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Photosynthesis by Isolated Chloroplasts

X. DEPENDENCE OF CARBON DIOXIDE ASSIMILATION ON THE PHOTOCHEMICAL REACTIONS OF CHLOROPLASTS*

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Until recently, the only experimentally documented photochemical activity of isolated chloroplasts was the Hill reaction (3) in which illuminated chloroplasts evolve oxygen in accordance with Equation 1:

$$A + H_2O \rightarrow AH_2 + \tfrac{1}{2}O_2 \quad (1)$$

where A represents a nonphysiological electron or hydrogen acceptor such as ferricyanide or benzoquinone (4).

Recent experiments (5–8) have shown that the Hill reaction is a fragment of a photosynthetic phosphorylation of the noncyclic type (9). For example, with ferricyanide as the electron acceptor, oxygen evolution is coupled with ATP formation, in accordance with Equation 2.[1]

$$4Fe^{3+}Cy + 2H_2O + 2ADP + 2P_i \rightarrow 4Fe^{2+}Cy + O_2 + 2ATP + 4H^+ \quad (2)$$

The recognition of the Hill reaction as an uncoupled photophosphorylation was paralleled (5–8) by the identification of the physiological counterpart of Reaction 2, the noncyclic photophosphorylation reaction in which oxygen evolution and ATP formation are linked with TPN reduction (Equation 3).

$$2TPN + 2H_2O + 2ADP + 2P_i \rightarrow 2TPNH_2 + O_2 + 2ATP \quad (3)$$

Isolated chloroplasts have also been found to form ATP by a cyclic photophosphorylation (5–8) in which ATP is the sole product of the photochemical reaction (Equation 4).

$$ADP + P_i \rightarrow ATP \quad (4)$$

In addition to photosynthetic phosphorylation recent work has also provided direct experimental evidence (10–14) for the often asserted but never previously demonstrated capacity of isolated chloroplasts to assimilate CO_2 photosynthetically to the level of carbohydrates. CO_2 assimilation by isolated chloroplasts was found to be a dark process (15) dependent on a photochemically generated "assimilatory power" comprising two components: $TPNH_2$ and ATP formed by the noncyclic and cyclic photophosphorylation reactions (Equations 3 and 4).

Noncyclic photophosphorylation (Equation 3) provides all the three expected products of the light phase of photosynthesis: O_2, $TPNH_2$, and ATP. Cyclic photophosphorylation (Equation 4) supplies only ATP and the participation of this reaction in CO_2 assimilation would be needed only if the ATP formed in Reaction 3 were insufficient for CO_2 assimilation to the level of carbohydrate.

The purpose of this article is to present evidence that in photosynthesis by isolated chloroplasts, assimilation of CO_2 to the level of sugar phosphates requires a properly balanced participation of both light reactions: cyclic and noncyclic photophosphorylation (Equations 4 and 3). In the present investigation the balance between the two light reactions was maintained by regulating the concentration of cofactors of cyclic photophosphorylation (16, 17).

EXPERIMENTAL

Methods—Broken chloroplasts from spinach (16) or sugar beet leaves (18) were used in all the experiments described herein. The broken chloroplasts were prepared with ascorbate (C_{1s} particles) as previously described (16). Chloroplast extract was prepared with 0.035 M NaCl and used without dialysis. CO_2 fixation was carried out at 20° in rectangular Warburg manometer vessels, flushed with argon gas before turning on the light (approximately 23,000 lux). 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 products 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 35 parts water.

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

RESULTS

Experimental documentation for the participation of cyclic photophosphorylation (Equation 4) in CO_2 assimilation by chloroplasts required use of a system different from the one described before in which $TPNH_2$ and ATP were already supplied

* Preliminary reports of this work have been published previously (1, 2).

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[1] The abbreviations used are: FMN, flavin mononucleotide; P_i, orthophosphate.

in excess, and a second reaction for generating ATP would be superfluous (*cf.* Trebst *et al.* (15), Table I). In the experiments to be described presently, evidence for the participation of cyclic photophosphorylation in CO_2 assimilation of isolated chloroplasts was obtained in a "catalytic" system, *i.e.* one in which, as in an intact cell, $TPNH_2$ and ATP were formed in catalytic amounts and CO_2 fixation was therefore possible only in the light while $TPNH_2$ and ATP were being continuously regenerated at the expense of absorbed light energy.

Effect of FMN on CO_2 Fixation—As shown in Table I, when the exogenous supply of ATP (2 μmoles) was replaced by a "catalytic" system containing 0.5 μmole of ADP, orthophosphate, and 0.3 μmole of TPN, CO_2 fixation was sharply reduced (Treatment B). However, the addition to the reaction mixture of an extremely minute quantity of FMN (0.001 μmole) greatly increased total CO_2 fixation (Treatment C). In the presence of this minute amount of FMN the capacity of chloroplasts to fix CO_2 was equal to, if not greater than, that in Treatment A in which 2 μmoles of exogenous ATP were supplied.

TABLE I

Effect of riboflavin phosphate (FMN) on CO_2 fixation dependent on regeneration of ATP in light

Each vessel contained in a final volume of 2.5 ml: broken chloroplasts (C_{1s}) containing 0.5 mg of chlorophyll; chlorophyll extract (CE) equivalent to 2 mg of chlorophyll; and the following in μmoles: tris(hydroxymethyl)aminomethane pH 7.5, 80; $MgCl_2$, 5; $MnCl_2$, 2; sodium ascorbate, 10; sodium phosphate, 5; reduced glutathione, 5; ribose 5-phosphate, 0.3; sodium carbonate-C^{14}, 10. In addition to the indicated ATP and ADP supplements, Treatment A included 2 μmoles of TPN and each of Treatments B and C, 0.3 μmole of TPN.

Treatment	Total $C^{14}O_2$ fixed
	c.p.m.
A. Control, 2 μmoles of ATP	232,000
B. 0.5 μmole of ADP	109,000
C. 0.5 μmole of ADP, 0.001 μmole of FMN	265,000

TABLE II

Effect of FMN concentration on CO_2 fixation by illuminated chloroplast fragments from sugar beets

The reaction mixture was the same as that described for Treatment B in Table I except that glutathione was omitted and each vessel contained, in addition to the indicated concentration of FMN, 0.3 μmole of glucose 1-phosphate instead of ribose 5-phosphate. Final volume 3.0 ml.

Treatment	FMN added	Total CO_2 fixed	Total C^{14} fixed as	
			Phosphoglyceric acid	Sugar phosphates*
	μmole/3 ml	*c.p.m.*	%	%
A	0	110,000	93	3
	0.0002	105,000	32	65
	0.0005	220,000	34	63
B	0.001	208,000	20	80
	0.005	231,000	10	85
	0.01	208,000	14	77
	0.1	275,000	60	35
C	0.5	164,000	75	24

* Sugar mono- and diphosphates and dihydroxyacetone phosphate.

The effect of FMN on CO_2 fixation was investigated in greater detail by varying the concentration of this cofactor of cyclic photophosphorylation. In addition to an increase in total CO_2 fixation, a striking correlation was observed between the concentration of added FMN and the pattern of carbon compounds formed. Table II shows that, depending on the concentration of FMN, the products of CO_2 assimilation were either predominantly sugar phosphates, which are taken here as a measure of a reductive (photosynthetic) assimilation pattern, or phosphoglyceric acid. Phosphoglyceric acid was the chief product of CO_2 assimilation when either no FMN (Treatment A) or a relatively large amount of FMN (0.5 μmole) (Treatment C) was added to the reaction mixture. At a range of low FMN concentration, from about 0.001 to 0.01 μmole per 3 ml, sugar phosphates were the predominant products of CO_2 assimilation (Treatment B). These effects of FMN concentration on the pattern of CO_2 assimilation are illustrated in Figs. 1 to 3.

Effect of Vitamin K_3 and Phenazine Methosulfate on CO_2 Fixation—Similar effects on total CO_2 fixation and the pattern of compounds formed were also observed by adding two other cofactors of cyclic photophosphorylation: vitamin K_3 and phenazine methosulfate. Typical results are shown in Table III. At the same molar concentration, FMN, vitamin K_3, or phenazine methosulfate produced comparable effects on total CO_2 fixation and on the relation between phosphoglycerate and sugar phosphates. The addition of small amounts of any one of the three cofactors of cyclic phosphorylation (0.01 μmole per 3 ml) increased total CO_2 fixation several times and gave sugar phosphates as the main products of photosynthesis. At a concentration of 0.3 μmoles per 3 ml the formation of sugar phosphate was markedly decreased and phosphoglyceric acid appeared as the principal product of CO_2 fixation.

DISCUSSION

The results of this investigation show that the addition of one of the cofactors of cyclic photophosphorylation, FMN, vitamin

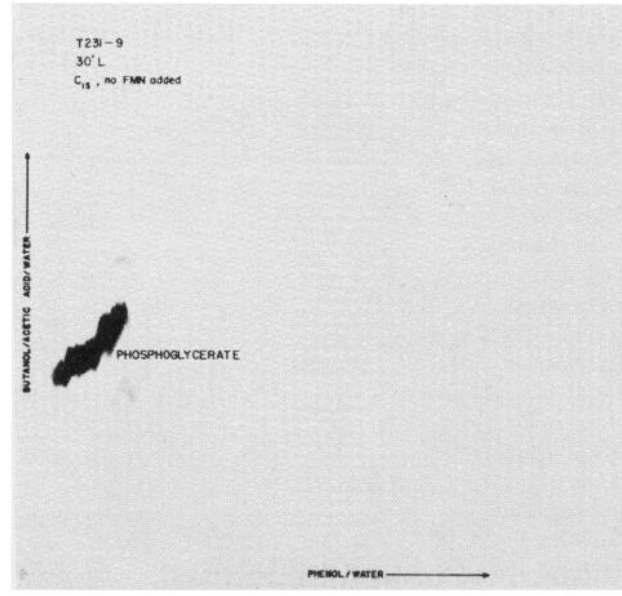

FIG. 1. Radioautograph of a chromatogram showing products of photosynthetic $C^{14}O_2$ assimilation by illuminated chloroplasts in the absence of added riboflavin phosphate (FMN). Other conditions as given in Table 2.

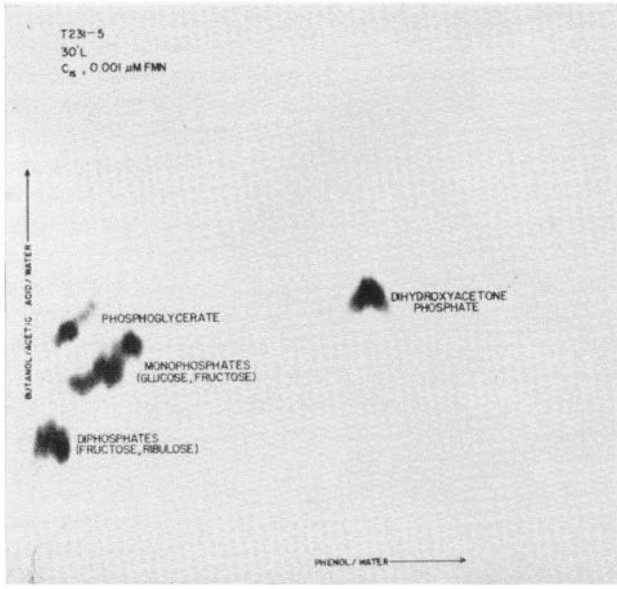

FIG. 2. Radioautograph of a chromatogram showing products of photosynthetic $C^{14}O_2$ assimilation by illuminated chloroplasts supplied with 0.001 μM riboflavin phosphate (FMN). Other conditions as given in Table 2.

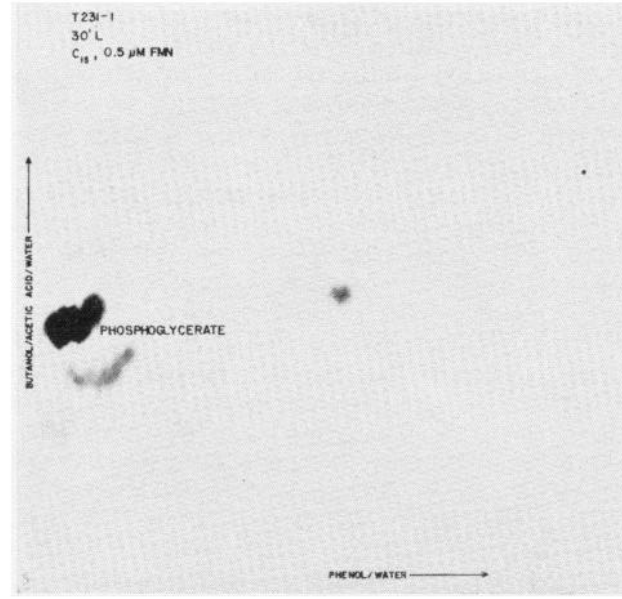

FIG. 3. Radioautograph of a chromatogram showing products of photosynthetic $C^{14}O_2$ assimilation by illuminated chloroplasts supplied with 0.5 μM riboflavin phosphate (FMN). Other conditions as given in Table 2.

K, or phenazine methosulfate, has a marked effect on CO_2 assimilation in a "catalytic" system in which CO_2 assimilation by chloroplasts depends on regeneration by light of assimilatory power, *i.e.* on $TPNH_2$ and ATP. Since there is no evidence to indicate that minute amounts of FMN, vitamin K, or phenazine methosulfate influence directly the enzymatic reactions responsible for CO_2 assimilation, it seems reasonable to seek an explanation of the observed results in the previously observed effects of these cofactors of cyclic photophosphorylation on the course of the light reactions (5, 17).

Without the addition of one of the cofactors of cyclic photophosphorylation, the light reactions of isolated chloroplasts are limited to noncyclic photophosphorylation (Equation 3). The ratio of $TPNH_2$ to ATP formed is 1:1. Adding a minute amount of FMN, vitamin K_3, or phenazine methosulfate to a noncyclic photophosphorylation system increases ATP formation without appreciably depressing oxygen evolution and the corresponding $TPNH_2$ accumulation (*cf.* Arnon *et al.* (17), Tables 3 and 4). The ratio of ATP to $TPNH_2$ formed becomes greater than 1. It appears likely that under these conditions cyclic photophosphorylation (Equation 4) is superimposed on the noncyclic process (Equation 3) and contributes additional ATP.

The shift from phosphoglycerate to sugar phosphates as the main products of CO_2 assimilation (compare Fig. 1 and Fig. 2) is explained by the additional ATP formed in the light by cyclic photophosphorylation as a result of adding one of its cofactors. Without this addition the light reaction is limited to noncyclic photophosphorylation (Equation 3) and fails to provide sufficient ATP for the reduction of CO_2 to sugars. It is concluded that the 1:1 ratio of ATP to $TPNH_2$ which characterizes Reaction 3 is insufficient for the reduction of CO_2 to the level of carbohydrate.

The need for more ATP than $TPNH_2$ in CO_2 assimilation is consistent with the view that there are two sites for phosphorylation but only one site for reduction in the formation of carbohydrates. The two phosphorylation reactions are: the phosphoribulokinase reaction (Equation 5) (19–22) and the phosphoglycerate kinase reaction (Equation 6). $TPNH_2$ is used only in the triosephosphate dehydrogenase reaction (Equation 7). All three enzymes concerned have been found in the chloroplast preparations used in these experiments.[2]

TABLE III

Effect of concentration of cofactors of cyclic photophosphorylation on CO_2 fixation by illuminated spinach chloroplasts

Experimental conditions as in Table II, except for the indicated additions of FMN, vitamin K_3, and phenazine methosulfate.

Cofactor added		Total $C^{14}O_2$ fixed	Total $C^{14}O_2$ fixed as	
			Phosphoglycerate	Sugar phosphates*
	μmole	c.p.m.	%	%
None		79,000		
FMN	0.01	318,000	15	69
	0.3	415,000	65	32
Vitamin K_3	0.01	530,000	16	77
	0.3	390,000	92	6
Phenazine methosulfate	0.01	310,000	8	86
	0.3	267,000	70	27

* Sugar mono- and diphosphates and dihydroxyacetone phosphate.

$$\text{Ribulose 5-phosphate} + \text{ATP} \xrightarrow{\text{phosphoribulokinase}} \text{ribulose 1,5-diphosphate} + \text{ADP} \quad (5)$$

$$\text{3-phosphoglyceric acid} + \text{ATP} \xrightarrow{\text{phosphoglycerate kinase}} \text{1,3-diphosphoglyceric acid} + \text{ADP} \quad (6)$$

$$\text{1,3-diphosphoglyceric acid} + TPNH_2 \xrightarrow{\text{triosephosphate dehydrogenase}} \text{glyceraldehyde 3-phosphate} + H_3PO_4 + TPN \quad (7)$$

[2] M. Losada, A. V. Trebst, and D. I. Arnon, manuscript submitted for publication.

When, in the absence of added cofactors of cyclic photophosphorylation, the light reactions of chloroplasts are limited to noncyclic photophosphorylation (Equation 3), the ATP formed is insufficient for both phosphorylating sites. As the ATP formed by Reaction 3 is used in the phosphorylation of ribulose 5-phosphate (Equation 5), the 1:1 ratio between ATP and $TPNH_2$ needed for the reduction of phosphoglycerate (Equations 6 and 7) begins to decrease. Eventually, in a "catalytic" system, this would bring the reduction of phosphoglycerate to a halt. Phosphoglycerate would then tend to accumulate (Fig. 1) as the ribulose diphosphate formed in Reaction 5 acts as a CO_2 acceptor and is subsequently cleaved by the carboxylase reaction (21–24).

The predominance of phosphoglyceric acid among the products of CO_2 assimilation when a larger amount of one of the factors of cyclic photophosphorylation is added to the reaction mixture (Fig. 3) is also explained by the previously observed effects of FMN, vitamin K, or phenazine methosulfate on noncyclic photophosphorylation (5, 17). When one of these cofactors is added to a noncyclic photophosphorylation system at the higher concentrations shown in Fig. 3 (also in Tables II and III), the noncyclic photophosphorylation is converted to the cyclic type (5, 17). Oxygen evolution and the accumulation of reduced TPN are suppressed, phosphorylation is sharply increased, and the principal product of the light reaction is ATP (*cf.* Arnon *et al.* (17), Figs. 7 and 8). Under these conditions the reduction of phosphoglycerate acid could not occur, since the triosephosphate dehydrogenase reaction (Equation 7) would be blocked by a lack of the reductant $TPNH_2$.

It appears, therefore, that the nonoccurrence of a reductive (photosynthetic) CO_2 assimilation resulting in the formation of sugar phosphate can be caused either by a shortage of ATP (Fig. 1) or of $TPNH_2$ (Fig. 3). In both cases phosphoglycerate would appear as the predominant product of CO_2 assimilation because its further assimilation would be blocked.

Although on the basis of present evidence the two sites for ATP action in CO_2 assimilation appear to be the phosphoribulose and the phosphoglycerate kinase reactions (Equations 5 and 6), there is a possibility that the second site for ATP action in the phosphorylation may be not the phosphorylation of phosphoglycerate but that of some unstable 6 carbon compound, which, *in vivo*, undergoes phosphorylation before reduction by $TPNH_2$ without breaking up into 2 moles of phosphoglycerate (25, 26).

In the experiments reported here the formation of sugar phosphates which is taken as a measure of photosynthetic CO_2 assimilation occurred only when a proper balance was maintained between cyclic and noncyclic photophosphorylation. In isolated chloroplasts this balance was maintained by adding different amounts of one of the catalysts of cyclic photophosphorylation. It is assumed that the intact cell has suitable physiological regulatory mechanisms for keeping the two reactions in balance.

SUMMARY

CO_2 assimilation in isolated chloroplasts was investigated in a "catalytic" system under three conditions: (1) when the photochemical phase was limited to noncyclic photophosphorylation, (2) when the photochemical phase was limited to cyclic photophosphorylation, and (3) when the photochemical phase included both cyclic and noncyclic photophosphorylations.

Under Conditions 1 or 2, CO_2 assimilation was limited almost entirely to the formation of phosphoglycerate. Sugar phosphates were the predominant products of CO_2 assimilation only in Condition 3. These results are interpreted as having been caused by a shortage of adenosine triphosphate (ATP) in Condition 1 and of reduced triphosphopyridine nucleotide ($TPNH_2$) in Condition 2; only in Condition 3 was a proper balance established between ATP and $TPNH_2$ formed at the expense of light energy, to make the formation of sugar phosphates possible.

The balance between the noncyclic and cyclic photophosphorylation necessary to bring about a photosynthetic (reductive) type of CO_2 assimilation was maintained by adding to the reconstituted chloroplast system minute amounts of one of the catalysts of cyclic photophosphorylation, riboflavin phosphate, vitamin K, or phenazine methosulfate.

REFERENCES

1. Arnon, D. I., *Proceedings of the IVth international congress of biochemistry, Vienna, 1958, Vol. 13*, Pergamon Press, Ltd., London, 1959, pp. 451–474.
2. Arnon, D. I., Trebst, A. V., and Losada, M., *Federation Proc.*, **18**, 183 (1959).
3. Hill, R., *Symposia Soc. Exptl. Biol.*, **5**, 223 (1951).
4. Warburg, O., *Heavy metal prosthetic groups and enzyme action*, Oxford University Press, London 1949, p. 213.
5. Arnon, D. I., Whatley, F. R., and Allen, M. B., *Science*, **127**, 1026 (1958).
6. Arnon, D. I., *Brookhaven Symposia in Biol.*, **11**, 181 (1958).
7. Avron, M., Krogmann, D. W., and Jagendorf, A. T., *Biochim. et Biophys. Acta*, **30**, 144 (1958).
8. Jagendorf, A. T., *Brookhaven Symposia in Biol.*, **11**, 236 (1958).
9. Arnon, D. I., *Nature*, **184**, 10 (1959).
10. Arnon, D. I., Presented at the Cell Symposium of the American Association for the Advancement of Science, Berkeley Meeting, 1954; *Science*, **122**, 9 (1955).
11. Allen, M. B., Arnon, D. I., Capindale, J. B., Whatley, F. R., and Durham, L. J., *J. Am. Chem. Soc.*, **77**, 4149 (1955).
12. Arnon, D. I., Allen, M. B., Whatley, F. R., Capindale, J. B., and Rosenberg, L. L., *Proceedings of the IIIrd international congress of biochemistry, Brussels, 1955*, Academic Press, Inc., New York, 1956, p. 227.
13. Gibbs, M., and Cynkin M. A., *Nature*, **182**, 1241 (1958).
14. Tolbert, N. E., *Brookhaven Symposia in Biol.* **11**, 271 (1958).
15. Trebst, A. V., Tsujimoto, H. Y., and Arnon, D. I., *Nature*, **182**, 351 (1958).
16. Whatley, F. R., Allen, M. B., and Arnon, D. I., *Biochim. et Biophys. Acta*, **32**, 32 (1959).
17. Arnon, D. I., Whatley, F. R., and Allen, M. B., *Biochim. et Biophys. Acta*, **32**, 47 (1959).
18. Whatley, F. R., Allen, M. B., Trebst, A. V., and Arnon, D. I., *Plant Physiol.*, in press.
19. Hurwitz, J., Weissbach, A., Horecker, B. L., and Smyrniotis, P. Z., *J. Biol. Chem.*, **218**, 769 (1956).
20. Horecker, B. L., Hurwitz, J., and Weissbach, A., *J. Biol. Chem.*, **218**, 785 (1956).
21. Jakoby, W. B., Brummond, D. O., and Ochoa, S., *J. Biol. Chem.*, **218**, 811 (1956).
22. Racker, E., *Arch. Biochem. Biophys.*, **69**, 300 (1957).
23. Calvin, M., *Proceedings of the IIIrd international congress of biochemistry, Brussels, 1955*, Academic Press, Inc., New York, 1956, p. 211.
24. Weissbach, A., Horecker, B. L., and Hurwitz, J., *J. Biol. Chem.*, **218**, 795 (1956).
25. Kandler, O., and Gibbs, M., *Plant Physiol.*, **31**, 411 (1956).
26. Gibbs, M., and Kandler, O., *Proc. Natl. Acad. Sci. U. S.*, **43**, 447 (1957).

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