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PHOTOSYNTHESIS

James A. Bassham

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by

James A. Bassham

I. Introduction

The process of photosynthesis in green plants has often been indicated

by the chemical equation:

$$CO_2 + H_2O \rightarrow (CH_2O) + O_2.$$
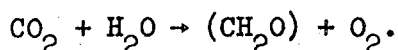
It has been recognized for many years that this equation must represent the

end result of a great many steps or biochemical reactions. Obviously, the

use of radioisotopes of carbon, hydrogen and oxygen suggests itself as a

method for following the course of these reactions since such tracer atoms

should enable one to label and subsequently to identify the intermediate

compounds. The long-lived carbon radioisotope, carbon fourteen, has indeed

proved to be of great value in tracing the pathway of carbon reduction during

photosynthesis and much of the subsequent discussion will be concerned with

its use.

The hydrogen radioisotope, H^3 or tritium, also has been used in studies

on the mechanism of photosynthesis, but such studies suffer from three serious

disadvantages. First, the hydrogen atoms of many chemical compounds encountered

in studies of photosynthesis may exchange with the hydrogen atoms of water.

Thus only the photosynthetic incorporation of tritium into non-exchangeable

positions can be studied. Secondly, the difference in mass between tritium

and hydrogen might be expected to produce rather important isotope discrimination, thus making interpretation of the results more difficult. Finally, the energy of the emitted β particle from tritium is so low that detection and counting are difficult. Despite these drawbacks, tritium has been used in studies of photosynthesis with some limited success.

Unfortunately, no radioisotope of oxygen with a half-life long enough to make it useful in biochemical experiments exists, and isotopic studies with oxygen have been confined to the use of the stable isotope, O^{18}.

Although it was formerly held that carbon reduction during photosynthesis resulted in the formation of carbohydrates (eq. 1), it is now known that energy derived from the conversion of light energy is used for the formation of amino acids and fats, and probably other products as well. Thus, a radioisotope of nitrogen could be employed in studies of the formation of nitrogen-containing compounds, but no useful radionitrogen is available.

Studies of the pathway of carbon reduction in which C^{14} was employed soon made apparent the fact that phosphate esters play a very important role on this pathway. Radiophosphorous, P^{32}, consequently has been utilized as an auxiliary tracer for the pathway of carbon reduction and has proved extremely valuable for this purpose. Quite a different use of P^{32} has been in studies of the formation of adenosine triphosphate in illuminated chloroplasts, and such studies have led to some understanding of the mechanism of formation of this important coenzyme, but a complete understanding of this mechanism remains a problem for the future.

One other radioisotope, sulfur thirty-five, deserves mention. Aside from its role in the synthesis of amino acids containing sulfur, there is now some evidence for the role of sulfate esters in lipid synthesis, and this

evidence has been obtained by studies which employed radiosulfur with photo-synthesizing algae. The proposal that thioctic acid might play some part in photosynthesis has as yet not been completely proved or disproved, and S^{35} may be employed in future studies of the role of thioctic acid or of other as yet unknown disulfide and sulfhydryl-containing compounds.

Techniques which have been used in studies with C^{14}, P^{32}, T (H^3), and S^{35} as tracers will be described. The experiments using these techniques are far too numerous to be described here, but some of the studies which the author feels to be of key importance in the present understanding of the biochemistry of photosynthesis and of closely related aspects of plant synthesis will be outlined as illustrations of the methods and as aids in understanding the current state of knowledge in this field.

Since many of the methods used in C^{14} tracer studies are applicable to the other tracers to be discussed and since the greatest advances have come from these studies, the use of C^{14} will be discussed in detail and in the sections on other tracers, C^{14} techniques will be referred to where appropriate.

B. Preparation of Plant Material

    I. Unicellular Algae

        1. Advantages

            Unicellular green algae, particularly Chlorella pyrenoidosa and Scenedesmus, have been extremely useful plants in studies of photosynthesis. This type of plant as compared with higher plants offers several advantages for such experiments. Unicellular algae may be maintained in a non-varying, steady state of growth for some months to permit a series of experiments with plant material of unchanging physiological state, whereas higher plants may

change significantly from day to day. The simplicity of the biological functions of unicellular algae enable the worker to focus attention more readily on the process of photosynthesis without concern for other processes such as inter-cellular transport, etc. Of particular importance is the fact that unicellular algae may be handled in liquid suspensions which may be readily subjected to physical and chemical manipulation such as pumping, aeration, adjustment of pH, addition of chemicals, and dilution of the biological material. The control of environmental conditions (and the subjection of the plant to sudden changes in environmental conditions) is therefore considerably easier than with higher plants. An important advantage of unicellular algae is the fact that a suspension of such plants may be killed very quickly if the suspension is poured into a suitable organic solvent such as methanol, and therefore the various enzymatic steps of the plant's metabolism can be brought to a halt quickly and simultaneously. Yet another useful property of unicellular algae is their very great photosynthetic rate, amongst the highest that has been measured for any plant.

2. Methods of Culture

Methods of culturing these algae vary considerably from one laboratory to another, not only with respect to their nutrition, but also with respect to conditions of illumination, temperature, density of the culture, and the variability that is permitted in these several factors. Experience in carbon labeling studies has shown that these variations affect the resulting pattern of carbon labeled compounds, in some cases causing important intermediate metabolites to occur in vanishingly small concentrations. It is easy to understand how an investigator unfamiliar with these variations

might have some difficulty in repeating the results of others working in this field.

Several different culture media have been employed by the authors and co-workers in carbon assimilation studies but the following one, reported by Myers (Myers, 1947), has been found to give algae with metabolic patterns similar in concentrations of intermediates to those obtained in much of the previous work in this field: To one liter of distilled water there are added 1.2 g KNO_3, 2.5 g $MgSO_4 \cdot 7H_2O$, 1.1 g KH_2PO_4, and 1 ml each of solution $\underline{A}$ and solution $\underline{B}$, described below. The carboy of nutrient solution is sterilized in an autoclave at $121^{\circ}C$ for 3 hours after which it is cooled and dispensed sterilely to the algae culture.

Solution $\underline{A}$ is made up by dissolving 59 g of N-hydroxyethylenediamine triacetic acid in 500 ml H_2O, adding 24.9 g $FeSO_4 \cdot 7H_2O$, diluting to 1.0 liter and aerating 16 hours. The solution is stored in the dark at about $10^{\circ}C$ and used as needed. Solution $\underline{B}$ is made up by adding 2.86 g H_3BO_4, 2.05 g $MnSO_4 \cdot 4H_2O$, 0.10 g $ZnCL_2$, 0.08 g $CuSO_4 \cdot 5H_2O$, and 0.02 g $H_2MoO_4 \cdot H_2O$ to one liter distilled H_2O.

Both variable density and constant density culture have been used for these experiments. In the case of the variable density cultures 1,100 ml of nutrient solution is inoculated with a liquid culture of the species desired in a low-form culture flask (circular, flat bottom, about 25 cm diameter) equipped with inlet and outlet tubes for adding nutrient and draining and for aerating with 4% CO_2 in air. The flasks are immersed in a water bath thermostated at $25^{\circ}C$, and illuminated through a transparent bottom panel by fluorescent lights (white, daylight type) which gives an intensity of 2,000 foot-candles at the bottom surface of the culture flasks. The algae are

allowed to photosynthesize and multiply until the culture density is about 3 cc wet packed volume of algae per liter of suspension. The algae are then harvested by withdrawing 900 ml of the suspension daily and adding 900 ml fresh nutrient each time. The culture soon reaches a "steady state" of growth in which the inoculum in the 200 ml left each day is sufficient, after 24 hours growth, to provide the same incoulum in the same volume the next day. The algae are therefore grown under identical conditions from day to day, although the effective light intensity varies over the 24-hour cycle decreasing as the algae suspension increases in density.

In order to overcome variation in light intensity, a constant density culture apparatus is used. In this case, the algae culture is grown in the space between two vertical concentric cylinders, sealed together at the top, the thickness of the culture suspension being 7.5 mm. Water at a thermostatically controlled temperature is circulated through the inside cylinder to maintain the algae suspension at a constant temperature. An inlet tube at the bottom of the space containing algae suspension is provided for entrance of nutrient solution and 4% CO_2 in air, and an outlet tube at the top permits overflow of the suspension and exit for the aerating gas. A magnetic stirrer at the bottom of the tube in addition to the bubbling action of the aerating gas, provides the necessary agitation to keep the algae from settling. The tube is illuminated by a vertical, circular bank of fluorescent lights which give an intensity of about 3,000 foot-candles at the outer surface of the algal suspension. An appendage with two windows on the outer cylinder provides a thickness of suspension through which a light beam is passed before striking a photocell. The optical path between the algae sus-

pension and the photocell may be altered so that the cell will be sensitive,

in one case to the amount of light absorption, or in the other case to the

amount of light scattering. Thus, the signal obtained from the photocell

may be made to indicate either the optical density of the suspension or approx-

imately the number of cells. The signal from the photocell is amplified and

used to control a solenoid-operated valve in such a manner as to admit fresh

nutrient solution to the tube when the density of the algal suspension exceeds

a predetermined value. The excess algal suspension overflows and the density

is maintained at a constant level. The overflow is not used for experiments,

but rather algal suspension is drained out through the nutrient inlet tube,

after which the tube automatically refills as the algae cells grow and divide.

3. Preparation for Experiment

The nutrient solutions used in the culturing of algae as described

in the preceding section contain a quantity of salts which often interfere with

the analysis of biochemical compounds from the algae following exposure to

the tracer element. Therefore, the harvested algal suspension is usually

centrifuged at about 1600 g, after which the algae are resuspended in the

buffer solution to be used during the experiment, or distilled water, and the

resulting suspension is again subjected to centrifugation. The algae are then

resuspended to a concentration of from 0.5% to 2.0%, depending on the nature

of the experiment. The solution in which they are suspended may be varied

and may be, for example, distilled water, 2.5×10^{-5} $\underline{M}$ $K_2HPO_4-KH_2PO_4$, for

short experiments (up to five minutes duration) where the investigator is not

concerned with possible diminution of amino acid labeling. A suspension in

10^{-3} $\underline{M}$ $(NH_4)_2HPO_4-NH_4H_2PO_4$ adjusted to pH 6.5 to 7.0 permits high rates of

carbon fixation with much labeling of amino acids during short periods of photosynthesis, and the subsequent chromatographic analysis is usually very good. For experiments of longer duration, Myer's Medium (preceding section) may be diluted five or ten fold and fortified with 1.0×10^{-3} to 3.0×10^{-3} $\underline{M}$ ammonium phosphate. For experiments of more than an hours' duration, it is advisable to make a calculation of the algal nutrient requirement for the period of the experiment and add a slight excess of the required minerals.

Once resuspended, the algae should be aerated at room temperature in weak light until used. It should be kept in mind that the conditions preceding the actual exposure to $C^{14}O_2$ will influence the subsequent labeling pattern, particularly in short exposures. A pattern approaching that of steady state growth conditions may be obtained by allowing the algae to photosynthesize in nutrient, diluted and fortified as described above, for 15 minutes in bright light (2000 to 5000 foot-candles) with a stream of 1% CO_2 in air bubbling through the suspension, and then proceeding with the exposure to carbon fourteen.

II. Higher Plants

A great many species of higher plants have been employed in photosynthesis experiments with tracer elements. In fact, the uptake of carbon fourteen has been used as a measure of photosynthesis rates of agricultural crops in the field (Zalensky, 1958). In the laboratory, plants with relatively small, easily extractable leaves and high photosynthesis rates lend themselves best to experimentation. Soybeans are a good example of a plant fulfilling these requirements and the soybean Glycine ussuriensis, variety Hawkeye, has been used in many experiments. These may be grown by planting seeds about 3 cm deep in sterilized, fertile soil (sandy loam) which is kept moist until ger-

mination. After germination the plants grow well if illuminated about 12 hours a day, given a "day" temperature of $25^{\circ}C$ and a "night" temperature of $18^{\circ}C$, and watered when the soil becomes dry and leaves begin to turn blue-green. The leaves may be used about 40 days after germination. Leaves of other plants of special interest may be used, for example, sugar cane and sugar beet. Where the leaf is too large to fit conveniently into an experimental vessel, it is necessary to employ an excised section of leaf.

III. Whole Chloroplasts and Chloroplast Fragments
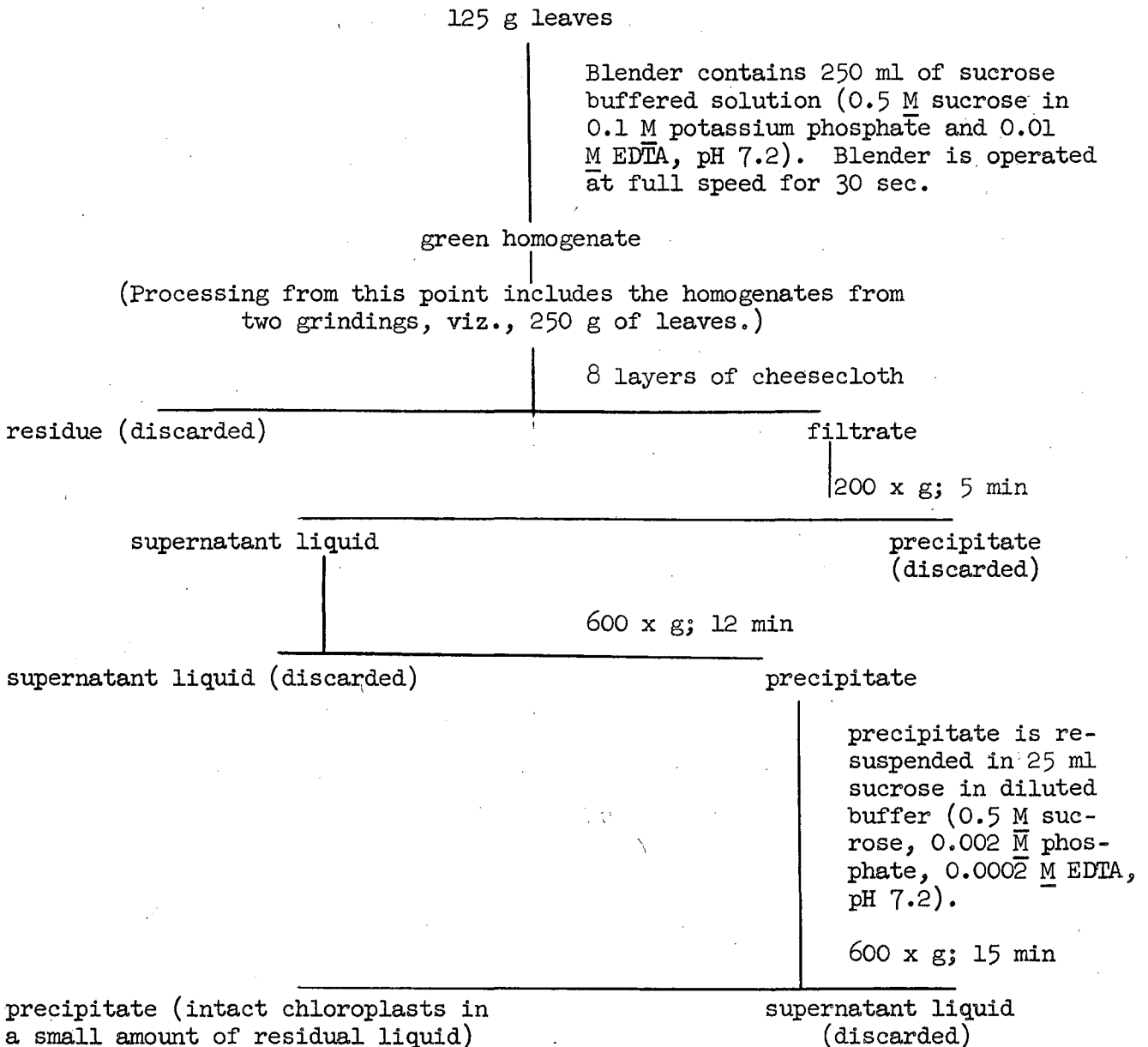
1. Whole Chloroplasts

Many studies with chloroplasts and chloroplast fragments with tracer carbon and also with radiophosphorous have been performed in recent years, particularly by Arnon and co-workers (Arnon, 1954a; Allen, 1957; Whatley, 1956; Trebst, 1958), who found many of the same carbon labeled compounds in chloroplasts as were isolated from whole cells photosynthesizing with radiocarbon. The most popular plant for chloroplast preparations for tracer studies seems to have been spinach. Spinach may be obtained from the market (freshness is important), grown in soil in pots or raised hydroponically. The source is frequently important for the results obtained from chloroplast preparations and hydroponically grown spinach have been reported to give the most active preparations (Gibbs, 1958).

The following methods for preparing spinach chloroplasts and sap have proved satisfactory (Holm-Hansen, 1959). Spinach leaves (Spinacea oleracea), obtained as fresh as possible from a commercial source, are used to prepare all fractions. For the preparation of whole chloroplasts and sap, the petioles are removed and the leaves are weighed. The leaves are washed thoroughly in cold tap water and drained as dry as possible. For the chloroplast prep-

aration, the leaves are drained dry by "tumbling" in a wire basket, while for
the preparation of sap the leaves are further dried by blotting with paper
towels. All subsequent operations are carried out at, or near, $0^\circ C$. All
glassware and centrifugation apparatus are precooled to about $0^\circ C$ unless other-
wise noted.

Two hundred and fifty grams of leaves are used for each experiment.
Grinding is performed in two batches, the leaves being cut into strips 0.5
cm wide. All centrifugations are performed in an International Portable
Refrigerated Centrifuge, Model PR-2. The flow diagram for a typical preparation
is given below:

125 g leaves

Blender contains 250 ml of sucrose buffered solution (0.5 $\underline{M}$ sucrose in 0.1 $\underline{M}$ potassium phosphate and 0.01 $\underline{M}$ EDTA, pH 7.2). Blender is operated at full speed for 30 sec.

green homogenate

(Processing from this point includes the homogenates from two grindings, viz., 250 g of leaves.)

8 layers of cheesecloth

residue (discarded)   filtrate

200 x g; 5 min

supernatant liquid   precipitate (discarded)

600 x g; 12 min

supernatant liquid (discarded)   precipitate

precipitate is re-suspended in 25 ml sucrose in diluted buffer (0.5 $\underline{M}$ sucrose, 0.002 $\underline{M}$ phosphate, 0.0002 $\underline{M}$ EDTA, pH 7.2).

600 x g; 15 min

precipitate (intact chloroplasts in a small amount of residual liquid)   supernatant liquid (discarded)

The material is resuspended in the residual liquid with the aid of a Potter-Elvehjem homogenizer equipped with a Teflon* pestle (Suspension C).

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*Teflon is duPont's trademark for its polyfluorohydrocarbon, tetrafluoroethylene.

2. Preparation of Sap

Centrifugations are performed at low and high speeds, respectively in an International Portable Refrigerated Centrifuge, Model PR-2, and a Spinco Ultracentrifuge, Model L, equipped with a No. 40 rotor. The leaves are cut into small pieces (ca 2 cm square) prior to the following treatment:

150-135 g leaves are placed in the center of five layers
of cheesecloth and the cheesecloth is drawn into a bag.

> The bag is dipped into liquid
> nitrogen for 30-60 sec.

frozen leaves

> The leaves are crushed with several
> blows of a hammer while still
> wrapped in the cheesecloth.

crushed leaves

> The leaves are placed in a precooled
> blender (-15°C). The blender is
> operated at full speed for 30 sec.

fine powder

> The powder is poured into a beaker
> and stirred constantly until a slurry
> is obtained. Approximately 30 min is
> required, and during this time the
> temperature of the mixture is never
> allowed to rise above $1-2^{\circ}$C.

green slurry

1,500 x g; 10 min

precipitate and crust   supernatant liquid
    (discarded)

20,000 x g; 15 min

clear supernatant liquid,   precipitate
greenish yellow. Yield: (discarded)
approximately 10 ml of
sap from 50 g leaves.

Further treatment of the clear supernatant liquid (sap): A suitable aliquot portion is set aside for the preparation of boiled sap (see below). Another 5 ml portion is adjusted to pH 7.2 at 25°C with 1 $\underline{N}$ KOH (taking careful note of the volume required). The rest of the supernatant liquid is maintained at 0°C and the pH is adjusted by the addition of a calculated amount of 1 $\underline{N}$ KOH using the volume determined in the previous step. (This preparation is found necessary because the sap pH has a large temperature coefficient; thus, the pH of the sap if adjusted to 7.2 at 0°C will drop to 6.8 when the sap is allowed to warm to room temperature.)

For control experiments the sap is heated in a boiling water bath for 5 min. The precipitate is removed by centrifugation and the clear, yellow supernatant liquid is allowed to cool to 25°C before the pH is adjusted to 7.2.

C. Experiments with Carbon Fourteen

I. Preparation of Radiocarbon Compounds

Carbon fourteen is usually obtained as $BaC^{14}O_3$ and is administered to plants either as an aqueous solution of bicarbonate or as carbon dioxide. A convenient specific radioactivity for most of the experiments in which the analytical techniques of two dimensional paper chromatography and radioautography to be described later are used is 75 μcuries/mg $BaC^{14}O_3$. For long exposures of the plant to C^{14} (five minutes or more), a specific activity of 10 μcuries/mg is often sufficient.

1. $HC^{14}O_3^-$
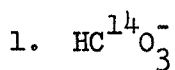
$BaC^{14}O_3$ is weighed and placed in a carbon dioxide generator M (Fig. 1) attached to a vacuum line, and M, S and P are evacuated. A convenient amount of $BaCO_3$ is 333 mg containing 25 millicuries of C^{14}. An amount of car-

bonate-free NaOH equal in moles to the $BaCO_3$ used (1.69 mmoles in the example given here) is placed in a 100 ml flask N after which the flask is attached to the vacuum line through stopcock B. With stopcocks C and E open and the stopcock H to the vacuum line closed, trap P is immersed in liquid nitrogen and the H_2SO_4 in the upper reservoir of the CO_2 generation vessel is run into the lower reservoir, generating gaseous CO_2 which is distilled into trap P. Spiral S, immersed in a bath of finely powdered solid CO_2 and isopropyl alcohol, is for the purpose of removing any impurities condensible at the temperature of this bath. Stopcock E is closed, A, B, F and G are opened, trap P is warmed to room temperature and the CO_2 is distilled into flask N, which is still frozen in liquid nitrogen. After distillation is completed, as indicated by the decrease in the reading on vacuum gage V, stopcock A is closed and together with flask N it is removed from the vacuum line. The contents (NaOH and CO_2) of flask N are warmed to room temperature and swirled together. The resulting solution of $NaHC^{14}O_3$ is diluted to the desired concentration, labeled, and stored at $-15^{o}C$ in stoppered bottles.

2. $C^{14}O_2$

For preparation of $C^{14}O_2$, vessel N and stopcock A in Fig. 1 are replaced by a loop L which is cooled in liquid nitrogen, evacuated and then closed off from the vacuum line by stopcock X. Exactly the same procedure may then be followed as in the preparation of $HC^{14}O_3$ (omitting NaOH) until the $C^{14}O_2$ is condensed in loop L, which is then closed by stopcock X. The CO_2 may be kept for a few days at room temperature in the loop, provided the internal pressure of the gas is less than atmospheric pressure.

3. Other Labeled Substrates

Compounds other than CO_2 or bicarbonate may be employed in some

experiments with photosynthesizing plants, provided they are able to penetrate the cell wall (in the case of whole cells). Carbon-fourteen labelled compounds such as formic acid, glucose, and glycollic acid have been used in experiments with plants. These compounds are usually purchased and made up to a solution of the desired strength. In some cases, the least expensive and quickest way to obtain a desired substrate for such experiments may be to allow the plant to photosynthesize with $C^{14}O_2$ of high specific activity for 20 to 60 minutes and then isolate the desired compound from the plant by the techniques of paper chromatography and radioautography which are described in sections II. 1. a., III and IV.

II. Administration of Carbon Fourteen During Photosynthesis

1. Algal Suspensions

a. Single Period-Single Condition

The simplest photosynthesis experiments are those in which the algae are exposed to carbon fourteen for a single short time (usually less than five minutes) under a single set of environmental conditions, and then killed. The suspension of algae, 0.5, 1.0, or 2.0 cc wet packed algae per 100 ml of weak phosphate or ammonium phosphate buffer (B. I. 3.) is allowed to photosynthesize with 1.0% CO_2 in air for ten minutes or more with a moderately bright light (2000 foot-candles) from each side of a vessel, no more than one cm thick. A vessel which holds about 50 ml is often used. The stream of CO_2 is then removed and a solution of C^{14}-labeled sodium bicarbonate is added. For 50 ml of 1.0% algae suspension (wet, packed cell volume/suspension volume) one ml of a solution of 0.026 $\underline{M}$ $NaHCO_3$ containing 400 μcuries of carbon fourteen is commonly used for 60 seconds photosynthesis with C^{14} in which case 20% to 60% of the radiocarbon would be incorporated into organic compounds. The

illumination vessel is stoppered and shaken in the light for the prescribed

period after which the algal suspension is quickly poured into the killing

reagent. The entire operation should be performed in a chemical fume hood or

closed box vented to the atmosphere outside the building.

Many variations of this simple experiment have been performed (Benson, 1950

Bassham, 1950; Buchanan, 1953). The pretreatment may be altered, for example,

so that the algal suspension is swept with a stream of CO_2-free air or nitrogen

for a few seconds prior to the exposure to C^{14}. This has the effect of increasin

the specific activity of the $HC^{14}O_3$ taken up during the exposure to C^{14} and also

increases the rate of fixation during the first few seconds of exposure due to

the build-up of CO_2 acceptor during the flushing period.

b.   Single Period-Multiple Condition

It is sometimes desirable to expose a number of samples of

algal suspension to C^{14} for the same period of time and under conditions which

are identical save for one systematically varied factor. One apparatus

designed for this purpose (Moses, 1958), provides for the continuous shaking

of 6 glass vessels while they are simultaneously illuminated from below.

After harvesting of the cells, washing and resuspension in medium, 0.7-0.8

ml of the cell suspension is pipetted into each of the vessels. These are

constructed from glass tubing of about 3.5 cm internal diameter and 7 cm long,

to which a flat circle of glass is sealed to form a bottom. One ml of liquid

in such a vessel forms a layer 1 mm deep, apart from miniscus effects. A

removeable lid is provided with an outlet and an inlet tube; the latter reaches

nearly to the bottom of the vessel (Fig. 2). During the "pre-adaptation"

period before the experiment, in which the cells are allowed to come to a

steady metabolic state, the cell suspension is shaken in the light for 30 min,
while air containing 1% (v/v) CO_2 is blown over the surface of the cell suspension.
The gas mixture is pre-wetted by bubbling through water to minimize evaporation
from the cell suspension and to serve as a control measure for the rate of
aeration (Fig. 3).

A rectangular glass water bath, fitted with inlet and outlet tubes to
permit a constant stream of cooling water to pass through it, is arranged over
a bank of eight 6-watt fluorescent lights. At the top of the water bath two
rails are fitted along which runs a small carriage consisting essentially of
four wheels fixed to a plate having six holes punched in it, in which six of
the glass incubation vessels are suspended so that the bottoms of the vessels
reach nearly to the bottom of the water bath. The whole carriage is made to
run back and forth along its rails by means of a connecting rod attached to
an eccentric on the drive shaft of a stirrer motor. The carriage oscillates
about 3 cm at approximately 250 cyc/min. The light intensity at the bottom
of the glass vessel is about 2000 foot-candles. While illumination is fairly
constant to each vessel, there is some variation between different positions
on the shaker. For this reason the samples are supplied with radioactive sub-
strate one at a time in a serial manner, each flask being moved to one part-
icular position on the shaker for the period of incubation with labeled sub-
strate.

Before the addition of labeled substrates any other additions, such as
mineral salts, are made, resulting in a total liquid volume of 0.8 ml. After
a definite incubation period with $HC^{14}O_3^-$, 4 ml of absolute ethanol are added
to kill the cells. The shaker is kept in motion while both additions are
made to ensure rapid mixing.

c. Multiple Period-Single Condition

There are several ways in which the investigator may expose algal suspensions to C^{14} for a series of different times under a single condition. Because of the great rate of the metabolic processes involved, it is often necessary to expose a considerable number of samples (10 to 30) for periods ranging from a few seconds to an hour. The system described above might be used in some cases but is cumbersome, particularly for very short exposures. A flow system has been used (Bassham, 1954) with success for periods ranging from one second to one minute. A stream of algal suspension was pumped through one mm diameter transparent tubing under illumination into a vessel containing killing reagent. At various selected points along the tubing a stream of $HC^{14}O_3^-$ solution was injected into the tubing, where it mixed with the algae stream. Good kinetic results were obtained from this experiment, but the method suffers from several limitations, one of which is the fact that it is difficult to expose the algae to C^{14} for more than 60 seconds owing to the high rate of flow required for good mixing and the resistance of the small tubing to flow through longer lengths. Therefore the method described in the next section is recommended for kinetic studies.

d. Multiple Point -- More than One Condition

The most versatile system for photosynthetic tracer studies with algae is the "steady state apparatus." This system permits the removal of many small aliquot samples from a reservoir of algae which are maintained under constant environmental conditions, or subjected to controlled changes in one condition during the course of the experiment.

If conditions are kept constant, and a sufficient time of exposure of the

algae to $C^{14}O_2$ of constant specific radioactivity is permitted (5 to 30 minutes),

then the primary products of CO_2 fixation during photosynthesis become "saturated"

with C^{14}. In other words the specific radioactivity of these compounds becomes

the same as the $C^{14}O_2$. Consequently the C^{14} contained in each compound under

such "steady state" conditions is a measure of the number of atoms of carbon it

contains and from this number and its chemical formula, the concentration of

the compound may be calculated.

The experimental system required for such a study consists essentially

of a closed gas circulating system in which the gas passes through the suspension

of algae that is contained in a vessel from which small samples may be taken

during the course of the experiment, (Bassham, 1956). Such a system is shown

schematically in Fig. 4. In its simplest form, all that is required is the

illumination vessel with a stopcock for the removal of samples, a pump for cir-

culation of gas, a reservoir to contain the $C^{14}O_2$ until it is added to the system,

and connecting tubing. Some provision must be made for admitting gas to the

system when a sample is taken in order to equalize the pressure, and this is

accomplished by an inlet bubbler at some point in the system which thereby

becomes the point at atmospheric pressure. The illumination vessel is main-

tained at slightly above atmospheric pressure to facilitate sample taking.

In addition to this basic system, a number of other devices are useful,

particularly for long experiments (ten minutes or more) and for studies at

steady state photosynthesis. The quantity of C^{14} in the system may be monitored

by allowing the circulating gas to pass through an ionization chamber attached

to a vibrating reed electrometer. The level of carbon dioxide can be observed

with a carbon dioxide analyzer, which detects the carbon dioxide in the gas

mixture by measuring its absorption of infra-red radiation. It is also useful to monitor the level of oxygen by means of an an analyzer which measures the paramagnetism of the circulating gas. Since the total volume of the system can be determined, the rate of C^{14} uptake, CO_2 uptake and oxygen evolution during the experiment can be determined. For long experiments, it may be necessary to increase the size of the system in order to have it contain enough $C^{14}O_2$ to last for the duration of the experiment, yet it may at the same time be desirable to be able to switch to a smaller system by means of stopcocks in order to determine the rate of gas exchange from time to time. The measurement of these rates is greatly facilitated by having the output signals from the gas-measuring instruments continuously recorded on an automatic recorder.

A number of refinements in the illumination vessel are also possible. Of great importance for even relatively simple experiments is the method of taking samples. A simple, two-way stopcock is not satisfactory, since an appreciable volume of algal suspension tends to be held up in the bore of any stopcock large enough to permit rapid taking of samples. A three-way stopcock in which the bore could be flushed with a stream of gas following the taking of a sample has been used, but suffers certain disadvantages of which the most serious is the difficulty in taking samples of uniform size. The most satisfactory arrangement is a small life valve operated by a solenoid which is energized through a time delay relay, the setting of which can be varied to provide the desired sample size. A stream of nitrogen enters the outlet tube at the bottom of the valve and flushes out residual algal suspension. With this arrangement, samples of uniform size may be taken every 2 seconds if desired.

The illumination vessel should be cooled with a water jacket on each side and should be equipped with a thermometer in the algal suspension, since with the bright lights often used in these studies considerable heat is generated in the algal suspension. If incandescent lamps are used as light sources, the light beam should pass through infra-red absorbing glass cooled by water before reaching the water-jacketed illumination vessel.

For long experiments, monitoring of the pH of the algal suspension is useful and may be accomplished by equipping the illumination vessel with a pair of pH electrodes.

In steady state experiments which the author has performed, the total time of a typical experiment was 40 minutes. The CO_2 pressure initially was 2% and did not go below 1% by the end of the experiment. The pH was controlled between 6.5 and 7.0 by the addition of small amounts of NH_4OH injected by needle through a rubber plug in the illumination vessel. A 0.5% suspension of Chlorella was employed in a diluted medium fortified with ammonium phosphate. Small samples (about 1.0 ml) of algal suspension were taken and killed immediately at times beginning at 5 seconds after addition of C^{14} to the system and continuing until forty minutes. The total volume of algal suspension at the start of the experiment was 80 ml and some 20 samples were taken.

In addition to simple time series, other experiments may be performed with this apparatus. For example, the light may be turned off at some time during the course of the exposure, samples being taken before and after this change at frequent intervals. Other environmental conditions such as CO_2 pressure may be altered, or chemicals such as specific enzymatic inhibitors may be injected. Using the method of calculation described at the beginning of

this section, it is possible to follow the changes in concentration of the metabolites brought about by the environmental change.

e. Substrates Other Than CO_2 and HCO_3^-

Solutions of carbon fourteen labeled substrates may be substituted for HCO_3^- solutions in any of the above procedures, provided the substrate is a substance capable of penetrating the cell wall. However, subsequent analysis by paper chromatography is often improved in this case by removing the excess substrate solution by rapid filtration prior to killing and extracting the algae. This rapid filtration may be accomplished by running the algae suspension onto a sintered glass funnel with a bed of diatomaceous earth and sucking the solution through the filter bed with a vacuum as indicated in Fig. 5. When the solution has just been sucked from the algae (about five seconds are required) a stopcock is turned, connecting the funnel with a different suction flask than the one into which the solution of labeled substrate ran, and hot 80% methanol-water (v/v) is poured over the algae, killing and extracting in one operation. The residue of filter pad and algae is further extracted with 20% methanol in water and the extracts are combined and analyzed as described in C. IV.

2. Higher Plants

a. Short exposures

One method for exposing the leaves of a higher plant to $C^{14}O_2$ for a short time (less than 2 seconds) is illustrated by the following procedure.

In a typical experiment (Bassham, 1954) a single excised trifoliate leaf from a soybean plant (var. Hawkeye) is placed in a circular flat illumination

chamber with a detachable face. The chamber is equipped with two tubes, the lower one leading through a two-way stopcock to a loop containing $C^{14}O_2$ (Fig. 6). A loosely tied thread (not shown in Fig. 6) leads from the leaf stem under the detachable face gasket, thence through a boiling ethanol bath and a glass tube to a weight. The illumination chamber is partially evacuated, both stopcocks are closed, and the clamps removed from the chamber, the detachable face remaining in position through atmospheric pressure. With the opening of the upper stopcock, the $C^{14}O_2$ is swept into the chamber by atmospheric pressure, the detachable face falls off and the leaf is pulled into boiling ethanol. An estimated exposure time of 0.4 second is obtained. The radioactive products are extracted and analyzed in the usual way. In other experiments, longer exposure times are obtained by holding the detachable face in position. The sugar phosphates obtained from the soybean extract are especially useful for degradation studies, since highly non-uniform labeling resulted from these short exposures to $C^{14}O_2$.

Another technique for obtaining very short exposure of an excised leaf to $C^{14}O_2$ has been reported by Doman, 1957. This method consists essentially of transferring a leaf from a chamber containing $C^{12}O_2$ to one containing $C^{14}O_2$ and then into a killing reagent by passing the leaf through a reservoir of mercury, over which the chambers are inverted. Through the use of this method, exposures of the leaf to periods less than a second were obtained.

b. Long exposures

Exposures of excised leaves to $C^{14}O_2$ for periods up to five minutes may be obtained without severe loss of photosynthetic activity even in bright light provided the stem of the leaf is immersed in water and the heat is

removed from the incident light beam by suitable filters. The CO_2 supply must, of course, be adequate to sustain photosynthesis for this period and it is sometimes desirable to circulate the gas to which the leaf is exposed through a cooling coil by means of a gas pump or fan.

Long exposures of higher plants to $C^{14}O_2$ (5 minutes to several days) are usually for the purpose of studying the pathway of reduced carbon into more advanced stages of plant synthesis, beyond the reduction of carbon dioxide to early photosynthetic intermediates. In such experiments, it is usually advisable to use an intact plant as the biological material and to enclose the entire plant in a gas tight chamber in which temperature, humidity and light are maintained at desired levels by suitable control devices. The conditions and degree of control vary so widely, according to the plant and the nature of the experiment that no general method can be given. It is often useful in such experiments to include a fan or gas circulating pump and C^{14} and/or CO_2 measuring devices such as those described under part C. II. 1. d.

c. Chloroplasts

Many studies have been carried out using bicarbonate labeled with carbon fourteen and chloroplasts or chloroplast fragments (Allen, 1957). In general these experiments have tended to verify the results obtained with whole cells, as well as to show that most of the reactions seen with whole cells do in fact take place in the chloroplast. The conditions necessary to obtain the most nearly complete duplication of the carbon fixing activity of whole cells appear to be those which are here described briefly (Holm-Hansen, 1959).

The apparatus described under II. 1. b. is used. The temperature at

which the experiments are carried out is about $18^{\circ}C$; the light intensity is 2,000 foot-candles. Appropriate volumes of the preparations of chloroplasts and sap are added, singly and in combination, to each reaction vessel. The total volume in each case amounts to 1.0 ml. Furthermore, except where otherwise noted, 0.05 ml of a cofactor solution is added to each flask. This solution contains, in a 0.05 ml aliquot portion, the following materials (values are in μmoles): $MgSO_4$, 4; $MnCl_2$, 1.6; ADP, 1; TPN, 0.02; DPN, 0.2; TPP, 1.4; and ascorbic acid, 2.5. Some reaction vessels contain other substrates. These additions are generally limited to 0.1 ml or less. The reaction is started by the addition of 0.1 ml of $NaHC^{14}O_3$ (0.0438 $\underline{M}$, 1 mc/ml). The pH of all solutions (cofactors and substrates) is adjusted to 7.2 just prior to use. Incubation is generally carried out for 30 min. The reaction is stopped by the addition to each flask of 4 ml of absolute alcohol at room temperature; the final concentration of alcohol was thus 80%.

III. Killing and Extraction

The killing reagent and temperature should be such that the plant material is penetrated and all enzymes are completely deactivated within one second or less, thus bringing to a halt the many biochemical reactions of the cell. Moreover, the conditions should not alter the chemical composition of the labeled biochemical intermediates which are the subject of the investigation. Finally, it is desirable that the killing reagent also may act as extraction solvent without producing "sticky" insoluble materials which absorb the radioactive compounds and thus prevent their extraction. These requirements exclude strongly acidic or alkaline extracting reagents and boiling water. The most satisfactory killing reagents for suspensions of algae

have been found to be methanol and ethanol, at room temperature (25°C) or at their boiling points, depending on the relative importance of quick killing versus gentle treatment of intermediate compounds. In any event, the following procedure appears to stop all the enzymatic steps of the carbon reduction cycle in photosynthesis in about one second, and there is at present no con- clusive evidence that any of the intermediates which have been identified by chromatography are altered during the killing and extraction with the exception of 1,5 diphospho-2-carboxy-3 ketopentitol. (However, some unstable compounds not yet isolated may be affected.)

After addition of radiocarbon in the form of $C^{14}O_2$ or $HC^{14}O_3^-$, and a period of photosynthesis, the algal suspension is run into an amount of methanol at 25°C which will give a mixture containing 80% methanol-20% water. The mixture is then allowed to cool and after 30 minutes the insoluble materials are removed by centrifugation. The supernatant solution is decanted and the solid residue is re-extracted with 20% methanol-80% water at 60°C for 10 minutes and the mixture is again centrifuged. The extracts are then combined and concentrated with a water vacuum and heating not in excess of 35°C to a volume of 2 or 3 ml. This material is carefully transferred with alcohol-water rinsing to a conical centrifuge tube in which it is reduced in volume to about 1 ml by evaporation with a nitrogen stream. The resulting thick sus-pension is extracted several times by mixing with petroleum ether. The layers are separated each time by centrifugation and the petroleum ether layer dis-carded or saved for investigation of the lipid components. The aqueous layer is then ready for analysis by paper chromatography. (This petroleum ether extraction is often omitted when the best chromatograms are not required.)

During the above procedures, determination is made of the amount of

radiocarbon in the various suspensions and extracts. This is done by taking

a small aliquot from each and spreading uniformly and drying on an aluminum

disc. The radioactivity of the disc is then determined by counting with a

Geiger-Müller tube equipped with a thin window (Mylar) less than 1 mg/cm^2 in

mass.

Leaves of Higher Plants

In experiments with leaves of higher plants, the leaf is taken from a

chamber, after exposure to $C^{14}O_2$, and plunged immediately into a boiling

solution of 80% methanol-20% water. The leaves are broken mechanically and

allowed to extract for 30 minutes. In an alternative procedure, the leaves,

after exposure to $C^{14}O_2$, are plunged into liquid nitrogen and ground with a

mortar and pestle to a fine powder which is then sprinkled into boiling methanol-

water solution. This method is recommended where the leaf is thick or for

some other reason difficult to extract. In either method, the resulting

leaf-alcohol-water mixture is treated in the same way as that described for the

algae mixtures, except that a small amount of phosphate buffer may be added to

the leaf extract to improve the chromatography.

IV. Analysis by Paper Chromatography and Radioautography

The most useful method of analyzing the carbon fourteen-labeled

compounds formed by the plant has been by means of two dimensional paper

chromatography and radioautography.

In order to achieve good chromatographic separation of intermediary

metabolites it is important that the amount of material to be analyzed be

kept small enough to avoid overloading. For this reason the extract from not

more than 20 mg (wet weight) of plant material should be applied to a single two-dimensional chromatogram. Moreover, this material should include not more than 300 μg of inorganic salts.

For most purposes, a sheet of Whatman No. 4 filter paper, about 46 to 57 cm is used. This paper is previously washed by soaking in 0.5% oxalic acid solution for 2 hours and then rinsing with distilled water 10 times, after which it is dried.

A measured quantity of extract is applied from a dropper or micropipette to an area of the paper about 8 by 20 mm at a distance of 60 mm from each edge of the paper and with the long axis of the area of application (origin) parallel to the short axis of the paper. As the extract is applied to the paper, drying is facilitated by a stream of air which may be warmed, provided the area of application (origin) is never allowed to become completely dry. When the last of the extract has been applied to the paper, the air stream should be stopped and the origin left damp. These precautions are required to reduce the possibility of "sticking" of the compounds to the origin during the subsequent chromatography due to formation of complexes between the compounds and the paper. The paper should then be placed in the chromatography box or other humidified place to prevent further drying.

For developing the chromatogram, the shorter edge of the paper, next to the origin, is folded 2 cm from the edge. The edges of two such papers are placed in a glass or stainless steel chromatography trough with a rod weight on top to hold them in place. The two papers hang from opposite sides of the trough over horizontal bars (to prevent siphoning) and with the origins below the point of contact of the bar with the paper. The entire assembly is placed

with the trough horizontal inside a vapor-tight box. Wooden or well insulated
boxes with double pane windows are recommended unless a well thermostated
room is available. The combined effect of a chromatography room thermostated
to a 3-degree range (22-25°C) and wooden boxes is satisfactory for maintenance
of constant temperature during development of the chromatogram. In practice,
it has been found useful to develop six to ten chromatograms in a single box,
as the space inside the box then becomes quickly saturated with the solvent
vapor.

After addition of 90-110 cc of solvent to each trough the box is closed
and the chromatograms allowed to develop in the longer dimension of the paper.
The solvent used in this first dimension is a neutral solvent made up of 72%
freshly distilled phenol and 28% distilled water by weight.

After 10-12 hours the solvent has reached the end of the paper and the
box is opened and the papers placed in a drying cabinet where they are allowed
to dry at room temperature. The drying cabinet should be blower-ventilated to
the outside atmosphere. The chromatography room should contain equipment to
protect people handling the wet chromatograms from possible deleterious physio-
logical effects of the solvent vapors. One solution to this problem is a
helmet covering eyes, nose, and mouth and supplied with a stream of fresh air.
Another method of protecting the worker is to place the entire chromatography
box and drier under a fume hood with a strong rate of air removal. A third
method is to vent the chromatography box directly to an exhaust fan which is
used to dry the papers in the box after they have been developed.

After drying, the papers are placed with their long edges (adjacent to
the origin) in troughs inside a box used only for the second solvent and about

90 cc of the solvent added. The second solvent, in this case, is made up immediately before using, by mixing equal parts of a solution of n-butanol (1,246 ml) + water (84 ml) and a solution of propionic acid (620 ml) + water (790 ml). The box is closed and the paper developed to the edge with this solvent (6-10 hours). The paper is removed and dried.

It should be noted that the above conditions were chosen as the best ones for separating from plant extracts groups of compounds having widely different solubilities and occurring in greatly varying concentrations. There are better systems for each specific group of compounds (i.e., amino acid, sugar phosphates, carboxylic acid, etc.)(Block, 1958). Moreover, if one wishes to obtain the best separation possible of phosphate esters with the above systems, one should allow the solvent to drip off the paper until two or three times as much movement of the compounds of interest has been obtained as can be obtained by allowing the solvent to travel only to the edge of the paper. The correct amount of time of development for this purpose may be followed by placing a small quantity of suitable mixture of colored indicators at the origin before development and observing their distance of travel.

After chromatography is complete, the papers are each stamped at the corners with radioactive ink and placed in contact in the dark with a sheet of single emulsion x-ray film, to determine the positions of the radioactive compounds. About 10^{-2} µg of C^{14} activity distributed over a 6 cm^2 area of the paper (an average-sized "spot") is sufficient to cause visible darkening of the film in 24 hours. This amount of radiocarbon emits 22,000 beta particles per minute, of which about 7,000 per minute escape from the paper, or 3,500 per minute on each side. With two weeks' exposure, 1/20 of this amount

can barely be detected. This is nearly the limit of sensitivity of detection by this method, since the darkening of the film by this number of beta particles is just discernible above the darkening due to cosmic radiation.

After development of the x-ray film, the positions of the radioactive compounds on the paper are indicated by darkened areas, or "spots," on the film. The radioactive ink and the corresponding darkening of the film permit exact coincidence between film and paper to be made any time after development. A typical radioautograph from a photosynthesis experiment is shown in Fig. 7.

In some cases, two or more compounds may overlap in position, even after chromatography, and it then becomes necessary to elute them from the paper and separate them by a second chromatography. This may be accomplished by choosing a different set of solvents or by first altering the compounds chemically and then rechromatographing the mixture. Thus, in a typical experiment, the sugar monophosphate area, which is of considerable importance in the photosynthetic carbon reduction cycle, is eluted, the compounds incubated at $37^{\circ}C$ with a phosphatase enzyme, and the free sugars rechromatographed on Whatman No. 1 filter paper with the two solvents described above.

There are available a large number of spray tests for identification of classes of compounds on paper chromatograms (Block, 1958). However, the amounts of material found on chromatograms of photosynthetic products are often below the limits of detection by these methods. Preliminary identification must usually be made by other methods. The position of the compound on the chromatogram provides two types of information. Since the stationary liquid phase is aqueous and the mobile phase organic, compounds moving short

distances in both solvents must have strong hydrophilic properties while those which move large distances are more lipophilic in nature.

The two solvent systems have about the same power to carry neutral substances; consequently, neutral compounds such as sugars and neutral amino acids move about as far in one direction as in the other and will be found somewhere along or near to a diagonal line drawn from the origin to the opposite corner of the chromatogram. However, since the first solvent is neutral and the second one acidic, there is a tendency for acidic substances to have their ionization repressed in the second dimension and consequently to move faster in the second direction than in the first. For that reason, acidic substances are usually found beyond the diagonal line in the second direction and basic substances short of it, these deviations from the diagonal being a rough measure of the compound's acidity or basicity.

The subsequent clues as to an unknown compound's identity usually come from eluting the substance from the paper, performing some chemical or enzymatic treatment, rechromatographing, and observing change in position, fragmentation into several compounds, or loss of radioactivity. For example, an unknown compound, suspected of being a sugar phosphate because of its position near the origin is eluted, phosphatased, and rechromatographed, along with added, unlabeled known sugars (about 50 μg of each). After development of the radioautograph, the paper is sprayed with a chemical solution which produces a color reaction wherever there is a carrier sugar compound. From the position of the unknown compound amongst the known compounds it is suspected of being a pentose. More of the original compound is phosphatased and run with various pentoses until it is found to co-chromatograph exactly with one

of them. In this case, both the position of the radioactive spot and the details of its shape (as revealed by the radioautograph) coincide exactly with the color produced from the carrier by the spray test. If further confirmation of the identity is desired, a freshly prepared unknown compound in the form of the free sugar is mixed with the authentic compound in solution and a derivative is prepared and recrystallized to constant specific activity or rechromatographed.

By such methods, as well as by other confirmatory chemical methods, a large number of compounds, which are labeled as the result of carbon assimilation and reduction by plants during photosynthesis and respiration with $C^{14}O_2$ present, have been identified, (Benson, 1951a, 1957a, 1957b). Many of these compounds are shown in Fig. 8.

V. Degradation of Products

Considerable information regarding the pathway of carbon reduction during photosynthesis has been derived from degradation of photosynthetic intermediates, (Bassham, 1954). These degradations usually have been performed on material isolated and purified by paper chromatography, with as much unlabeled carrier compound added as is necessary for carrying out the chemical reactions of the degradation procedure.

VI. Results and Interpretation

1. The carbon reduction cycle

The interpretation of results obtained by application of the techniques which have been described in the preceding sections has led to a number of conclusions of which the best established probably are contained in

the present concept of the pathway of carbon dioxide incorporation and reduction via the carbon reduction cycle. This cycle is shown in Fig. 9. The results which led to its elucidation may be summarized by the following statements (Bassham, in press).

The intermediate compounds of the cycle were identified as the substances which are labeled during short exposures of the plant (less than three minutes) to $C^{14}O_2$ during photosynthesis.

Kinetic studies indicated that 3-phosphoglyceric acid (PGA) was the first of the compounds stable to these methods of killing and analysis to be labeled with carbon fourteen.

Degradation studies of intermediates obtained from such short exposures of the plant to $C^{14}O_2$ indicated triose and hexose phosphates are formed from PGA by a route similar to or identical with that shown in the cycle. Other degradation studies gave results consistent with the pathway of conversion of triose and hexose phosphates through heptose phosphates to ribulose diphosphates shown in the cycle.

Multiple point -- more than one condition experiments (C. II. 1. d.) showed that when CO_2 pressure is suddenly decreased during photosynthesis, the concentration of ribulose diphosphate (calculated as described in C. II. 1. d.) suddenly increases while the concentration of PGA decreases (Wilson, 1955). This indicated the presence of a reaction of carbon dioxide with RuDP to produce PGA. In other experiments in which the concentration of carbon dioxide was kept constant but the light was suddenly stopped, the concentration of PGA rose suddenly while that of ribulose diphosphate rapidly decreased to zero (Bassham, 1956). This result (Fig. 10) indicated that compounds carrying

chemical energy derived from light are required for the utilization of PGA and for the formation of RuDP. This observation is consistent with the proposed reduction of PGA to triose phosphate via enzymatic reactions requiring reduced triphosphopyridine nucleotide and adenosine triphosphate (derived from photochemical reactions) and the formation of ribulose diphosphate from ribulose monophosphate via an enzymatic reaction requiring adenosine tri-phosphate.

The suspected intermediate compound, a 1,5 diphospho-2-carboxy-3-keto-pentitol which is too unstable to be isolated by the usual methods of killing and analysis was isolated in very small quantities through the use of special low temperature killing techniques and chromatography (Moses, in press).

Kinetic experiments under steady state conditions (C. II. 1. d.) have shown that the greater part (80-95%) of carbon dioxide taken up during photo-synthesis as measured externally is incorporated via the carbon reduction cycle.

2. Carboxylation via C_3- C_1 addition

A second major route for the incorporation of carbon dioxide during photosynthesis is via the carboxylation of a three carbon compound. The exact mechanism of this reaction is not known but the pathway shown in Fig. 11 seems the most likely.

It can be seen that since this pathway begins with PGA, formed through the carbon reduction cycle, the introduction of carbon dioxide in the C_1 - C_3 addition leads to four carbon compounds labeled in the two carboxy carbon atoms in short experiments, since in such experiments, the carboxyl carbon of the C_3 compound is already labeled.

This 1,4 distribution of label has been verified by chemical degradation

of malic acid obtained from such experiments. The carbon introduced by C_1 to C_3 addition probably is only 5-10% of the total carbon reduced, but this results in 20-40% of all the carbon reduced passing through the four carbon products of this reaction. Since PGA and consequently the phosphoenolpyruvic acid are almost completely labeled with C^{14} after one minute's exposure of the plant to $C^{14}O_2$, malic acid and aspartic acid are among the most prominently labeled compounds in experiments of this duration.

3. Formation of Primary Metabolites for Further Synthesis

While no sharp distinction can be made between photosynthetic reduction of carbon dioxide to "primary metabolites" and further reactions of these metabolites in the synthesis of the various plant constituents, there are certain compounds that appear to be formed at very great rates during photosynthesis and all are labeled to a considerable extent during exposures of the plant to $C^{14}O_2$ of 1 to 5 minutes duration. These substances undoubtedly serve as the building blocks from which the plant constructs the other compounds of which it is made. In algae these substances include, in addition to the compounds of the carbon reduction cycle itself, several amino acids and carboxyllic acids, polysaccharides and precursors of polysaccharides (uridine diphosphoglucose). Some of these substances are listed in Table I, together with very approximate estimates of the rates of flow of carbon through these compounds in Chlorella photosynthesizing in 1% CO_2 in dilute nutrient solution at nearly saturating light intensity and $25°C$. The amino acids listed are known to serve as precursors for other amino acids and together with these other amino acids, as precursors for protein synthesis. Glutamic and aspartic acids may also serve as precursors in nucleic acid synthesis.

The exact pathway to fatty acid synthesis is not known, but the necesssary precursor, acetyl coenzyme A could arise either by oxidation of pyruvic acid, or more likely, in the reductive environment of the photosynthetic cell, by splitting of malic acid into two carbon units, acetyl Co a and glyoxylic acid. The glyoxylic acid may be used in the synthesis of glycine and glycolic acid or further reduced to acetic acid (in the form of acetyl Co A).

The carbohydrates, including polysaccharides are no doubt derived from intermediates of the carbon reduction cycle (sugar phosphates) and from uridinediphosphoglucose, which in turn is formed from the sugar phosphates. Furthermore, the glycerol and galactose (Benson, 1958a) moities of fats are probably derived from the sugar phosphates of the carbon reduction cycle by well known reactions.

Thus, the early stages of synthesis of all the important plant constituents may be traced to labeled compounds formed during short periods of exposure of photosynthesizing plants to $C^{14}O_2$. It is not important to attempt to delineate which of these reactions are "photosynthetic" and which are "non-photosynthetic" since all are probably accelerated in the light, yet all are essentially "dark" reactions in that they can occur in the dark if provided with coenzymes derived from the light. The light is the ultimate source of chemical energy which the plant cell employs in the form of coenzymes to carry out reductions and other reactions requiring energy. The coenzymes triphosphopyridine nucleotide (reduced form) and adenosine triphosphate are the principal coenzymes presently known to be used in this role.

4. Experiments with Isolated Chloroplasts

The radioautograph obtained from experiments with C^{14} and chloro-

plasts, with and without the addition of sap (see section B. III. 1.) show
that when the sap from spinach cells is added to the chloroplasts, a pattern
of carbon fixation very similar to that found with intact cells is obtained.
The presence of the sap produces a very great increase in the rate of CO_2 fix-
ation over that obtained with chloroplasts alone, indicating that either the
sap contains enzymes lost from the chloroplasts during their usual method of
preparation or else that the products of primary carbon fixation on the chloro-
plasts must be removed by further reactions mediated by enzymes of the sap in
order for the primary reactions to take place. In view of the high rate of
conversion of PGA to products other than sugar phosphates of the carbon
reduction cycle observed in the kinetic steady state studies (VI. 3. Table I),
again the latter hypothesis is not unreasonable. The preparation of chloro-
plasts which are capable of fixing and reducing carbon dioxide at a rate equal
to that of chloroplasts _in vivo_ remains an important problem for the future
as does the problem of demonstrating which of the reactions found in the _in vivo_
studies can be performed by isolated chloroplasts without the addition of any
enzymes or cofactors except those demonstrably present in chloroplasts in
intact cells.

D. Experiments with Phosphorous Thirty-Two

    I. Methods of Exposure and Analysis by Chromatography

        1. General Conditions

            The identification of many of the compounds of the carbon reduction
cycle has been aided by studies with photosynthesizing algae and P^{32}-labeled
phosphate. The methods employed in these studies are in general very similar
to those described for studies with C^{14}, and the reader is referred to the

appropriate sections of part C of this discussion for the details. A typical experiment may be described as follows:

Unicellular algae, <u>Chlorella</u> or <u>Scenedesmus</u>, grown and harvested under standard conditions B. I., are suspended to a concentration of 1% (volume wet packed cells/volume of suspension) in distilled water, to which may be added small amounts (to 10^{-5} $\underline{M}$) phosphate buffer, pH 7 and, if desired, other nutrient solutions not containing phosphate. The algal suspension is placed in an illumination vessel (C. II. 1. a.) where the algae are allowed to photosynthesize with a stream of 1% $C^{12}O_2$ in air bubbling through the suspension and an illumination of 2000 to 6000 foot-candles from each side. After five minutes or more of normal photosynthesis, 0.5 millicuries (per 100 ml of algal suspension) of carrier-free KH_2PO_4 solution is added while the stream of 1% CO_2 is continued. After a prescribed period of time (for example one minute or five minutes), the suspension of algae is run onto a filter (Fig. 5) where the medium is rapidly removed and the algae killed and extracted (C. II. 1. e.).

The subsequent analysis by chromatography and radioautography is carried out in the same manner as with C^{14} experiments (C. III. and IV.) except that due regard for the shorter half-life (14 days) and higher energy β particles (1.7 Mev) must be observed. In particular, it is important that paper chromatography in contact with exposing x-ray film be shielded from other x-ray film.

The same techniques of identification as were used for C^{14}-labeled compounds may often be used for P^{32}-labeled compounds. However, many of the substances important in photosynthesis contain phosphorous in the form of phosphate ester groups, so that the label is of course lost when the compound is treated with a phosphatase and rechromatographed. For this reason,

since treatment with phosphatase and rechromatography is one of the most useful

tools of identification, it is sometimes useful to perform double labeling

experiments with P^{32} and C^{14} so that the portion of the phosphate ester

remaining after treatment with phosphatase may still be located with radio-

autography.

Such a double-labeling experiment may serve another purpose of identifi-

cation provided that the algae are left in contact with C^{14} and P^{32} of con-

stant specific activity for a sufficiently long time to saturate the early

intermediates of carbon fixation with both isotopes, (C. II. l. d.) In this

case, from the known specific activities of the two isotopes and from the

experimentally determined number of counts in each isotope, the ratio of

phosphorous to carbon atoms in an unknown molecule may be determined. This

method was used as corroborating evidence in the identification of ribulose

diphosphate (Benson, 1951b). The algae, <u>Scenedesmus</u>, were left in contact

with P^{32}-labeled phosphate for 12 hours and then allowed to photosynthesize

with $C^{14}O_2$. The P^{32} radioactivity of the resulting chromatograph may be

determined by shielding out the weaker C^{14} radioactivity and multiplying

the count by an independently determined shielding factor, while the radio-

carbon activity may be determined most accurately by waiting for three months

(six P^{32} half lives) for the decay of P^{32} and then counting the remaining

radioactivity.

    2. Specific Experiments and Results

        a. Short Exposures to P^{32}

        The incorporation of P^{32}-labeled phosphate into organic

esters has been studied by allowing the radioisotope to be in contact with the

algae only a short time while the algae were photosynthesizing with ordinary CO_2. The radioautographs of P^{32}-labeled products of one minute of such exposure are shown in Fig. 12. It can be seen that the incorporation of P^{32} is accelerated by the light and that the principal products of its incorporation are PGA, sugar monophosphates and diphosphates and phosphonucleotides, (Goodman, 1953, 1955).

b. Changes in Phosphorous-labeled Compounds with Changing Conditions

By allowing algae to photosynthesize for long periods with radiophosphate and then changing some environmental condition (C. II. 1. d.) transient changes in concentrations of these compounds have been studied (Bradley, 1955). In particular, the changes accompanying the transition from light to dark and from dark to light were studied. In general the changes in ribulose diphosphate and in PGA were as would be predicted from the studies with carbon fourteen (VI. 1.) and the proposed carbon reduction cycle. Although adenosine triphosphate (ATP) is one of the cofactors formed in the light, the changes in the concentration of this coenzyme during light-dark transitions are dependent on other conditions as well, since the photosynthetic formation of ATP on turning on the light is to a varying extent offset by an increased utilization of ATP by the carbon reduction cycle. Consequently the light-dark transition may be accompanied by either an increase or decrease, or an increase followed by a decrease in the concentration of ATP.

II. Measurement of Photosynthetic Phosphorylation in Chloroplasts

Arnon (1954b) demonstrated the photosynthetic incorporation of P^{32} into organic phosphate in whole spinach chloroplasts. The P^{32}-labeled organic phosphate was identified as adenosine triphosphate and the process of

its formation in the light called "photosynthetic phosphorylation." The experimental conditions used by Arnon and coworkers were reported as follows:

All operations are carried out in the cold. Spinach leaves are ground in 0.35 $\underline{M}$ NaCl or 0.5 $\underline{M}$ glucose, and whole chloroplasts are separated by centrifugation at 1000 g for 7 minutes. The chloroplasts are washed once and resuspended in NaCl or glucose solution.

The reaction is carried out in Warburg manometer vessels of conventional design. To the main compartment are added 15 μmoles of a mixture of potassium and sodium phosphate, pH 7.2, 10 μmoles of sodium ascorbate, except as otherwise noted, and an acceptor for the newly esterified phosphate, consisting of either 10 μmoles of AMP, or 1 μmole of AMP with 1% glucose and 75 units of hexokinase (Kunitz, 1946). In the side-arm is placed 5 μmoles of phosphate, pH 7.1, containing P^{32}. The vessels are then chilled in ice. A suspension of chloroplasts in 0.5 $\underline{M}$ glucose or 0.35 $\underline{M}$ sodium chloride is pipetted into the main compartment and the volume brought to 3 ml by the addition of cold glucose or sodium chloride solution. Unless otherwise stated, an aliquot of chloroplasts is taken to give 2 mg of chlorophyll per vessel. The vessels are attached to manometers and shaken at 15° in a refrigerated waterbath. The reaction is started by tipping the P^{32} solution in and turning on the light.

The reaction is terminated by turning off the light and adding 0.3 ml of 20% trichloroacetic acid to each vessel. Chloroplast debris is removed by contrifugation, the precipitate washed with 2% trichloroacetic acid, and the supernatant liquid and washings are made up to 5 ml. These operations are carried out in the cold.

"Organic phosphate" is measured by the radioactivity which remains after

the removal of the inorganic phosphate as magnesium ammonium phosphate (Umbreit, 1949). Blank runs show that insignificant amounts of radioactive inorganic phosphate remain in solution after this procedure.

Radioactive phosphorus is estimated by pipetting 1 ml samples into shallow plastic cups, evaporating to dryness under an infrared lamp with the aid of a current of warm air, and counting under a thin-window Geiger counter of conventional design. The total amount of P^{32} added to each vessel is 500,000-1,000,000 c.p.m.

The studies of photosynthetic phosphorylation in chloroplasts have been extended, both by the use of P^{32} and by other means (Allen, 1958). Some of the conclusions may be summarized as follows:

Photosynthetic phosphorylation in chloroplasts proceeds at a rate which could easily supply the number of molecules of ATP required by the postulated carbon reduction cycle (three per CO_2 molecules reduced) at the rates of CO_2 reduction observed in vivo.

Under some conditions there appears to be two types of photosynthetic phosphorylation. In one of these, one molecule of ATP is produced for each molecule of triphosphopyridine nucleotide formed in the light. The other type of photosynthetic phosphorylation is a "cyclic" type in which some of the photosynthetically formed reduced triphosphopyridine nucleotide (TPNH) is concommitantly oxidized to oxidized triphosphopyridine nucleotide (TPN$^+$) by the (OH) fragment (whatever its chemical species may be) from the photochemical splitting of water.

One of the most important problems for the future is the elucidation of the chemical mechanism of these reactions, and it is likely that the appli-

cation of tracer methods, both C^{14} and P^{32} may play an important part in the

solution of this problem.

E. Experiments with Tritium (H^3)

 I. Methods

 Within limitations imposed by the low energy of the β particle

emitted by tritium, the same techniques of administration to suspensions of

algae and analysis by paper chromatography and radioautography as were used

in the case of C^{14} may be employed with tritium. The tracer has been admin-

istered as T_2O in experiments reported so far, but could also be administered

as T_2 gas to hydrogen adapted algae. In the experiment described below (Moses,

1958) very large amounts of radioactivity (1 curie per sample) requiring very

great safety precautions were employed in order that the resulting paper

chromatograms would contain T-labeled compounds with enough energy emitted to

prepare a radioautograph in a reasonable period of time. However, Wilson

(1958) has reported a method for preparing a scintillation autograph of

paper chromatograms obtained from exposures of photosynthesizing algae to

T_2O which permit using smaller amounts (50 mcuries) which could be substi-

tuted for the method of radioautography which has been used for C^{14} and in

Moses' experiments which were carried out under the following conditions:

 The apparatus used for exposure is the same as described in part

C. II. 1. c. and provides for the shaking of six glass vessels while they are

simultaneously illuminated from below. After harvesting of the cells (Chlorella

see part B), washing with distilled water, and resuspending in distilled

water or other medium, 0.7 to 0.8 ml of the cell suspension is pipetted into

each vessel. The cell suspension is shaken in the light for 10 to 30 minutes,

while 1% CO_2 in air is blown over the surface.

Before the addition of labeled substrates any other additions, such as mineral salts, are made, resulting in a total liquid volume of 0.8 ml. At the start of the experimental period, 0.2 ml of ^3H_2O (specific activity, 5 C/ml, prepared by oxidation of tritium gas) are added to the cell suspension, and after a definite incubation period 4 ml of absolute ethanol are added to kill the cells. The shaker is kept in motion while both additions are made to ensure rapid mixing. Unless $NaH^{14}CO_3$ is also used as a substrate, the vessels are kept flushed with the gas mixture during the incubation period.

The suspension of cells in 80% ethanol at the end of the incubation period is allowed to stand at room temperature for 2 hours, and then is centrifuged. The residues are extracted with 2 ml of 20% (v/v) ethanol for 1 hour, and with 2 ml of water overnight. The extracts are pooled and the cell residues discarded. The extracts are evaporated to dryness in vacuo below 40°. The dry residue after evaporation is redissolved in 5 ml of 20% ethanol and again evaporated to dryness; this is repeated twice more, and the residue finally dissolved in less than 1 ml of 20% ethanol, solution being aided by placing one or two small glass beads in the flask and using these to loosen material from the walls. The purpose of the repeated evaporations is to remove tritium present in compounds in exchangeable positions, leaving the isotope present only in nonexchangeable locations. Were this not done it is likely that almost every compound in the cells would have been labeled. All volatile radioactive materials are trapped and properly disposed of in accordance with safety procedures. Owing to the large quantities of radioactivity used, all the operations described above, from the time the

vessel containing labeled substrate is first opened until the final evaporation is completed, are performed within a sealed "glove box." A rapid draught of air is maintained through the box by a suction fan which is vented through the roof of the building, and the vacuum pump used for the evaporations, even though outside the box, is exhausted back into the main vent. The whole system inside the box, in addition to the room air, is very carefully monitored to ensure that no radioactive substances escape from the confines of the box and the venting system.

    II. Results

        While a number of compounds which are found to be labeleled experiments with photosynthesizing algae and $C^{14}O_2$ were also found to be labeled in these exposures of photosynthesizing algae to T_2O, the most striking effect was the appearance of T-labeled glycolic acid which appeared on the radioautographs as by far the most strongly labeled compound. In control experiments in the dark, glycolic acid was only slightly labeled. The role of glycolic acid reduction in photosynthesis is not known. It has been suggested (Tolbert, 1958) that glycolic acid may act as a hydrogen carrier between chloroplasts, in which photosynthetic reduction takes place, and other parts of the cell. The glycolic acid would be formed by the reduction of glyoxylic acid in the chloroplast and would be oxidized outside the chloroplast to glyoxylic acid, which could either be recycled to the chloroplast or be converted to glycine. Glyoxylic acid could arise in the chloroplast by the splitting of malic acid as suggested earlier (C.VI.3.).

F. Experiments with Sulfur Thirty-five

    Few experiments have been reported in which radiosulfur has been used

in the study of photosynthesis. Since S^{35} would probably be administered

in the form of $S^{35}O_4^{-2}$ ion in solution, the experimental procedure for exposing

photosynthesizing algae to this isotope would be similar to that employed

in the administration of P^{32}-labeled phosphate (C.II.1.e. and D.I.). Since

the energy of the emitted β^- particle is similar to that of the β^- particle

from C^{14}, techniques of radioautography and counting of radioactivity which

were employed in the analysis of radiocarbon compounds may also be employed

in studies with S^{35}.

An interesting example of the use of S^{35} in studies of photosynthesizing

algae has been reported by Benson (1958b, c). In these studies, a sulfolipid

was identified as a major constituent in green plants and photosynthetic

organisms. It was first detected in S^{35}-labeled algae where it contains

almost all of the ethanol-soluble sulfur. Radiochemical identification of

the unknown compound using S^{35}-, P^{32}-, and C^{14}-labeled Chlorella has established

its structure as 3'-O-oleyl-1'glyceryl β-D-galactopyranoside-6-sulfate. The

structure of the lipid is based upon radiochromatographic observations of

its derivatives and hydrolysis products. With C^{14}-labeled algae the sulfolipid-C^{14}

was easily isolated. The C^{14} radioactivity of the glycerol, fatty acid,

and sugar sulfate defined the carbon composition of these hydrolysis products.

A comparison with P^{32}-labeled algal lipids established the singularity of

the sulfolipid.

It is apparent that the sulfolipid is actually a sulfate ester of mono-

acylglyceryl galactoside. Phytosynthesis of the sulfolipids, concomitant

with formation of the other plant galactolipids, provides evidence for their

biochemical relationship.

G. Present Status and Future Problems

The carbon reduction cycle (Fig. 9) represents the accumulation of a large amount of information regarding the major metabolic pathways of photosynthesizing plants and a tentative elucidation of some 12-15 important steps in the fixation and reduction of carbon dioxide during photosynthesis. Coupled with the information regarding the formation of the cofactors required to drive the cycle, adenosinetriphosphate and reduced triphosphopuridine-nucleotide, which has resulted primarily from studies with chloroplasts and P^{32} as well as with non-tracer methods, these findings permit a hypothesis to be made for perhaps the majority of enzymatic steps following the primary photochemical conversion of light energy to chemical energy. Even within this body of knowledge, however, there exist a number of important problems and questions. Additional proof of the mechanism of some of the steps in the carbon reduction cycle is needed in order that it may be established without question. This is particularly true of the carboxyllation reaction, where it has not been established with certainty that all the CO_2 incorporated via addition of CO_2 to ribulose diphosphate leads to PGA as the only product. The alternative proposal in this case is that some of the addition product of the carboxyllation reaction may be reduced directly to the sugar phosphate level without passing through PGA as an intermediate. Aside from the carbon reduction cycle, the other pathways of carbon reduction are even less clearly known. For example, the mechanism of the addition of CO_2 to a C_3 compound is not known as to the identity of the C_3 acceptor or the C_4 product. The route by which glutamic acid is formed is not known with certainty. The fate of malic acid and fumaric acid, once formed, is not known.

The further use of radiocarbon and radiophosphorous is indicated as a means of solving these and other problems. One principal line of investigation may be to continue the search for unstable intermediates and intermediates occuring at very small concentrations under conditions used thus far. New killing techniques and methods of analysis (low temperature, rapid analysis through centrifugal chromatography, etc.) are suggested as methods for discovering unstable intermediates. A different selection of experimental conditions (nutrient, light intensity, temperature, CO_2 tension, chemical inhibitors) may lead to the accumulation of intermediates previously occuring in very small concentrations.

Another promising line of investigation appears to be in the application of quantitative methods to steady state studies. It should be possible to measure and calculate the rate of flow of carbon through each of the intermediate compounds of the various pathways by studying their rate of labeling and steady state concentrations simultaneously. Thus it may be possible to resolve the question of the carboxyllation reaction by measuring the rate of flow of carbon through PGA and comparing this rate with the overall rate of carbon uptake by the photosynthesizing plant.

The role of glycolic acid requires further experiments both with C^{14} and with tritium. It is also possible that tritium may prove useful in investigations of the formation and utilization of the pyridinenucleotides.

The mechanism of formation of adenosine triphosphate from adenosine diphosphate is being actively studied in many laboratories using P^{32} and other means and it is to be expected that the details of photosynthetic phosphorylation will be understood in a few years.

The use of S^{35}, rather limited to date in photosynthetic studies, should lead in time to increased knowledge of the phytosynthetic pathways by which lipids and proteins are made.

Finally, it may be mentioned that other radioactive isotopes, not yet employed, may prove valuable in studying some phases of photosynthesis. For example, the roles of certain trace elements, known only to be required for plant growth at present, might be determined through the use of suitable radioisotopes.
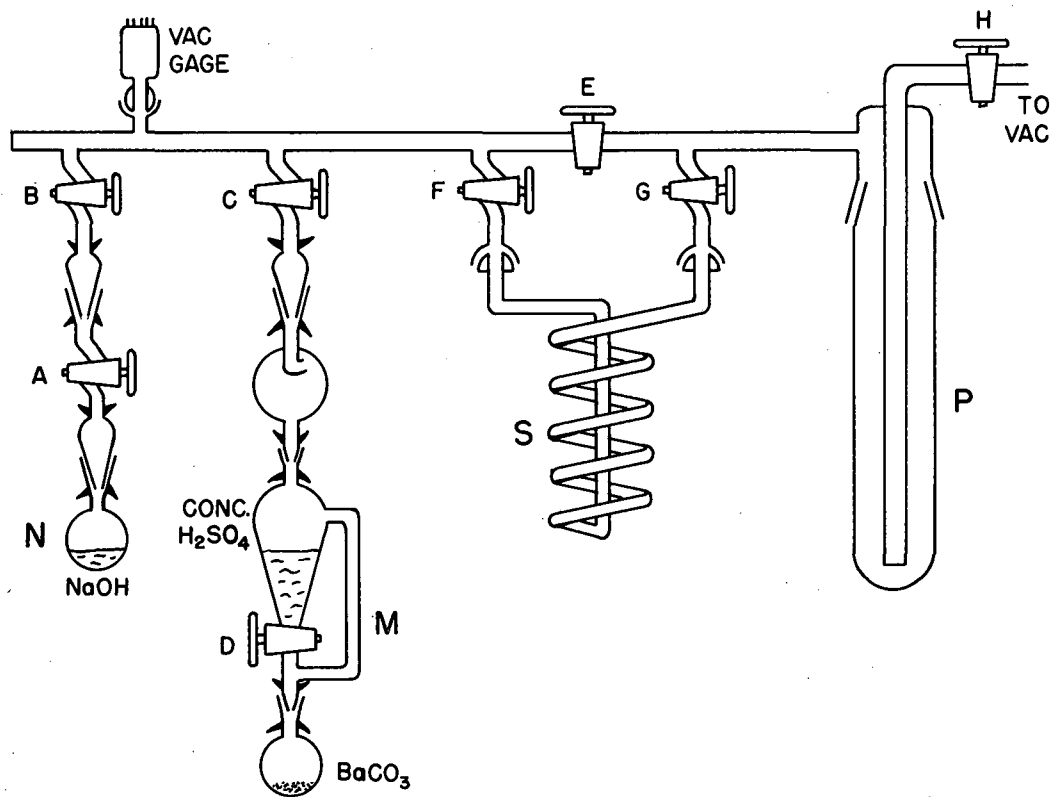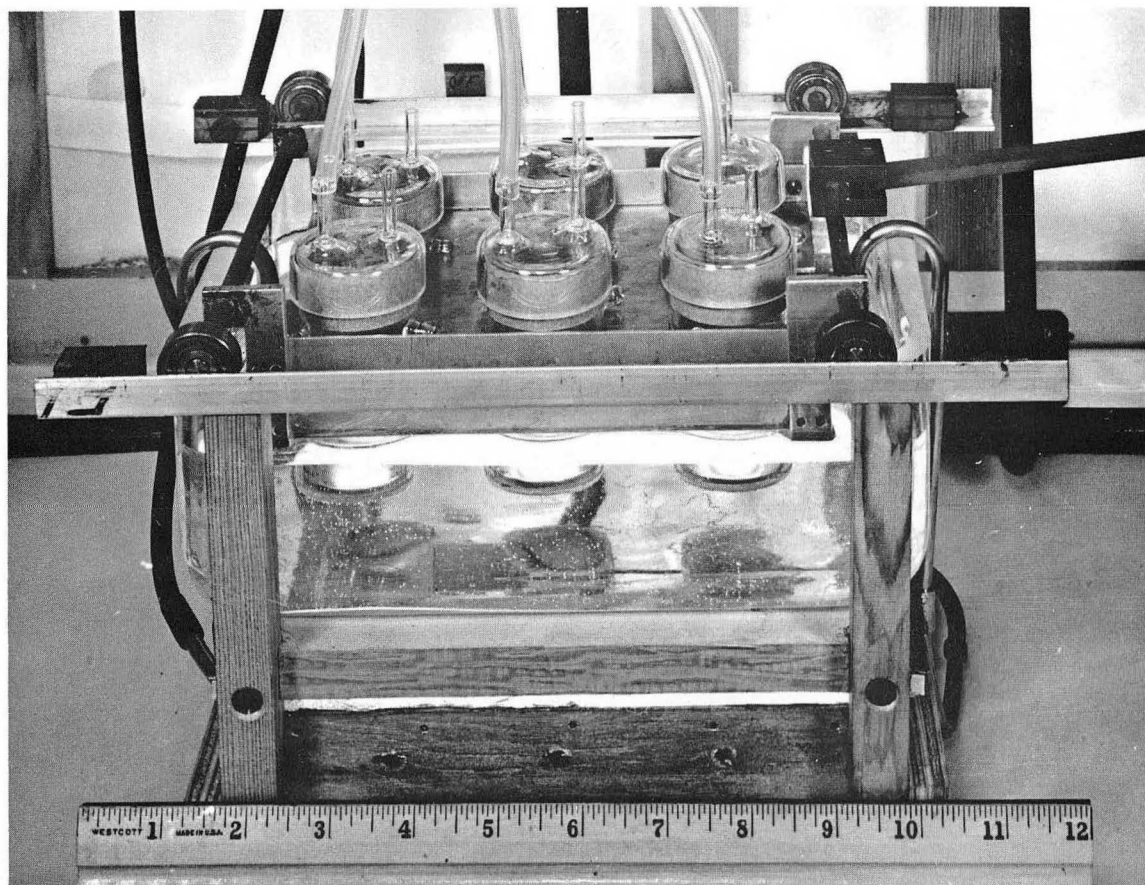
Fig. 1. An apparatus for preparing $HC^{14}O_3^-$ and $C^{14}O_2$.
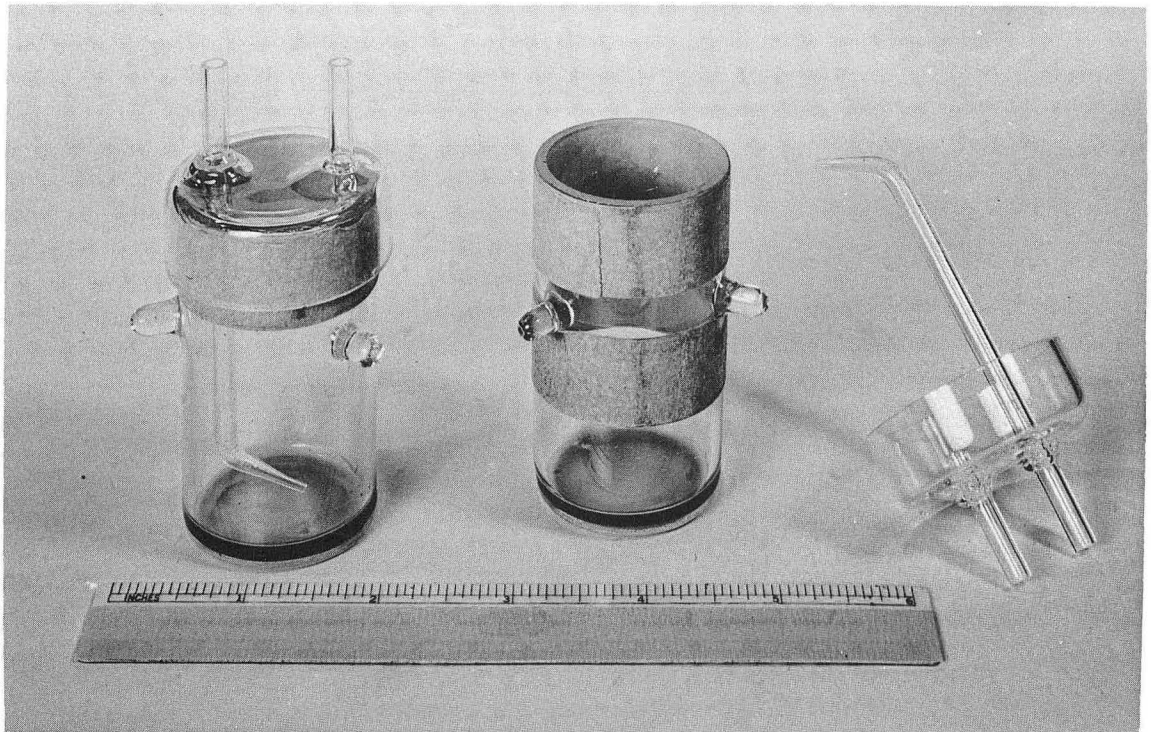
ZN-1867

Fig. 2. Illumination vessel for exposing algal suspensions to
$HC^{14}O_3^-$ under several conditions.

ZN-2147

Fig. 3. Apparatus for exposing algal suspensions to $HC^{14}O_3^-$ under several conditions.

C^{14}

$C^{14}O_2$
SPIRAL

CO_2

O_2

ALGAE CELL

P
PUMP
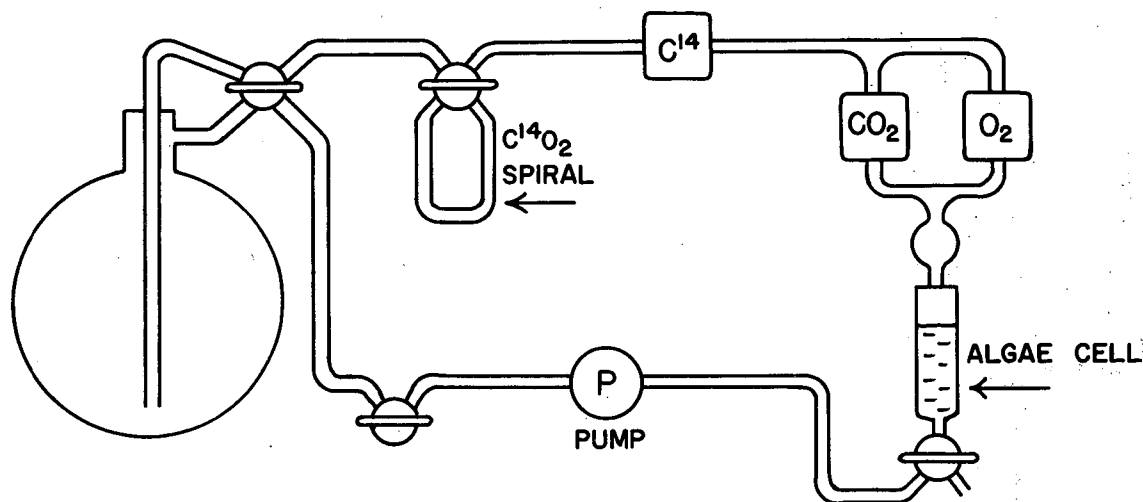
MU-14886

Fig. 4. Apparatus for steady state photosynthesis studies.

C^{14} indicates ionization chamber

CO_2 and O_2 represent carbon dioxide and oxygen analyzers respectively

ZN-415

Fig. 5. Apparatus for exposing algae to C^{14}-labeled organic substrates or to P^{32}-labeled phosphate.
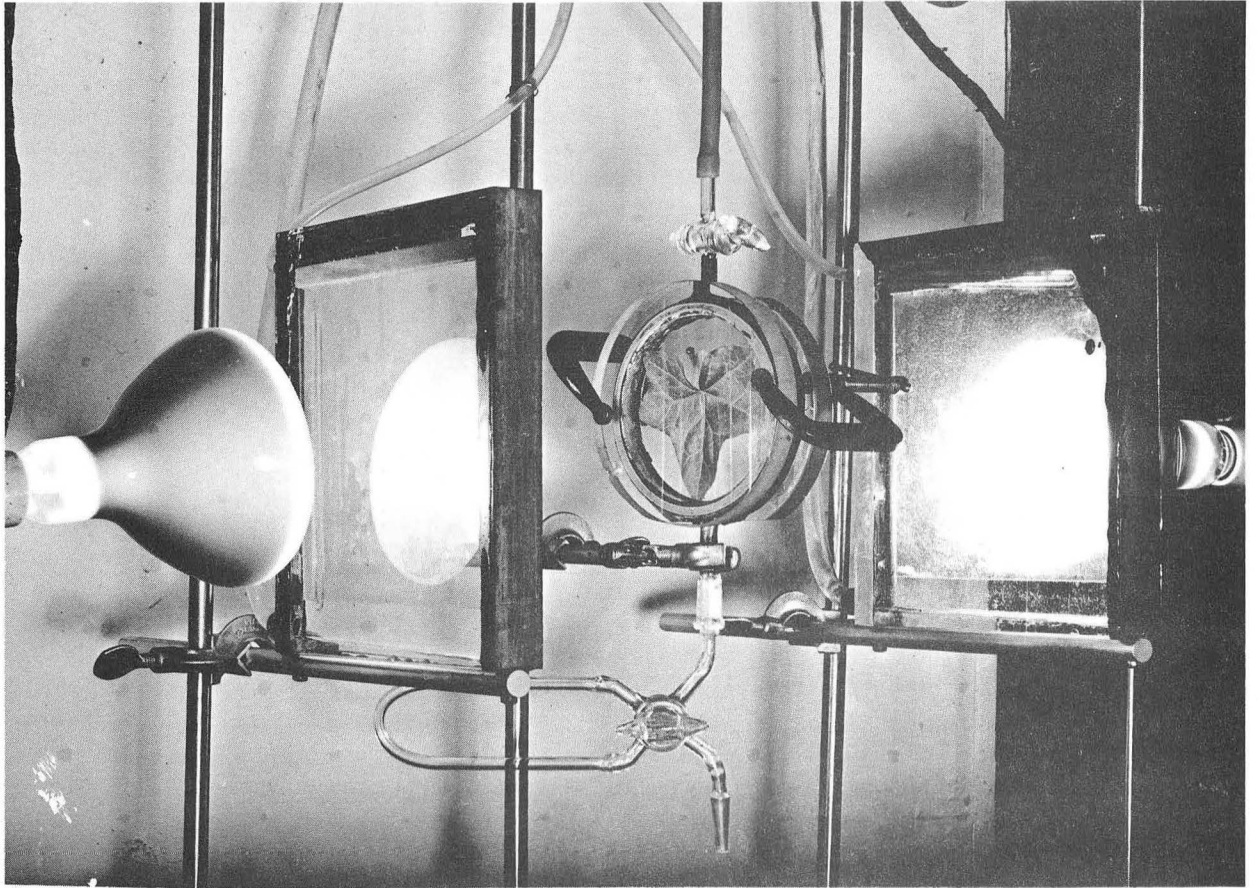
ZN-952

Fig. 6. Apparatus for exposing leaf to $C^{14}O_2$.
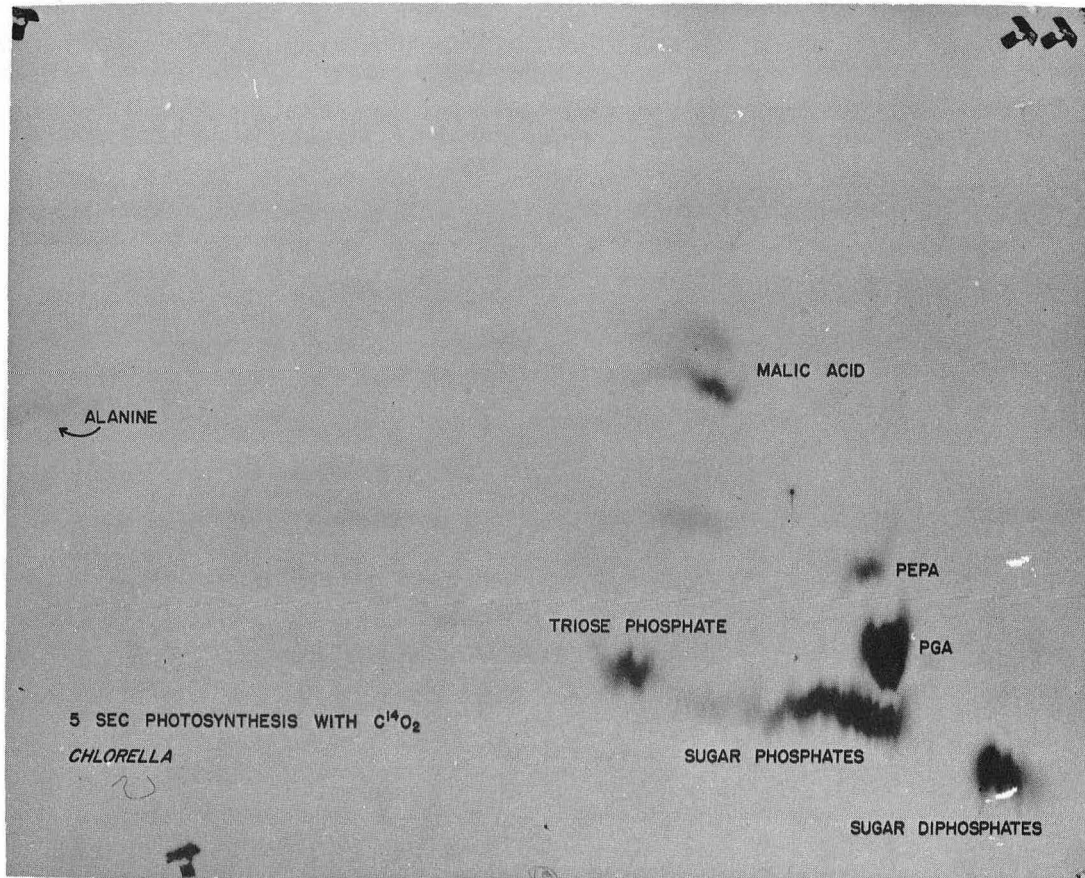
ZN-1968

Fig. 7. Radioautograph of chromatograms from 5 second exposure
of photosynthesizing algae to $C^{14}O_2$.
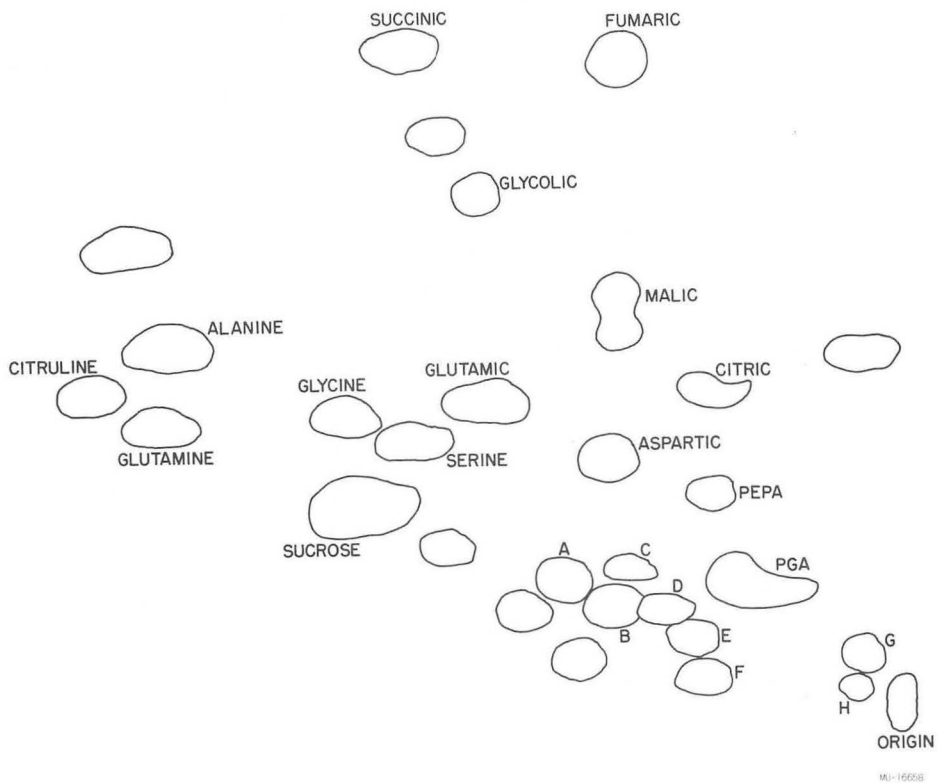
Fig. 8. Chromatographic map of some compounds commonly labeled with C^{14} in photosynthesis experiments with $C^{14}O_2$.

PEPA = phosphoenolpyruvic acid
A = dihydroxyacetone phosphate
B = ribose-5-phosphate and ribulose-5-phosphate
C = glutathione
D = fructose-6-phosphate and mannose-6-phosphate
E = glucose-6-phosphate and sedoheptulose-7phosphate
F = uridine diphosphoglucose
G = ribulose disphosphate and fructose diphosphate
H = 1, 5 diphospho-2-carboxy-3-keto-pentitol

Unlabeled spots are unidentified
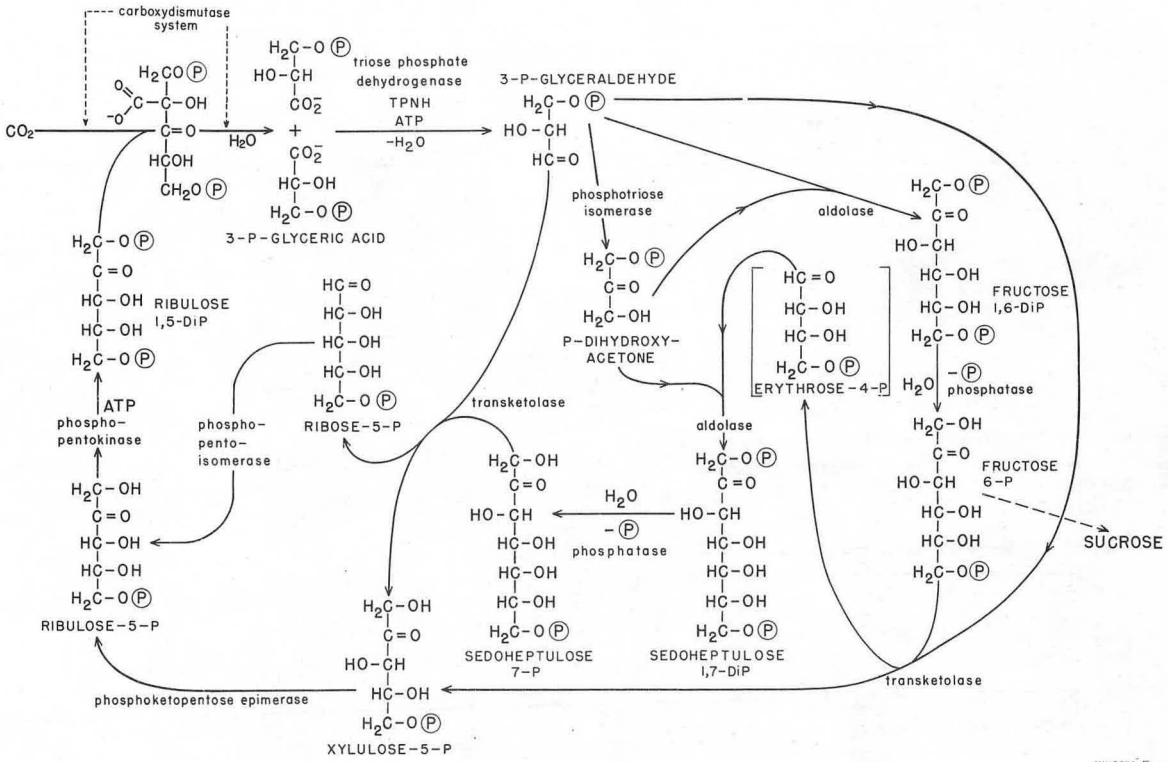
Fig. 9. The carbon reduction cycle of photosynthesis.
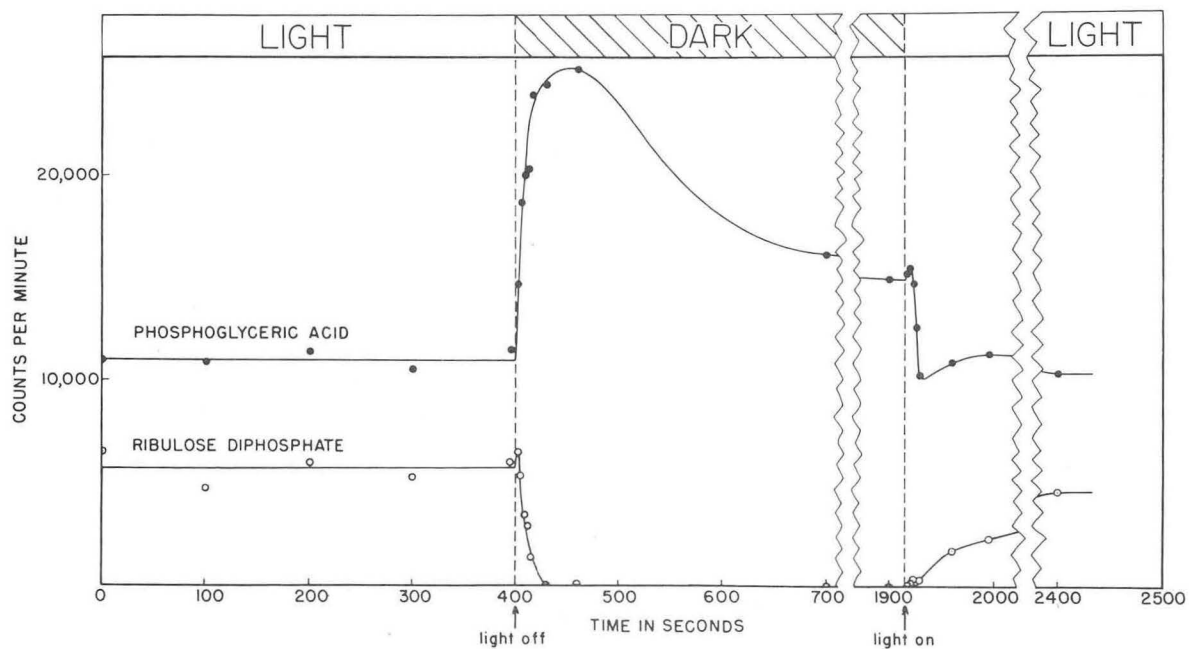
Fig. 10. Changes in concentrations of intermediates during light-dark transition.
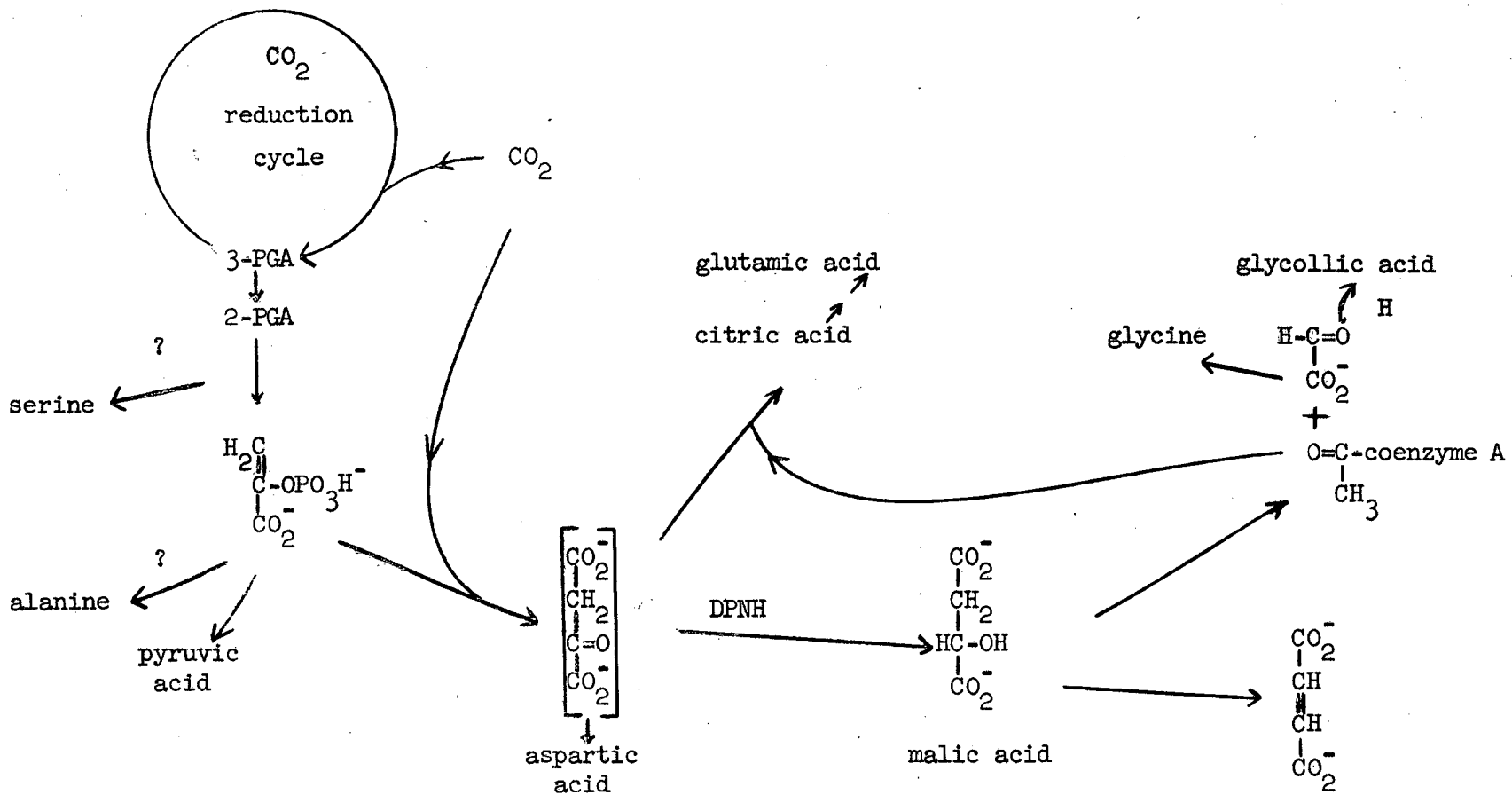
Fig. 11. Carboxylation via C_3-C_1 addition.
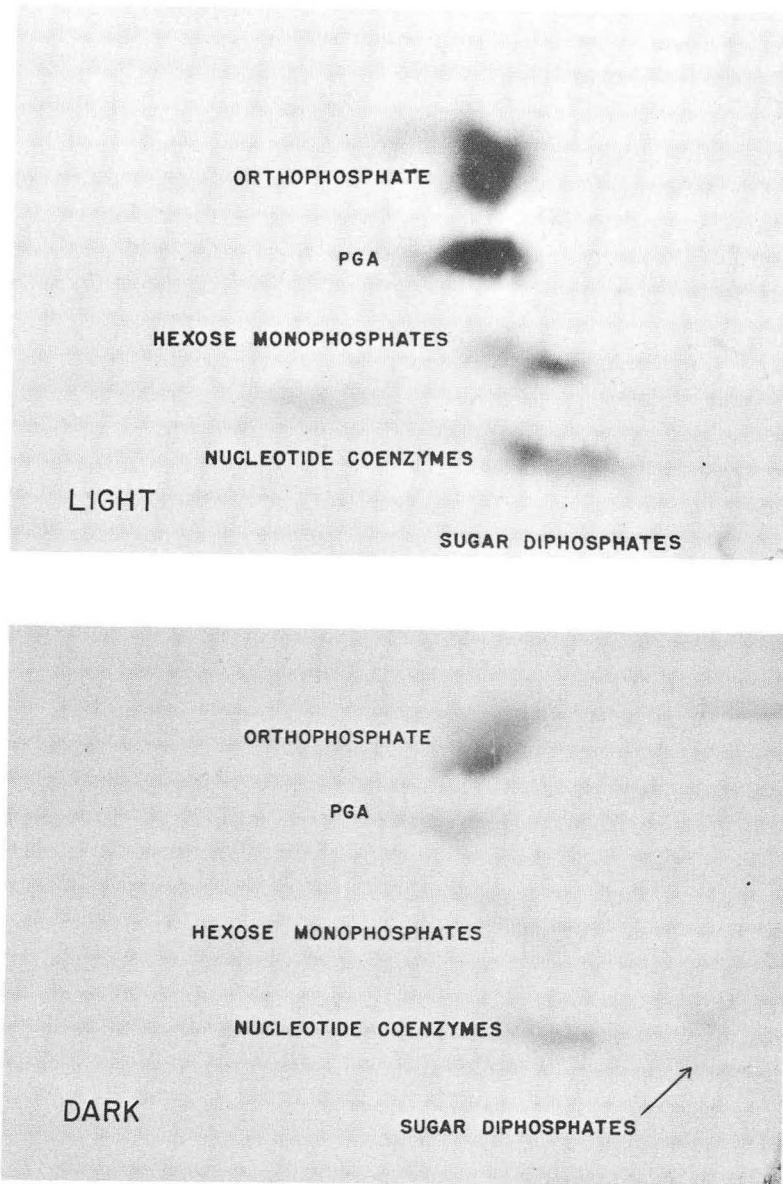
ZN-363

Fig. 12. Fixation of P^{32} by <u>Scenedesmus</u> in one minute.

TABLE I

CONCENTRATIONS OF INTERMEDIATES AND APPROXIMATE

RATES OF FLOW OF CARBON THROUGH INTERMEDIATES

(Preliminary Results)

Compound	Concent. μmoles of carbon*	Flow of carbon through compound. μmoles of carbon/min.
PGA	3	10
Malic Acid	5	2.5
Alanine	6	2.5
Aspartic acid	4	1.5
Serine	3	1
Glutamic acid	10-20	1-2

*Concentration of compound in μmoles per cm^3 of algae times number of carbon atoms in molecule.

REFERENCES

Allen, M. B., Whatley, F. R., Rosenberg, L. L., Capindale, J. B., and Arnon, D. I. Photosynthetic Carbon Dioxide Fixation by Broken Chloroplasts. Research in Photosynthesis. New York, Interscience Publishers, Inc., 1957.

Allen, M. B., Whatley, F. R., Arnon, D. I.: Photosynthesis by Isolated Chloroplasts. VI. Rates of Conversion of Light into Chemical Energy in Photosynthetic Phosphorylation, Biochim. & Biophys. Acta. 27, 16 (1958).

Arnon, D. I., Whatley, F. R. and Allen, M.B.: Photosynthesis by Isolated Chloroplasts. II. Photosynthetic Phosphorylation, the Conversion of Light into Phosphate Bond Energy, J. Am. Chem. Soc. 76, 6324 (1954a).

Arnon, D. I., Allen, M. B. and Whatley, F. R.: Photosynthesis by Isolated Chloroplasts, Nature 174, 394 (1954b).

Bassham, J. A., Benson, A. A. and Calvin, M.: The Path of Carbon in Photosynthesis. VIII. The Role of Malic Acid, J. of Biol. Chem. 185, 781 (1950).

Bassham, J. A., Benson, A. A., Kay, L. D., Harris, A. Z., Wilson, A. T., and Calvin, M.: The Path of Carbon in Photosynthesis. XXI. The Cyclic Regeneration of Carbon Dioxide Acceptor, J. Am. Chem. Soc., 76, (1760) (1954)

Bassham, J. A., Shibata, K., Steenberg, K., Bourdon, J., Calvin, M.: The Photosynthetic Cycle and Respiration: Light-dark Transients, J. Am. Chem. Soc. 78, 4120 (1956).

Bassham, J. A. and Calvin, M.: The Path of Carbon in Photosynthesis. Handbuch der Pflanzenphysiologie, Part II., Vol. 5. Berlin-Gottingen-Heidelberg: Springer (in press).

Benson, A. A., Bassham, J. A., Calvin, M., Goodale, T. C., Haas, V. A. and Stepka, W.: The Path of Carbon in Photosynthesis. V. Paper Chromatography and Radioautography of the Products, J. Am. Chem. Soc. 72, 1710 (1950).

Benson, A. A., Bassham, J. A., Calvin, M.: Sedoheptulose in Photosynthesis by Plants, J. Am. Chem. Soc. 73, 2970 (1951a).

Benson, A. A.: Identification of Ribulose in $C^{14}O_2$ Photosynthesis Products, J. Am. Chem. Soc. 73, 2971 (1951b).

Benson, A. A.: Sugar Phosphates, Paper and Column Chromatography, in Methods In Enzymology, Volume 3. Edited by Sydney P. Colowick, and Nathan O. Kaplan. New York, Academic Press, Inc. (1957a).

Benson, A. A. and Calvin, M.: Intermediates of Photosynthesis: Isolation and Degradation Methods, in Methods in Enzymology, Volume 4. Edited by Sydney P. Colowick and Nathan O. Kaplan. New York, Academic Press, Inc. (1957b).

Benson, A. A., Wiser, R., Ferrari, R. A. and Miller, J. A.: Photosynthesis of Galactolipids, J. Am. Chem. Soc. 80, 4740 (1958a).

Benson, A. A. and Wiser, R.: Plant Physiology 33, XVII (1958) presented at the annual meeting of the American Society of Plant Physiologists, Bloomington, Indiana. August (1958b).

Benson, A. A.: Distribution of the Plant Sulfolipid. A paper presented at the annual meeting of Plant Chemists, National Institutes of Health, Bethesda, Maryland, October 17 (1958c).

Block, R. J., Durrum, E. L. and Zweig, G.: Paper Chromatography and Paper Electrophoresis. New York, Academic Press Inc. (1958).

Bradley, D. F.: Phosphate Transients in Photosynthesis, Arch. Biochem. & Biophys. 68, 172 (1957).

Buchanan, J. G., Lynch, V. H., Benson, A. A., Bradley, D. F. and Calvin, M.: The Path of Carbon in Photosynthesis. XVIII. The Identification of Nucleotide Coenzymes, J. of Biol. Chem. 203, 935 (1953).

Doman, N. G.: Study of the First Products of Photosynthesis with the Aid of Radioactive Isotopes. Proceedings of the All-Union Scientific Conference on the Use of Radioactive and Stable Isotopes and Radiation in National Economy and Science, April 4-12, 1957. Moscow (1958).

Goodman, M., Bradley, D. F. and Calvin, M.: Phosphorous and Photosynthesis. I. Differences in the Light and Dark Incorporation of Radiophosphate, J. Am. Chem. Soc. 75, 1962 (1953).

Goodman, M.: Fractionation of Phosphates from Scenedesmus by Anion Exchange J. Am. Chem. Soc. 77, 4257 (1955).

Gibbs, M.: Reported at meeting of American Society of Plant Physiologists, August 24-28, 1958, Bloomington, Indiana.

Holm-Hansen, O., Pon, N. G., Nishida, K., Moses, V., and Calvin, M.: Uptake and Distribution of Radioactive Carbon from Labeled Substrates by Various Cellular Components of Spinach Leaves (in press).

Kunitz, M. and McDonald, M. R.: Crystaline Hexokinase (Heterophosphatase). Methods of Isolation and Properties, J. Gen. Physiol. 29, 393 (1946).

Moses, V. and Calvin, M.: The Metabolism of 2-Carboxy-4-Ketopentitol Diphosphate, Biochem. & Biophys. Acta (in press).

Moses, V.: Photosynthesis Studies with Tritiated Water (in press).

Myers, J.: Culture Conditions and the Development of the Photosynthetic Mechanism. V. Influence of the Composition of the Nutrient Medium, Plant Physiology 22, 590 (1947).

Tolbert, N. E.: Brookhaven Biology Conference Report 11, (1958), in press.

Trebst, A. V., Tsujimoto, H. Y., Arnon, D. I.: Separation of Light and Dark Phases in the Photosynthesis of Isolated Chloroplasts, Nature 182, 351 (1958

Umbreit, W. W., Burns, R. H. and Staufer, J. F.: Manametric Techniques and Tissue Metabolism. Minneapolis, Minnesota. Burgess Publishing Co. (1949).

Wilson, A. T., Calvin, M.: The Photosynthesis Cycle. CO_2 Dependent Transients, J. Am. Chem. Soc. 77, 5948 (1955).

Wilson, A. T.: Tritium and Paper Chromatography, Nature 182, 524 (1958).

Zalensky, O. V., Voznesensky, V. L., Semikhatova, O. A.: Photosynthesis Studies by Quantitative Radiometric Methods. July 7, 1958 -- prepared for presentation at Second United Nations International Conference on the Peaceful Uses of Atomic Energy, Geneva, 1958.