Phosphoglyceric Acid Formation by Carbon Dioxide Fixation in Plant Extracts

By E. W. FAGER
The Institute of Radiobiology and Biophysics (Fels Fund), University of Chicago, Chicago, Illinois

(*Received* 31 *October* 1953)

INTRODUCTION

It is now generally agreed that the carboxyl group of phosphoglyceric acid is the first stable site of carbon dioxide fixation in normal photosynthesis (Calvin & Benson, 1948; Benson *et al.* 1950; Fager & Rosenberg, 1950, 1952). Investigation of the details of this process is made much easier if a cell-free system, obtained from plant materials, which will mediate the formation of phosphoglyceric acid by carbon dioxide fixation is available. Employing such a system removes the difficulties of cell-wall permeability and reduces the number of undefined changes which the metabolizing intact cell may make in added substances. Previous papers (Fager, 1952*a, b*) have reported progress in the examination of such a system. This paper presents recent findings which have considerably increased the information concerning both the carboxylation enzyme and the acceptor whose carboxylation leads to the formation of phosphoglyceric acid.

MATERIALS AND METHODS

The material used in the experiments cited in Table 1 was prepared exactly as described in the previous paper (Fager, 1952*b*). All other experiments described were done with a combination of a chloroplast preparation from spinach and an aqueous extract from algae. Conditions used in preparing these are discussed below.

Preparation of chloroplast material

One hundred grams of spinach (*Spinacia oleracea*) were washed and placed in the refrigerator for 3–4 hr. When thoroughly chilled, it was ground in an ice-cold mortar with sand and 50 ml. of ice-cold sorbitol:borate buffer. The sorbitol:borate buffer used throughout the work was prepared as follows: 0·05 mole of sorbitol, 0·05 mole of boric acid and 0·01 mole KCl were dissolved in about 900 ml. of water. The pH was adjusted to 6·9 with NaOH and the solution was made to 1 l. Just before use, the required volume was flushed with N_2; cysteine hydrochloride to give a concentration of 2×10^{-3}M was added and the pH was adjusted to 6·9. Cysteine was omitted in those cases where oxidants or sulphydryl poisons were to be tested. Various other buffers were tried but none gave as good results (Clendenning & Gorham, 1950*a*).

The resulting mush was squeezed through heavy canvas and then centrifuged (4000 *g*, 3 min., refrigerated centrifuge). The supernatant which contained the chloroplasts and chloroplast fragments was decanted and centrifuged at higher speed (13 000 *g*, 15 min., refrigerated centrifuge). The supernatant from this treatment was discarded; the precipitate was suspended in 50 ml. of fresh buffer, centrifuged down as before and finally taken up in 10 ml. of fresh buffer. This suspension was forced through a Pyrex sintered plate (M, max. pore diameter 10–15 μ.) under N_2 pressure. When kept in ice under N_2, it slowly lost its activity but gave consistent results for an hour or more; when frozen and stored at $-20°$ it lost about 30% of its activity overnight. Because of this lability, it was prepared immediately before use. The chlorophyll concentration was in the range 1·3–1·7 mg./ml. This preparation is denoted as 'chloroplasts' in this paper.

The spinach used for this preparation was purchased in the market. Two types of spinach with quite different aspects are available in the Chicago area: from about October until June the material agrees with the description of var. Nobel (Burpee catalogue: erect; somewhat pointed, rather thin, moderately crinkled leaves); during the summer it has the aspect of var. Long Standing Bloomsdale (Burpee catalogue: low rosettes; dark green, rather thick, very crinkled leaves). The Nobel-like material gave excellent preparations in which fixation of $^{14}CO_2$ by the chloroplast preparation alone represented only 0–5% of the fixation by the complete system (cf. Table 1). The Bloomsdale-like material had a variable blank ranging from 5 to 30% of the fixation by the complete system and in general was less efficient in promoting tracer fixation in phosphoglyceric acid; qualitatively the blank activity and the phosphoglyceric acid-forming activity appear to vary inversely. Using the production of acid from Hill solution (ferric oxalate:ferricyanide) by illuminated chloroplasts as the test method, Clendenning & Gorham (1950*b*) have reported comparable differences in activity between different varieties of tomato, cucumber, etc.

The fixation of $^{14}CO_2$ by chloroplast material alone did not lead to the formation of phosphoglyceric acid and was over in less than 2 min., while the fixation leading to phosphoglyceric acid continued for 15–20 min. Therefore, a correction for the blank was made in all cases in which it represented over 5% of the total fixation.

Extract from algae

The alga, *Scenedesmus obliquus* D3, was grown in the light, in inorganic medium (Brown, Fager & Gaffron, 1948), at 25–30°, with shaking and with 4% CO_2 in air as the gas phase. When the algae had grown to a concentration of 0·03–0·04 ml./10 ml., they were harvested by centrifuging at low speed (1000 *g*, 5 min.) and were washed by suspension in sorbitol:borate buffer (without cysteine) and recentrifuging. They were then suspended in fresh buffer to a concentration of 0·04 ml./ml. and kept at 15–20°. Air was

bubbled through the suspension until used. 5 ml. portions were transferred to the exposure tubes (made by flattening the lower 7·5 cm. of 20 × 150 mm. Pyrex culture tubes until the internal distance between the walls was 3–4 mm.; the tubes were then cut off to a length of 10 cm.) and illuminated (light intensity about 500 ft.c.) for 20 min. at 20° while being flushed with a rapid stream of N_2. The tubes were then agitated in a boiling-water bath for 1 min. This treatment caused the algae to coagulate. The suspension was poured through a dense plug of glass wool into a Pyrex sintered filter (M plate) and forced through the latter with N_2. The resulting clear, colourless solution was kept in ice under nitrogen until used. It could be concentrated by lyophilizing or stored at −20° without appreciable loss of activity. However, the usual practice was to prepare 30–40 ml. of the extract in the morning and use it that afternoon. This preparation is denoted as 'extract' in this paper.

A combination of this 'extract' and 'chloroplasts' prepared as described above always fixed tracer carbon dioxide; different preparations fixed a similar amount under standard conditions and over 90 % of the fixation was in the carboxyl carbon atom of phosphoglyceric acid.

Conditions of tracer fixation

All experiments were done at 14–16°. Except for experiments on the effect of concentration, 2 ml. of 'extract' and 0·5 ml. of 'chloroplasts' were used in each exposure tube. Tracer was added as 0·5 ml. of 0·03M-$Na_2{}^{14}CO_3$ (about 5×10^6 counts/min. when measured as $CaCO_3$ at infinite thickness in the gas flow counter used for all experiments. The ^{14}C was obtained as barium carbonate from the Isotopes Division, U.S. Atomic Energy Commission). Contact with tracer was for 15 min. except for the experiments on the time course of fixation. Illumination was used in most cases because it resulted in 30–50 % more fixation; it had no determinable effect upon the proportion of tracer fixed in phosphoglyceric acid. The light intensity was about 500 ft.c. The reaction was terminated by pouring the material into 25 ml. of boiling water to which sufficient H_2SO_4 had been added to give a final pH of 4–5. Boiling was continued for 4 min. The coagulated solids were filtered off and the filter paper was thoroughly washed. The clear filtrate was acidified to approx. pH 3, air was blown through for 20 min. to remove unfixed tracer carbon dioxide, the pH was then adjusted to $6·8 \pm 0·2$ and the samples were diluted to 60 ml. They were stored in the refrigerator under Hutner's bactericide (Hutner & Bjerknes, 1948).

Analytical methods

Radioactivity estimation. Total activity fixed was determined by wet combustion (Van Slyke & Folch, 1940) of a 10 ml. portion which had been dried under air at room temperature. The evolved CO_2 was collected in NaOH and precipitated from this, after buffering with NH_4Cl, as $CaCO_3$. The precipitate was washed, dried, weighed, and then counted in a gas-flow counter as a 1 sq.cm. sample of 30–40 mg. All counts reported are corrected for the portion of the total solution which was combusted and the weight of $CaCO_3$ thus obtained, as well as self-absorption and background count. Geometry was constant. Counting error was ±5 %. All observations are based on at least two experiments made with different preparations.

Phosphopyruvic and pyruvic acids. A suitable portion (20 ml.) of the solution to be analysed was mixed with 2 ml. of 0·09M sodium pyruvate (pH approx. 6·5), bactericide (Hutner & Bjerknes, 1948) was added, and the mixture was incubated overnight at 37° with intestinal phosphatase (Armour Laboratories, Chicago, Ill.). Sufficient conc. HCl to make the final solution 1N was then added, followed by 10 ml. of 0·03M 2:4-dinitrophenylhydrazine in 2N-HCl. The reaction mixture was warmed on steam and, after cooling, extracted with ethyl acetate (40, 20, 20 ml.). The ethyl acetate solution was washed with water (15, 15 ml.) and then extracted with 0·5M-$NaHCO_3$ (25, 25, 25 ml.). The bicarbonate solution was acidified with conc. HCl (5 ml.) and extracted with ethyl acetate (20, 20, 20 ml.). The ethyl acetate solution was washed with water (15, 15 ml.), filtered into a tared beaker and dried, first under air and then in a vacuum desiccator, and weighed. The yield was 70–90 % of the theoretical based on the added pyruvic acid. The hydrazones were counted as 1 sq.cm. samples of 30–40 mg. The observed counts were corrected for sample size, self-absorption, background count and theoretical weight of hydrazone based on the pyruvic acid added as carrier. This method of analysis would include any other labelled keto or aldehydo acids present. However, evidence presented in the previous paper (Fager, 1952*b*) indicates that the analytical results correspond to phosphopyruvic acid.

Phosphoglyceric and glyceric acids. A suitable portion (20 ml.) of the solution to be analysed was mixed with 5 ml. of 0·05M sodium phosphoglycerate (prepared from barium phosphoglycerate, Nutritional Biochemicals Corp., Cleveland, Ohio; pH about 6·5), bactericide (Hutner & Bjerknes, 1948) was added, and the mixture was incubated overnight at 37° with intestinal phosphatase. It was then run through a cation-exchange resin column (1·5 g. of Nalcite HCR, National Aluminate Corp., Chicago, Ill.), followed by 30 ml. of water. The combined effluent was then run through an anion-exchange resin column (1·5 g. of Amberlite IR-4B, Resinous Products Corp., Philadelphia, Pa.), followed by 30 ml. of water. The anions were eluted from the resin by 25 ml. of 3 % aqueous NH_3 followed by 30 ml. of water. The excess of NH_3 was removed from the eluate by concentration at reduced pressure. The resulting solution (pH about 6–7) was cooled in the refrigerator for 15 min., 0·5 ml. of 0·5M periodic acid was added and the mixture left in the refrigerator for 60 min. Conc. HCl (5 ml.) and $NaHSO_3$ (100 mg.) were then added to decompose the iodate and excess of periodate. The hydrazone of glyoxylic acid was formed by the addition of 15 ml. of 0·03M 2:4-dinitrophenylhydrazine in 2N-HCl. The mixture was warmed on steam, cooled and extracted, dried, weighed and counted exactly as described for the pyruvic acid analysis. The yield of hydrazone was 70–80 % of the theoretical based on the added phosphoglyceric acid. Observed counts were corrected for sample size, self-absorption, background counts and theoretical weight of hydrazone based on the phosphoglyceric acid added as carrier. This method of analysis would include all labelled keto or aldehydo acids present. Therefore, the true radioactivity present in phosphoglyceric acid is represented by that observed in this analysis minus that observed in the analysis for phosphopyruvic acid. Although the radioactivity of all labelled polyhydroxy acids with adjacent hydroxyl groups would be included, evidence that the analytical results correspond to phosphoglyceric acid was presented in the previous paper (Fager, 1952*b*).

The analytical method measures radioactivity in the carboxyl carbon atom plus the α carbon atom of phosphoglyceric acid. Decarboxylation of the hydrazones indicated

that all (within experimental error) of the activity was in the carboxyl group. Analyses in which the exchange resin treatment was omitted gave the same results as those done by the method described above, but the yields of glyoxylic acid 2:4-dinitrophenylhydrazone were not as high.

Preparations

Hydroxypyruvic acid. This substance was made according to the method of Sprinson & Chargaff (1946). Potassium was removed by a cation-exchange resin and bromide was removed by addition of a slight excess of freshly precipitated, washed silver carbonate to the acid solution. Excess of silver ion was removed by a cation-exchange resin, and the solution of acid was then taken to pH 6·2 with NaOH and concentrated to 0·2 M at low temperature under reduced pressure. Periodic acid oxidation of a portion (excess of periodate was titrated with arsenite) indicated a 90% yield of sodium hydroxypyruvate.

Phosphoglycollic acid. Phosphoglycolic acid was obtained inadvertently during an attempt to make phosphohydroxypyruvic acid. Na_3PO_4, $12H_2O$ (2·8 g.) was dissolved in 20 ml. of water and added to a solution of the sodium salt of bromopyruvic acid (1·67 g.) in 5 ml. of water. After standing overnight, the solution was diluted to 1 l. and $CaCl_2$ (1·7 g./20 ml. water) was added. The precipitated calcium phosphate was centrifuged off and the supernatant was concentrated at reduced pressure to 125 ml. A considerable ppt. formed on warming. This was filtered off warm and dried *in vacuo* over $CaCl_2$. The yield was 0·5 g. (Found: C, 9·5; P, 12·8; Ca, 24·5. Calc. for $C_2H_2O_6PCa_{1\cdot5}$, $2H_2O$: C, 9·7; P, 12·5; Ca, 24·1%.)

Phosphoglycolaldehyde. Calcium α-glycerophosphate was made according to the method of King & Pyman (1914). Analysis of the product, dried *in vacuo* over $CaCl_2$, indicated a dihydrate rather than the anhydrous salt which they reported. The calcium was removed from 1·0 g. of this salt by passage through a column of cation-exchange resin. This acid solution (40 ml.) was cooled in the refrigerator for 15 min. and 10 ml. of 0·5 M periodic acid was added. After 60 min. in the refrigerator, titration of a portion with arsenite indicated completed oxidation. Barium hydroxide equivalent to the periodate (20 ml. of 0·25 N) was added and the crystalline ppt. of barium iodate was filtered off. Barium hydroxide was then added to pH 6, the solution was cooled and a slight precipitate was removed by filtration. An equal volume of ethanol was added and the resulting ppt. was centrifuged off, dissolved in 20 ml. of water and reprecipitated by the addition of an equal volume of ethanol. After drying *in vacuo* over $CaCl_2$, the yield was 0·6 g. (Found: C, 7·9; P, 9·7; Ba, 42·0. Calc. for $C_2H_3O_5PBa$, $3H_2O$: C, 7·3; P, 9·5; Ba, 41·7%.) Using a somewhat different procedure, Fleury, Courtois & Desjobert (1948) obtained a tetrahydrate.

A solution of the free acid, after removal of barium, gave a strong positive test for aldehyde with fuchsin reagent. This solution contained no inorganic phosphate, but 43% of the bound phosphate was liberated by 10 min. digestion with N-HCl at 100°, whereas none was liberated by similar treatment of the original α-glycerophosphate.

Vinyl phosphate. 2-Chloroethanol (6·4 g.) was phosphorylated in dry dimethylaniline (100 ml.) at 0° by the addition of phosphorus oxychloride (13·8 g.) dissolved in dimethylaniline (50 ml.). The reaction mixture was decomposed with water, ice and Na_2CO_3 and the dimethylaniline was separated. Inorganic phosphate was removed with magnesia mixture and the 2-chloroethyl phosphate was precipitated as the barium salt by addition of barium acetate, followed by sufficient ethanol to give a 50% solution. After being dried *in vacuo* over $CaCl_2$, analysis indicated the dihydrate. (Found: P, 9·2; Ba, 41·7. Calc. for $C_2H_4O_4ClPBa$, $2H_2O$: P, 9·3; Ba, 41·3%.) Using somewhat different preparative conditions, Plimmer & Burch (1929) obtained the monohydrate, and Fischer & Pfähler (1920) obtained the trihydrate. Barium was removed from a portion of the dihydrate (1·7 g.) by passage through a column of cation-exchange resin. This acid solution was dried under air at room temperature and then *in vacuo* over $CaCl_2$. The resulting semi-crystalline solid was taken up in absolute methanol (40 ml.) and refluxed for 1 hr. with KOH in methanol (1·7 g./30 ml.) under dry nitrogen. The methanol was then removed at reduced pressure and the residue was taken up in 30 ml. of ice-cold water containing sufficient cation-exchange resin (Nalcite HCR) to make the pH approx. 7. The resin was filtered off and washed, and the combined filtrate and washings were made to 100 ml. An analysis for chloride indicated that 34% of the bound chlorine had been ionized. The solution contained no inorganic phosphate but hydrolysis for 10 min. at 100° with N-HCl liberated 50% of the bound phosphate, which the chloride analysis had indicated might be present in an acid-labile form. Neither β-chloroethyl phosphate nor glycolphosphate liberate any phosphate under similar conditions. A strong odour of acetaldehyde was evident when the material was hydrolysed with N acid and crystals formed immediately in a droplet of 2:4-dinitrophenylhydrazine solution held above the hydrolysis vessel. No attempt was made to isolate the vinyl phosphate because it was wanted in solution and the evidence for its presence seemed adequate.

RESULTS

Effect of heat treatment

Certain observations made during the course of the investigations suggested that although the fraction which was termed 'enzyme' in the previous paper (Fager, 1952*b*) contained several active enzymes, its function in the system which forms phosphoglyceric acid was to supply the carbon dioxide acceptor. The carboxylation enzyme appeared to be associated with the 'chloroplasts'. In order to test this hypothesis, the experiments shown in Table 1 were performed. It was at once evident that the 'enzyme' was indeed the source of the carbon dioxide acceptor, and that the latter was unharmed by a heat treatment that coagulated the protein. In fact, the coagulated protein could be filtered off and the clear filtrate still retained most of the original activity. This heat treatment inactivated the enolase so that the ratio of radioactivity in phosphoglyceric acid/radioactivity in phosphopyruvic acid had a value of about 40 instead of the value of about 4 usually obtained. Other enzymes must also have been inactivated for fixation in phosphoglyceric acid represented over 95% of the total fixation as compared to 60–70% with the unheated material.

Table 1. *Effect of heat treatment*

All vessels: N_2 atmosphere; 2 ml. of 'enzyme' + 0·5 ml. of 'chloroplasts', both in 0·05M sorbitol:borate buffer (pH 6·9, 0·01M-KCl, 0·002M cysteine); 0·5 ml. of 0·03M-Na_2CO_3 containing ^{14}C; 15 min. illumination at 15–16°; light intensity about 500 ft.c. The 'enzyme' and 'chloroplasts' were prepared as described in the previous paper (Fager, 1952*b*). Values are in corrected counts/min. × 10^{-2}.

Treatment	Total fixation of ^{14}C	Fixation of ^{14}C into phosphoglyceric acid	Fixation of ^{14}C into phosphopyruvic acid
None	125	81	17
'Enzyme' replaced by buffer	4	0	0
'Chloroplasts' replaced by buffer	1	0	0
'Enzyme' heated for 1 min. under nitrogen in a boiling water bath	119	115	3
'Chloroplasts' heated, as above	1	0	0
'Enzyme' + 'chloroplasts' heated together, as above	1	0	0
'Enzyme' replaced by algae 'extract'	50	47	1

As an extension of this observation, it was found that a hot-water extract of pre-illuminated algae could replace the material prepared from spinach. The 2 ml. of 'enzyme' from spinach was equivalent to 16 g. of spinach; the 2 ml. of 'extract' from algae was equivalent to 80 mg. of algae. The reduction in weight of plant material by a factor of 200 reduced the phosphoglyceric acid-forming activity by only a factor of about 2·5. The amount of fixation is still very small when considered in terms of normal photosynthesis, but in terms of dark fixation following pre-illumination this reaction represents about 2% of that obtained with intact algae (Gaffron, Fager & Rosenberg, 1951). Furthermore, the pattern of fixation is identical; 95% of the total is in phosphoglyceric and phosphopyruvic acids, though the second compound contained a greater proportion in the case of the intact algae because the enolase had not been inactivated.

An experiment was done to determine the location of the labelled fixation product: 0·5 ml. of 'chloroplasts', 2·0 ml. of 'extract' and 0·5 ml. of the labelled carbonate solution were mixed and allowed to stand for 15 min. The mixture was then centrifuged and the clear supernatant was decanted from the precipitated 'chloroplasts'. The two fractions were poured separately into boiling dilute sulphuric acid and subsequent analysis was as usual. The supernatant contained 85–95% of the total fixed tracer, indicating that the product was not strongly bound to the chloroplast fragments, the fixation probably having occurred at the surface.

Effect of pretreatment

It was never possible with spinach to test satisfactorily the effect of various pretreatments of the leaves; there was too much variation in absolute activity between batches or even between different plants to make quantitative comparisons. However, it was observed that illumination overnight under an atmosphere of 4% carbon dioxide in air generally gave material devoid of activity, that use of air alone gave much better results and that use of nitrogen seemed to bring about some further improvement. It was, of course, impossible to tell whether the effect was on the 'chloroplasts' or on the 'enzyme' or on both. With the algae, portions of the same suspension could be subjected to different pretreatments and portions of the same 'chloroplasts' could be used with the different samples of 'extract'. Table 2 shows the results obtained.

Table 2. *Effect of pretreatment upon fixation*

All vessels: N_2 atmosphere; 2 ml. of algae 'extract', 0·5 ml. of 'chloroplasts', both in 0·05M sorbitol:borate buffer (pH 6·9, 0·01M-KCl, 0·002M cysteine); 0·5 ml of 0·03M-Na_2CO_3 containing ^{14}C; 15 min. illumination at 15–16°; light intensity about 500 ft.c. The extracts were prepared exactly as described in section on Materials and Methods, except that different gases were used during the 20 min. pre-illumination. All extracts were thoroughly flushed with N_2 before use. Values are in corrected counts/min. × 10^{-2}.

Gas used during pre-illumination	Total fixation of ^{14}C	Fixation of ^{14}C into phosphoglyceric acid
Nitrogen	48	44
Air	51	17
4% carbon dioxide + 96% nitrogen	15	0

It is quite clear that the effect is upon the substance which reacts with carbon dioxide to form phosphoglyceric acid and that the amount of this substance present decreases as the amount of carbon dioxide available during the preceding illumination is increased. It is also seen that anaerobic conditions favour the formation of this acceptor, whereas the presence of oxygen apparently favours the formation of other substances which can fix carbon dioxide. These results explain why spinach used as purchased gave variable results, whereas illumination overnight under air before use led to greater uniformity. In order to keep them fresh, the leaves were laid in about 5 mm. of water and a constant stream of air saturated with water was passed over them during the illumination. It

may be reasonably supposed that the result was a moderate carbon dioxide limitation and a consequent formation of acceptor.

Kinetics of the fixation

Three variables, time of contact with tracer, concentration of 'chloroplasts' and concentration of 'extract', were investigated.

As Table 3 shows, the fixation continues for a surprisingly long time although after 16 minutes the relative amount of ^{14}C incorporated into phosphoglyceric acid decreases. This may be because the rate of formation of this compound decreases as the acceptor is used up while other substances which can fix carbon dioxide are slowly formed by the 'chloroplasts' or because side reactions which transform phosphoglyceric acid become more important as the carboxylation reaction proceeds and the concentration of this compound increases.

Table 3. *Time course of the fixation of carbon dioxide*

All vessels: N_2 atmosphere; 2 ml. of algae 'extract', 0·5 ml. of 'chloroplasts', both in 0·05 M sorbitol: borate buffer (pH 6·9, 0·01 M-KCl, 0·002 M cysteine); 0·5 ml. of 0·03 M-Na_2CO_3 containing ^{14}C; 15–16°; light intensity about 500 ft.c. Values are in corrected counts/min. $\times 10^{-2}$.

Time of contact with tracer (min.)	Total fixation of ^{14}C	Fixation of ^{14}C into phosphoglyceric acid
4	11	11
8	22	22
16	51	46
24	63	54
32	85	70

Table 4. *Effect of concentration of 'chloroplasts' or 'extract' of algae*

All vessels: N_2 atmosphere; amounts of 'chloroplasts' and algae 'extract' as shown, both in 0·05 M sorbitol: borate buffer (pH 6·9, 0·01 M-KCl, 0·002 M cysteine); 0·5 ml. of 0·03 M-Na_2CO_3* containing ^{14}C; 15 min. in the dark at 15–16°. Values are in corrected counts/min. $\times 10^{-2}$.

Expt.	Amount of 'chloroplasts' (ml.)	Amount of 'extract' (ml.)	Amount of buffer (ml.)	Total fixation† of ^{14}C
A	1·0	2·0	0·0	51
B	0·5	2·0	0·5	29
C	0·25	2·0	0·75	15
D	0·125	2·0	0·875	8
E	0·5	4·0	0·0	25
F	0·5	2·0	2·0	15
G	0·5	1·0	3·0	8
H	0·5	2·0	0·0	40
I	0·5	2·0	0·0	57‡

* Carbon dioxide is not limiting at this concentration.
† >90 % of total fixation was in phosphoglyceric acid.
‡ Illuminated; light intensity about 500 ft.c.

The values given in Table 4 represent amounts of tracer carbon dioxide fixed in a definite time (15 min.), not rates. However, as Table 3 shows, these are related to the rate of fixation for the first 15–20 min. The dependence of the rate of carbon dioxide fixation upon 'extract' concentration (Expts. *E–G*) was expected, but the fact that this rate also shows good proportionality to 'chloroplasts' concentration (Expts. *A–D*) was unexpected. This situation will be examined in more detail in the Discussion.

Properties of the enzyme ('chloroplasts')

The fixation of carbon dioxide was completely inhibited by the presence of 10^{-3} N-Cu^{2+}. This suggested the participation of sulphydryl groups, presumably in the enzyme.

More definite evidence was obtained by the use of *p*-chloromercuribenzoate (Barron & Singer, 1945). This poison was added to the 'chloroplasts' (0·5 ml.) and the mixture was allowed to stand 10 min. at 15° before addition of 'extract' and tracer. Controls were treated in an identical manner except for omission of the poison. The fixation was done in the dark. The following results were obtained: 3×10^{-3} M poison obliterated the fixation; 6×10^{-4} M reduced it to 40 % of the control; 10^{-4} M (Table 5) reduced it to 60 % of the control (concentrations were calculated on the basis of the volume of 'chloroplasts' plus poison, not the final volume). The addition of 10 times the equivalent of cysteine, after 8 min. contact with poison, gave complete reversal of the inhibition by the lowest concentration of poison (Table 5) but was less effective against the higher concentrations. Illumination seemed to be nearly as effective a reversal agent (Table 5) as the addition of cysteine.

Table 5. *Properties of 'chloroplasts'*

All vessels: N_2 atmosphere; 2 ml. of algae 'extract', 0·5 ml. of 'chloroplasts', both in 0·05 M sorbitol: borate buffer (pH 6·9, 0·01 M-KCl); 0·5 ml. of 0·03 M-Na_2CO_3 containing ^{14}C; 15 min. in dark at 15–16°. Values are in corrected counts/min. $\times 10^{-2}$.

Pre-treatment of 'chloroplasts'	Total fixation of ^{14}C
None	38*
p-Chloromercuribenzoate, 10^{-4} M†	22*
p-Chloromercuribenzoate, 10^{-4} M,† followed by 10^{-3} M cysteine	37*
p-Chloromercuribenzoate, 10^{-4} M,† followed by illumination‡	35*
Cyanide, 10^{-3} M	11§

* >90 % of total fixation was in phosphoglyceric acid.
† Concentration of poison calculated on the basis of volume of 'chloroplasts' plus poison, not final volume. The 'chloroplasts' were in contact with the poison for 10 min. before addition of 'extract' and tracer.
‡ Illuminated during fixation for 15 min.
§ About 75 % of total fixation was in phosphoglyceric acid.

When the 'chloroplasts' were flushed with oxygen for 15 min. and then with nitrogen, the fixation was reduced to 70 % of the control, which had been flushed only with nitrogen. The addition of cysteine (final concentration 10^{-3} M) restored the activity of the oxygen-treated material to 95 % of the control.

Cyanide is an effective inhibitor of the fixation in the system being studied; 10^{-3} M reduced fixation to 30 % of the control (Table 5). It also reduced the proportion of the total fixation present as phosphoglyceric acid from 95 to 75 %. These results are exactly what would be expected on the basis of previous work with intact algae; 2×10^{-3} M cyanide introduced in the light 1 min. before termination of the pre-illumination and addition of tracer, reduced total fixation to 17 % of the control and reduced the proportion of radioactivity in water-soluble substances (mostly phosphoglyceric acid) from 95 % of the total to 34 % of the total (Gaffron *et al.* 1951). The greater effect in the intact algae is reasonably ascribable to the higher concentration of cyanide and to the longer contact time with poison before the addition of tracer.

Properties of the acceptor ('extract')

The acceptor presumed to be in the algae 'extract' is a rather stable substance. The material can be kept at −20° for several weeks and at least overnight at 25° without loss of activity. A solution containing it can be flushed with oxygen or treated with 3 % (w/v) hydrogen peroxide at pH 7 without effect. It can be heated under nitrogen for 1 min. at 100° at pH 1·4 (Table 6) or pH 4 or pH 9·5 without appreciable loss of activity. Hydrolysis with 0·5 N hydrochloric acid or 0·5 N sodium hydroxide under the same conditions gave inconsistent results, apparently because of the complications introduced by the high salt concentration resulting when the solutions were neutralized.

The active substance is a relatively small molecule since after dialysis of 5 ml. of 'extract' in a Visking sausage casing against 5 ml. sorbitol: borate buffer for 10 hr. at 5° it was evenly distributed on both sides of the membrane.

It is strongly adsorbed on alumina; 10 mg. of alumina removed 85 % of the activity from 3 ml. of 'extract' when shaken with it for 20 min. at 10°.

The substance is an anion. It is not adsorbed during passage through a cation-exchange resin column (Nalcite HCR) and none passes through a column of anion-exchange resin (Amberlite IR-4B). About 60–70 % of the original activity can be recovered by elution from the anion-exchange resin with 0·05 N sodium hydroxide (Table 6).

The substance was first suspected of being a phosphate ester on the basis of the inhibition of the fixation by fluoride and the strong inhibition shown by phosphate esters, such as phosphoglyceric acid and ribose 5-phosphate, when employed in concentrations above 10^{-3} M (Fager, 1952*b*). The inhibitory action of these latter compounds affected particularly the fixation in phosphoglyceric and phosphopyruvic acids; total fixation was reduced by a factor of 3–4 while fixation in these compounds was reduced by 10 times as large a factor. Proof that it is a phosphate ester was obtained by employing intestinal phosphatase. Samples were kept overnight at 25°; one with 5 mg. of phosphatase added, the other without this enzyme. Both of the samples were under a nitrogen atmosphere and contained bactericide (Hutner & Bjerknes 1948). After 15 hr., they were heated for 1 min. in boiling water and then cooled and mixed with tracer carbonate and freshly prepared 'chloroplasts'. The sample which had not been treated with phosphatase fixed as much tracer in phosphoglyceric acid as did a portion which had been kept at −20° overnight. The sample which had been subjected to phosphatase hydrolysis fixed only 0–5 % of this amount (Table 6).

There seem to be four reasonable alternative explanations of these results: the 'extract' supplies a source of energy, probably in the form of labile phosphate bonds; or there is in the 'extract' a phosphate ester which can be carboxylated, perhaps reductively, to yield phosphoglyceric acid; or the 'extract' contains phosphoglyceric acid and the fixation represents only an exchange between the carboxyl of this compound and the added tracer carbon dioxide; or the fixation is a combination of the first-stated with one of the others. These alternatives were tested as described below and no combination as yet employed has functioned as an acceptor of carbon dioxide leading to the formation of phosphoglyceric acid.

Table 6. *Properties of the 'extract' of algae*

All vessels: N_2 atmosphere; 2 ml. of 'extract', 0·5 ml. of 'chloroplasts', both in 0·05 M sorbitol: borate buffer (pH 6·9, 0·01 M-KCl, 0·002 M cysteine); 0·5 ml. of 0·03 M-Na_2CO_3 containing ^{14}C; 15 min. illumination at 15–16°; light intensity about 500 ft.c. Values are in corrected counts/min. $\times 10^{-2}$.

Pre-treatment of 'extract'	Total fixation of ^{14}C*
None	50
1 min., 100°, pH 1·4	49
Passed through cation-exchange resin (Nalcite HCR)	42
Passed through cation-exchange resin (Nalcite HCR) and through anion-exchange resin (Amberlite IR-4B)	<1
Eluted from anion-exchange resin (Amberlite IR-4B)	37
Phosphatase overnight	<1

* >90 % of total fixation was in phosphoglyceric acid.

Adenosine triphosphate (sodium salt, Pabst Laboratories, Milwaukee, Wisc.) at 10^{-3} M acts as an inhibitor (Fager, 1952*b*). At 10^{-4} M and lower concentrations it caused no fixation when tested by itself; it had no effect upon the fixation promoted by the 'extract'; it did not cause fixation in the presence of any of the potential acceptors (cf. following paragraph) which were added; nor did it promote an exchange involving the carboxyl of added phosphoglyceric acid. The phosphate-bond energy might, of course, be present in a different form, but in a recent publication Strehler (1953) has shown that the level of adenosine triphosphate in green plants is very sensitive to illumination. He has suggested that it is intimately involved in photosynthetic carbon dioxide fixation. It does not seem to be involved in the fixation of carbon dioxide by the cell-free system with which this paper is concerned.

In testing phosphate esters as possible acceptors a range of concentrations from 10^{-4} M to 10^{-6} M was employed; higher concentrations produced inhibition of the fixation in phosphoglyceric acid. A consideration of two-carbon compounds suggests that the phosphate esters of glycolaldehyde and acetaldehyde are perhaps the most likely. The first would require a reductive carboxylation (2H); the second could form phosphoglyceric acid directly by appropriate addition of carbonic acid or bicarbonate ion. Unfortunately, neither 2-phosphoglycolaldehyde nor vinyl phosphate showed any ability to fix carbon dioxide at any concentration tested. It has recently been shown (Racker, Haba & Leder, 1953) that hydroxypyruvic acid can function as a source of 'active' glycolaldehyde. This acid was, therefore, tested, but was also found to be inactive in the system being studied. As mentioned in an earlier section, attempts to prepare phosphohydroxypyruvic acid were unsuccessful. Two other possibilities, both of which would require reductive carboxylations (4H), are 2-phosphoglycollic acid and phosphoglyoxal. The first was tested and had no effect; attempts to prepare the second were unsuccessful. A mixture of sugar phosphates (supplied by Dr R. S. Bandurski, Division of Biology, The California Institute of Technology) containing those ketoses, ribulose and sedoheptulose, which have recently been suggested (Bassham, 1953) as possible sources of the acceptor, was tried and found to be inactive. In this connexion it should be noted that the spinach 'enzyme' described in the previous paper (Fager, 1952*b*) has been shown (A. Magdali in the laboratory of Dr B. Vennesland, Department of Biochemistry, University of Chicago) to contain an active enzyme complex which transforms ribose 5-phosphate into ribulose phosphate, heptulose phosphate and triose phosphate (Axelrod, Bandurski, Greiner & Jang, 1953). However, the addition of ribose 5-phosphate at concentrations from 10^{-4} M to 10^{-6} M to a combination of this 'enzyme' and 'chloroplasts' did not increase the fixation of carbon dioxide into phosphoglyceric acid; at higher concentrations the ribose 5-phosphate acted as a strong inhibitor.

Practically every low molecular weight compound which can exist as a phosphate ester has been proposed as the acceptor at one time or another (Bassham, 1953; Calvin *et al.* 1951; Gaffron *et al.* 1951). The ones tested seemed the most likely, but none of them served to fix carbon dioxide in phosphoglyceric acid. It is of course possible that one of them is the acceptor present in the 'extract' but that in its 'active' form it has the phosphate in a different position or is combined with some other substance.

The suggestion that the fixation is actually an exchange involving the carboxyl of phosphoglyceric acid appeared strengthened by the observed stability of the acceptor. However, 3-phosphoglyceric acid did not show any ability to fix tracer carbon dioxide either by itself or with added adenosine triphosphate; nor did it enhance fixation by the 'extract' in any concentration from 10^{-4} to 10^{-7} M. Perhaps even more convincing evidence is the observation that the 'extract' prepared from algae exposed to 4% carbon dioxide in nitrogen (Table 2) fixed no tracer in phosphoglyceric acid. These algae surely contained this substance in the steady state concentration present during photosynthesis and in the form utilized in this process. The same algae at the same concentration, the same conditions of preparation of the 'extract' and of fixation, and the same methods of analysis were used to obtain all the results shown in Table 2 so that it seems very unlikely that an 'active' phosphoglyceric acid would, if present, have been destroyed in one case and not in the other. Further evidence against exchange was provided by the use of labelled phosphoglyceric acid. Algae in the same concentration as that used for the preparation of the 'extract' were pre-illuminated and then allowed to fix tracer carbon dioxide for 1 min. in the dark. The resulting labelled phosphoglyceric acid (Gaffron *et al.* 1951) was extracted with boiling water. Two ml. of this extract were mixed with 0·5 ml. of 'chloroplasts' and 0·5 ml. of 0·03 M non-isotopic sodium carbonate and nitrogen was passed over the solution and then through sodium hydroxide for 15 min. Sulphuric acid sufficient to give a pH of about 2 was then added and the solution was boiled, the evolved carbon dioxide being collected in the sodium hydroxide. Calcium carbonate precipitated from this trapping solution in the usual manner contained 0–1% of the tracer present in the original extract. Analysis of the sulphuric acid solution showed the presence of 98–103% of the labelled phosphoglyceric acid originally present. Because the

amount of algae, the conditions of pre-illumination, and the conditions of tracer fixation which were used for the preparation of the labelled phosphoglyceric acid were identical with those used to prepare the usual 'extract', and to study tracer fixation by it, it seems reasonable to assume that the concentration and form of the labelled phosphoglyceric acid would have been identical with any 'active' phosphoglyceric acid which might have been the cause of fixation of tracer carbon dioxide by the 'extract' employed in the work described in this paper. The evidence, therefore, is against an exchange as the source of tracer fixation. In this connexion it is of interest to note that although Clendenning, Waygood & Weinberger (1952) demonstrated the presence of other carboxylases in varying amounts in leaf macerates, they were unable to find evidence for any enzyme or combination of enzymes which would decarboxylate phosphoglyceric acid.

DISCUSSION

It may be useful to list the properties of the system which seem well established. The carboxylation enzyme is associated with the 'chloroplasts'. The enzymic activity is inhibited by reagents which block sulphydryl groups. Cyanide acts as an inhibitor in a manner closely parallel to its effect on carbon dioxide fixation by pre-illuminated intact algae. The carbon dioxide acceptor is a relatively stable phosphate ester which can be extracted from pre-illuminated algae (or from a 47·5–60% (v/v) acetone precipitate from the soluble portion of spinach macerates) by hot water. The fixation is not the result of an exchange reaction involving the carboxyl group of phosphoglyceric acid. Fixation in phosphoglyceric acid precedes that in phosphopyruvic acid (Fager, 1952*b*), the latter being formed by enolase activity. The rate of fixation is dependent upon both the concentration of 'extract' and the concentration of 'chloroplasts' and the rate is increased by illumination.

As was shown in the section on *Kinetics*, the rate of the carbon dioxide fixation reaction is proportional to either the concentration of 'extract' or the concentration of 'chloroplasts' when the other is held constant. A consideration of Expts. *B*, *F* and *H* (Table 4) provides additional evidence that the rate is proportional to the product of the concentrations of these two materials. All three of these experiments had the same amount of 'extract' and of 'chloroplasts' but differed in total volume: *B*, 3·5 ml.; *F*, 5 ml.; *H*, 3 ml. Using *H* as a basis, the expected values of the amount of radioactivity fixed in Expts. *B* and *F* would be $(3/3{\cdot}5)^2 \times 40 = 29$ and $(3/5)^2 \times 40 = 14$, respectively. Agreement with expectation is very close. The dependence of the rate on the concentration of 'extract' seems reasonable, but its dependence upon the concentration of 'chloroplasts' seems much less so if the enzymic activity of this preparation represents the carboxylation step in photosynthesis; for in order to keep up with the observed rate of photosynthesis in intact cells the carboxylation enzyme must have a large turnover number. The simplest explanation would be that the carboxylation enzyme system present in the 'chloroplasts' is very sensitive and that almost all of it is destroyed during the preparation; the remnant would then act as a limiting factor. Alternatively, the 'chloroplasts' may perform a reduction and the rate may be limited by the proportion of the enzyme which is in the reduced form. However, the fact that illumination increases the fixation in this sytem by only 30–50% (cf. Table 4, Expts. *H* and *I*), whereas in other cases, such as those involving the pyridine nucleotides (Arnon, 1951; Tolmach, 1951; Vischniac & Ochoa, 1951), the increase may be as much as tenfold, makes this alternative appear less probable; for one might reasonably expect that light would tend to maintain the enzyme in its reduced form. The increased fixation in phosphoglyceric acid which does result from illumination may indicate either that light promotes the reduction of small amounts of a precursor to the fully reduced acceptor or that it 'activates' the carboxylation enzyme by forming sulphydryl groups (cf. Table 5). If it is only the latter, this probably does not represent the light reaction which drives photosynthesis.

If, as suggested in the preceding paragraph, no reduction is involved in the carboxylation reaction, the acceptor formed by pre-illumination being fully reduced, then there will be several possibilities for the acceptor of which perhaps the most likely may be vinyl phosphate, or a ketose (perhaps ribulose) phosphate (Bassham, 1953). The evidence available at present is against the participation of either of these compounds as acceptors in the system being studied. If a reduction is involved in the fixation of carbon dioxide, the small effect of light being due to disruption of the hydrogen-transport system, then the most likely acceptors would seem to be phosphoglycolaldehyde or phosphoglycollic acid or phosphoglyoxal. Not only do the two compounds which have been tested (the first two) not give any evidence of being acceptors, but fixation of carbon dioxide by all of them would presumably give rise to a derivative of hydroxypyruvic acid. It would be hard to reconcile the intermediate formation of a hydroxypyruvate derivative with the observation that although there is an active hydroxypyruvic acid reductase in the soluble portion of leaf macerates (Stafford & Magdali, 1953) this enzyme has been shown (A. Magdali, Laboratory of Dr B. Vennesland, Department of Biochemistry, University of Chicago) not to occur in the fraction from spinach

which is most active in regard to carbon dioxide fixation into phosphoglyceric acid.

The following appears to the writer to be the most consistent picture which can be formed on the basis of the information available at present. Pre-illumination of the algae results in the formation of a fully reduced acceptor (a phosphate ester) which can form phosphoglyceric acid by the fixation of carbon dioxide when activated by the carboxylation enzyme. This enzyme needs free sulphydryl groups in order to function properly, but it does not perform any reduction though it may promote an intramolecular oxidation-reduction in the acceptor molecule. Such a picture implies that photosynthetic reduction is not directly involved in the formation of phosphoglyceric acid but occurs elsewhere in the cycle. This is consistent with a suggestion which we made earlier (Gaffron *et al.* 1951) on the basis of studies of the formation of phosphoglyceric acid by intact cells: namely, that there is only a single photochemical reduction step in photosynthesis and that this step is the reduction of phosphoglyceric acid to the level of carbohydrate. All of the observations on the carbon cycle of photosynthesis now available are explicable in terms of such a hypothesis.

SUMMARY

1. Further work on the cell-free system from plant materials which forms phosphoglyceric acid by carbon dioxide fixation has shown that the precipitate which was termed 'enzyme' in a previous paper (Fager, 1952*b*) is in fact the source of the carbon dioxide acceptor and that the carboxylation enzyme is present in the 'chloroplasts'.

2. The enzyme is dependent upon free sulphydryl groups for activity and the carboxylation is inhibited by cyanide in a manner parallel to the effect of this poison on intact algae.

3. A hot-water extract of pre-illuminated algae has been found to be a reliable source of the acceptor. When this extract is used, over 90% of the total fixation is found in the form of phosphoglyceric acid.

4. The acceptor is a relatively stable phosphate ester.

5. Vinyl phosphate, phosphoglycollic acid, phosphoglycolaldehyde and ketose phosphates do not function as acceptors in the system as studied.

6. Evidence is presented to show that the fixation is not an exchange reaction involving the carboxyl group of an 'active' phosphoglyceric acid.

7. It is suggested that there is only a single photochemical reduction step in photosynthesis and that this step is the reduction of phosphoglyceric acid to the level of carbohydrate.

The author is indebted to Mr Hans Troll for assistance in the experimental portion of this work and to Drs J. Franck, H. Gaffron and J. L. Rosenberg for many valuable discussions of the observations and their interpretation.

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