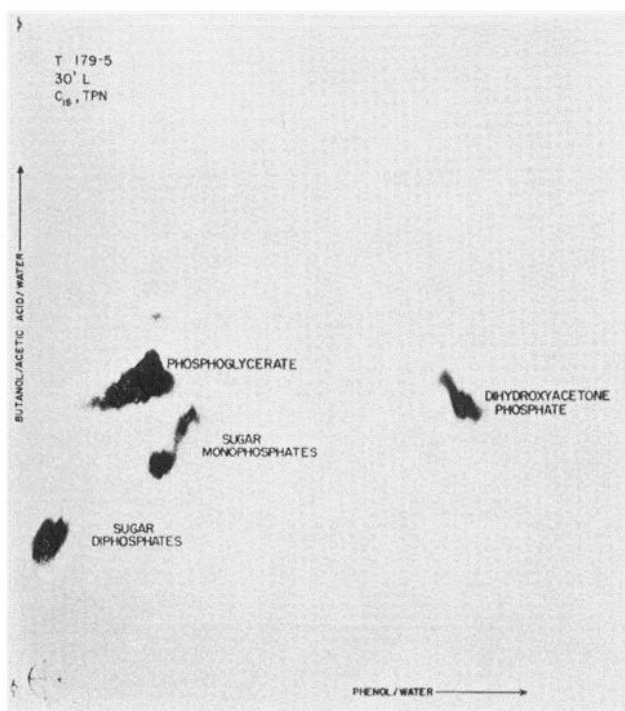

FIG. 2. Radioautograph of a chromatogram showing products of photosynthetic CO_2 assimilation by illuminated chloroplasts in the presence of 1 μmole of DPN. Other conditions as given in Table III.
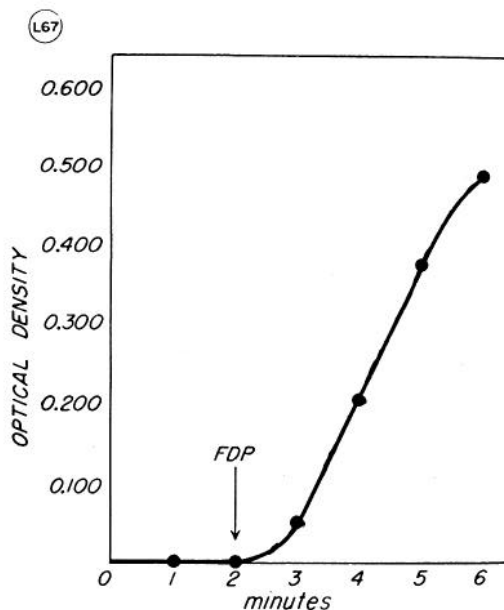

FIG. 4. Aldolase in chloroplasts. The reaction mixture (3.0 ml final volume) contained chloroplast extract equivalent to 1 mg of chlorophyll, and in micromoles: Tris buffer, pH 8.3, 100; cysteine, 9; sodium arsenate, 15; and DPN, 0.3. At the time indicated by the arrow 3 μmoles of fructose diphosphate were added.

chloroplasts strengthens the view that a reversal of the glycolytic pathway is involved in the reaction of CO_2 to the level of sugar. As was emphasized by Krebs (44), Reaction 8, by a hydrolytic removal of phosphate from fructose-di-P, circumvents an energy barrier when the glycolytic pathway is functioning as a synthetic rather than a degradative pathway in carbohydrate metabolism.
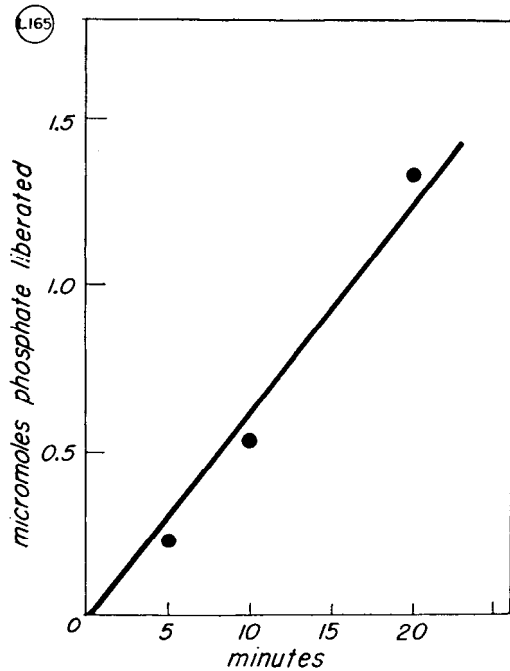
Fig. 5. Fructose-diphosphatase in chloroplasts. The reactio mixture (1 ml final volume) contained chloroplast extract equiva lent to 1 mg of chlorophyll, and in micromoles: Tris buffer, pH 8.0, 100; cysteine, 6; magnesium chloride, 5; manganese chloride, 2; and fructose diphosphate, 10.

III. Regenerative Phase

Experiments with the reconstituted "catalytic" chloroplast system have also yielded evidence for the regenerative phase of CO_2 assimilation, *i.e.* enzymatic rearrangements of carbohydrates or related compounds, to form the pentose monophosphate needed in the carboxylative phase. The dependence of CO_2 assimilation on these "primer" substances in the regenerative phase was indicated by experiments with chloroplasts prepared from plants in which starch and other sugar reserves were depleted. The depletion was accomplished by transferring the intact plants, kept in the same nutrient solution in which they were grown, to darkness for a period of 95 hours before harvesting leaves for the isolation of chloroplasts. As shown in Table IV, chloroplasts prepared from such carbohydrate-depleted leaves were unable to fix CO_2 unless supplemented with solubilized starch or catalytic amounts of glucose 1-phosphate. The addition of starch was effective only in the presence of inorganic phosphate, suggesting that phosphorylation of starch and its cleavage to sugar-phosphate were involved.

Table V shows that with chloroplasts from carbohydrate-depleted leaves the "priming" of CO_2 assimilation was also obtained by adding to the reaction mixture catalytic amounts of one of a number of phosphorylated sugars or related compounds, some of which have previously been found to increase CO_2 fixation by "broken" chloroplasts (41, 42). It seems justifiable to conclude that chloroplasts have the enzyme systems necessary for catalyzing the rearrangements of the added primer substances to give pentose monophosphate which is needed for the carboxylative phase of CO_2 assimilation. In normal leaves pentose monophosphate would be formed at the expense of the carbohydrate reserves available at the beginning of each period of photosynthesis. However, as shown in Table IV, the supply of the CO_2

acceptor or its precursor was limiting CO_2 fixation even in chloroplasts isolated from normal leaves. Unlike the whole chloroplasts (17, 19), CO_2 fixation in broken or reconstituted chloroplasts was greatly increased by, and often failed to occur without, the addition of the CO_2 acceptor or its precursor.

IV. CO_2 Fixation by Isolated Chloroplasts and by Intact Leaves

Since the chloroplast is the site of complete photosynthesis in the leaf, namely the site of CO_2 assimilation to the level of carbohydrates and of simultaneous oxygen evolution, it is of interest to compare the rate of CO_2 assimilation by chloroplasts *in situ* with that by chloroplasts removed from the cell. To be meaningful, the comparison must be made between the rate of CO_2 assimilation by isolated chloroplasts and that of the same batch of parent leaf material from which the chloroplasts were removed. Experiments of this kind were carried out in this laboratory by

TABLE IV

CO_2 fixation by chloroplasts isolated from carbohydrate-depleted leaves

Reaction mixture as in Table II, except that ribulose-di-P was omitted and 0.1 μmole of FMN was used. Sodium phosphate was omitted in treatments 4, 6, and 8. Two μmoles of ATP were added in treatments 4 to 8, 0.3 μmole of glucose-1-P in treatments 3 and 8, and 90 μg of "soluble" starch in treatments 2, 6, and 7. The normal leaves were harvested at 4 p.m., the carbohydrate-depleted leaves were kept in the dark for 95 hours before harvest.

	$C^{14}O_2$ fixed	
	Source of chloroplasts	
Substance added	Normal leaves	Carbohydrate-depleted leaves
	c.p.m.	*c.p.m.*
1. None................	100,000	12,000
2. Starch...............	153,000	82,000
3. Glucose-1-P..........	230,000	240,000
4. None................	32,000	10,000
5. PO₄.................	59,000	10,800
6. Starch...............	38,000	16,000
7. Starch + PO₄........	100,000	63,000
8. Glucose-1-P..........	164,000	170,000

TABLE V

Effect of different "primer" substances on CO_2 fixation by illuminated isolated chloroplasts

Chloroplasts were isolated from carbohydrate-depleted leaves (Table IV). Reaction mixture as in Table II, except that ribulose-di-P was omitted. Of each of the 6- or 5-carbon primer substances, 0.3 μmole and of each of the 3-carbon primer substances 0.6 μmole were added as indicated.

Primer substance added	$C^{14}O_2$ fixed	Primer substance added	$C^{14}O_2$ fixed
	c.p.m.		*c.p.m.*
None................	6,000	6-Phosphogluconic acid..	90,000
Ribose-5-P...........	191,000	Glyceraldehyde-3-P......	138,000
Fructose-1,6-di-P....	116,000	3-Phosphoglycerate......	127,000
Fructose-6-P.........	106,000	2-Phosphoglycerate......	85,000
Glucose-6-P..........	116,000	Fructose................	74,000
Glucose-1-P..........	100,000	Glucose................	58,000

TABLE VI

*CO_2 fixation by isolated spinach chloroplasts and by parent leaves**

Whole normal leaves, freshly harvested from the greenhouse, were exposed to $C^{14}O_2$ in a glass chamber, and illuminated at 20° with the same light intensity as the isolated chloroplasts. At the end of illumination the leaves were killed in boiling 80% ethanol and the radioactivity counted in the ethanol-soluble and insoluble fractions. CO_2 fixation by illuminated chloroplasts was measured, as described in ref. 42, Table IV (fructose-di-P treatment).

Experiment	CO_2 fixed/hr/mg chlorophyll		CO_2 fixed by chloroplasts/ CO_2 fixed by parent leaves
	Whole leaves	Isolated chloroplasts	
	μmoles	*μmoles*	*%*
A	16.4	4.5	26.4
B	20.2	5.2	25.8
C	30.7	6.0	19.5

* Data of F. R. Whatley and M. B. Allen.

Drs. F. R. Whatley and M. B. Allen. As shown in Table VI the rate of CO_2 fixation by isolated chloroplasts was, on a unit chlorophyll basis, about 20 to 25% of that in the intact parent leaf. It should be pointed out, however, that the rates of CO_2 assimilation in these detached spinach leaves (high in starch) were lower than maximal rates reported for other leaves or for Chlorella cells (45). Photosynthesis in detached leaves varies inversely with the stored carbohydrates and ceases altogether when these reach a high concentration (46, 47).

DISCUSSION

The results of this investigation support the conclusion that carbohydrate synthesis from CO_2 by isolated chloroplasts occurs by the same dark reactions of the reductive carbohydrate cycle which has been observed in intact cells, whether photosynthetic or not. The dark reactions of carbohydrate synthesis take place in the colorless, water-soluble fraction of chloroplasts and are driven by "assimilatory power," consisting of ATP and $TPNH_2$, the two compounds which are formed photochemically by the green fraction of chloroplasts in two reactions: cyclic and noncyclic photophosphorylation (Equations 1 and 2).

The inability of chloroplasts, when freed from mitochondrial contamination, to respire (20, 48) rules out the contribution of respiration to the energy requirements of CO_2 assimilation by isolated chloroplasts. ATP and $TPNH_2$ formed by light appear to suffice for accomplishing the dark conversion of CO_2 to carbohydrates. From the work with isolated chloroplasts, there is neither valid evidence nor a theoretical need for postulating a photochemical formation of a special, strong reductant capable of a direct reduction of CO_2 to the level of carbohydrate by some unknown reaction peculiar to photosynthesis (see, for example, 49, 50).
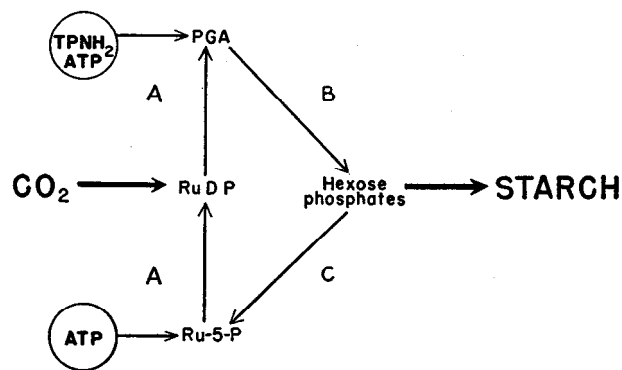
Of the three phases of the reductive carbohydrate cycle (Fig. 6) only two, the carboxylative and reductive, require an input of chemical energy derived from the conversion of trapped light energy. The carboxylative phase depends on light for ATP but requires no "reducing power." The energy for this phase of CO_2 assimilation can thus be provided by cyclic photophosphorylation (Equation 1), which generates ATP but no $TPNH_2$. This was demonstrated by first using cyclic photophosphorylation for the formation of ribulose-di-P in the absence of CO_2, and then

carrying out the carboxylation reaction in the absence of phosphorylation (Table I). By contrast, with ribose-5-P as the CO_2 acceptor, no significant carboxylation occurred without a concurrent photophosphorylation (cf. 51).

The site of action for both components of assimilatory power, $TPNH_2$ and ATP, is the reductive phase of CO_2 assimilation in chloroplasts, when PGA is reduced to sugar phosphate (Fig. 6). The light reaction of chloroplasts which supplies both $TPNH_2$ and ATP, with a concomitant evolution of oxygen (Equation 2), is noncyclic photophosphorylation. The mechanism of noncyclic photophosphorylation, as it is now envisaged (16), explains, without invoking the photolysis of water theory, why CO_2 assimilation is accompanied by oxygen evolution in green plants but not in photosynthetic bacteria.

The regenerative phase of CO_2 assimilation in chloroplasts, when molecular interconversions of hexose phosphate form storage carbohydrates and pentose monophosphate, appears to be independent of a photochemically generated driving force. The known enzyme reactions involved in this phase do not require ATP or $TPNH_2$. The demonstrated convertibility of a large number of "primer" substances, including starch, to what is probably pentose monophosphate (Tables IV and V), leaves little doubt that chloroplasts contain the enzymes required for the conversion of hexose phosphate to storage carbohydrates and pentose monophosphate.

The investigation of the enzymatic composition of chloroplasts has thus far revealed only one enzyme which seems to be peculiar to photosynthetic tissues: the TPN-linked triosephosphate dehydrogenase. This enzyme was previously found in leaves of sugar beets, spinach, sunflower, and tobacco (37, 38) and, independently by Gibbs, in pea leaves (39). Its cyclic emergence in leaves is induced by light (52). With the sole exception of a low activity in an induced barley albino mutant (11), this enzyme was never reported as occurring in nongreen tissues. By contrast, as already mentioned, ribulose-di-P carboxylase is not

Carbohydrate synthesis by isolated chloroplasts.

FIG. 6. Condensed diagram of the reductive carbohydrate cycle in chloroplasts. The cycle consists of three phases. In the carboxylative phase (A), ribulose-5-P is phosphorylated to ribulose-di-P which then accepts a molecule of CO_2 and is cleaved to 2 molecules of PGA; in the reductive phase (B) PGA is reduced and converted to hexose phosphates; in the regenerative phase (C) hexose phosphate is converted into storage carbohydrates (starch) and into the pentose monophosphate needed for the carboxylative phase. All the reactions of the cycle occur in the dark. The reactions of the carboxylative and reductive phases are driven by ATP and $TPNH_2$ formed in the light.

specific to photosynthetic tissues; it is also known to occur in non-chlorophyllous cells (12, 13, 9, 11).

The characteristic occurrence of the TPN-linked triosephosphate dehydrogenase in photosynthetic tissues is in accord with the observed specificity of TPN in the reduction of PGA by illuminated chloroplasts (Figs. 2 and 3). These results add to the evidence for the specificity of TPN in photosynthesis of green plants, as previously observed in cyclic and noncyclic photophosphorylation (53, 14, 40, 34).

The rate of CO_2 assimilation by isolated chloroplasts (54, 42) was found to be substantial when compared with that observed in the parent leaf tissue. The retention by isolated chloroplasts of 20 to 25% of the rate of CO_2 fixation of the parent leaf tissue (Table VI), compares favorably with some other extracellular activities of isolated cellular components as, for example, with the loss of 80 to 90% in respiration of disrupted yeast cells (Krebs, 55). It is possible that future improvements of experimental techniques will result in further increases of the rate of CO_2 fixation by isolated chloroplasts. However, once chloroplasts have been found to be capable of a substantial assimilation of CO_2 to sugars outside the cell, the characterization of the isolated photosynthetic apparatus acquires intrinsic interest, regardless of the currently demonstrable rate of CO_2 fixation. (In the case of cyclic photophosphorylation, improved experimental conditions gave, after 3 years of experimentation, rates of over 170 times higher than those initially obtained (56–58).)

The results of this investigation invite comparison with those of Fager (59). His special chloroplast preparation from spinach leaves "by itself exhibited no carbon dioxide-fixing power in the light or in the dark" (59). CO_2 was fixed by a protein fraction ("enzyme"), which he later found (60) to be a source of the CO_2 acceptor. The rate of CO_2 fixation was increased when the "enzyme" was illuminated in the presence of the chloroplast preparation. The addition of DPN or TPN had no effect, whereas that of ATP was inhibitory to CO_2 fixation in both light and dark. The fixation of CO_2 did not proceed beyond phosphoglycerate. Thus Fager's results provided no evidence for a reductive (photosynthetic) CO_2 assimilation in his chloroplast preparations but suggest that these contained a ribulose-di-P carboxylase system. Why that system had become inhibited by added ATP cannot be explained by the results of our investigation.

SUMMARY

CO_2 assimilation by "broken" and reconstituted chloroplasts was investigated in a "catalytic system" that was dependent on the products of the light reactions of photosynthesis.

Evidence is presented for the operation in isolated chloroplasts of a reductive carbohydrate cycle similar to that found by other investigators in photosynthetic and nonphotosynthetic cells. Chloroplasts were found to have enzymes catalyzing three phases of the cycle: the carboxylative phase, which includes the formation and subsequent carboxylation of ribulose diphosphate; the reductive phase, which includes reduction to hexose phosphate by a reversal of glycolysis; and the regenerative phase, which includes molecular rearrangements resulting in the formation of pentose monophosphate needed for the carboxylative phase.

The dark reactions of the reductive carbohydrate cycle in isolated chloroplasts depended on the two light reactions, cyclic and noncyclic photophosphorylation, for the formation of reduced triphosphopyridine nucleotide required in the reductive phase, and of adenosine triphosphate required in the carboxylative and reductive phases.

Chloroplasts isolated from carbohydrate-depleted leaves were unable to fix CO_2, but regained this capacity on addition of one of a number of carbohydrates or related compounds.

Although chloroplasts contained both the tri- and diphosphopyridine nucleotide-linked triosephosphate dehydrogenases, only the triphosphopyridine nucleotide-linked enzyme was effective in the light-dependent assimilation of CO_2. This enzyme emerges as the only distinctive feature, known so far, of the reductive carbohydrate cycle in chloroplasts.

REFERENCES

1. RUBEN, S., *J. Am. Chem. Soc.*, **65**, 279 (1943).
2. THIMANN, K. V., *Science*, **88**, 506 (1938).
3. LIPMANN, F., *Advances in Enzymol.*, **1**, 148 (1941).
4. QUAYLE, J. R., FULLER, R. C., BENSON, A. A., AND CALVIN, M., *J. Am. Chem. Soc.*, **76**, 3610 (1954).
5. WEISSBACH, A., HORECKER, B. L., AND HURWITZ, J., *J. Biol. Chem.*, **218**, 795 (1956).
6. JAKOBY, W. B., BRUMMOND, D. O., AND OCHOA, S., *J. Biol. Chem.*, **218**, 811 (1956).
7. RACKER, E., *Arch. Biochem. Biophys.*, **69**, 300 (1957).
8. CALVIN, M., *Proceedings of the third international congress of biochemistry, Brussels, 1955*, Academic Press, Inc., New York, 1956, p. 211.
9. VISHNIAC, N., HORECKER, B. L., AND OCHOA, S., *Advances in Enzymol.*, **19**, 1 (1957).
10. RACKER, E., *Nature*, **175**, 249 (1955).
11. FULLER, R. C., AND GIBBS, M., *Plant Physiol.*, **34**, 324 (1959).
12. TRUDINGER, P. A., *Biochem. J.*, **64**, 274 (1956).
13. AUBERT, J. P., MILHAUD, G., AND MILLET, J., *Ann. inst. Pasteur*, **92**, 515 (1957).
14. ARNON, D. I., WHATLEY, F. R., AND ALLEN, M. B., *Science*, **127**, 1026 (1958).
15. AVRON, M., KROGMANN, D. W., AND JAGENDORF, A. T., *Biochim. et Biophys. Acta*, **30**, 144 (1958).
16. ARNON, D. I., *Nature*, **184**, 10 (1959).
17. ARNON, D. I., ALLEN, M. B., AND WHATLEY, F. R., *Nature*, **174**, 394 (1954).
18. ARNON, D. I., Paper presented at the Cell Symposium, American Association for the Advancement of Science, Berkeley Meeting, 1954; *Science*, **122**, 9 (1955).
19. ALLEN, M. B., ARNON, D. I., CAPINDALE, J. B., WHATLEY, F. R., AND DURHAM, L. J., *J. Am. Chem. Soc.*, **77**, 4149 (1955).
20. ARNON, D. I., ALLEN, M. B., AND WHATLEY, F. R., *Biochim. et Biophys. Acta*, **20**, 449 (1956).
21. GIBBS, M., AND CYNKIN, M. A., *Nature*, **182**, 1241 (1958).
22. TOLBERT, N. E., *Brookhaven symposia in biology, No. 11*, Brookhaven National Laboratory, Upton, N.Y., 1958, p. 271.
23. SACHS, J., *Lectures on the physiology of plants*, Clarendon Press, London, 1887.
24. PFEFFER, W., *Physiology of plants*, Clarendon Press, London, 1900.
25. HILL, R., *Symposia Soc. Exptl. Biol.*, **5**, 223 (1951).
26. BROWN, A. H., AND FRANCK, J., *Arch. Biochem.*, **16**, 55 (1948).
27. BENSON, A. A., AND CALVIN, M., *Annual review of plant physiology, Vol. 1*, Annual Reviews, Inc., Palo Alto, California, 1950, p. 25.
28. RABINOWITCH, E., *Annual review of plant physiology, Vol. 3*, Annual Reviews, Inc., Palo Alto, California, 1952, p. 229.
29. LUMRY, R., SPIKES, J. D., AND EYRING, H., *Annual review of plant physiology, Vol. 5*, Annual Reviews, Inc., Palo Alto, California, 1954, p. 271.
30. ARNON, D. I., *Brookhaven symposia in biology, No. 11*, Brookhaven National Laboratory, Upton, N.Y., 1958, p. 181.
31. TREBST, A. V., TSUJIMOTO, H. Y., AND ARNON, D. I., *Nature*, **182**, 351 (1958).
32. TREBST, A. V., LOSADA, M., AND ARNON, D. I., *J. Biol. Chem.*, **234**, 3055 (1959).

33. ARNON, D. I., *Proceedings of the IVth international congress of biochemistry, Vienna, 1958, Vol. 13*, Pergamon Press, Ltd., London, 1959, pp. 451–474.
34. WHATLEY, F. R., ALLEN, M. B., AND ARNON, D. I., *Biochim. et Biophys. Acta*, **32**, 32 (1959).
35. SUMNER, J. B., *Science*, **100**, 413 (1944).
36. HURWITZ, J., WEISSBACH, A., HORECKER, B. L., AND SMYRNIOTIS, P. Z., *J. Biol. Chem.*, **218**, 769 (1956).
37. ARNON, D. I., in W. D. MCELROY AND H. B. GLASS (Editors), *Phosphorus metabolism, Vol. II*, Johns Hopkins Press, Baltimore, 1952, pp. 67–79.
38. ARNON, D. I., *Science*, **116**, 635 (1952).
39. GIBBS, M., *Nature*, **170**, 164 (1952).
40. ARNON, D. I., WHATLEY, F. R., AND ALLEN, M. B., *Biochim. et Biophys. Acta*, **32**, 47 (1959).
41. ARNON, D. I., ALLEN, M. B., WHATLEY, F. R., CAPINDALE, J. B., AND ROSENBERG, L. L., *Proceedings of the third international congress of biochemistry, Brussels, 1955*, Academic Press, Inc., New York, 1956, p. 227.
42. WHATLEY, F. R., ALLEN, M. B., ROSENBERG, L. L., CAPINDALE, J. B. AND ARNON, D. I., *Biochim. et Biophys. Acta*, **20**, 462(1956).
43. RACKER, E., AND SCHROEDER, E. A. R., *Arch. Biochem. Biophys.*, **74**, 326 (1958).
44. KREBS, H. A., *Bull. Johns Hopkins Hosp.*, **95**, 19 (1954).
45. HILL, R., AND WHITTINGHAM, C. P., *Photosynthesis*, Methuen and Company, Ltd., London, 1955, p. 152.
46. SAPOSCHNIKOFF, W., *Ber. deut. botan. Ges.*, **9**, 293 (1891); **11**, 391 (1893).
47. EWART, A. J., *J. Linnean Soc.* London, **31**, 364 (1896).
48. JAMES, W. O., AND DAS, V. S. R., *New Phytologist*, **56**, 325 (1957).
49. STREHLER, B. L., *Arch. Biochem. Biophys.*, **43**, 67 (1953).
50. GAFFRON, H., FRY, B. A., AND PEEL, J. L., (Editors), *Autotropic Micro-organisms*, Cambridge University Press, London, 1954, p. 163.
51. TREBST, A. V., LOSADA, M., AND ARNON, D. I., *J. Biol. Chem.*, **235**, 840 (1960).
52. HAGEMAN, R. H., AND ARNON, D. I., *Arch. Biochem. Biophys.*, **57**, 421 (1955).
53. ARNON, D. I., WHATLEY, F. R., AND ALLEN, M. B., *Nature*, **180**, 182 (1957).
54. WHATLEY, F. R., ALLEN, M. B., TREBST, A. V., AND ARNON, D. I., *Plant Physiol.*, in press.
55. KREBS, H. A., in D. M. GREENBERG (Editor), *Chemical pathways of metabolism, Vol. I*, Academic Press, Inc., New York, 1954, p. 134.
56. ALLEN, M. B., WHATLEY, F. R., AND ARNON, D. I., *Biochim. et Biophys. Acta*, **27**, 16 (1958).
57. AVRON, M., JAGENDORF, A. T., AND EVANS, M., *Biochim. et Biophys. Acta*, **26**, 262 (1957).
58. JAGENDORF, A. T., AND AVRON, M., *J. Biol. Chem.*, **231**, 277 (1958).
59. FAGER, E. W., *Arch. Biochem. Biophys.*, **41**, 383 (1952).
60. FAGER, E. W., *Biochem. J.*, **57**, 264 (1954).

Photosynthesis by Isolated Chloroplasts: XI. CO2 ASSIMILATION IN A RECONSTITUTED CHLOROPLAST SYSTEM

M. Losada, A. V. Trebst and Daniel I. Arnon