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Authors

Bassham, J.A.
Kirk, Martha.

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University of California

Ernest O. Lawrence Radiation Laboratory

Berkeley, California

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J. A. Bassham and Martha Kirk

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SYNTHESIS OF COMPOUNDS FROM $^{14}CO_2$ BY CHLORELLA IN THE DARK

FOLLOWING PREILLUMINATION[1]

J.A. Bassham and Martha Kirk

In 1947, Benson and Calvin ([1]) found that the dark fixation of $^{14}CO_2$ by Chlorella could be greatly stimulated by preillumination, and shortly thereafter they reported that the principal products after 1 min in the dark following preillumination were phosphoglyceric acid, malic acid and alanine ([2]). These products are similar to some of those found after short periods of photosynthesis ([3]). Gaffron et al. ([4]) also reported the formation of PGA following preillumination. Calvin and Massini ([5]) performed another type of experiment which gives information about the changes occurring in the plant when the light is turned off. They permitted the plant to photosynthesize for some time with $^{14}CO_2$ until all the intermediates of the early carbon fixation pathway were completely labeled with carbon 14, at which time they took samples for subsequent analysis. Then they turned off the light and took more samples in the dark. They found that the radioactivity in PGA increased rapidly while that in ribulose diphosphate dropped rapidly. A similar experiment by Wilson and Calvin ([6]) showed that, when the light was left on but the CO_2 pressure lowered, the radioactivity in PGA declined rapidly while that in ribulose diphosphate rapidly increased. From these two experiments it was concluded that during photosynthesis ribulose diphosphate is carboxylated and the resultant product gives two molecules of 3-phosphoglyceric acid. This

conclusion was strengthened by studies with cell-free extracts and isolated enzyme systems (7, 8, 9, 10). Further study of the light-dark transients by Bassham et al. (11) showed that at the very shortest times (5 sec) after the light was turned off the rate of increase of PGA (in moles) approached twice the rate of CO_2 uptake (in moles) in the light just prior to the experiment. This was considered to be another indication of the formation of two molecules of PGA per CO_2 molecule taken up, at least in the dark immediately following photosynthesis. By 15 sec, though, the rate of increase of PGA has already decreased at least 50%, and between 30 and 60 sec the increase stops and the PGA starts to decline.

Since experiments of this type show only the changes in total concentration but not the kinetics of the newly incorporated carbon, it would be an advantage to do parallel experiments in which the $^{14}CO_2$ is added only upon turning off the light, particularly if some quantitative comparison could be made between the rate of carbon uptake in the light and the rate of appearance of labeled carbon in the dark immediately following illumination. Development of quantitative techniques for doing studies of $^{14}CO_2$ fixation during photosynthesis (12, 13) made such a study more feasible. We shall report here the results of a study of the appearance of ^{14}C in specific compounds immediately following illumination and shall describe also a light-dark transient study in which the algae were permitted to take up ^{14}C until the intermediates of the carbon cycle were saturated, before the light was turned off.

Tamiya, Miyachi and Hirokawa (14) measured the capacity of the plant to fix carbon 14 dioxide in the dark following preillumination, which they call R. They found that cyanide inhibits the reaction between R and CO_2 and also accelerates the non-photochemical decay of R. Since it has been found that cyanide reacts irreversibly with ribulose diphosphate in the

plant, thereby destroying its ability to react with carbon dioxide, we are inclined
to think that the major part of the CO_2 fixing capacity of preilluminated
algae (R) is represented by ribulose diphosphate, together with those
cofactors that bring about the formation of ribulose diphosphate in the
dark following illumination. For example, ATP would cause a phosphorylation
of some initial ribulose monophosphate to give ribulose diphosphate, thereby
contributing to R. Another portion of R would be the carbon fixation by
phosphoenolpyruvate and TPNH to form malic acid. Miyachi et al. (15) not
only showed that R decays rapidly in the dark and is quite independent of the
levels of TPNH and DPNH, but produced a very interesting fact that the levels
of these reduced cofactors actually increase in the absence of CO_2 when the
light is turned off. Miyachi (16) studied the effect of sulfhydryl reagents
on R. Although p-chloromercuribenzoic acid, like iodoacetic acid and arsenite,
lowered the level of R, it did not affect the absolute rate constant for
the reaction between R and CO_2. Since purified carboxydismutase (8), the
enzyme responsible for carboxylation of ribulose diphosphate to give two
molecules of PGA, is highly sensitive to this reagent, Miyachi concluded
that R could not be identical with ribulose diphosphate. Isolated carboxy-
dismutase, however, appears to have much less activity than that required for
carboxylation in vivo, and there are many other lines of evidence which lead
us to believe that the in vivo operation of the carbon reduction cycle must
have certain characteristics different from those of the isolated enzymes (17).

In the studies of R, the Chlorella have usually been preilluminated in
the absence of CO_2, whereas in the work reported here, the Chlorella are
allowed to photosynthesize under steady state conditions prior to the
administration of carbon 14 dioxide. Consequently, a direct comparison between
the results of the two types of experiments is not possible. We believe, however,

that the details of the kinetics of ^{14}C fixation into specific compounds which we report here will be of use in the general problem of interpreting the results of preillumination experiments.

MATERIAL AND METHODS

Chlorella pyrenoidosa were grown in continuous culture tubes as described previously (19). After being centrifuged and washed once with the medium given in Table I, they were centrifuged again and resuspended in this medium to a concentration of 25 ml packed volume per liter, and placed in the illumination cell of a steady state apparatus similar to that described previously (12). This apparatus has been modified, however, to include both automatic pH control and automatic density control. The algae suspended in the nutrient given in Table I have a pH of 4.75. The pH control is set at 6 and 0.1 N ammonium hydroxide flows in automatically until the pH reaches the set value, after which time it is added automatically from time to time as the algae photosynthesize, take up ammonium ion and tend to become acidic. All other nutrients are added automatically in response to a signal from a photoresistor which measures the light coming through the algae. The addition medium is the same as that in which the algae were initially suspended (Table I). Also, algae suspension is automatically withdrawn from the cell when the volume exceeds a certain amount.

Two experiments were performed: Experiment 1 was a light-dark transient study in which the algae were allowed to photosynthesize in the presence of ^{14}CO$_2$ until the intermediates of the carbon cycle were saturated, then the light was turned off with samples being taken periodically during and after the illumination. Experiment 2 was a preillumination experiment in which carbon 14 was added as both ^{14}CO$_2$ and H^{14}CO$_3^-$, in such a proportion as to give immediately the final specific activity of radiocarbon in both

TABLE I

Nutrient solution for Chlorella

KH_2PO_4	2.0 mM
$MgSO_4$	2.0 mM
$Ca(NO_3)_2$	0.02 mM
$FeSO_4$-Versen-ol	0.1 mM in Fe
*Modified Arnon's A-4 trace	2 ml/1
NH_4VO_3 (0.023 g/1)	1 ml/1

*Modified Arnon's A-4- stock solution

$CoCl_2 \cdot 6H_2O$	.168 mM
H_3BO_3	46.0 mM
$MnCl_2 \cdot 4H_2O$	50.0 mM
$ZnSO_4 \cdot 7H_2O$	.771 mM
$CuSO_4 \cdot 5H_2O$	.316 mM
MoO_3 (99.5%)	.100 mM

solution and gas phase (12). In experiment 1 the incident and transmitted intensities from the photospot were 64,800 and 810 lux, respectively. In experiment 2 the incident and transmitted intensities were 140,400 and 1,620 lux. In both experiments light intensities from the blue and white fluorescent tubes were 7,830 lux incident and 86 lux transmitted. In the light-dark transient experiment (1), the temperature was controlled automatically at 12°C. In the preillumination experiment the temperature was controlled at 20°C. These differences in light intensity and in temperature mean that the two experiments are not strictly comparable. In experiment 1, (light-dark transient) the algae were placed in the steady state apparatus and allowed to photosynthesize for 24 hrs before the start of the exposure to radiocarbon. The times after the administration of carbon are given in the figures, in the results section. In experiment 2, the preillumination experiment, the algae were placed in the steady state apparatus and allowed to photosynthesize in the presence of 1.9% CO_2 in air for 3 1/2 hrs before the start of the experiment. Just prior to the start of the experiment this gas system was closed and the per cent CO_2 allowed to decline due to photosynthesis to 0.6%. The light was turned off and the apparatus, already in a dark room, was shrouded with a black cloth. At the same instant, a loop containing 4.4 cc of $^{14}CO_2$ was added to the system (total gas and liquid volume was 440 cc) and at the same time 1.0 ml of $H^{14}CO_3^-$ solution, 0.06 mMoles and 1.98 mC per cc was injected through a hypodermic needle into the algae suspension. Because of the rapid bubbling of the cycling gas, through the algae suspension, mixing is considered to be almost instantaneous - from experiments with dyes we estimate about 1 sec. In experiment 2 (preillumination experiment) samples were taken every 5 sec, as shown in Fig. 4.

All samples were analyzed by two dimensional paper chromatography and

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radioautography as described previously (3). In order/to see all possible
stable compounds, no extractio were performed, and the concentrated
killed algae suspension was placed directly on the paper chromatogram.
After two dimensional paper chromatography and radioautography with
x-ray film, the individual spots as well as the origin were counted by
a double G-M tube which counts on both sides of the paper simultaneously.

During the course of both experiments the rates of photosynthesis
were determined just prior to administering the ^{14}C by using the
small gas volume of the steady state apperatus (12) and measuring the slopes
of CO_2 and oxygen change (12, 13). After addition of ^{14}C, the levels of
^{14}C and CO_2 were monitored giving continuous values for the specific
radioactivity.

RESULTS

Results of experiment 1 (light-dark transient) are shown in Figs.
1, 2 and 3. In all results given in this paper, the term "micromoles of ^{14}C"
means the number of micromoles of carbon total corresponding to the measured
amount of ^{14}C when the specific activity is that of the $^{14}CO_2-^{12}CO_2$
administered to the algae. In other words, it is the measured amount of ^{14}C
divided by the ratio of ^{14}C to $^{14}C + ^{12}C$. This method of expression is
convenient for comparing the flow of labeled carbon with the total flow
of carbon.

As in earlier detailed light-dark transient studies (11), the level of
PGA rises rapidly and then falls whereas the level of ribulose
diphosphate falls off to 0. However, in the earlier studies because of the
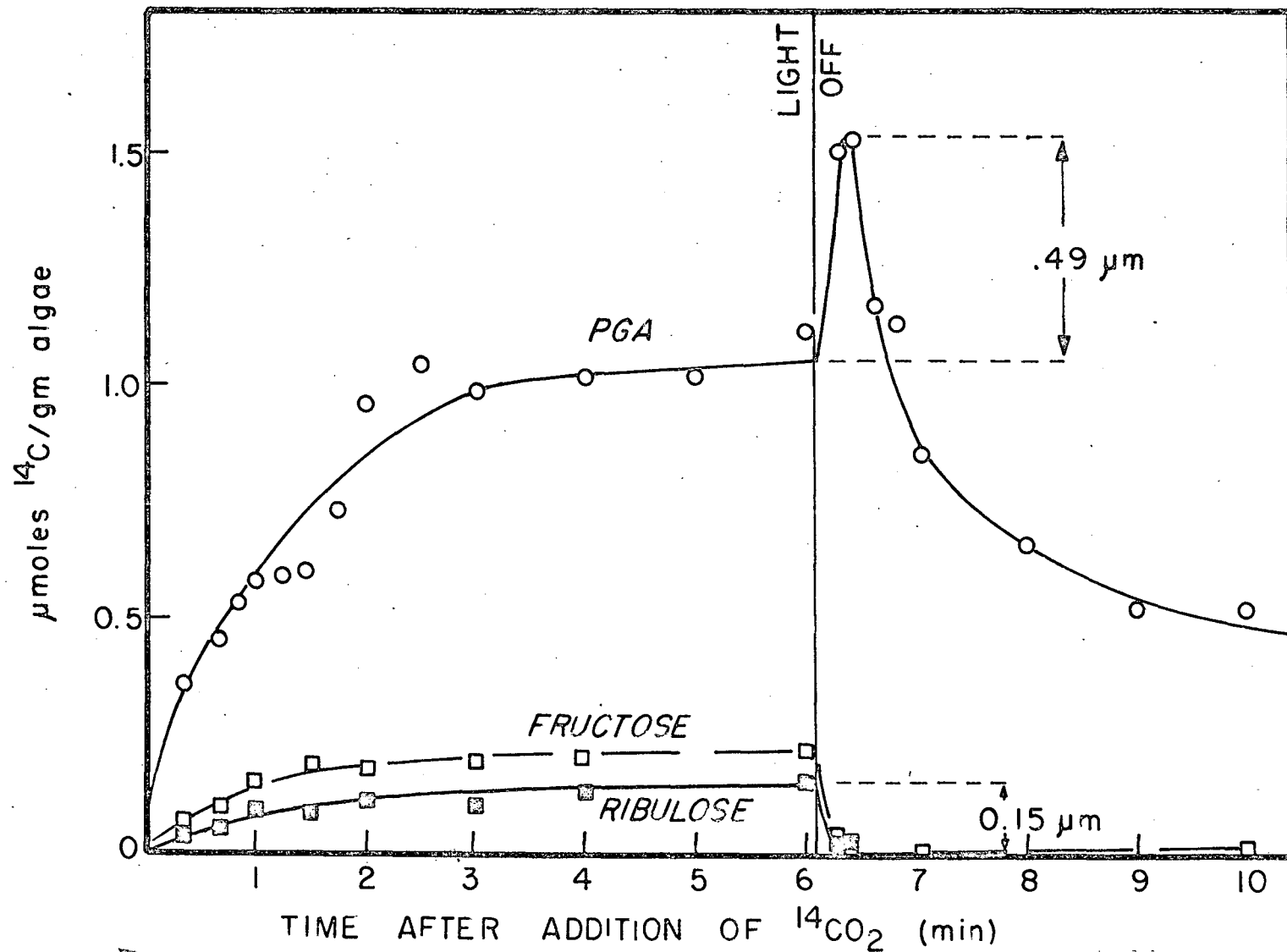different physiological conditions, the ribulose diphosphate was

Fig. 1. Light-dark transients, experiment 1 (see text). Changes in ^{14}C labeling of compounds in <u>Chlorella</u> when illumination stops. PGA = 3-phospho-glyceric acid. "Fructose" and "ribulose" refer to fructose-1,6-diphosphate and ribulose-1,5-diphosphate, respectively.
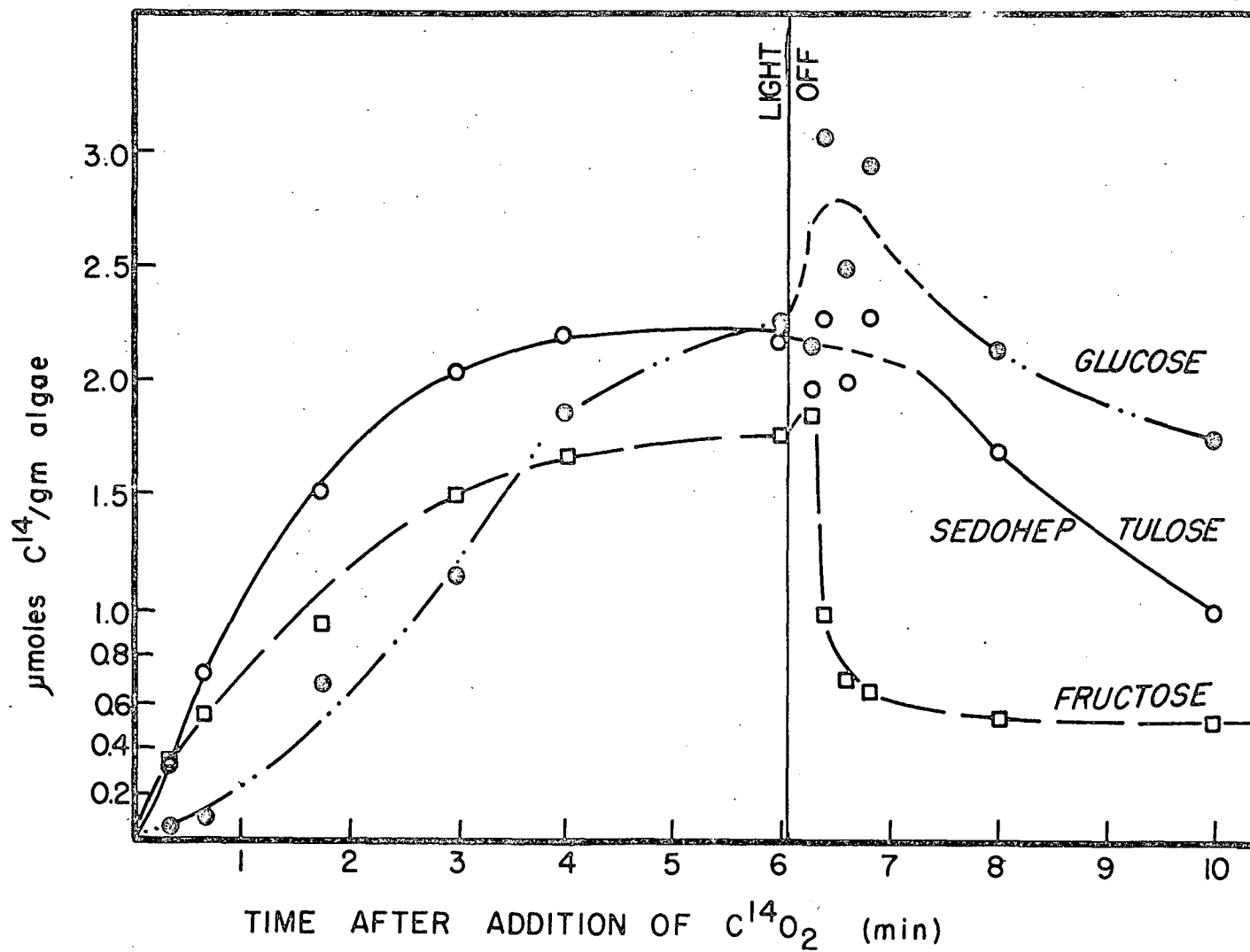
Fig. 2. Light-dark transients (experiment 1). "Glucose", "sedoheptulose" and "Fructose" refer to the sugar monophosphates.
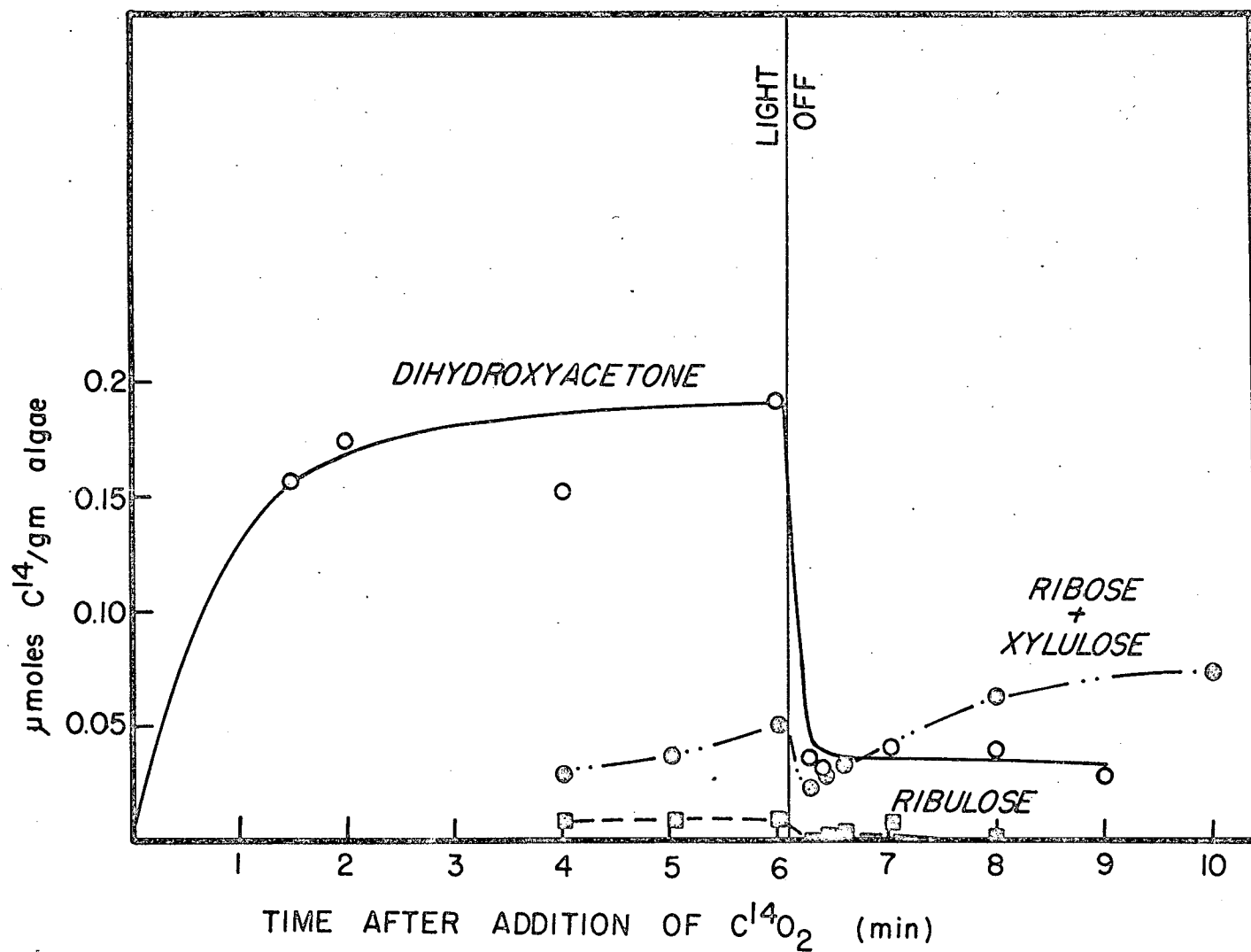
MU-27064

Fig. 3. Light-dark transients (experiment 1). "Dihydroxyacetone",

"ribose + xylulose" and "ribulose" refer to the sugar monophosphates.

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the only significant pool of diphosphates. The fructose diphosphate and
sedoheptulose diphosphate were almost negligible in concentration. In the present
study, however, the amounts of these other diphosphates are significant
and it is interesting to see/their concentration also falls to 0 when the light
is turned off.

The results of the preillumination experiment, experiment 2, are
shown in Fig. 4, 5 and 6. Compounds and areas not shown, and the amount of
radiocarbon in micromoles/cc algae found in them at 100 sec are as follows:
Pyruvic acid, .014; uridine diphosphoglucose, .013; sucrose, .035; the
origin, .042. Except for phosphoenolpyruvic acid, whose behavior paralleled
that of PGA, these other substances were first detected at 15 sec and increased
more or less linearly to the value just given. In Fig. 5 sugar monophosphates
which have not been resolved in this experiment are plotted together. We could
detect no traces of sugar diphosphates despite the fact that radiocarbon
presumably must pass through fructose diphosphate on the way to the other
sugar monophosphates and sucrose. From the light-dark transient study, however,
we have seen that its concentration approaches 0 when the light is turned
off.

In looking at the total ^{14}C fixed in experiment 2, it must be remembered
that the radiocarbon must find its way through any unstable or volatile
compounds before it reaches the stable compounds seen by paper chromatography.
We have previously reported (12) that the pool of such compounds is of the
order of 1-1.5 micromoles of carbon. This includes pools of bicarbonate
within the cell and possible unstable substances such as CO_2-enzyme
complexes. Assuming a pool of only 0.8 micromoles of unstable compounds, and
assuming the initial rate of flow of carbon through the system was the
same that existed when the light was on, namely, 10 micromoles of carbon
per cc of algae per min we have calculated a correction to show what
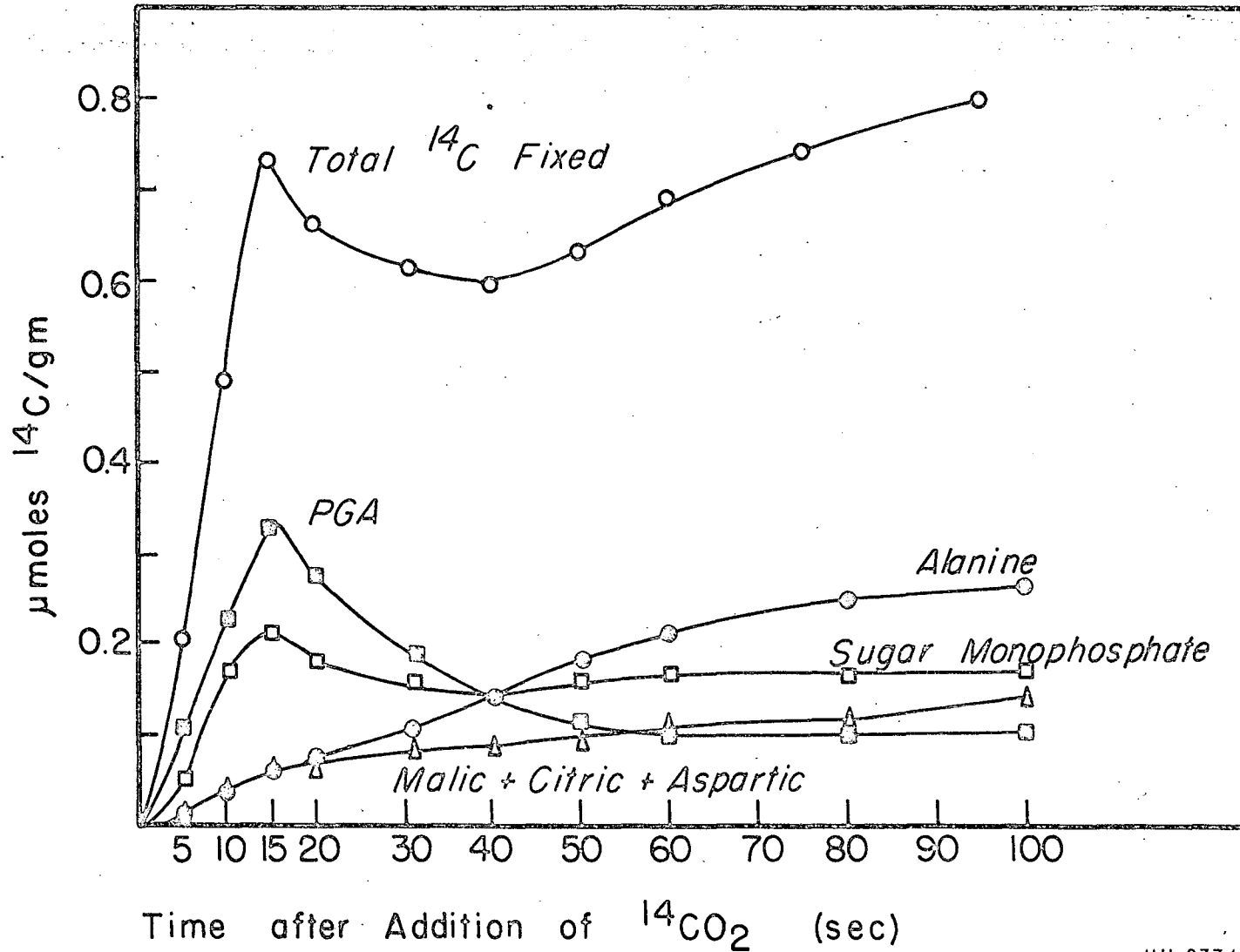
Fig. 4. Fixation of ^{14}C into compounds in Chlorella following preillumination,
(experiment 2, see text). PGA = 3-phosphoglyceric acid; sugar monophosphates
include monophosphates of fructose, glucose, sedoheptulose, ribose and ribulose.
"Total ^{14}C fixed" includes all radioactive compounds, including origin found
on the chromatogram.
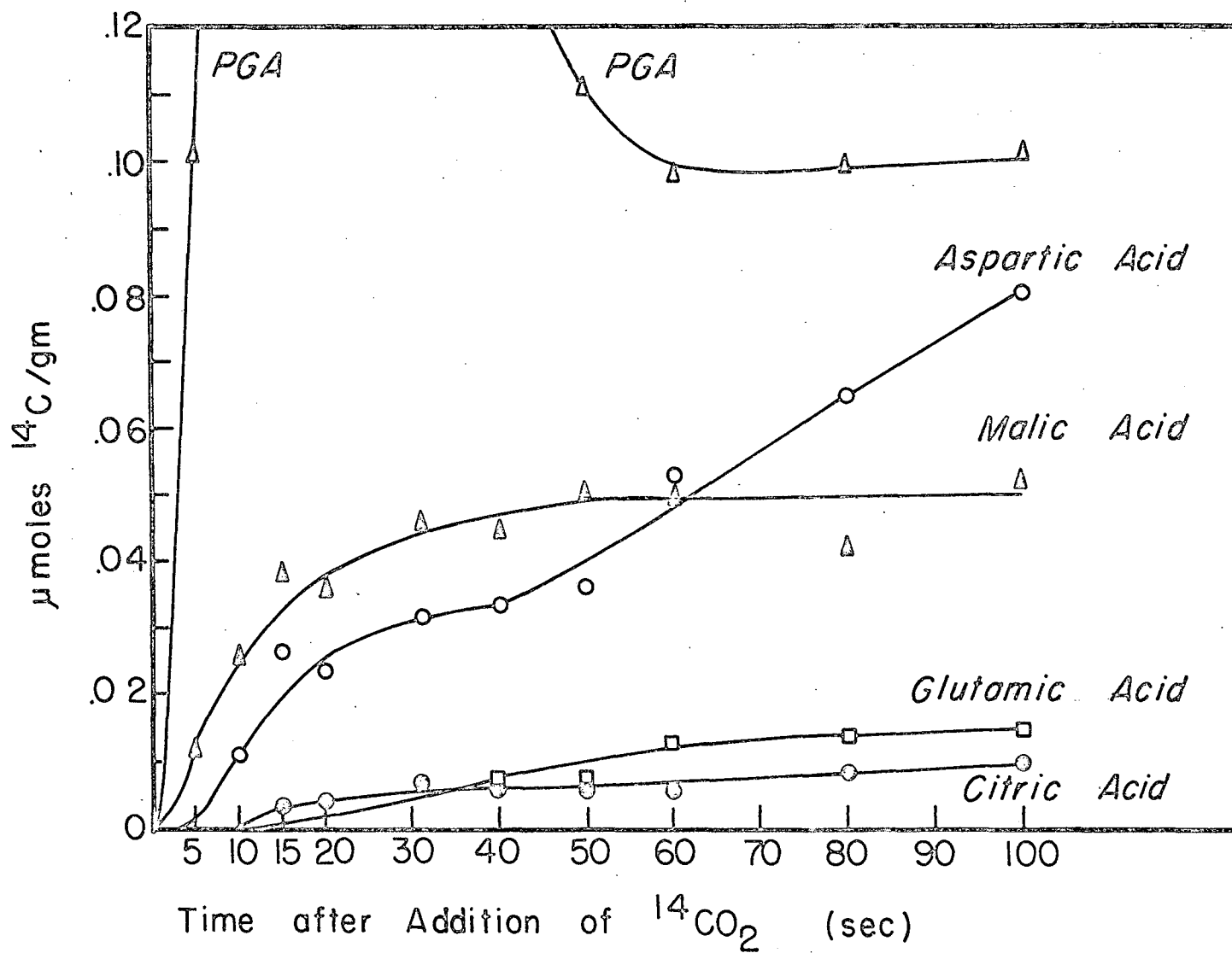
MU-27742

Fig. 5. Fixation of ^{14}C into compounds in Chlorella following preillumination (experiment 2, see text).
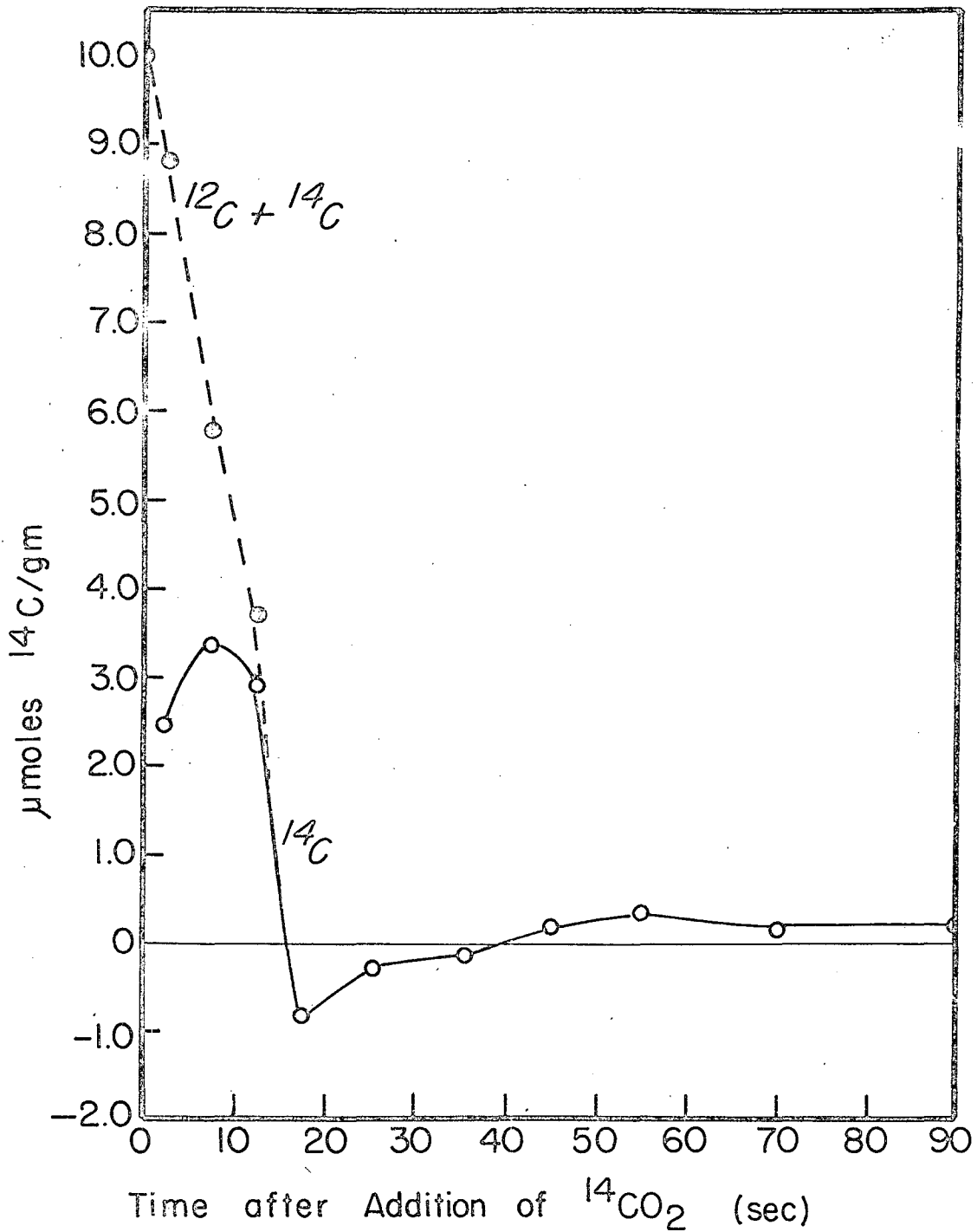
Fig. 6. Rate of total $^{14}CO_2$ fixation in experiment 2 (preillumination experiment). "^{12}C + ^{14}C" refers to estimated total CO_2 fixation. See text for explanation of calculation.

the total ^{14}C fixation rate would be if there were no unstable pools preceding stable compounds. This rate is shown as a dashed line in Fig. 6 along with the solid line depicting the observed rate of ^{14}C uptake based on the measured 5 sec increments. It is clear from Fig. 6 that the rate of fixation falls very rapidly when the light is turned off in the presence of 2% CO_2.

DISCUSSION

The results of the light-dark transient study (Fig. 1) show that when the light is turned off, the increase in PGA (.49 micromoles) is too great to be accounted for by carboxylation of the ribulose diphosphate present at the moment the light was turned off (.15 micromoles). Carboxylation of .15 micromoles ribulose diphosphate would give 0.19 micromoles of PGA, if two molecules of PGA were formed by each carboxylation. The additional PGA might be made in one of two ways: 1) More ribulose monophosphate might be phosphorylated with ATP left over from the light or with other sources of high energy phosphate and subsequently the resulting ribulose diphosphate could be carboxylated. The total drop in diphosphates (ribulose, sedoheptulose, and fructose) is sufficient to account for all the rise in PGA if there were some mechanism for transfer of C-1 phosphate from fructose diphosphate and sedoheptulose diphosphate to ribulose-5-phosphate. Such a mechanism is not known, however. 2) When the light is turned off oxidation of sugar phosphates via triose phosphate dehydrogenase could take place, giving rise to PGA. The drop in dihydroxy-acetone phosphate (Fig. 3) may indicate oxidation of triose phosphate, conversion of triose to pentose monophosphate (which might then be phosphorylated and carboxylated), or conversion to other sugars. All these possibilities should be viewed in the light of the results of the experiment 2, the preillumination experiment.

From Fig 6 we can see that the peak of radioactivity in PGA in the preillumination is caused in part by the rising specific activity of carbon entering the PGA pool, as well as the accumulation of radiocarbon in this pool with time. The downward trend of the PGA concentration in experiment 1 and PGA labeling in experiment 2 are both caused by the conversion of PGA to alanine as well as to malic, citric, aspartic acids, and other products. In addition it appears that some of the radioactivity in the PGA must be lost as evolved carbon dioxide since the total activity declines for a time before it reaches a slow but steady rate of increase due to carboxylation of phosophoenolpyruvic acid, giving, eventually, aspartic, malic, glutamic and citric acids.

One of the most interesting results from the preillumination experiment is the fairly rapid incorporation of ^{14}C into sugar monophosphates (mostly hexose and sedoheptulose monophosphate) even at 10 sec exposure to $^{14}CO_2$. At 10 sec the PGA carboxyl carbon, whose pool size has been estimated at about 1 micromole (in an experiment at comparable light intensity, 12) has itself only become about 20% labeled. This result strongly suggests the possibility that one of the products of carboxylation is a form of bound phosphoglyceric acid, which is reduced to the level of sugar phosphate without ever coming free and equilibrating with the phosphoglyceric acid pool. There have been previous indications of this possibility, including the finding (18) that during a kinetic study of the products of carbon 14 fixation in Scenedesmus the percentage radiocarbon appearing in PGA extrapolated to only about 75% at the time of addition of the ^{14}C. It has been suggested (17) that most or all of the steps of the carbon reduction cycle (19) of photosynthesis may take place on a multifunctional enzyme system which has the capacity for transferring carbon compound moieties from one functional site to another.

In this proposal, ribulose-1,5-diphosphate would react with a thiazole grouping on the enzyme similar to thiamine pyrophosphate to give a phosphoglycoaldehyde addition compound plus a phosphoglyceryl moiety which is transferred to a disulfhydryl function of the enzyme. The phosphoglycoaldehyde addition compound is then said to be carboxylated, phosphorylated, perhaps by thiamine triphosphate, reported by Yusa [20] to be in plants, and reduced, after which it is also transferred to a disulfhydryl function of the enzyme, giving another 3-phosphoglyceryl moiety. Hydrolysis of these 3-phosphoglyceryl enzyme complexes gives PGA and disulfhydryl enzyme. Otherwise, they can react to give disulfide enzyme and free dihydroxyacetone phosphate, which can then react with another phosphoglyceryl sulfhydryl enzyme to give fructose-1,6-diphosphate and a disulfide grouping on the enzyme. Other well known reactions catalyze the transfer of glycoaldehyde moieties from fructose-6-phosphate forming at the same time 4-phosphoerythryl sulfide, sulfhydryl enzyme.

If this proposal is correct, or even similar to the true mechanism, the cyclic carbon fixing power (R) of preilluminated algae may depend not only on levels of ribulose diphosphate, but also to some extent on some reduced functional group of the enzyme, or on some reducing cofactor which may not be identical with reduced pyridine nucleotide.

The formation of malic, citric, glutamic, and aspartic acids in the dark indicates that the carboxylation of phosphoenol pyruvic acid is not dependent on cofactors from the light but is probably only light-stimulated by the presence of more phosphoenolpyruvic acid (derived from the cycle) in the light than in the dark. Formation of malic acid, which requires TPNH, does appear to stop after about one minute. Aspartic acid labeling presumably results from transamination of the C_1-C_3 carboxylation product, either by glutamic acid or by unlabeled aspartic acid.

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